DOCTOR OF PHILOSOPHY

Studies on the antihaemostatic repertoire of the bloodsucking fly, Stomoxys calcitrans

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By: Philip Jenkin
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Abstract

Investigations into the antihaemostatic properties of the salivary glands and midgut regions of the bloodsucking insect, *Stomoxys calcitrans*, revealed an interesting repertoire of activities.

The reservoir zone of the anterior midgut region was demonstrated to contain a potent anti-thrombin, confirmed with chromogenic substrate analysis, and monitored using the Activated Partial Thromboplastin Time (APTT) assay. Further investigations revealed the anti-thrombin activity may correspond to protein bands 26kDa and 34kDa in size, which it is proposed may form a protein complex. The haematological requirements for a *secondary inhibition* system are discussed, and the evolutionary and digestive advantages of these activities are proposed.
The midgut reservoir zone and salivary glands were also demonstrated to inhibit complement-mediated haemolysis. Midgut surfaces, such as the glycocalyx, and the chitin of the peritrophic matrix, would provide an ideal environment for complement to undergo contact activation. It is suggested that the inhibitor provides protection for the delicate endothelial cells, whose membranes are extremely active during and after a blood meal, and to ensure that erythrocytes contained within the blood meal remain intact.

The salivary glands were demonstrated to contain a FXa inhibitor, also confirmed using a chromogenic substrate assay. In addition the presence of a putative apyrase activity is reported, demonstrated using degenerate primers of a highly conserved sequence to screen a cDNA library. Preliminary (unpublished) studies also demonstrate the inhibition of ADP-induced platelet aggregation, indicative of apyrase activity. The demonstration of a potent salivary vasodilator, antagonistic to the vasoactive effects of adrenaline, is also reported. Abundancy screening and suppressive subtractive hybridisation techniques identified a putative genetic sequence from a cDNA library. The gene fragment demonstrated extremely high correlation to antigens identified in the saliva of the tsetse fly, and the venoms of wasps, hornets and fire ants.

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Introduction

The title of this research project, **"Studies into the Antihaemostatic Repertoire of the blood-sucking fly *Stomoxys calcitrans*"**, encompasses the large, complex and developing field of mammalian haemostasis. It also examines the various mechanisms which haematophagous insects have employed to overcome these defences in order that they may successfully feed on blood.

Haematophagy seems to have evolved independently in at least 13 different families of insect[2] along two main routes[72] and among the >14,000 species and 400 genera of haematophagous arthropod.[3] The term is derived from the Greek *haimat-* (for blood) and *-phagia* (the eating of).[71] Blood is a rich source of protein and has a very high nutritional value, it is not suprising therefore that this valuable resource was exploited by those insects which regularly encountered it.

The bloodsucking habit is believed to have evolved along two main routes.[72] The first route involves a prolonged association between the insect and its vertebrate host, most frequently in its burrow or nesting place. The initial impetus for the insect to form this association is likely to have involved shelter from the elements and an abundant supply of food in the form of organic debris littering the nest. It is believed that physiological adaptations slowly started to develop in response to regular but accidental ingestion of meals containing sloughed skin from the host. With the

discovery of this nutritionally favourable feedsource it would not have been long before behavioural adaptations occurred permitting the occasional direct feeding from the host.[73] This closer feeding association of the insect on the host would have put it in the ideal environment and proximity to encounter a chance blood meal at sites of accidental injury. Indeed some insects, for example the non-biting flies *Hydrotaea armipes* and *Fannia benjamini*, will opportunistically feed on blood from wounds caused by tabanids.[77] Blood is a superior source of nutrition than sloughed skin, and it is far easier to digest, thus having encountered this meal by chance the evolutionary pressure is on to utilise this as a primary foodsource.

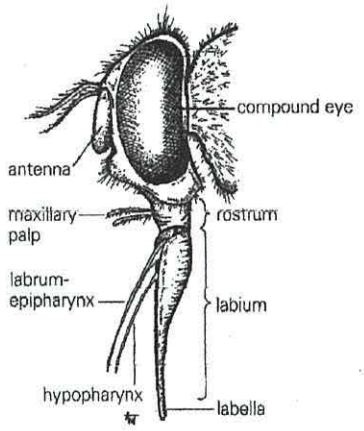
The innate value of blood to the host organism is great, it is obviously a valuable and essential resource with many functions, and as such it is obviously not freely available to the insect for feeding. Ordinarily the blood circulates in a closed system of vessels of which the inner surface, the *tunica intima*, comprises a single layer of endothelial cells which eventually forms the capillaries. These vessels perfuse all tissues of the body and supply blood to them.[117] Therefore to ensure that the insect can take a bloodmeal at regular intervals, so as not to be reliant merely upon chance encounters of blood, it must be able to penetrate the skin and vasculature of the host to make available this resource. The evolution and adaptation of the insect's mouthparts to facilitate this process was therefore a natural progression in this direction, and it is believed that the mouthparts underwent a progressive functional development. This would have progressed from the simple opening-up of old scabs in the initial stages, as is seen with *Philaematomyia lineata*,[82] through to much more specialised adaptations allowing the insect to penetrate unbroken skin.[73]

The second route by which haematophagy is believed to have evolved is through the dietary adaptation of insects which had already evolved mouthparts for piercing, but in this case their intended use was initially for plant-feeding or entomophagy. Chance encounters of a bloodmeal from vertebrate hosts, perhaps through the close association of other insects which formed its intended prey, may have led to a change in dietary preference.[73] The anatomical adaptations already in place would have facilitated a change in feeding habit and dietary preferences. An example of this is where the moth, *Calpe eustrigata*, deliberately uses its sharp and unusually modified proboscis to take a bloodmeal when this structure was initially evolved to pierce fruit skins.[73] However it would appear that not all such encounters are haematophagous in nature, the entomophagous flower bug *Anthocoris nemorum* has been noted to pierce human skin without any evidence of having ingested a bloodmeal.[73] It seems logical that factors other than the potential availability of a bloodmeal are involved in the evolution, or not, of the bloodsucking habit.

Another factor which must be taken into consideration is the ability of the insect to utilise and digest the blood meal once it has fed. For instance, it is argued that the ancestors of the *hemipteran* bugs evolved as sap-suckers, and as such would have not required proteolytic trypsin activity which would have been lost over time. Obviously proteolytic activity is an essential requirement in the efficient utilisation and digestion of a bloodmeal, and it was therefore a necessity that these bugs re-acquire this ability having subsequently acquired the haematophagous habit.[73] It is argued that cathepsins, which were already present intracellularly in lysosomes, were utilised extracellularly as digestive proteinases.[73] It would appear that this was evolutionarily

more favourable than completely re-evolving trypsin activity. The metabolic pathway and synthetic apparatus were already in place, the only requirement being a re-routing and modification in the utilisation of the end-product.[73]

Fig 1.1: The mouthparts of *S.calcitrans*



Not only have successful haematophagous insects had to evolve specialised mouthparts for the physical process of obtaining the bloodmeal itself, and digestive adaptations to accommodate the high protein content of the meal, they must also be able to overcome the haemostatic response of the host organism. If the blood meal clots around the mouthparts of the insect it will be unable to feed again, and it will starve. If blood clots in the gut of the insect, not only will this cause a physical problem by "blocking" the system, the digestive enzymes will not be able to function as efficiently if they cannot be mixed adequately with the meal. Hence the potential nutritional benefit derived from the meal is lost. Therefore these insects have evolved a series of mechanisms with which to overcome the host haemostatic response. They are involved both during the feeding process itself, to reduce the feeding time required (and hence the risk of being swatted by the host). Also post-feeding, helping to maintain the blood meal in a fluid state for subsequent digestion.[2]

Haemostasis may be defined as the balance of processes which allow blood to remain in a fluid state and flow freely within the confines of the circulatory system. Vascular damage triggers physiological events, termed the haemostatic response, which serve

to restrict and eventually stop blood loss. The haemostatic mechanism is not a single biological pathway, but the product of a balance of complex interactions between a number of distinct systems.

Fig 1.2:
Systems Involved in Haemostasis

The Vascular System
Blood Platelets
The Coagulation Cascade
The Fibrinolytic System
The Complement System
The Kinin System

These systems have complex cascades of reactions, feedback and control mechanisms, and interactions with themselves and other biological systems to maintain a steady-state of dynamic equilibrium. As such there is a constant low-level of activity of all components at any one time, and it is only when this equilibrium is disturbed that one or more components of the system will fully activate. This is generally achieved through a massive feedback amplification of one or more of the constituent parts, which in-turn brings in to play other components of the response.

The characterisation of individual antihaemostatic substances from a variety of arthropods has revealed a tremendous diversity in their structure and function. What is perhaps more intriguing is that ***anticoagulants***, ***vasodilators*** and ***platelet anti-aggregating factors*** have been found in every blood-feeding arthropod that has been examined.[2] Conservation of all three of these main antihaemostatic activities during evolution demonstrates that coagulation, vasoconstriction and platelet aggregation each individually pose a significant obstacle to successful haematophagy.

The mechanisms underlying these systems in the mammalian host, and the ways haematophagous animals have found to overcome them, are many and varied.

Mammalian Vasoconstriction and Haematophagous Salivary Vasodilators

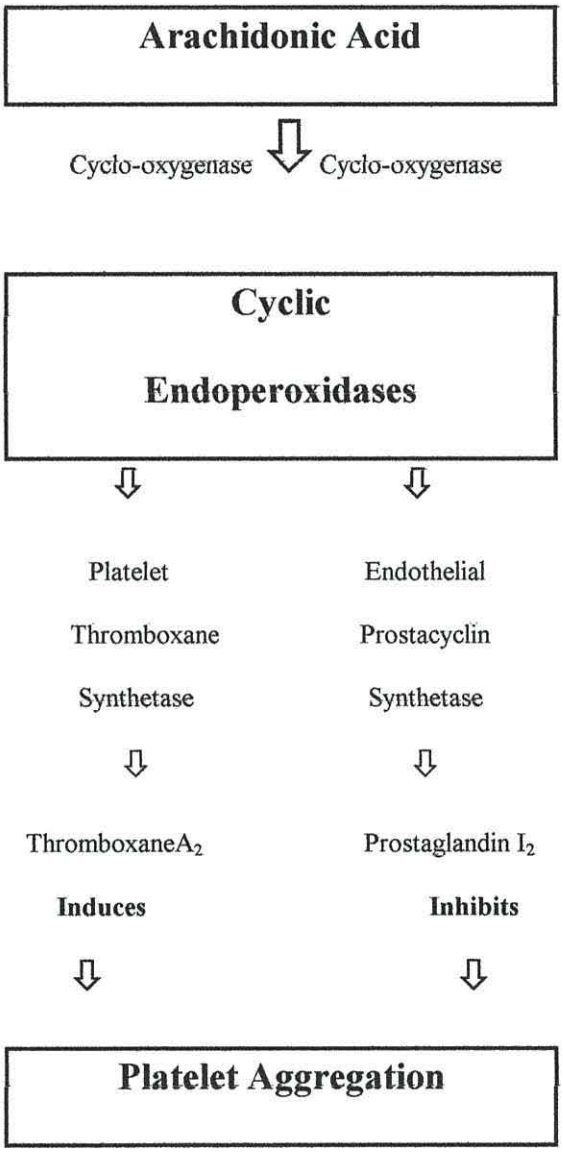
Immediate vasoconstriction of the injured blood vessel occurs at the site of injury, together with a reflex constriction of the small arteries and arterioles. This is the primary vascular response, due to muscular contractions of the vessel wall, which limits immediate blood loss at the site of injury. When there is widespread damage from a significant injury this vascular reaction helps to prevent exsanguination. Capillaries, which have no smooth muscle component, are unable to constrict at the site of injury, instead it is believed that constriction of the precapillary sphincter limits blood flow to the area, and hence limits blood loss. Reduced blood flow to the area of injury facilitates the contact activation of platelets and coagulation factors, and therefore the vascular system can be regarded as the first main component of the haemostatic response.

Vasoactive amines and thromboxane A_2, released from platelets, and the fibrinopeptides liberated during the coagulation cascade, may also have vasoconstrictive properties. Therefore although it is the primary system to play a part in the response, it also interacts and feeds-back with those other systems throughout the whole response.

Endothelial vasoactive substances are thought to be of much greater significance in the response of blood vessels to local changes while perivascular nerves are more concerned with the integration of blood flow to the whole organism. Therefore while vasoconstriction at the site of injury is under local neural and hormonal control, the latter system in the context of evolution of haematophagy is most important.[30] This vaso-activity may be mediated by various compounds, many of which are derived

from platelets, therefore, in addition to playing a primary role in haemostasis and coagulation, platelets are also an important source of inflammatory mediators, including potent vasoactive substances.[29]

Fig 1.1.3: Arachidonic Acid Metabolism[1]



Arachidonic acid, a polyunsaturated fatty acid normally bound to membrane phospholipids, is liberated by the enzyme **phospholipase A_2** which is released from activated platelets. Both vascular endothelial cells and platelets contain biochemical pathways for the metabolism of arachidonic acid. The platelet enzymes **cyclo-oxygenase** and **thromboxane synthetase** convert the free arachidonic acid into **thromboxane A_2**. This has a very short half-life (≈30 secs) before it is converted into its stable derivative thromboxane B_2.[1] Thromboxane A_2, in addition to being a platelet aggregating agent, is a potent *vasoconstrictor*.

The enzyme **prostacyclin synthetase** (vascular endothelial cells) is responsible for the production of **prostaglandin I_2** (PGI_2), also from arachidonic acid. This too has vaso-active properties, but unlike thromboxane A_2, PGI_2 is a potent vasodilator.[1]

Nitric oxide (NO), a small non-organic compound also referred to as **endothelium-derived relaxing factor (EDRF)**, also plays a very important role in mammalian haemostasis. In addition to its main role as a potent endogenous vasodilator and platelet aggregation inhibitor, NO also has a role in inflammation, neurotransmission, immunity, and thrombosis.[31,32] NO is produced under normal physiological conditions primarily in vascular endothelial cells, by the iso-enzyme endothelial nitric oxide synthase (eNOS). However it is now known that NO synthase is expressed in a variety of other tissues, including platelets.[35] The primary stimulus for the production of NO is thought to be the frictional force, or "shear stress", caused by blood flowing through the vessel.[33] ADP is abundantly released by haemolysed red blood cells, and is known to stimulate the release of NO.[43] Once produced NO acts by way of guanylate cyclase, which causes an increase in the intracellular concentration of cyclic guanosine monophosphate (cGMP), resulting in the antiplatelet effects, and in this context, smooth muscle cell relaxation and hence vasodilation.[34]

Therefore in normal circumstances the balance between NO, thromboxane A_2 and PGI_2 helps to regulate local vascular smooth muscle activity, and hence vasoactivity. Histamine and serotonin (5-hydroxytryptamine), also released during platelet degranulation are other mediators that directly induce changes in vascular permeability.[29] The salivary vasodilators already discovered vary widely in their mode of action, having been identified as prostaglandin-like molecules in ticks, nitric oxide-binding proteins in triatomine bugs, and novel vasoactive peptides in sandflies and mosquitoes.[4] However it has only been relatively recently that this diverse range of vasodilators has been investigated in the saliva of haematophagous arthropods, and it is very likely that other novel compounds have yet to be discovered.

Nitrovasodilators

Since 1980 it has been observed that acetylcholine, and other vasodilators, have been able to stimulate the release of a very labile compound from the endothelium which caused the relaxation of vascular smooth muscle cells.[94] Nitric oxide (NO), a small non-organic compound also referred to as endothelium-derived relaxing factor (EDRF), plays a very important role in mammalian haemostasis and vasodilation. A nitrosyl-heme protein which reversibly binds NO, named nitrophorin, has been found in the salivary glands of the bug *Rhodnius prolixus*.[36] It was found to interact with histamine, released at the site of feeding. The histamine was scavenged by the NO-bound nitrosyl-heme protein, and displaced the bound nitric oxide it was transporting.[37] The effect of this is two-fold, the molecule has removed histamine from the site of injury/feeding, an immune mediator involved in the inflammatory response, and it has also released NO, a potent vasodilator and platelet anti-aggregating compound. The benefits of this dual-action to the haematophagous insect are obvious. However, in order to be able to release NO bound to the nitrophorin, it is logical that *Rhodnius prolixus* must be able to synthesise both of these components. It has been found that they are both indeed produced, separately, and then combined in the luminal cavity where the nitrophorins are stored.[38] However, it is not just the nitric oxide system which is a target for the vaso-active salivary molecules of haematophagous insects.

Novel Peptides

A peptide extracted from the saliva of *Lutzyomia longipalpis*, the sand fly, has also been shown to have novel vaso-active properties.[44] Moro and Lerner[94] demonstrated that the potent vasodilator isolated was a specific pituitary adenylate cyclase activating peptide type I receptor agonist. Adenylate cyclase is present on the inner surface of the plasma membrane of most animal cells, and is the enzyme responsible for catalysing the reaction of ATP⇨cAMP + PP_i. Therefore by acting as an agonist for this receptor, the peptide initiates a response which leads to relaxation of the smooth muscle and hence vasodilation. It has also been shown that this peptide is the most persistent and potent vasodilator known.[94]

Also the saliva of the mosquito *Aedes aegypti* has been reported to contain a vaso-active peptide with pharmacological properties typical of a tachykinin. (the Kinins, as stated earlier, are a group of endogenous low mol.weight vaso-active peptides) Further investigation revealed this to actually consist of 2 peptides, named *Sialokinin-I* and *Sialokinin-II*, differing only in the first amino-acid of the sequence.[45] Other members of the mosquito genus were also shown to have similar vasodilators with tachykinin and catechol oxidase/peroxidase activity.[47]

Oxidases and Peroxidases

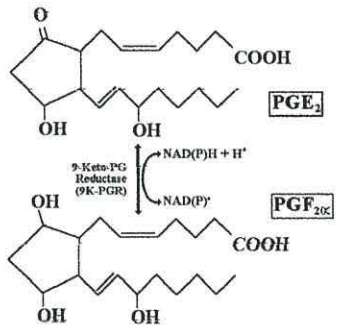
It has been observed that an enzyme with NADPH oxidase activity, isolated from *Anopheline* mosquitoes, is capable of degrading catecholamines and serotonin released at the site of injury. The removal of these endogenous vasoconstrictors is thought to occur via the production of H_2O_2 (Ribeiro, unpublished observations).[3]

Prostaglandins

Historically the name "prostaglandin" is derived from the prostate gland, the molecules having first been identified in human semen over 50 years ago.[97] Prostaglandins are produced in many tissues, however, and the salivary glands and/or saliva of the ticks *Ixodes dammini*[22,52], *Hyalomma excavatum*[95], *Ambylyomma americanum*[94] and *Boophilus microplus* have been demonstrated to contain potent vasoactive prostaglandins. It is believed that these, in addition to increasing the blood flow at the site of feeding, may also be involved in a generalised suppression of lymphocytes observed in tick-infested animals.[22,96]

A.americanum was demonstrated to contain prostaglandins $PGF_{2\alpha}$ and PGE_2 by HPLC/Bioassay and GC-Mass Spectrophotometer, whereas *I.dammini*, *H.excavatum* and *B.microplus* were demonstrated to only contain PGE_2. The prostaglandin PGE_2 is known to be a potent vasodilator, and it has been shown that in some tissues PGE_2 and $PGF_{2\alpha}$ are able to undergo interconversion.[97]

Fig 1.4: The Interconversion of PGE_2 and $PGF_{2\alpha}$



There are obviously a wide and diverse range of vaso-active molecules utilised by haematophagous insects for the purposes of feeding. The enormous potential of vasodilators in the treatment of hypertension and related conditions, and the money that this market generates, ensures that drug companies are always on the lookout for novel new vasodilators. It is only relatively recently that such a diverse range of vasodilators have been discovered in the saliva of haematophagous arthropods, and it is likely that other novel compounds have yet to be discovered.

Mammalian Platelet Aggregation & Haematophagous Inhibitors

Platelet aggregation can be triggered by many routes, including exposure to collagen, ADP, thromboxane A_2, or thrombin, and therefore any of these mechanisms are suitable targets for the inhibition of platelet aggregation. Most haematophagous arthropods have a salivary apyrase, an enzyme which hydrolyses the pyrophosphate bonds of nucleoside tri- and di-phosphates, which will degrade any free ADP or ATP at the site of injury. Normal intracellular concentrations of these nucleotides are 1,000 fold higher than extracellular levels, therefore rupture of cells at the site of injury releases these nucleotides and their presence acts as strong biological indicators of tissue damage. Apyrase may also have a dual function by inhibiting the inflammatory response at the site of feeding by lowering ATP levels (which are involved in neutrophil activation) and via neutrophil-mediated degradation[42] of the apyrase end-product AMP into adenosine (a potent anti-inflammatory substance). This has obvious benefits for haematophagous insects, since the inflammatory response would be a major factor in alerting the host organism to its presence. It is no suprise therefore that, given the central role ADP plays in aggregation, an apyrase of some form has been found in most haematophagous arthropods that have been investigated.[3]

Fig 1.5 Apyrase Reaction

$$ATP \Rightarrow ADP + Pi$$

$$ADP \Rightarrow AMP + Pi$$

However, despite the frequent occurrence of an apyrase in the saliva of most species, other mechanisms of inhibiting platelet aggregation exist. Restrictions in the size of this document do not allow for these to be discussed in any further detail, but the following table gives a complete summary of platelet aggregation inhibitors.

Table 1.1.1: Summary of Platelet Aggregation Inhibitors

Species	Activity	Reference
Ticks		
Ornithodorus moubata	Apyrase	49
	Moubatin	50 & 51
Ixodes dammini	Apyrase	22
	PGI_2 (?)	52
Bugs		
Rhodnius prolixus	Apyrase	55
	Nitric Oxide	54
Triatoma pallidipennis	Disintegrin	56
Fleas		
Oropsylla bacchi	Apyrase	57
Orchopea howardii	Apyrase	57
Xenopsylla cheopis	Apyrase	57
Mosquitoes		
Anopheles freeborni	Apyrase	58
Anopheles stephensi	Apyrase	58
Anopheles albimanus	Apyrase	59
Anopheles gambiae s.l.	Apyrase	59
Aedes aegypti	Apyrase	60
Deerfly		
Chrysops spp.	Disintegrin	46
Blackfly		
Simulium vittatum	Apyrase	61
Sand Flies		
Lutzomyia longipalpis	Apyrase	62
Phlebotomus papatasi	Apyrase	63
Phlebotomus argentipes	Apyrase	63
Phlebotomus perniciosus	Apyrase	63
Tsetse		
Glossina morsitans	Apyrase	64

Anticoagulants and The Blood Coagulation Cascade

In the first half of this century it was widely accepted, due largely to the work of Schmidt (1872) and Morawitz (1905), that the exposure of damaged tissues to plasma was the initiating event in blood coagulation.[138,117,48] This substance was initially named Tissue Thromboplastin until the term Tissue Factor was later introduced. The mechanism was generally accepted, and early work suggesting an alternative pathway was initially rationalised to conform to this theory. However as the years went by mounting evidence forced a reassessment of the coagulation mechanism, and in 1964 the "traditional" coagulation cascade was proposed.[83&84] This traditional picture, comprising the *intrinsic*, *extrinsic* and *common* pathways, has been the mainstay of how we understand the coagulation mechanism in the years that followed, and it accurately portrays the sequence of reactions which are observed to occur *in vitro*.

The blood coagulation system is composed of a series of functionally specific plasma proteins which interact in a highly ordered and regulated predetermined sequence. These complex interactions, between protease zymogens, enzymes and co-factors culminate with the sole objective of the conversion of soluble fibrinogen into a meshwork of insoluble covalently cross-linked fibrin strands, which binds, consolidates, and stabilises the primary haemostatic plug.

The factors are, by convention, referred to by an internationally agreed system represented by roman numerals. They all exist as zymogens in the circulating blood, with the exception of tissue factor (TF), an integral subendothelial membrane glycoprotein which is "compartmentalised" and only comes into contact with blood after vascular injury. The TF:VIIa complex is considered to be the main initiator of

the coagulation cascade *in vivo*, but due to TFPI-mediated feedback inhibition - amplification of the initial response, through the actions of factors VIII, IX and XI, is required to sustain the cascade.[48] Therefore, although the "traditional picture" of the intrinsic and extrinsic pathways of coagulation are considered correct for *in vitro* purposes, the interactions that actually occur *in vivo* are not quite so simplistic.

Following injury to the tissue, in addition to providing a surface for the adhesion and activation of platelets, the exposed subendothelium (collagen, basement membranes and lipopolysaccharides) activates Factor XII and, more importantly, causes the release of tissue factor which is compartmentalised in the subendothelium and fully activates FVII. These actions can be regarded as the "triggers" which initiate the traditional intrinsic and extrinsic pathways, respectively.

The Extrinsic Pathway

At any point in time, small quantities of factor VII are converted to VIIa by trace amounts of activated factors Xa, IXa, XIIa and thrombin. However, despite "activation" factor VIIa still remains in a relatively inactive form. It is only when FVIIa forms a complex with tissue factor that its procoagulant catalytic activity increases over 300,000 fold.[104]

Tissue factor (TF) is a large lipoprotein complex synthesised as an integral cell membrane protein. There are little or no intracellular stores, and although rapid synthesis of TF can be induced by a variety of cytokines including tumour necrosis factor and the interleukins, regulation of TF expression is not fully understood.[104] It is

believed that TF is a member of the cytokine receptor family, even though no other structurally similar proteins are known. TF consists of a large N-terminal extracellular domain and a short (21 residue) cytoplasmic C-terminal domain, separated by a hydrophobic transmembrane domain. The prime function of the extracellular domain is to act as a receptor for plasma factor VII and VIIa, the cytoplasmic domain is thought to be involved with cell signalling, and plays no part in the coagulation activities. It is believed that changes in cell membrane state may be involved in the cellular regulation of TF activity, because the phospholipid configuration of the membrane is a vital factor in the procoagulant activity of TF.[1]

TF can, under the right stimulation and circumstances, be synthesised by endothelial cells and peripheral blood monocytes. However it is not normally found, or synthesised, in cells which are in contact with the circulating blood. TF is compartmentalised, that is to say it is selectively expressed in many tissues, including the epidermis, vascular adventitia, mucosal epithelium organ capsules, myocardium, cerebrum, lungs and placenta.[1] It is found at its highest concentration in the 3 latter sites, which are particularly sensitive and important locations, where effective haemostasis is of the utmost importance. In general terms, TF expression occurs in tissues in a pattern which creates a "haemostatic envelope" around structures, in order to rapidly activate the blood coagulation system should a breach occur.[104]

Following its release at the site of injury, TF is able to complex with both the active and inactive forms of factor VII with equal affinity, producing the TF:VIIa and TF:VII complexes, respectively. The TF-bound zymogen of factor VII (TF:VII) is rapidly

converted to the active form (TF:VIIa) by trace amounts of thrombin and factors IXa, Xa and XIIa. The enzymatic procoagulant activity of factor VIIa and TF:VII complex is greatly enhanced by forming the TF:VIIa complex, and it is this TF:VIIa complex which is then capable of activating both factors IX and X (the latter reaction being the main role). Activated factor Xa then forms the beginning of the common pathway.

The Intrinsic Pathway

Tissue damage causes the subendothelium (collagen, basement membranes and lipopolysaccharides) to become exposed to the blood and the coagulation factors. Although factor XII plays the central role in the initial stages of activation in the intrinsic pathway, membrane localisation of factor XI, prekallikrein and high molecular weight kininogen (HMWK) are also important in the early stages. This enables all of the contact activation factors to be localised at the point of initialisation.

Prekallikrein and HMWK circulate freely in the plasma as a 1:1 complex. Once the negatively charged surface of the collagen is exposed to the blood some of the circulating prekallikrein is bound to the activating surface by HMWK. Inactive factor XI is also bound to the surface by HMWK, and factor XII is adsorbed onto the surface and undergoes a conformational change, but is **not** activated. This conformational change in factor XII activates a small amount of the prekallikrein present, to kallikrein, which is released as a free molecule into the plasma. This free kallikrein converts the surface bound factor XII to its active form XIIa, a process termed

reciprocal proteolytic activation. Factor XIIa, once formed, is able to auto-activate more factor XII zymogen into XIIa.

The generation of activated factor XIIa and kallikrein causes the digestion of the surface-bound HMWK releasing the vasoactive peptide bradykinin. Some references cite inactive factor XII, conformationally changed inactive factor XII and functionally active factor XII as factors XII, αXIIa and βXIIa, respectively.[74]

The inactive factor XI, which until now was bound to the activating surface by HMWK, is activated to XIa by factor XIIa. Factor XIa then cleaves factor IX activating it to produce factor IXa. Factor IXa (a small proportion of which may have been generated through the extrinsic pathway) then combines with factor VIIIa*, factor X and Ca^{2+} on the phospholipid membrane exposed on the platelet to form the **tenase complex**. This complex then cleaves factor X activating it to produce factor Xa. The production of factor Xa marks the beginning of the common pathway.

The Common Pathway

Factor Xa combines with factor Va*, Ca^{2+} and prothrombin on the phospholipid membrane exposed by the platelet. This forms the *prothrombinase complex*, which in-turn activates the prothrombin molecule, producing thrombin. Thrombin has many functions, but its main role is to remove fibrinopeptides A+B from the soluble fibrinogen molecule, causing the precipitation of insoluble fibrin. Thrombin also activates factor XIII to XIIIa. The precipitated fibrin strands spontaneously aggregate to form a weak meshwork of fibres, which are then covalently bound together by the thrombin activated factor XIIIa. Thrombin also has a positive-feedback amplification

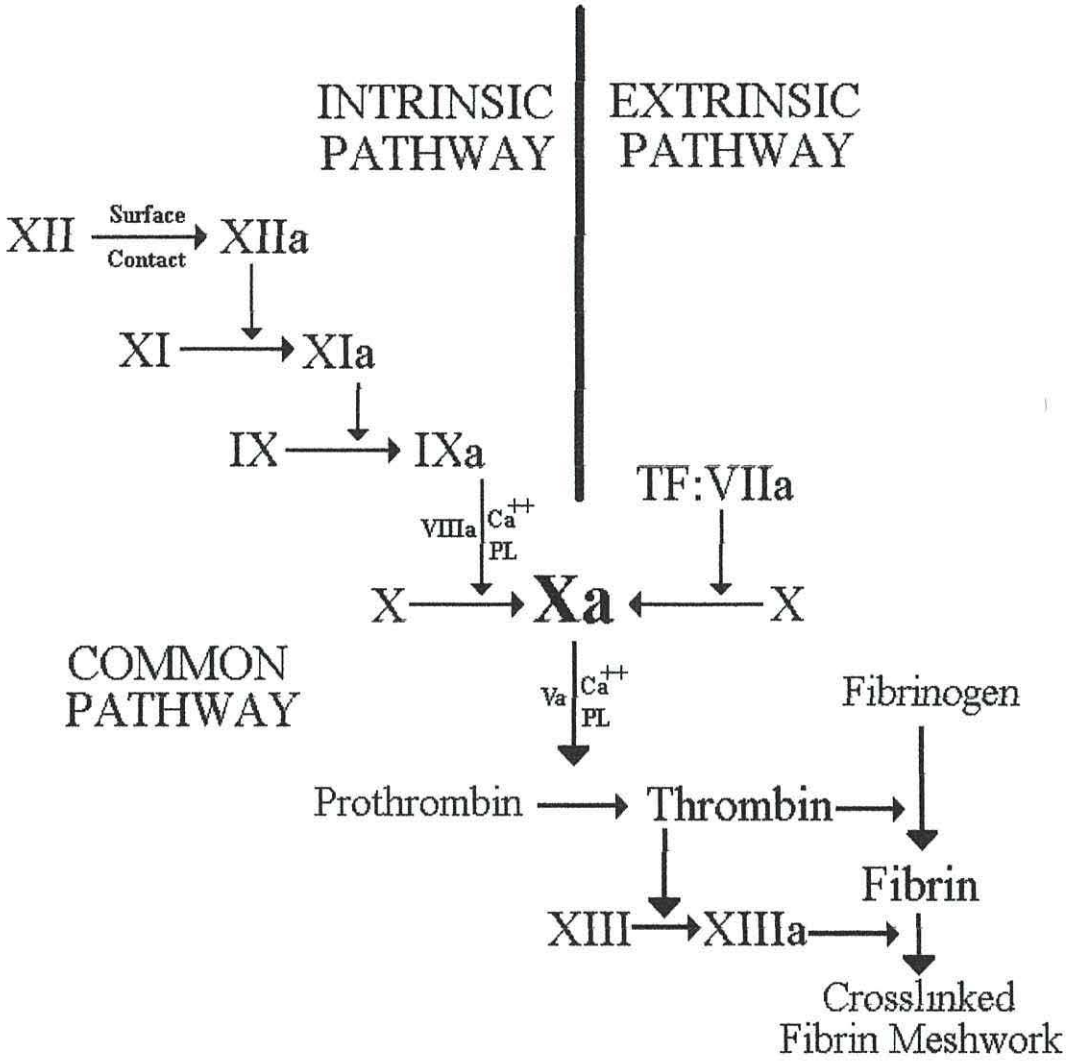
role in activating more factor XI, of the intrinsic pathway, hence amplifying the whole response, maintaining the "flow" of the coagulation cascade once it has been initialised.

The Co-Factors

*Factors V and VIII are co-factors which are also activated by thrombin, and are present in active form (Va and VIIIa) at a low baseline level due to trace amounts of thrombin present normally. Factor VIII circulates in the blood bound to von-Willebrands Factor (vWF) which acts as a carrier molecule. Once coagulation has been initiated, and thrombin has been formed, it has a positive-feedback "amplification" activity activating more of the endogenous factor V and VIII, to Va and VIIIa, respectively. However, as thrombin levels increase, the thrombin-activated factor Va and factor VIIIa start then to become degraded by thrombin. Therefore, although thrombin initially acts to amplify the process, this is not a process which runs uncontrolled. In this way, and through other control systems, the cascade is prevented from becoming a runaway reaction, and so is kept in-check.

The whole process is in a state of dynamic equilibrium at resting state, with the factors self-activating at a low level, with natural inhibitors balancing this process. The amplification that occurs at the site of tissue damage must not be allowed to run uncontrolled, and hence the natural inhibitors to the system are also activated upon accumulation of activated procoagulant factors within the cascade. These inhibitors may act on the factors directly, or on their products (i.e. fibrin), however a summary of their activities would be beyond the scope of this text.

Fig 1.1.6: The Traditional Blood Coagulation Cascade



Rhodnius prolixus has been demonstrated to contain an anti-thrombin in its gut, termed Prolixin-G, and also an anti-VIIIa molecule in the saliva, Prolixin-S.[14,15,16] There has been very little work done to follow-up the proposed gut-derived anti-thrombin prolixin-G in the literature, having been mentioned initially in one paper, by Hellman & Hawkins, in *Nature* in 1965.[14] Plasminogen activators have been reported in *Rhodnius prolixus*, *Hirudu medicinalis*[100] and *Eutriatoma maculatus*,[102] and fibrinolytic activity has been reported in larval haemolymph extracts from *Rhodnius*

prolixus[93,99,101] although the latter case is likely to have little bearing on the process of haematophagy.

Crude extracts from *Dermacentor andersonii* have also been shown to have a possible anti-Va activity, the mechanism here again is directed at the intrinsic pathway, and in this case it is also believed that an anti-VIIa activity also exists[19]. This particular tick also has an anti-thrombin activity demonstrated from an egg extract,[20] although it is difficult to see the relevance of an anti-thrombin requirement in egg production. It is much more likely to represent a physiologically relevant anti-serine protease used in the egg during development.

A broad-spectrum serine protease inhibitor was isolated and partially characterised from the tick *Boophilus microplus*,[18] which is capable of inhibiting any of the activated serine proteases in the coagulation cascade. In this case the molecule was shown to have activity against the enzymes trypsin and chymotrypsin also.

Saliva from the tick *Ixodes scapularis* also contained a substance which inhibited the intrinsic pathway, but not the extrinsic pathway.[22] The exact nature and specificity of this inhibitor remains to be elucidated, but this observation does not necessarily rule-out the presence of an anti-thrombin. The saliva of another tick, *Rhipicephalus appendiculatus*, has been shown to possess an anti-prothrombinase activity.[23] This is a variation on the anti-Xa theme, where instead of inhibiting factor Xa directly, the activity is directed at the prothrombinase complex, in which Xa forms a complex with prothrombin, Ca^{2+}, phospholipid and factor Va. It is only once the factors are formed

in this complex that the inhibitory activity is apparent, and the conversion of prothrombin to thrombin is prevented.

The soft tick *Ornithodorus moubata*, has been shown to have a salivary anti-IXa activity, which would behave *in-vivo* in a similar manner to FVIIIa inhibition. Supplementary to this anti-IXa activity *O. moubata* has also been shown to have anti-thrombin and anti-Xa activity. The utilisation of inhibitors at all 3 of these pivotal points in the coagulation cascade, any one of which would be effective in its own right, leads to a extremely effective and overpowering anticoagulant strategy indeed.

The observation that leech saliva, in addition to having the potent anti-thrombin *hirudin*, contain compounds which prolong clotting via the intrinsic pathway.[92] These compounds, extracted from medicinal leeches (*Hirudu medicinalis*), are demonstrated to be inhibitors of plasma kallikrein, kininase and kinin-like activities. The inhibition of plasma kallikrein, which causes contact activation in association with factor XIIa, is located at the beginning of the intrinsic pathway. This activity, on its own, would be insufficient for an effective anti-haemostatic strategy.

The purpose of the intrinsic pathway is primarily to amplify the stimulus of the extrinsic pathway (TF:VIIa initiation). Although the intrinsic pathway can initiate itself, through the contact activation process, the "extrinsic" thrombin would activate factor XI and therefore bypass the effect on any kallikrein inhibitors present. Such an activity would aid in the suppression of contact activation, but would only really be effective when used in conjunction with other coagulation inhibitors.

Table 1.1.2: Anticoagulant activity in haematophagous arthropods and leeches.[2]

Species	Site Of Action (name)	Tissue
Mosquitoes		
Aedes aegypti	Anti-Xa	SG[5]
Aedes albopictus	Anti-Xa (prothrombinase)	SG[6]
Armigeres subalbatus	Anti-Xa	SG[6]
Culex quinquefacsiatus	Anti-Xa	SG[6]
Anopheles albimanus	Anti-Thrombin	SG[6]
Anopheles gambiae	Anti-Thrombin	SG[6]
Anopheles freeborni	Anti-Thrombin	SG[6]
Anopheles quadrimaculatus	Anti-Thrombin	SG[6]
Anopheles stephensi	Anti-Thrombin	SG[6]
Tsetse		
Glossina austeni	Anti-Thrombin	SG[7]
Glossina morsitans	Anti-Thrombin	SG[8,9]
Glossina techinoides	Not Identified	SG[10]
Blackflies		
Simulium argus	Anti-Thrombin	SG[11]
	Anti-Xa	SG[11]
Simulium metallicum	Anti-Xa	SG[11]
Simulium ochraceum	Anti-Xa	SG[11]
Simulium vittatum	Anti-Thrombin	SG[11,12]
	Anti-Xa	SG[11,12]
Other Flies		
Culicoides variipennis		SG[2]
Tabanus bovinus	Anti-Thrombin (Tabanin)	SG[13]
Hemiptera		
Cimex lectularius	X⇨Xa Inhibitor	SG[2]
Rhodnius prolixus	Anti-Thrombin (Rhodniin)	WB(Gut)[14,15]
	Anti-VIIIa (Prolixin S)	SG[14,16]
Triatoma infestans	Anti-Thrombin (Triatomin)	SG[13]
Triatoma maculata	Anti-Thrombin (Maculatin)	Gut[17]
	Not Identified	SG[17]
Hard Ticks		
Boophilus microplus	Serine Protease Inhibitor	WB (?S)[18]
Dermacentor andersonii	Anti-Va and VIIa	SG[19]
	Anti-Thrombin	EE[20]
Ixodes ricinus	Anti-Thrombin (Ixin)	WB[21]
	Anti-Xa (Ioxdin)	WB[13]
Ixodes scapularis	Intrinsic Pathway Inhibitor	S[22]
Rhipicephalus appendiculatus	Anti-Prothrombinase	SG[23]
Soft Ticks		
Argas persicus	Not Identified	SG, Gut[24]
	Anti-Xa (Argasin)	WB[13]
Ornithodorus moubata	Anti-Xa (TAP)	WB[25]
	Anti-Thrombin	SG, Gut[20]
	Anti-IXa	SG[20]
Medicinal Leeches*		
Haementeria ghiliani	Anti-Xa (Ghilanten)	SG[26]
Haementeria officinalis	Anti-Xa (Antistasin)	SG[27]
Hirudu medicinalis	Anti-Thrombin (Hirudin)	SG[28]

Adapted from Stark K.R. & James A.A. *Parasitology Today* **12;11:**430-437

As of yet there is no evidence in the literature of any antihaemostatic molecules derived from *Stomoxys calcitrans*.[111] It is one of the most widely distributed members of the subfamily *Stomoxyinae,* belonging to the family *Muscidae*. It is a diurnal, facultative, haematophagous fly and was observed in the United States as early as 1776.[79] And although humans are regularly attacked by *S.calcitrans*, which is considered to be a nuisance, humans never form the primary food source.[69] It is an insect associated primarily with horses, camels and livestock such as bovines, and is considered to be a serious pest to the livestock industry. In the United States alone it has been estimated that *Stomoxys calcitrans* is responsible for an annual loss of more than $400 million in the beef industry alone.[66]

The impetus, which drove the investigations that started this research project, were based upon an observation - that whilst the antihaemostatic repertoire of many haematophagous species had been painstakingly investigated, there was not a single recent study in this area performed on the bloodsucking fly, *Stomoxys calcitrans*. Infact the only paper to specifically mention *S.calcitrans* in this field of investigation, actually claimed there was no salivary anticoagulant activity.[111] This may have contributed to the reason why it has been overlooked for such a long time.

Initial investigations were stimulated by the observation that red blood cells (erythrocytes) remain intact in the reservoir zone of *S.calcitrans* after a bloodmeal, while the contents of the lumen are waiting to be digested in the posterior midgut regions. Furthermore, the fact that they remain in a fluid state with no visible sign of a clot present, suggests some form of antihaemostatic activity. These observations,

combined with the fact that the midgut of *S.calcitrans* contains a type-II peritrophic matrix, produced by specialised cells posterior to the proventriculus.[112] The function of this structure is thought mainly to be a protective one, since the physiology of the insect gut prevents it from producing mucin to protect the surface of the lumen. However the peritrophic matrix contains chitin, and the luminal surface of the microvilli, the cells lining the gut wall, contain a glycocalyx - a carbohydrate-rich electron dense layer.[113,114] These structures, the chitin and the negatively charged surface of glycoproteins, create a perfect environment for the contact activation of the coagulation and complement systems. Given that such an environment exists, one would expect to see clot formation in the midgut lumen, and the accompanied lysis of red blood cells due to membrane damage by complement, and to a lesser extent the secondary actions of platelets during the contraction phase of clot formation.

Studies by *Lester* and *Lloyd* in 1929 demonstrate that tsetse flies with their salivary glands removed can still take a successful blood-feed from a host, although lethal blood clots did form in their mouthparts.[115] Similarly *Rhodnius prolixus* was able to feed from a live rabbit after surgical intervention to remove its salivary glands.[116] However, the ability to make a single successful feed has little relevance when it is considered that numerous bloodmeals may be required by some haematophagous insects before sufficient protein has been acquired for egg production. *Stomoxys calcitrans* is one such insect.[73] The evidence was all pointing towards an as-of-yet undiscovered antihaemostatic repertoire in the bloodsucking fly, *Stomoxys calcitrans*.

Chapter 1

Preliminary Screening & Studies of Antihaemostatic Activity in *Stomoxys calcitrans*

1.1 Introduction

1.2 Materials and Methods

Preliminary coagulation assays, using the ACL1000 coagulometer

Manual methods to determine anticoagulant activity from crude extract

Coagulation Investigations & Factor Specific Assays

Chromogenic Substrate Inhibition Analysis.

Sample Preparation & Partial Purification of the Midgut Antithrombin.

Determination and Characterisation of Vasoactive (Vasodilatory) Activity from the Salivary Glands of *Stomoxys calcitrans*

Determination and Characterisation of Anti-Complement Activity from the Salivary Glands & Midgut of *Stomoxys calcitrans*

1.3 Results

1.4 Discussion

1.1 Introduction

The aim of this research project was to investigate the potential antihaemostatic mechanisms employed by the haematophagous insect, *Stomoxys calcitrans*. Haemostasis may be defined as the balance of processes which allow blood to remain in a fluid state and flow freely within the confines of the circulatory system. Injury or damage to a tissue and hence the underlying blood vessels can lead to a rapid loss of blood. Vascular damage triggers physiological events, termed the *haemostatic response*, that restrict and eventually stop this loss. The haemostatic mechanism is not a single biological pathway, but the product of a balance of complex interactions between a number of distinct systems[104] :-

- The Vascular System
- Blood Platelets
- The Coagulation System
- The Fibrinolytic System
- The Complement System
- The Kinin System

There is a fine balancing act between keeping blood in a fluid state so that it may be pumped around the body efficiently, and ensuring that any "leaks" in the system are adequately dealt with. Too far one way and the blood will not clot, causing internal bleeding and eventual death. Too far the other way and clots will form in the blood

vessels, occluding them, and depriving the tissues they supply of oxygen and nutrients, also leading to death or disablement.

These systems have complex cascades of reactions, feedback and control mechanisms, and interactions with themselves and other biological systems, to maintain a steady-state of dynamic equilibrium. As such there is a constant low-level of activity of all components at any one time, and it is only when this equilibrium is disturbed that one or more components of the system will fully activate.

This is generally achieved through a massive feedback amplification of one or more of the constituent parts, which in-turn brings in to play other components of the response. These reactions proceed in a highly controlled manner until the cause of the initial stimulus is rectified, and feedback controls eventually return the system to the normal resting state. One of the most common mechanisms used to overcome host haemostasis by haematophagous animals, and perhaps the easiest to measure outside of the clinical laboratory, would be that of the blood coagulation inhibitors.

Aims and Objectives

This chapter summarises the investigations performed in demonstrating the presence of salivary and midgut anticoagulant activities from *S.calcitrans*. These investigations, combined with the identification of various physical properties, aim to eluicidate the specific mechanism through which the inhibitors are thought to exert their effect. The overall objective is to examine the inhibitory potential of the salivary gland and midgut homogenate samples on the mammalian haemostatic system.

1.2 Materials and Methods

Modification of the Automated PT and APTT Coagulation Assays

Table 1.2.1: Automated Dilutions #1

Sample	Volume Normal Pool (µl)	H_2O (µl)	Soln (µl)
Machine	500	0	0
Blank	400	100	0
Test	400	0	100

(a) Three 500µl plasma sample solutions were prepared as shown in the table. The first was normal human control plasma which acted as the machines internal control. The blank sample contained 400µl of normal human control plasma sample which was diluted with 100µl water, and the test sample contained 400µl of plasma diluted with 100µl of 0.05% Triton X-100 in 150mM NaCl solution. The samples were tested to determine the automated prothrombin (PT) and activated partial thromboplastin (APTT) coagulation times. See table 1.2.1 above.

Table 1.2.2: Automated Dilutions #2

Sample Cups		Composition (µl)		
Rotor	Sample	Plasma	NaCl	X100
1	NP	700	0	0
2	ABN	700	0	0
3	O	350	350	0
4	X	350	0	350

(b) Three 700µl plasma solutions were also prepared, as shown in the table. The first was normal human control plasma which acted as the machines internal control. The blank sample (O) contained 350µl of normal human control plasma sample which was diluted with 350µl 150mM NaCl, and the test sample (X) contained 350µl of plasma diluted with 350µl of 0.05% Triton X-100 in 150mM NaCl solution. These were also tested to determine PT and APTT coagulation times. See table 1.2.2 above.

Preliminary Activity and Machine Carryover Investigation

Table 1.2.3: Preliminary Activity Testing

Sample Cups		Composition (µl)		
Rotor	Sample	Plasma	Spl	X100
1	NP	700	0	0
2	Test	350	350	0
3	Control	350	0	350

Three 700µl plasma solutions were prepared as shown in the table. The first was normal human control plasma which acted as the machines internal control. The test sample contained 350µl of normal human control plasma sample mixed with 350µl of 1 gut.µl^{-1} concentration of sample homogenate suspended in 0.05% Triton X-100 150mM NaCl. The control sample contained 350µl of plasma diluted with 350µl of 0.05% Triton X-100 in 150mM NaCl solution. The samples were loaded into the machine and tested to determine their INR (PT), APTT and Fibrinogen levels (run A) and then the same samples were retested immediately afterwards (run B) to determine if the machine control values were accurately reproducible - to indicate if any sample carryover was occuring with the machine. See table 1.2.3 above.

Preliminary Quantification (Automated Method)

Table 1.2.4: Preliminary Quantification

Sample		Volumes (µl)			
No	Titre (1/x)	NP	H_2O	Spl. d.d.	Total
1	0	350	300	50	700
2	2	350	300	50 ↻	700
3	4	350	300	50 ↻	700
4	8	350	300	50 ↻	700
5	16	350	300	50 ↻	700
6	32	350	300	50 ↻	700
7	64	350	300	50 ↻	700
8	128	350	300	50 ↻	700

Doubling dilutions of sample were prepared to a titre of 1/128 as shown in the table. The resulting solutions gave a titre range from 1/2 through 1/128 as shown in the table. To this was added 300µl of diluent and 350µl of normal plasma. The sample rack was then placed immediately in the ACL1000 for a full coagulation screen (run A). The samples were then retested immediately after these assays were completed (run B) to give a comparison for the purposes of reproducibility. See table 1.2.4.

Preliminary Activity Quantification (Manual Method)

50µl of water were pipetted into each of the 10 sample tubes & 2 control tubes. 50µl of neat (1 gut.µl^{-1}) homogenate were then added to the first of the sample tubes, and doubling dilutions were carried out as before, discarding the final aliquot. This was then repeated to obtain a second set of 12 tubes, and they were all placed on ice. Manual testing was then carried out individually on each tube.

Manual Prothrombin Time (PT) Assay

The tube to be tested was removed from the ice bath and incubated for 30 secs in the 37°C water bath before 50µl of normal human control plasma were added, and mixed. This was incubated for 1 minute after which 200µl of thromboplastin reagent (kept on ice) were added and a stopwatch started simultaneously. The tube was mixed, and inspected under good light for signs of coagulation whilst continuously moving through a 70° angle in and out of the water at a steady rate (the "manual coagulation technique"). The time at which the first distinct clot appears is reported. The control tube time is used to construct the Prothrombin Time Ratio (PTR), or International Normalised Ratio (INR), where the subsequent test samples are compared in a ratio to the control time, or internationally corrected control time, respectively.

Manual Activated Partial Thromboplastin Time (APTT) Assay

The tube to be tested was removed from the ice bath and incubated for 30 secs in the 37°C water bath before 50µl of normal human control plasma were added, and mixed. This was allowed to incubate for a further 1 minute at which point 100µl of cephalin reagent were added and a stopwatch started simultaneously. The tube was mixed with a quick shake, and allowed to incubate for a further 5 minutes. After exactly 5 minutes 100µl of 25mM $CaCl_2$ were added to the tube and a stopwatch was immediately started. The tube was inspected under good light for signs of coagulation, using the technique described earlier. The control tube time is then used to construct the Activated Partial Thromboplastin Time Ratio (APTR), where the subsequent test samples are compared in a ratio to the control time.

Modification of the Manual APTT Coagulation Assay

One bottle of lyophilised IL-Normal Human Control Plasma was reconstituted with 3ml of demineralised water, mixed, and allowed to stand at room temperature for 30 minutes. With regular mixing 50µl aliquots of reconstituted plasma were dispensed into each of 50 borosilicate glass coagulation tubes and placed in the -20°C freezer. Approximately 500µl of the fresh normal human control plasma which remained were stored at 4°C for no longer than 8 hours.

Table 1.2.5: Modified Manual Coagulation Assay

Tube No.	Plasma (µl) Frozen	Plasma (µl) Fresh	Test Solution (50µl)
1-3	50		H_2O
4-6		50	
7-9	50		150mM NaCl
10-12		50	
13-5	50		0.05% Triton X-100
16-18		50	
19-21	50		0.05% Triton X-100 and 150mM NaCl
22-24		50	

Prior to testing a bottle of IL-Cephalin reagent were reconstituted with 4ml demineralised water and allowed to stand at room temperature for 30 minutes to reconstitute, before being placed on ice ready for use. The frozen plasma samples were removed from the freezer and placed immediately into a 37°C waterbath and allowed to incubate for 1 minute. 50µl of test solution (see table 2.2.3) were added, mixed thoroughly, and an APTT coagulation assay was performed. Tests were repeated to obtain results in triplicate and the procedure was repeated, substituting the frozen plasma sample with 50µl of fresh reconstituted plasma to obtain normal APTT control values. See table 1.2.5 above.

Temperature Sensitivity Range of Anticoagulant Activity

40 midguts were homogenised in 200µl tritonX-100 150mM NaCl solution and centrifuged at 13,000 rpm for 10 mins. The supernatant was removed and made upto 1000µl with the same solution. 50µl aliquots were pipetted into 16 borosilicate glass coagulation tubes, labelled, and placed into the -20°C freezer. Each tube was removed from the freezer, incubated for 1 min in a waterbath at 37°C, and placed into a waterbath of the appropriate temperature for 10 minutes. It was then returned to the 37°C waterbath for a further 1 minute. 50µl of freshly prepared normal human control plasma were then added to the tube, mixed thoroughly, and incubated for another minute at 37°C. The manual APTT coagulation assay was then performed as normal. This process was repeated to obtain results, in steps of 10°C, for temperatures in the range 30°C to 100°C inclusive.

Coagulation Factor VIII Assay

Table 1.2.6: Factor VIII Assay Sample Positions

Rotor No.	Spl. Titre	Volumes (µl)			
		Sample	IL-Cal	CCF	FD
DIL			-	-	400
POOL			100	-	400
1			-	100	400
2		100 H_2O	-	-	400
3	Ctl	50 H_2O	-	-	450
4		25 H_2O	-	-	475
5		100	-	-	400
6	1:64	50	-	-	450
7		25	-	-	475
8		100	-	-	400
9	1:32	50	-	-	450
10		25	-	-	475
11		100	-	-	400
12	1:16	50	-	-	450
13		25	-	-	475
14		-	100	-	400
18		500µl	FVIII Def.	Plasma	

18 sample tubes were prepared (see Table 1.2.6) in addition to 2 reagent wells which contained the factor diluent and diluted calibration plasma, in the DIL and POOL locations respectively. Rotor position 14 contained the same dilution of calibration plasma as the POOL position, to provide an internal control for the coagulometer, and position 18 contained the factor VIII deficient plasma which is the basis for the test. Rotor positions 2-4 contained 100µl of plasma diluted 50:50 with demineralised water. Positions 5-13 contained 100µl of plasma diluted 50:50 with sample homogenate of the appropriate titre, and position 1 contained 100µl of Clot Control F plasma diluted 50:50 with demineralised water. The ACL1000 was programmed for a high curve factor VIII assay, the calibration standard correction factor for that batch of plasma was entered, and the samples were tested. See table 1.2.6 above.

Thrombin Time Assay

Table 1.2.7: Thrombin Time Assay Dilutions

Test	Titre	Volumes (µl)		
		Plasma	X100	Sample
Control	0	50	50	0
1A	2	50	0	50
1B	2	50	0	50
2A	4	50	0	50
2B	4	50	0	50
3A	8	50	0	50
3B	8	50	0	50
Control	0	50	50	0
4A	16	50	0	50
4B	16	50	0	50
5A	32	50	0	50
5B	32	50	0	50
Control	0	50	50	0
6A	64	50	0	50
6B	64	50	0	50
7A	128	50	0	50
7B	128	50	0	50
Control	0	50	50	0
7C	128	50	0	50
7D	128	50	0	50
8A	256	50	0	50
8B	256	50	0	50
9A	512	50	0	50
9B	512	50	0	50
Control	0	50	50	0
10A	1024	50	0	50
10B	1024	50	0	50
Control	0	50	50	0

The stock solution of thrombin reagent was reconstituted with 5.7ml demin. water and dispensed into 100µl aliquots in the -80°C freezer. When required a 100µl aliquot was removed from the freezer and 900µl of Imidazole Buffer (pH 7.3) were added to the frozen pellet. This was allowed to thaw, mixed quickly, and placed in the fridge. Thrombin is extremely labile and must be controlled at regular intervals, it can be kept for upto 1 hour at 4°C.

50µl doubling-dilutions of the sample homogenate were prepared with titres between 1/2 and 1/1024 as described in the previous PT and APTT manual assays. The samples were labelled according to the table, and kept on ice until testing commenced. The first control tube was removed from the ice bath, placed in the 37°C water bath, and allowed to acclimatise for 30 secs before adding 50µl of normal human control plasma and 100µl Imidazole buffer. The solution was mixed and allowed to incubate for a further 60 seconds, at which point 100µl of thrombin reagent (kept on ice) were added and a stopwatch started simultaneously. The tube was mixed thoroughly and agitated according to the standard coagulation technique. The time taken for full clot formation to occur was measured. The Thrombin Time Ratio (TTR) was then calculated by comparing the sample times to the control thrombin time in a similar manner to the PTR and APTR assays. See table 1.2.7 above.

Salivary Gland Anticoagulant Assay

25 salivary glands were dissected and homogenised in 200µl of Triton X-100 150mM NaCl. 50µl of this homogenate were added to 50µl of normal human control plasma, mixed and incubated at 37°C for exactly 1 minute before carrying-out an APTT coagulation assay. This was repeated with a further two

50µl aliquots to give coagulation results in triplicate, and duplicate control values were obtained using 50µl of Triton X-100 150mM NaCl solution instead of homogenate.

Chromogenic Substrate Inhibition Analysis

The chromogenic substrate stock solutions were reconstituted in sterile deionised water to obtain a concentration of 0.9mmol/L according to directions provided, and stored on ice. Stock solutions (in 50mM Tris Buffer pH 8.3) were prepared of the selective thrombin inhibitor *I-2581* (120µmol/L), and the FXa inhibitor *Soyabean Trypsin Inhibitor* (3µg/ml). These were then stored on ice until required. A 4% solution of citric acid was prepared, and 100µl of this were added to each reaction to prevent oxidation from affecting the results during the period of testing. The salivary gland or midgut homogenates were prepared (as outlined in appendix) immediately prior to use, in 50mM Tris Buffer pH 8.3, and maintained on ice.

The following reaction tubes were prepared, and incubated for 5 minutes at 37°C. See table 1.2.8 below.

Table 1.2.8: Chromogenic Assay Tubes

Test	Homogenate		50mM	Inhibitors		4%	Enzyme		Sub. 300µM	
No.	SG	RZ	Tris	STI	I-2581	C.A.	FXa	FIIa	S-2765	S-2238
1	50µl	-	200µl	-	-	100µl	50µl	-	200µl	-
2	-	50µl	200µl	-	-	100µl	50µl	-	200µl	-
3	-	-	250µl	-	-	100µl	50µl	-	200µl	-
4	50µl	-	200µl	-	-	100µl	-	50µl	-	200µl
5	-	50µl	200µl	-	-	100µl	-	50µl	-	200µl
6	-	-	250µl	-	-	100µl	-	50µl	-	200µl
7	50µl	-	100µl	100µl	-	100µl	50µl	-	200µl	-
8	-	50µl	100µl	100µl	-	100µl	50µl	-	200µl	-
9	-	-	150µl	100µl	-	100µl	50µl	-	200µl	-
10	50µl	-	100µl	100µl	-	100µl	-	50µl	-	200µl
11	-	50µl	100µl	100µl	-	100µl	-	50µl	-	200µl
12	-	-	150µl	100µl	-	100µl	-	50µl	-	200µl
13	50µl	-	100µl	-	100µl	100µl	50µl	-	200µl	-
14	-	50µl	100µl	-	100µl	100µl	50µl	-	200µl	-
15	-	-	150µl	-	100µl	100µl	50µl	-	200µl	-
16	50µl	-	100µl	-	100µl	100µl	-	50µl	-	200µl
17	-	50µl	100µl	-	100µl	100µl	-	50µl	-	200µl
18	-	-	150µl	-	100µl	100µl	-	50µl	-	200µl

The absorbance was then measured on a spectrophotometer at 405nm, using 50mM Tris Buffer (pH 8.3) as the blank, and the % activities calculated using control tubes (shaded) as the 100% value.

Proteolytic & Anticoagulant Activity Profile of the Reservoir & Opaque Zones

50 midgut (reservoir zone) samples were homogenised in 200µl 0.05% Triton X-100 150mM NaCl solution, centrifuged at 13,000 rpm for 10 minutes, and the supernatant made upto 500µl. Similarly 50 opaque zones were homogenised in 200µl and made upto 500µl with the same solution. The samples were then prepared as follows:

(a) A 50µl aliquot from each of the opaque and reservoir zone homogenates were tested for anticoagulant activity, this was repeated to obtain duplicate APTT results for each zone. **(b)** Two 50µl aliquots of each homogenate were pipetted into separate 1.5ml labelled eppendorf tubes, together with 650µl of Tris buffer pH8.0 and 600µl of 0.05% Triton X-100 150mM NaCl solution. The solutions were then mixed thoroughly, transferred to appropriately labelled tubes containing 5mg of azocoll reagent, and mixed again to create an even suspension. The samples were then incubated at 37°C for 1h, mixing thoroughly at 15 minute intervals, before centrifugation at 13,000 rpm for 5 minutes. The supernatant was carefully removed, using a disposable plastic pasteur pipette, and the absorbance at 520nm was measured on a spectrophotometer. **(c)** 300µl of water were added to each of four 1.5ml Eppendorf tubes. The remaining 300µl of opaque zone homogenate were added to the first tube and mixed thoroughly. A 300µl aliquot of this mixture were removed and added to the following tube, which was repeated to create a series of doubling dilutions, discarding the final aliquot. Ten individual 50µl aliquots from each of the four diluted opaque zone homogenates were each pipetted into 36 suitably labelled 1.5ml Eppendorf tubes. Two tubes from each dilution were removed and frozen immediately in liquid nitrogen and stored at -20°C, the remaining eight tubes per dilution were placed on ice. One tube per dilution was incubated for 10 minutes at 30°C, then immediately 650µl of Tris buffer pH8.0 and 600µl of 0.05% Triton X-100 150mM NaCl solutions were added, and mixed thoroughly. The contents of the tube was transferred to another containing 5mg of azocoll reagent, mixed thoroughly to suspend,

and incubated at 37°C for 1 hour, mixing thoroughly at 15 minute intervals. This was repeated with the remaining 7 sets of tubes, incubating one tube per dilution for 10 minutes at temperatures from 40°C to 100°C in steps of 10°C. The two frozen sample dilutions were defrosted for 1 minute at 37°C and then incubated at 30°C and 40°C to compare post freeze-thaw cycle activity to the fresh sample data.

Protease Inhibitor Trial : Proteolytic and Anticoagulant Activity Profile

Fresh stock solutions of benzamidine (50mg.ml^{-1}), trypsin inhibitor (10mg.ml^{-1}) and the combined chymotrypsin/trypsin (0.1mg.ml^{-1}) inhibitors were prepared, and kept refrigerated. 200 opaque zones were freshly dissected, homogenised with 200µl 0.05% Triton X-100 150mM NaCl, centrifuged at 13,000 for 10 minutes at 4°C. The supernatant was removed and made upto 2,000µl in a borosilicate glass tube which were kept on ice and used as quickly as possible. The sample dilutions were made up in seperate, suitably labelled, 1.5ml Eppendorf tubes and allowed to incubate at room temperature for 10 mins. 100µl of the final 600µl solution were pipetted into a suitably labelled 0.5ml Eppendorf tube and placed on ice for coagulation testing. The remaining 500µl were added to 650µl of Tris buffer pH8.0 *containing 150µl of 0.05% Triton X-100 150mM NaCl solution*, to make 1,300µl in total. This was* then added to a 1.5ml Eppendorf tube containing 5mg azocoll reagent and the proteolytic assay performed as previously, incubating for 30 minutes at 37°C. A series of control tubes blanks were prepared, and used to zero the spectrophotometer before taking readings of the appropriate sample.

Table1.2.9: Protease Inhibitor Trial : Sample Preparation Table

Tube	Volumes (µl)								
	Inhibitors			Samples		Diluents			Total
	B.A.	T.I.	T.C.	Trypsin	O.Zone	H_2O	Remove	Add*	Vol.
1	-	-	-	-	50	550	100	800	1,300
2	-	-	-	-	50	550	100	800	1,300
3	-	-	-	50	-	550	100	800	1,300
4	-	-	-	50	-	550	100	800	1,300
5	50	-	-	-	50	500	100	800	1,300
6	40	-	-	-	50	510	100	800	1,300
7	30	-	-	-	50	520	100	800	1,300
8	20	-	-	-	50	530	100	800	1,300
9	10	-	-	-	50	540	100	800	1,300
10	50	-	-	-	50	500	100	800	1,300
11	40	-	-	-	50	510	100	800	1,300
12	30	-	-	-	50	520	100	800	1,300
13	20	-	-	-	50	530	100	800	1,300
14	10	-	-	-	50	540	100	800	1,300
15	-	50	-	-	50	500	100	800	1,300
16	-	50	-	-	50	500	100	800	1,300
17	-	10	-	-	50	540	100	800	1,300
18	-	10	-	-	50	540	100	800	1,300
19	-	-	50	-	50	500	100	800	1,300
20	-	-	50	-	50	500	100	800	1,300
21	-	-	10	-	50	540	100	800	1,300
22	-	-	10	-	50	540	100	800	1,300
23	50	-	-	50	-	500	100	800	1,300
24	40	-	-	50	-	510	100	800	1,300
25	30	-	-	50	-	520	100	800	1,300
26	20	-	-	50	-	530	100	800	1,300
27	10	-	-	50	-	540	100	800	1,300
28	-	50	-	50	-	500	100	800	1,300
29	-	10	-	50	-	540	100	800	1,300
30	-	-	50	50	-	500	100	800	1,300
31	-	-	10	50	-	540	100	800	1,300
32	50	-	-	-	-	550	100	800	1,300
33	40	-	-	-	-	560	100	800	1,300
34	30	-	-	-	-	570	100	800	1,300
35	20	-	-	-	-	580	100	800	1,300
36	10	-	-	-	-	590	100	800	1,300
37	-	50	-	-	-	550	100	800	1,300
38	-	10	-	-	-	590	100	800	1,300
39	-	-	50	-	-	550	100	800	1,300
40	-	-	10	-	-	590	100	800	1,300

Key : **B.A.** Benzamidine **T.I.** Trypsin Inhibitor **T.C.** Trypsin-Chymotrypsin Inhibitor

* 650µl of Tris buffer pH8.0 containing 150µl of 0.05% Triton X-100 150mM NaCl solution.

Table 1.2.10: Control Tubes for Protease Inhibitor Trial

Ctrl for Tube	Volumes (µl)								Total Vol.
	Inhibitors			Samples		Diluents			
	BA	TI	TC	Trypsin	O.Zone	H_2O	Remove	Add*	
1-2	-	-	-	-	50	550	100	800	1,300
3-4	-	-	-	50	-	550	100	800	1,300
5,10	50	-	-	-	50	500	100	800	1,300
6,11	40	-	-	-	50	510	100	800	1,300
7,12	30	-	-	-	50	520	100	800	1,300
8,13	20	-	-	-	50	530	100	800	1,300
9,14	10	-	-	-	50	540	100	800	1,300
15,16	-	50	-	-	50	500	100	800	1,300
17,18	-	10	-	-	50	540	100	800	1,300
19,20	-	-	50	-	50	500	100	800	1,300
21,22	-	-	10	-	50	540	100	800	1,300
23	50	-	-	50	-	500	100	800	1,300
24	40	-	-	50	-	510	100	800	1,300
25	30	-	-	50	-	520	100	800	1,300
26	20	-	-	50	-	530	100	800	1,300
27	10	-	-	50	-	540	100	800	1,300
28	-	50	-	50	-	500	100	800	1,300
29	-	10	-	50	-	540	100	800	1,300
30	-	-	50	50	-	500	100	800	1,300
31	-	-	10	50	-	540	100	800	1,300
32	50	-	-	-	-	550	100	800	1,300
33	40	-	-	-	-	560	100	800	1,300
34	30	-	-	-	-	570	100	800	1,300
35	20	-	-	-	-	580	100	800	1,300
36	10	-	-	-	-	590	100	800	1,300
37	-	50	-	-	-	550	100	800	1,300
38	-	10	-	-	-	590	100	800	1,300
39	-	-	50	-	-	550	100	800	1,300
40	-	-	10	-	-	590	100	800	1,300
Coag	-	-	-	-	-	600	100	-	-

Ammonium Sulphate Protein Precipitation : Method 1

500 midguts were homogenised in 250µl of 0.05% Triton X-100 150mM NaCl, centrifuged at 13,000 rpm for 10 minutes, and the supernatant was removed and made up to 1,250µl. 750ml of a saturated (5.7M) solution of $(NH_4)_2SO_4$ were prepared at room temperature and used to make the following dilutions in 0.5ml Eppendorf tubes.

Table 1.2.11: Ammonium Sulphate Stock Preparation Dilutions

Tube No.	$(NH_4)_2SO_4$ Stock			Combined Sample			Total Vol. (µl)
	ml Sat. $(NH_4)_2SO_4$	ml H_2O	Stock %	Vol. Stock (µl)	Sample (µl)	Overall % $(NH_4)_2SO_4$	
Test 1	75.0	125.0	37.50	400	100	30	500
2	87.5	112.5	43.75	400	100	35	500
3	100.0	100.0	50.00	400	100	40	500
4	112.5	87.5	56.25	400	100	45	500
5	125.0	75.0	62.50	400	100	50	500
6	137.5	62.5	68.75	400	100	55	500
7	150.0	50.0	75.00	400	100	60	500
8	162.5	37.5	81.25	400	100	65	500
9	175.0	25.0	87.50	400	100	70	500
10	187.5	12.5	93.75	400	100	75	500
11	200.0	0.00	100.00	400	100	80	500

Table 1.2.12: Ammonium Sulphate Precipitation Investigation - Control Tubes

Control Tubes	$(NH_4)_2SO_4$ Stock (%)	Vol. (µl) Stock	H_2O (µl)	Final %	Add µl Water	Add µl Sample	Total Vol.
Neat	-	0	400	0	500	100	1,000
Norm	-	0	400	0	100	-	1,000
C-30	30	150	250	37.5	600	0	1,000
C-35	35	175	225	43.75	600	0	1,000
C-40	40	200	200	50	600	0	1,000
C-45	45	225	175	56.25	600	0	1,000
C-50	50	250	150	62.5	600	0	1,000
C-55	55	275	125	68.75	600	0	1,000
C-60	60	300	100	75	600	0	1,000
C-65	65	325	75	81.25	600	0	1,000
C-70	70	350	50	87.5	600	0	1,000
C-75	75	375	25	93.75	600	0	1,000
C-80	80	400	0	100	600	0	1,000

The 100µl of sample homogenate were added first, followed by the 400µl of $(NH_4)_2SO_4$ solution, mixed and allowed to stand at room temperature for 1 hour. The tubes were then centrifuged at 13,000 rpm for 10 minutes and the 500µl of supernatant were removed and diluted to 1ml with water. The precipitated protein pellet was then resuspended in 1ml of water using the pipette to mix thoroughly. The anticoagulant activity of each solution was measured, using 50µl aliquots for triplicate APTT assays. Two 20µl aliquots of the remaining solution were then used in a duplicate **Bio-Rad** protein assay.

Ammonium Sulphate Protein Precipitation : Method 2

750 midguts were homogenised in 200µl of 0.05% Triton X-100 150mM NaCl solution, centrifuged for 10 mins at 13,000 rpm, and the supernatant made upto 750µl. Stock solutions of Ammonium Sulphate (5.7M) and Barium Chloride (1.52M) were prepared, and used to make the following dilutions. After protein precipitation had occurred in the initial 250µl sample, the supernatant was removed and the precipitate resuspended in 1,000µl of water. 200µl of the supernatant were added to 600µl of $BaCl_2$ solution and centrifuged to remove the precipitate which forms. The supernatant of this solution, together with the resuspended protein samples, were then used for coagulation testing.

Table 1.2.13: $(NH_4)_2SO_4$ Protein Precipitation Investigation - $BaCl_2$ Neutralisation Dilutions

Tube	Stock (µl)		Sample			Take	Stock (µl)			Final
No.	5.7M AS	H_2O	µl	% AS	Total	µl	1.52M BC	H_2O	Vol.	µl
t1	200.0	0	50	80	250	200	600.0	0	600	800
t2	187.5	12.5	50	75	250	200	562.5	37.5	600	800
t3	175.0	25.0	50	70	250	200	525.0	75.0	600	800
t4	162.5	37.5	50	65	250	200	487.5	112.5	600	800
t5	150.0	50.0	50	60	250	200	450.0	150	600	800
t6	137.5	62.5	50	55	250	200	412.5	187.5	600	800
t7	125.0	75.0	50	50	250	200	375.0	225	600	800
t8	112.5	87.5	50	45	250	200	337.5	262.5	600	800
t9	100.0	100.0	50	40	250	200	300.0	300	600	800
t10	87.5	112.5	50	35	250	200	262.5	337.5	600	800
t11	75.0	125.0	50	30	250	200	225.0	375	600	800
t12	62.5	137.5	50	25	250	200	187.5	412.5	600	800
NS	0	200	50	0	250	200	0.0	600	600	800
c1	200.0	50.0	0	80	250	200	600.0	0	600	800
c2	187.5	62.5	0	75	250	200	562.5	37.5	600	800
c3	175.0	75.0	0	70	250	200	525.0	75.0	600	800
c4	162.5	87.5	0	65	250	200	487.5	112.5	600	800
c5	150.0	100.0	0	60	250	200	450.0	150	600	800
c6	137.5	112.5	0	55	250	200	412.5	187.5	600	800
c7	125.0	125.0	0	50	250	200	375.0	225	600	800
c8	112.5	137.5	0	45	250	200	337.5	262.5	600	800
c9	100.0	150.0	0	40	250	200	300.0	300	600	800
c10	87.5	162.5	0	35	250	200	262.5	337.5	600	800
c11	75.0	175.0	0	30	250	200	225.0	375	600	800
c12	62.5	187.5	0	25	250	200	187.5	412.5	600	800
NC	0	250	0	0	250	200	0.0	600	600	800

Ammonium Sulphate Protein Precipitation : Method 3

225 midguts were homogenised in 200µl of 0.05% Triton X-100 150mM NaCl, centrifuged at 13,000 rpm for 10 minutes, and the supernatant was then removed and made up to 3,215µl in a borosilicate glass coagulation tube. 750ml of a saturated (5.7M) solution of $(NH_4)_2SO_4$ were prepared at room temperature and used to make the following dilutions in 1.5ml Eppendorf tubes to give an activity of 14 gut equivalents per assay.

Table 1.2.14: Ammonium Sulphate Protein Precipitation (Method 3) Dilutions:

Tube	$(NH_4)_2SO_4$ Stock			Combined Sample			Total Vol.
No.	ml Sat. $(NH_4)_2SO_4$	ml H_2O	Stock %	Vol. Stock (µl)	Sample (µl)	Overall % $(NH_4)_2SO_4$	(µl)
Test 1	75.0	125.0	37.50	800	200	30	1,000
2	87.5	112.5	43.75	800	200	35	1,000
3	100.0	100.0	50.00	800	200	40	1,000
4	112.5	87.5	56.25	800	200	45	1,000
5	125.0	75.0	62.50	800	200	50	1,000
6	137.5	62.5	68.75	800	200	55	1,000
7	150.0	50.0	75.00	800	200	60	1,000
8	162.5	37.5	81.25	800	200	65	1,000
9	175.0	25.0	87.50	800	200	70	1,000
10	187.5	12.5	93.75	800	200	75	1,000
11	200.0	0.00	100.00	800	200	80	1,000
Ctrl's	$(NH_4)_2SO_4$	H_2O	%	Water	Sample	$(NH_4)_2SO_4$	Total Vol.
Filter1	0	200	0	800	200	0	1,000
Filter2	0	200	0	800	200	0	1,000
Neat1	0	200	0	-	200	0	200
Neat2	0	200	0	-	200	0	200

The 200µl of sample homogenate were added first, followed by the 800µl of $(NH_4)_2SO_4$ solution, mixed and allowed to stand at room temperature for 1 hour. The tubes were then centrifuged at 13,000 rpm for 10 minutes and the supernatant removed. The precipitated protein pellet were then resuspended in 500µl of water using the pipette to mix thoroughly. Both the resuspended protein pellet solution, and the $(NH_4)_2SO_4$ supernatant solutions were then filtered separately through a Centricon-50 filter and the residue from each sample resuspended in 100µl demineralised water.

80µl of this resuspended solution were transferred to a 0.5ml Eppendorf tube and was diluted with a further 80µl of water. The anticoagulant activity of this solution was measured, using the three 50µl aliquots available for triplicate APTT assays. Two 3µl aliquots of the remain 20µl of "neat" resuspended solution were then used in a duplicate **Bio-Rad** protein assay.

Ion Exchange Chromatography : Batch Method 1

Six different pH buffers (1.0M) were prepared (**Table 1.2.15**) each one in 0.1M NaCl solution. A further six buffers were prepared, also 1.0M strength, but in 2.0M NaCl solution. 100mg of QAE Sephadex A-50 anion exchange gel were weighed into each of six labelled 1.5ml Eppendorf tubes. This was repeated using 100mg of CM Sephadex C-25 cation exchange gel. 1ml of each of the 2.0M NaCl buffer were added to the appropriate tubes, and the gels were allowed stand for 1 hour to swell and exchange counter-ions. The tubes were then washed 3 times, at 10 minute intervals, by centrifugation followed by removal and replacement of supernatant, and resuspension. A further 10 washes were carried out, as above, using the 0.1M NaCl solution. On the final wash all of the supernatant were removed and no replacement was carried out. 500 midguts were homogenised in 400µl of 0.05% Triton X-100 150mM NaCl solution and centrifuged at 13,000rpm for 30 minutes. The supernatant were removed, made upto 1ml, and filtered through a Centricon-100 size exclusion filter. This filtrate was then passed through a Centricon-30 filter, and the residue from this were resuspended in 800µl of water. 50µl of this sample were added to each tube, followed by 500µl of appropriate 0.1M NaCl pH buffer.

Table 1. 2.15: Ion Exchange Chromatography - Batch Method 1 Dilutions

Tube	Ion-Exchange Gel Type	Buffer (0.1M in 0.1M NaCl)			Sample (µl)
		pH	Type	µl	
1 2	Anionic Cationic	4.0	Citrate-Phosphate	500	50
3 4	Anionic Cationic	5.0	Citrate-Phosphate	500	50
5 6	Anionic Cationic	6.0	Citrate-Phosphate	500	50
7 8	Anionic Cationic	7.0	Citrate-Phosphate	500	50
9 10	Anionic Cationic	8.0	Phosphate	500	50
11 12	Anionic Cationic	9.0	Tris	500	50
13 14 15 16 17 18	Control Tubes Without Gel	4.0 5.0 6.0 7.0 8.0 9.0	Citrate-Phosphate Citrate-Phosphate Citrate-Phosphate Citrate-Phosphate Phosphate Tris	500 500 500 500 500 500	50 50 50 50 50 50
19 20	Normal Coag. Control	Water Only		500 500	0 0

Specific Activity and pH Stability Investigation

Table 1.2.16: pH Buffer Dilutions:

Tube No.	Buffer Solutions			Volume Sample Homogenate
	Buffer	pH	Volume	
1	PO_4-Citrate	4.0	250µl	50µl
2	PO_4-Citrate	5.0	250µl	50µl
3	PO_4-Citrate	6.0	250µl	50µl
4	PO_4-Citrate	7.0	250µl	50µl
5	PO_4	8.0	250µl	50µl
6	Tris-HCl	9.0	250µl	50µl
Ctrl	Water		250µl	50µl

100 midguts were homogenised in 200µl 0.5% Triton X-100 150mM NaCl solution and the homogenate were centrifuged at 13,000 rpm for 30 mins. The supernatant solution were removed and made-up to 400µl and mixed thoroughly. 250µl of the appropriate pH buffer was added to the Eppendorf tubes, and labelled accordingly, as shown in **Table 1.2.16** above. 50µl of homogenate were added to each of these tubes, mixed thoroughly, and a stopclock started. The tubes were incubated for 1 hour at room temperature, centrifuged at 13,000 rpm for 10 minutes, and filtered through a Centricon-30 filter. The residue on the filter was then resuspended in 300µl of demineralised water for subsequent **Bio-Rad** protein quantification (see **appendix j**), SDS-PAGE (see **appendix k** and **appendix l**) and coagulation testing. Any acid-precipitation of protein which occurred in the low pH samples, after the centrifugation step, were resuspended in water with vigorous vortex mixing. This was then centrifuged and resuspended in 300µl of water and used for the subsequent SDS-PAGE, coagulation and **Bio-Rad** protein assay testing, as with the other samples.

Protein Quantification (Using Commercial **Bio-Rad** Test Kit Reagents)

100µl of sample were pipetted into each of the Eppendorf tubes together with 700µl of water and 200µl of Bio-Rad reagent dye. A standard curve was also constructed for protein concentrations ranging from $1\mu g.ml^{-1}$ - $30\mu g.ml^{-1}$. Following the addition of dye the samples were vortex mixed and allowed to incubate at room temperature for 15 minutes, to allow colour development to occur. The absorbance of the standards and samples were then measured at 595nm using the blank sample to zero the spectrophotometer. A standard curve was constructed and the protein concentrations of the sample unknowns were determined from this. (See **appendix j** for full details).

Table 1.2.17: Specific Activity and pH Stability Investigation - Protein Quantification Volumes

Tube	Volume $0.2mg.ml^{-1}$ Protein Std. (µl)	Volume H_2O (µl)	Volume Dye (µl)	Total Vol. (µl)	Protein Conc. ($\mu g.ml^{-1}$)
1	120	680	200	1,000	30
2	100	700	200	1,000	25
3	80	720	200	1,000	20
4	60	740	200	1,000	15
5	40	760	200	1,000	10
6	20	780	200	1,000	5
7	16	784	200	1,000	4
8	12	788	200	1,000	3
9	8	792	200	1,000	2
10	4	796	200	1,000	1
BLK	0	800	200	1,000	0

Table 1.2.18: Specific Activity and pH Stability Investigation - Test Dilutions

Tube	Sample Volume (µl)	Volume H_2O (µl)	Volume Dye (µl)	Total Vol. (µl)	Sample Source
A	100	700	200	1,000	pH 4.0 ss
B	100	700	200	1,000	pH 5.0 ss
C	100	700	200	1,000	pH 6.0 ss
D	100	700	200	1,000	pH 7.0 ss
E	100	700	200	1,000	pH 8.0 ss
F	100	700	200	1,000	pH 9.0 ss
G	100	700	200	1,000	H_2O ss
H	100	700	200	1,000	pH 4.0 ppt
I	100	700	200	1,000	pH 5.0 ppt

Coagulation Activity

A 50µl aliquot of sample were used to perform an APTT coagulation assays, which was repeated with a further 50µl sample to obtain results in duplicate. The coagulation and protein assay data were combined to give values for specific activity.

SDS-PAGE Analysis (Laemmli)[139]

Nine 20µl aliquots of running buffer were pipetted into 0.5ml Eppendorf sample tubes, into which 20µl of sample were added and mixed thoroughly. The samples were then used for standard tris-glycine SDS PAGE analysis (see **appendix k** and **appendix l**), loading gels A and B in the following order:

Fig 1.2.1: SDS-PAGE Gel Running Order

A	M	1	2	M	3	M	4	5
B	M	4	5	6	7	M	8	9

Key
1 = pH 4.0 ppt 2 = pH 5.0 ppt 3 = H_2O Sample 4 = pH4 sample 5 = pH5 sample 6 = pH6 sampl
7 = pH7 sample 8 = pH8 sample 9 = pH9 sample M = High Mol.Wt. Markers

Ion Exchange Chromatography : Batch Method 2

Six different pH buffers (0.1M) were prepared, each one in 0.1M NaCl solution. A further six buffers were prepared, also 0.1M strength, but in 2.0M NaCl solution. 100mg of QAE Sephadex A-50 anion exchange gel were weighed into each of six labelled 1.5ml Eppendorf tubes. This was repeated using 100mg of CM Sephadex C-25 cation exchange gel. 1ml of each of the 2.0M NaCl buffer were added to the appropriate tubes, and the gels were allowed stand for 1 hour to swell and exchange counter-ions. The tubes were then washed 3 times, at 10 minute intervals, by centrifugation followed by removal and replacement of supernatant, and resuspension. A further 10 washes were carried out, as above, using the 0.1M NaCl solution. On the final wash all of the supernatant were removed, with no replacement stage. 500 midguts were homogenised in 400µl of 0.05% Triton X-100 150mM NaCl solution and centrifuged at 13,000rpm for 30 minutes. The supernatant were removed, made upto 1ml, and filtered through a Centricon-100 size exclusion filter. This filtrate were then passed through a Centricon-30 filter, and the

residue from this were resuspended in 800µl of water. 50µl of this sample were added to each tube, followed by 500µl of the appropriate 0.1M NaCl pH buffer (see **Table 1.2.19**).

Table 1.2.19: Ion Exchange Gel Chromatography (Method 2) Dilutions

Tube	Ion-Exchange Gel Type	Buffer (0.1M in 0.1M NaCl) pH	Type	µl	Sample (µl)
1 2	Anionic Cationic	4.0	Citrate-Phosphate	500	50
3 4	Anionic Cationic	5.0	Citrate-Phosphate	500	50
5 6	Anionic Cationic	6.0	Citrate-Phosphate	500	50
7 8	Anionic Cationic	7.0	Citrate-Phosphate	500	50
9 10	Anionic Cationic	8.0	Phosphate	500	50
11 12	Anionic Cationic	9.0	Tris	500	50
13 14 15 16 17 18	Control Tubes Without Gel	4.0 5.0 6.0 7.0 8.0 9.0	Citrate-Phosphate Citrate-Phosphate Citrate-Phosphate Citrate-Phosphate Phosphate Tris	500 500 500 500 500 500	50 50 50 50 50 50
19 20	Normal Coag. Control	Water Only		500 500	0 0

The tubes were vortex mixed and left to stand at room temperature for 15 minutes, mixing at 3 minute intervals, and then left to stand for 15 minutes without centrifugation to allow the gel to settle. The supernatant were removed, filtered through a Centricon-30, and the residue were resuspended in 200µl of water and frozen at -20°C. A further 500µl of 0.1M NaCl pH buffer were added to the gels, vortex mixed, and allowed to settle-out. The wash supernatant were removed, filtered, and frozen as before. Finally 500µl of 2.0M NaCl pH buffer were added to the gels to elute the sample, mixed, removed and filtered as before. The samples were then removed from the freezer to be tested for coagulation activity, protein content (Bio-Rad assay) and run on an SDS-PAGE gel.

Sephadex G100 Size Exclusion Gel Chromatography: Preliminary Investigation

Approximately 1-2g of Sephadex G100 were weighed carefully into a 250 ml beaker and an excess of 0.1M phosphate buffer pH7.0 150mM NaCl solution were added. The mixture was stirred slowly, using a magnetic stirrer, for a minimum of 30 mins until an even slurry consistency was obtained. The beaker was then removed and allowed to stand for a further 30 minutes to allow the gel to settle-out. The supernatant solution was removed using a disposable pasteur pipette and replaced with fresh buffer. The mixture was then remixed for a further 5 minutes, allowed to settle again, and a final wash performed as before. After removing the final supernatant buffer solution the gel were poured into a small 10cm benchtop column, which was then assembled and mounted on a retort stand. The inlet tube at the top of the column apparatus was then immersed in a 1 litre conical flask containing 750ml of the filtered phosphate buffer solution, at a level below the height of the column. The flask was then placed on a shelf approximately 20cm above the column assembly and the phosphate buffer was allowed to wash through the column for a minimum of 1 hour under the influence of gravity, topping up the reservoir flask when appropriate.

During this time 500 midguts were homogenised in 250µl of 0.05% Triton X-100 150mM NaCl solution, transferred to a 0.5ml Eppendorf tube and centrifuged at 13,000 rpm for 30 minutes. The supernatant was removed using a 200µl Gilson pipette and transferred to another 0.5ml Eppendorf tube.

Following the 1 hour column conditioning, the reservoir flask was lowered to the same level as the column to halt the flow. The inlet line was removed quickly but carefully from the mobile phase buffer and placed into the sample homogenate, taking care not to introduce bubbles into the system. The sample tube was then raised to a level above the column and the homogenate was drawn into the inlet line. The sample tube was immediately lowered to the same level as the column once all of the homogenate had been loaded into the line, ensuring again that no bubbles enter the system, and the line was then returned quickly into the reservoir buffer. The reservoir flask was then replaced in its position above the column to restart the flow through the column, and a stopclock was started simultaneously. Fractions were collected at 1 minute intervals in appropriately labelled 0.5ml Eppendorf tubes, which were capped and weighed before freezing at -20°C. One hundred 0.5ml Eppendorf tubes were weighed

to determine an average value, and these data were then used to calculate the volume of liquid collected per fraction, and hence the flow rate of the column throughout the experiment.

The frozen fractions were removed individually from the freezer, defrosted for 2 mins in a 37°C waterbath and transferred to a Centricon-50 filter unit. The sample was cold filtered at 3,000 rpm using the Fisons Chilspin centrifuge and the residue were resuspended in 175µl of demineralised water. 50µl of the resuspended solution were then used for an APTT coagulation assay, which were repeated using the remaining two 50µl aliquots to obtain results in triplicate. 20µl of the remaining 25µl of sample were mixed with 20µl of x2 concentration loading buffer and these were used to run an SDS-PAGE gel to obtain a protein profile of the fractions collected.

Sample Preparation

25 midguts were manually homogenised for 10 minutes in 200µl of 150mM NaCl 0.05% Triton X-100 solution. The homogenate were transferred to a 1.5ml Eppendorf tube and centrifuged at 13,000 rpm for 10 minutes. The supernatant solution was transferred to an ultracentrifuge tube, balanced using 150mM NaCl, and centrifuged at 50,000 rpm for 2 hours at 4°C. The supernatant solution was carefully removed, without disturbing the pellet, and filtered through a Centricon-100 size-exclusion filter. The filtrate collected from this was then filtered through a Centricon-30, and the filter residue was resuspended in 100µl of 150mM NaCl.

Gel Preparation

2g Sephadex G-100 size exclusion gel were reconstituted for 30 minutes in a beaker containing 100ml of 150mM NaCl solution with continuous stirring. The stirring was stopped and the gel allowed to settle for a further 60-90 minutes. It was then washed by carefully removing the supernatant, and replacing with a similar volume of 150mM NaCl solution. This was repeated again, without the final replacement stage, to provide washed reconstituted gel for the column.

Column Preparation

A straight graduated 3mm diameter 2ml volume pipette was thoroughly cleaned and rinsed in ultrapure water and mounted on a retort stand. A chit was cut to the correct size and inserted in the top of the pipette, it was carefully pushed down with a piece of plastic HPLC tubing until it was firmly held in place near the bottom of the pippette. The Sephadex G-100 gel suspension were then loaded into the column, carefully to avoid bubble formation, using a disposable 10ml plastic syringe and some HPLC tubing. This was allowed to settle for 48 hours, during which time 150mM NaCl solution were allowed to flow through the column under the influence of gravity. This was achieved using a glass funnel as a reservoir mounted above the column which was connected via a short piece of plastic tubing to the top of the pipette. Once the column had settled and packed-down to its final volume the sample homogenate was injected.

System Setup

The base of the column is attached to a UV detector set at 215nm, which in turn is connected to a chart recorder and fraction collector. Each of the fractions collected comprise 10 drops, the *mean volume* of each drop was *calculated* as 26.7µl. The settings for the chart recorder were:

Chart Speed :	**1mm.min^{-1}**
Zero Suppress :	**0**
Range :	**1 Volt**

Sample "Injection"

The saline reservoir was emptied using a 10ml syringe and a length of plastic HPLC tubing, leaving a small amount of liquid above the gel column. This was allowed to run through until the gel was almost exposed to air, at which point 100µl of sample were carefully pipetted onto the surface above the gel. The stopclock was started immediately. The sample was allowed to run into the gel, until the gel surface was almost exposed again, then normal saline mobile phase was then added and the funnel reservoir reassembled above the pipette column.

Determination and Characterisation of Vasoactive (Vasodilatory) Activity from the Salivary Glands of *Stomoxys calcitrans*

Aortic Ring Preparation

2 Male Dunkin-Hartley Guinea Pigs (250-500g) were killed with a blow to the back of the head, followed by exanguination under running water. The ribcage was removed and the thoracic aorta exposed. The aorta was cleared of connective tissue *in situ* and then excised. The endothelial lining of one aorta was then mechanically removed by gently rubbing the luminal surface with a softwood stick. The remaining aorta was left with the luminal surface intact.

5mm rings were then cut from each, and mounted in 50ml, 20ml, and 5ml tissue baths containing Krebs bicarbonate solution in distilled water. The composition was as follows :

NaCl	118.4 mM
KCl	4.7 mM
$NaHCO_3$	24.9 mM
$MgSO_4.2H_2O$	1.2 mM
$KH_2PO_4.2H_2O$	1.2 mM
Glucose	11.6 mM
$CaCl_2.2H_2O$	2.5 mM

The tissues were maintained at 37°C throughout the procedure, with a continual stream of 95% O_2 and 5% CO_2 bubbling gently up through the solution. A tension of 1g was applied to all preparations, and the isometric tension was recorded using Dynamometer UF1 transducers attached to a Lectromed Multitrace 8 Recorder. The tissues were then allowed to equilibrate for 30 minutes before various drugs, or the salivary gland homogenate preparation, were added directly into the aerated buffer medium.

Cumulative Concentration-Response and Standard Curve Preparation

Cumulative concentration-response curves were constructed, in order to determine the submaximal concentration suitable for preconstriction, using stock solutions of :-

Noradrenaline	(NA)	4.8mg in 10ml of 5% Ascorbic Acid
Phenylephrine	(PE)	5.0mg in 10ml of 5% Ascorbic Acid
Adrenaline	(ADR)	10.2mg in 10ml of 5% Ascorbic Acid

These stock concentrations of NA, PE and ADR were then used to preconstrict the aortic rings to a predetermined level, arbitarily indicated on the chart paper. When the tension had risen to a plateau, the cumulative concentration-response curves of the salivary gland homogenate were then determined. The response to single dose of Adenosine, a powerful vasodilator, was also measured. This was used as a comparison to determine the relative vasodilator activity of the salivary gland homogenate, and to act as a control - demonstrating vasodilation in any tissues which did not respond to salivary gland homogenate. Responses were measured as the peak relaxation or contraction (mg). The relaxation (vasodilatory) responses were then converted into % of the peak plateau height immediately prior to adding the homogenate. The drugs used were all prepared in a 5% solution of ascorbic acid to prevent oxidation of the drug in solution prior to use.

Determination and Characterisation of Anti-Complement Activity from the Salivary Glands and Midgut of *Stomoxys calcitrans*

CH100 Calibration Curve

30ml of 3% freshly washed sheep erythrocytes were prepared, in BBS. The follow dilutions were then made:

Materials & Equipment	
5x Barbitone Buffered Saline (BBS)	Sheep Erythrocytes in BBS
0.04% Ammonia Soln.	GuineaPig Serum
Goat Anti-SheepRBC Serum	Spectrophotometer
(541nm)	

Table 1.2.20: Complement Heamolysis (CH_{100}) Test - Calibration Dilutions

Tube No.	Volume (µl)			Overall % RBC
	3% RBC	NH_4OH Soln.	BBS	
1	0	1,000	1,000	0
2	50	1,000	950	0.15
3	100	1,000	900	0.30
4	150	1,000	850	0.45
5	200	1,000	800	0.60
6	250	1,000	750	0.75
7	300	1,000	700	0.90
8	350	1,000	650	1.05
9	400	1,000	600	1.20
10	450	1,000	550	1.35
11	500	1,000	500	1.50
12	550	1,000	450	1.65
13	600	1,000	400	1.80
14	650	1,000	350	1.95
15	700	1,000	300	2.10
16	750	1,000	250	2.25
17	800	1,000	200	2.40
18	850	1,000	150	2.55

A suitable volume of erythrocytes (300µl) were then selected for use in the following assays.

Preparation of 3% Sensitised Erythrocytes

30ml of 6% erythrocytes were freshly prepared in BBS, and divided into two 15ml aliquots. To the first aliquot was added 100µl of anti-sheep goat serum. To the remaining 15ml of 6% cells were added 100µl of BBS. Both samples were then incubated at 37°C for 15 minutes, centrifuged at 3,100 rpm, and the supernatant were carefully removed. The cells were washed once in BBS, and then made-up to 30ml in BBS to give 3% stock solutions of normal, and sensitised sheep erythrocytes, which were stored on ice and used fresh.

Table 1.2.21: CH_{50} Test Dilutions for Quantification of Guinea Pig Serum Complement Activity

Tube No.	3% Sensitised RBC			G.P.Serum		Post-Incubation	
	RBC	BBS	Total	Vol (µl)	Titre	BBS	Total
1	300	600	900	100	1/10	1,000	2,000
2	300	600	900	100	1/20	1,000	2,000
3	300	600	900	100	1/40	1,000	2,000
4	300	600	900	100	1/80	1,000	2,000
5	300	600	900	100	1/160	1,000	2,000
6	300	600	900	100	1/320	1,000	2,000
7	300	600	900	100	1/640	1,000	2,000
8	300	600	900	100	1/1280	1,000	2,000
9	300	600	900	100	1/2560	1,000	2,000
10	300	600	900	100	1/5120	1,000	2,000

A suitable titre of guinea pig serum (1/40) was then selected for use in the final assays.

Table 1.2.22: Complement Haemolysis Inhibition (CHI) Assay Dilutions

Tube	Guinea Pig Serum		BBS	Salivary Gland Homogenate			Total	Post Incubation		
No.	(µl)	Titre	(µl)	(µl)	Titre	Protein	(µl)	RBC	BBS	Total
1	100	1/40	150	100	Neat	-	350	300	1350	2000
2	100	1/40	150	100	1/2	-	350	300	1350	2000
3	100	1/40	150	100	1/4	-	350	300	1350	2000
4	100	1/40	150	100	1/8	-	350	300	1350	2000
5	100	1/40	150	100	1/16	-	350	300	1350	2000
6	100	1/40	150	100	1/32	-	350	300	1350	2000
7	100	1/40	150	100	1/64	-	350	300	1350	2000
8	100	1/40	150	100	1/128	-	350	300	1350	2000
Tube	Guinea Pig Serum		BBS	Reservoir Zone Homogenate			Total	Post Incubation		
No.	(µl)	Titre	(µl)	(µl)	Titre	Protein	(µl)	RBC	BBS	Total
9	100	1/40	150	100	Neat	-	350	300	1350	2000
10	100	1/40	150	100	1/2	-	350	300	1350	2000
11	100	1/40	150	100	1/4	-	350	300	1350	2000
12	100	1/40	150	100	1/8	-	350	300	1350	2000
13	100	1/40	150	100	1/16	-	350	300	1350	2000
14	100	1/40	150	100	1/32	-	350	300	1350	2000
15	100	1/40	150	100	1/64	-	350	300	1350	2000
16	100	1/40	150	100	1/128	-	350	300	1350	2000

Control Tubes

Tube	Guinea Pig Serum		BBS	Salivary Gland Homogenate			Total	Post Incubation		
No.	(µl)	Titre	(µl)	(µl)	Titre	Protein	(µl)	RBC	BBS	Total
17	100	1/40	150	100	Neat	-	350	-	1650	2000
18	100	1/40	150	100	1/2	-	350	-	1650	2000
19	100	1/40	150	100	1/4	-	350	-	1650	2000
20	100	1/40	150	100	1/8	-	350	-	1650	2000
21	100	1/40	150	100	1/16	-	350	-	1650	2000
22	100	1/40	150	100	1/32	-	350	-	1650	2000
23	100	1/40	150	100	1/64	-	350	-	1650	2000
24	100	1/40	150	100	1/128	-	350	-	1650	2000
Tube	Guinea Pig Serum		BBS	Reservoir Zone Homogenate			Total	Post Incubation		
No.	(µl)	Titre	(µl)	(µl)	Titre	Protein	(µl)	RBC	BBS	Total
25	100	1/40	150	100	Neat	-	350	-	1650	2000
26	100	1/40	150	100	1/2	-	350	-	1650	2000
27	100	1/40	150	100	1/4	-	350	-	1650	2000
28	100	1/40	150	100	1/8	-	350	-	1650	2000
29	100	1/40	150	100	1/16	-	350	-	1650	2000
30	100	1/40	150	100	1/32	-	350	-	1650	2000
31	100	1/40	150	100	1/64	-	350	-	1650	2000
32	100	1/40	150	100	1/128	-	350	-	1650	2000

1.3 Results

Modification of the Automated PT and APTT Coagulation Assays

The homogenisation solution used to prepare the salivary gland and midgut homogenates contained the surfactant Triton X-100 at a concentration of 0.05% in isotonic saline. Some stages of the coagulation cascade are membrane-dependent, and therefore it was necessary to ensure that this would not interfere with the coagulation assay(s). The first set of results compares the coagulation results for the homogenisation solution, with those of a control substance (water). The automated assays were performed on an Instrumentation Laboratories (IL) ACL1000 coagulometer.

Table 1.3.1: Results of the Preliminary Controls

Sample	Vol (µl) Norm. Pool	H_2O (µl)	Soln (µl)	Coagulation Times (secs)				Mean Time (secs)			
				PT		APTT		PT		APTT	
				1	2	1	2	C	T	C	T
Machine	500	0	0	15.3	15.3	35.8	35.8	16.6	17.45	44.75	43.5
Blank	400	100	0	16.6	16.6	44.4	45.1	PTR		APTR	
Test	400	0	100	17.4	17.5	42.1	42.6	1.05		0.97	

Key :- C = Mean Control Time PTR = Prothrombin Time Ratio
T = Mean Test Time APTR = Activated Partial Thromboplastin Ratio

The results at this plasma:sample ratio were satisfactory. The following results examine the effect of isotonic saline & Triton X-100 solution on the coagulation assays at a different plasma:sample ratio. Normal & Abnormal Std samples were run for QC purposes.

Table 1.3.2: Results of Preliminary Controls - Adjusted Volumes

Sample Cups		Fib	Composition (µl)			Intrinsic Pathway		Extrinsic Pathway	
Rotor	Sample	mg/dl	Plasma	NaCl	X100	APTT	APTR	PT	PTR
1	NP	2.69	600	0	0	33.6	1.12	14.5	1.06
2	ABN	1.58	600	0	0	73.9	2.47	35.4	2.98
3	O	1.29	300	300	0	54.6	1.82	20.8	1.61
4	X	1.35	300	0	300	52.6	1.76	23.1	1.82
Corellation*		1.05				0.96*	0.97*	1.11*	1.13*
Machine Control		2.87	-	-	-	33.8	-	14.4	-

Key :- O & NaCl =150mM NaCl solution X-100 = 150mM NaCl & 0.05% Triton X-100
PT (Prothrombin Time) APTT (Activated Partial Thromboplastin Time)
INR (Prothombin Time Ratio) APTR (Activated Partial Thromboplastin Ratio)
NP (Normal Control Plasma) ABN (Abnormal Control Plasma)
*** Correlation calculated as the (Value Sample X/Value Sample O) 1.0 ± 0.2**

The results of both preliminary tests with the 0.05% Triton X-100 solution were satisfactory.
Machine carryover is sometimes noticed when using snake venom preparations, in these cases the machine needs acid cleaning between runs. It was now necessary to determine whether this was the case, to help plan subsequent methodology. Neat concentrations of sample homogenate were used.

Table 1.3.3: Results of Preliminary Sample Activity and Machine Carryover Investigation

Sample	Fibrinogen		Extrinsic Pathway				Intrinsic Pathway			
			PT		INR		APTT		APTR	
	A	B	A	B	A	B	A	B	A	B
Control	1.28	1.45	22.0	21.8	1.72	1.69	55.0	55.0	1.84	1.84
Test	**	*	**	*	**	*	*	*	*	*
NP Machine	2.75	2.83	14.4	14.3			33.6	33.6		

Key * Not coagulated (Time went beyond the extended range of machine)
** Coag. Error 3 Third criteria algorithm is not passed (difference between the 2 thresholds is exceeded, i.e. non-phasic curve).

The results show that no machine carryover can be observed, and therefore additional acid-wash stages between testing runs is not deemed necessary. The following assays provide some preliminary data on the quantification of anticoagulant activity, using both the automated and manual methods.

Table 1.3.4: Results of Preliminary Activity Quantification (using the Automated Method):

Sample		Volumes (µl)			Extrinsic Pathway				Intrinsic Pathway			
No	Titre	NP	H_2O	Spl.	PT		INR		APTT		APTR	
	(1/x)			d.d.	A	B	A	B	A	B	A	B
1	0	350	300	50	21.1	21.4	1.64	1.67	54.6	54.8	1.82	1.83
2	2	350	300	50 ♨	**	*	**	*	*	*	*	*
3	4	350	300	50 ♨	22.8	**	1.79	**	*	*	*	*
4	8	350	300	50 ♨	21.4	**	1.67	**	***	*	***	*
5	16	350	300	50 ♨	21.1	27.6	1.64	2.23	**	*	**	*
6	32	350	300	50 ♨	21.3	24.3	1.65	1.93	65.8	*	2.20	*
7	64	350	300	50 ♨	21.1	23.3	1.64	1.83	60.0	**	2.00	**
8	128	350	300	50 ♨	21.0	22.0	1.63	1.72	56.4	63.1	1.88	2.11
NP					14.3	14.4			33.0	33.3		

Key
- * Not coagulated
- ** Coag. Error 3: Third criteria algorithm not passed, non-phasic curve.
- *** Coag. Error 1: Curve passes first threshold, not the 2nd before end of time.

The results (above) demonstrate that the automated method is an unsuitable assay to use routinely, because the machine has trouble photometrically determining the end-point with the crude homogenate.

Table 1.3.5: Results of the Modified Manual APTT Coagulation Assay at Various Conditions

Tube No.	Plasma Type	Test Solution	APTT Coagulation Times (secs) #1	#2	#3	Mean	F/F Ratio
1-3	Frozen	H_2O	53	52	53	53	1.0
4-6	Fresh		53	53	53	53	
7-9	Frozen	150mM NaCl	52	53	53	53	1.0
10-12	Fresh		53	52	53	53	
13-15	Frozen	0.05% Triton X-100	54	54	54	54	1.0
16-18	Fresh		55	54	54	54	
19-21	Frozen	NaCl & Triton X-100	54	54	55	54	1.0
22-24	Fresh		54	54	54	54	

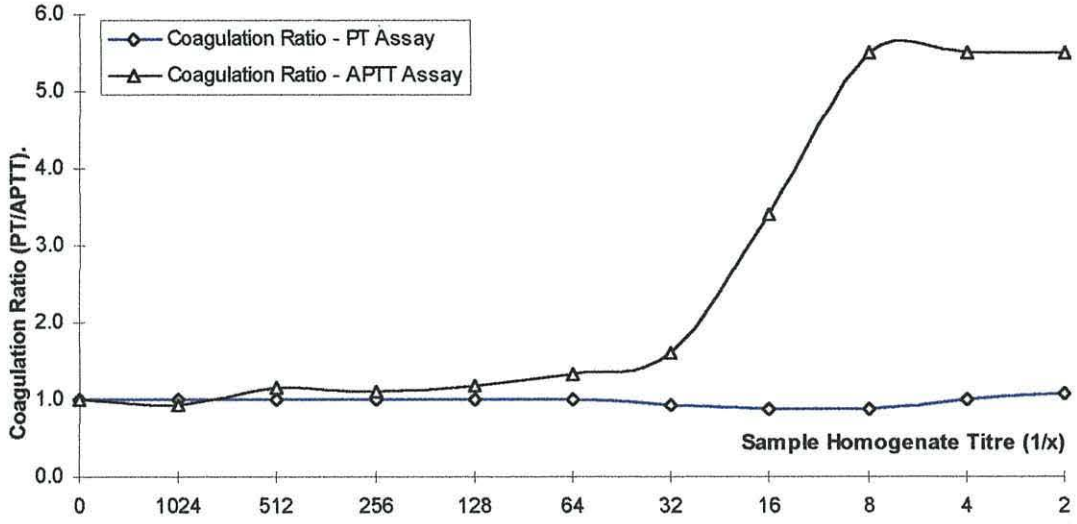
The results (above) demonstrate that the manual coagulation assays are not adversely affected by the presence of Triton X-100, and may be suitable candidates for a routine coagulation assay. Preliminary activity quantification investigations (below) demonstrate the assays are capable of producing results, because the end-point is measured in a different way to the automated technique.

Table 1.3.6: Results of the Preliminary Activity Quantification (Manual Method)

Titre	Intrinsic Pathway			Extrinsic Pathway		
(1/x)	Control	PT (Secs)	Ratio (PTR)	Control	APTT (Secs)	Ratio (APTR)
0	26	26	1.00	63	63	1.00
1024	⇩	26	1.00	⇩	58	0.92
512	⇩	26	1.00	⇩	72	1.14
256	⇩	26	1.00	⇩	69	1.10
128	⇩	26	1.00	⇩	74	1.17
64	⇩	26	1.00	⇩	83	1.32
32	26	24	0.92	66	105	1.59
16	⇩	23	0.88	⇩	224	3.39
8	⇩	23	0.88	⇩	>360	>5.50
4	⇩	26	1.00	⇩	>360	>5.50
2	⇩	28	1.08	⇩	>360	>5.50

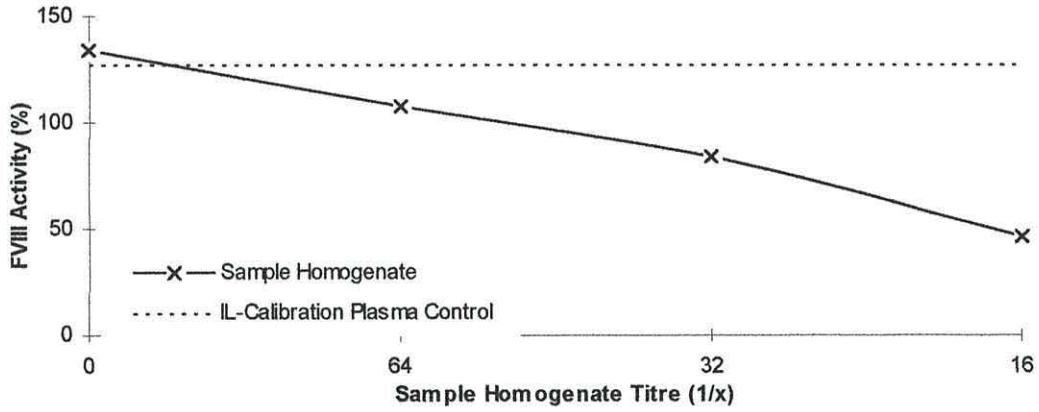
The results (above) demonstrate a divergence in activity between the two coagulation assays for the midgut derived anticoagulant. The APTT assay, an indicator of the intrinsic pathway, shows clear and marked prolongation rising from baseline (63 secs) to >360 seconds as the concentration of homogenate increased. However the PT assay, an indicator of the extrinsic pathway, appears to be unaffected and seldom varies above baseline activity (≈25 secs). The results are summarised graphically in Fig.1.3.1.

Fig 1.3.1: Anticoagulant Activity of Sample Homogenate using the Manual PT/APTT Method:



The APTT assay is a measure of the intrinsic pathway, one component of which is the Factor VIII molecule. Reduced FVIII activity causes the bleeding disorder Haemophilia A, and the FVIII assay is a common investigation in the clinical haematology laboratory. It was decided to perform this assay to gather some more data about the activity of the midgut-derived anticoagulant.

Fig 1.3.2: Factor VIII Assay Sample Activity Results



The results demonstrate a prolongation of the FVIII assay (described as % FVIII activity) with increasing concentration of homogenate. The assay utilised FVIII-depleted plasma mixed with midgut homogenate & control plasma. However the results are inconclusive given that the assay is an APTT-based method and inhibitors to components of the final (common) pathway may also show this effect.

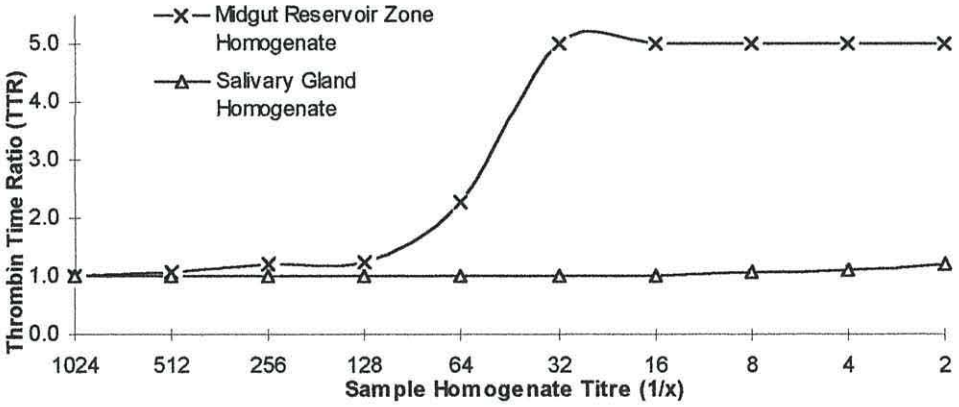
To exclude inhibitors from the final (common) pathway, a Thrombin Time (TT) test was performed. The test measures the coagulation time of standard plasma after the addition of the active thrombin enzyme. The absence of prolongation would suggest an activity further back in the pathway, such as a FVIIIa, FIXa or FXa inhibition, wheras prolongation would demonstrate direct thrombin inhibition.

Table 1.3.7: Thrombin Time Assay Results

Test	Titre	Volumes (µl)			Results	
	(1/x)	Plasma	X100	Sample	TT secs	TT Ratio
Control	0	50	50	0	22	1.0
1A	2	50	0	50	>180	>5.0
1B	2	50	0	50	>180	>5.0
2A	4	50	0	50	>180	>5.0
2B	4	50	0	50	>180	>5.0
3A	8	50	0	50	>180	>5.0
3B	8	50	0	50	>180	>5.0
Control	0	50	50	0	23	1.0
4A	16	50	0	50	>180	>5.0
4B	16	50	0	50	>180	>5.0
5A	32	50	0	50	>180	>5.0
5B	32	50	0	50	>180	>5.0
Control	0	50	50	0	24	1.0
6A	64	50	0	50	55	2.29
6B	64	50	0	50	54	2.25
7A	128	50	0	50	31	1.29
7B	128	50	0	50	28	1.17
Control	0	50	50	0	20	1.0
7C	128	50	0	50	26	1.30
7D	128	50	0	50	26	1.30
8A	256	50	0	50	24	1.20
8B	256	50	0	50	24	1.20
9A	512	50	0	50	22	1.10
9B	512	50	0	50	21	1.05
Control	0	50	50	0	21	1.0
10A	1024	50	0	50	21	1.00
10B	1024	50	0	50	21	1.00
Control	0	50	50	0	21	1.0

The results demonstrate prolongation of the TT assay with the midgut (reservoir zone) homogenate, but no observed prolongation with the salivary gland homogenate. These observations suggest an anti-thrombin activity in the midgut, but exclude this mode of action with the salivary gland samples. The results are demonstrated graphically below:

Fig 1.3.3: Thrombin Time Coagulation Assay Results with Midgut and Salivary Gland Homogenate Samples



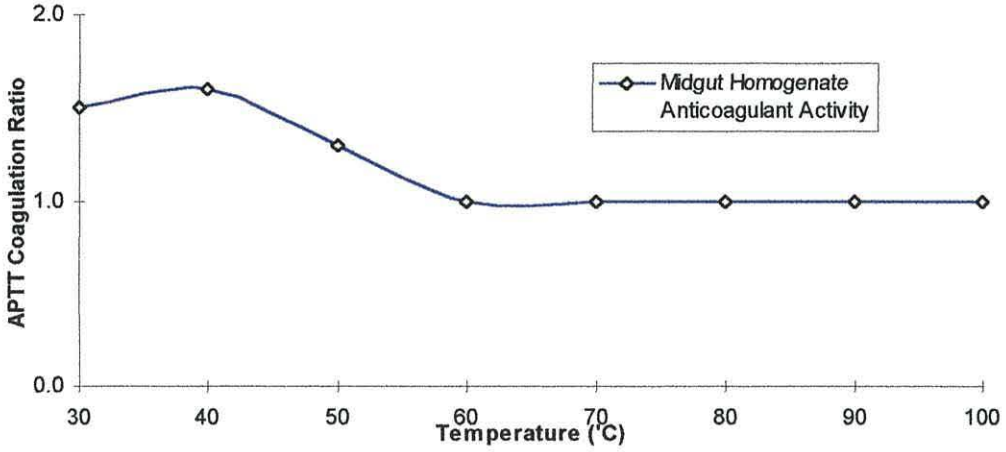
Having provisionally determined some activity profiles for the anticoagulants from both homogenates, the manual APTT assay was selected as most appropriate for the follow-up investigations.

Table 1.3.9: Modified Manual APTT Coagulation Assay Results

Tube No.	Plasma Type	Test Solution	APTT Coagulation Times (secs) #1	#2	#3	Mean	F/F Ratio
1-3	Frozen	H_2O	53	52	53	53	1.0
4-6	Fresh		53	53	53	53	
7-9	Frozen	150mM NaCl	52	53	53	53	1.0
10-12	Fresh		53	52	53	53	
13-15	Frozen	0.05% Triton X-100	54	54	54	54	1.0
16-18	Fresh		55	54	54	54	
19-21	Frozen	NaCl & Triton X-100	54	54	55	54	1.0
22-24	Fresh		54	54	54	54	

The results were satisfactory, providing a fresh:frozen ratio of 1.0 in each case. This allowed further investigations to procede, such as the temperature sensitivity range of the midgut-derived antithrombin:

Fig 1.3.4: Temperature Sensitivity Range of Midgut Homogenate Anticoagulant Activity, measured using the Manual APTT Assay



The results (above) demonstrate anti-thrombin activity from the midgut homogenate is severely affected at temperatures in excess of 40°C, showing no activity at temperatures of 60°C and above.

Table 1.3.10: Salivary Gland Anticoagulant Assay

Tube	Coagulation Data APTT	APTR	% Prolongation
Control #1	58	1.00	0
Test #1	76	1.31	31
Test #2	74	1.28	28
Test #3	75	1.29	29
Control #2	58	1.00	0
Mean Control	58	1.00	0
Mean Test	75	1.29	29 %

200µl of salivary gland homogenate contained 25 salivary gland equivalents. One 50µl aliquot therefore contains the equivalent of 6.25 salivary glands, causing an average 29% prolongation of the APTR. Therefore (assuming a linear relationship) an estimated 21.55 salivary gland equivalents would be required to double the APTR of 50µl of normal human control plasma, the volume used in one APTT assay. One salivary gland pair would be capable of the anticoagulation of 4.64µl standard plasma, equivalent to between 8.4µl and 9.3µl of whole blood (assuming HCT=0.45 to 0.50). A volume of blood with an appropriate order of magnitude for a medium-sized bloodmeal.

Having demonstrated anticoagulant activity in the salivary glands and midgut reservoir zone regions of *S.calcitrans*, and having obtained preliminary data suggesting a possible mode of action, it was necessary to further confirm and identify these activities. A chromogenic assay, comprising the purified coagulation factor(s) with a specific labelled substrate, in the presence & absence of the proposed inhibitor, would determine the specific inhibitory activity for each of the homogenate samples. The results of these investigations for thrombin and FXa activity are summarised below:

Table 1.3.11: Results of Chromogenic Assay Investigations

Test No.	Sample S G	Sample R Z	Inhibitor STI (FXa)	Inhibitor I-2581 (FIIa)	Enzyme/Substrate FXa S-2765	Enzyme/Substrate FIIa S-2238	Absorbance (405nm) A_{405} Test #1	Test #2	Mean Value
1	✓				✓		0.274	0.267	0.271
7	✓		✓		✓		0.211	0.207	0.209
13	✓			✓	✓		0.214	0.219	0.217
3					✓		0.898	0.884	0.891
2		✓			✓		0.771	0.783	0.777
8		✓	✓		✓		0.331	0.317	0.324
14		✓		✓	✓		0.614	0.631	0.623
9			✓		✓		0.347	0.312	0.330
15				✓	✓		0.788	0.797	0.793
Test No.	Sample S G	Sample R Z	Inhibitor STI (FXa)	Inhibitor I-2581 (FIIa)	Enzyme/Substrate FXa S-2765	Enzyme/Substrate FIIa S-2238	Absorbance (405nm) A_{405} Test #1	Test #2	Mean Value
4	✓					✓	0.796	0.808	0.802
10	✓		✓			✓	0.752	0.808	0.780
16	✓			✓		✓	0.303	0.291	0.297
6						✓	0.821	0.816	0.819
5		✓				✓	0.316	0.331	0.324
11		✓	✓			✓	0.308	0.311	0.310
17		✓		✓		✓	0.281	0.277	0.279
12			✓			✓	0.813	0.815	0.814
18				✓		✓	0.319	0.322	0.321

Table 1.3.12: Results Expressed by Substrate

Test No.	Sample S G	Sample R Z	Inhibitor STI (FXa)	Inhibitor I-2581 (FIIa)	Enzyme/Substrate FXa S-2765	Enzyme/Substrate FIIa S-2238	%Activity (% of Max A_{405}) Mean (%)	Interpretation of Test Result
1	✓				✓		30.4	SG alone on FXa
7	✓		✓		✓		23.5	SG & FXa Inhib. on FXa
13	✓			✓	✓		24.4	SG & FIIa Inhib. on FXa
3					✓		100.0	100% FXa Activity
2		✓			✓		87.2	RZ alone on FXa
8		✓	✓		✓		36.4	RZ & FXa Inhib. on FXa
14		✓		✓	✓		69.9	RZ & FIIa Inhib. on FXa
9			✓		✓		37.0	FXa Inhib. on FXa
15				✓	✓		89.0	FIIa Inhib. on FXa
Test No.	Sample S G	Sample R Z	Inhibitor STI (FXa)	Inhibitor I-2581 (FIIa)	Enzyme/Substrate FXa S-2765	Enzyme/Substrate FIIa S-2238	Activity (% of Max) Mean %	Interpretation of Test Result
4	✓					✓	97.9	SG alone on FIIa
10	✓		✓			✓	95.2	SG & FXa Inhib. on FIIa
16	✓			✓		✓	36.3	SG & FIIa Inhib. on FIIa
6						✓	100.0	100% Thrombin Activity
5		✓				✓	39.6	RZ alone on FIIa
11		✓	✓			✓	37.9	RZ & FXa Inhib. on FIIa
17		✓		✓		✓	34.1	RZ & FIIa Inhib. on FIIa
12			✓			✓	99.4	FXa Inhib. on FIIa
18				✓		✓	39.2	FIIa Inhib. on FIIa

The results demonstrated inhibitory activity against activated coagulation factor X (FXa) from the salivary gland homogenate, as is common in many haematophagous insects. The midgut derived anticoagulant was demonstrated to have anti-thrombin activity, confirming the results of the TT assay.

The reservoir zone of *S.calcitrans* resides next to the proteolytically active opaque zone in the digestive system. It is important to determine the relative proteolytic:anticoagulant activities from the reservoir zone, not only to demonstrate predominance for each activity, but also to assess the proteolytic activity which would be encountered for any subsequent purification procedure. The results below demonstrate <0.1% of total midgut proteolytic activity resides in the reservoir zone, and that no anti-thrombin activity is detected in the opaque zone whatsoever. They also demonstrate the temperature sensitivity range of the proteolytic activity, and the effect freeze-thaw cycles have upon this activity.

Table 1.3.13: Proteolytic & Anticoagulant Profile of the Midgut Reservoir & Opaque Zones

Tube No.	Midgut Zone	Coagulation Assay Results				
		APTT#1	APTT#2	Mean	APTR	ΔAPTR.Gut^{-1}
1(a)	Reservoir	164	165	164.5	3.10	0.420
2(a)	Opaque	47	44	45.5	0.86	-0.028
Ctrl	Blank	53	53	53	1.00	

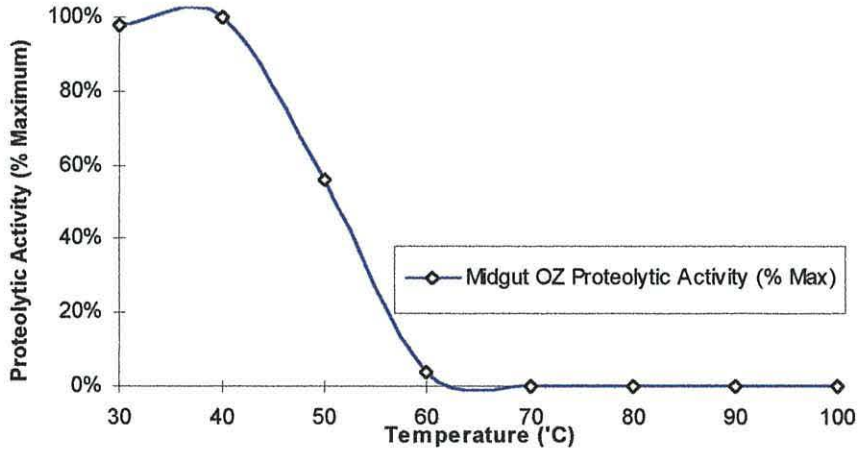
Tube No.	Midgut Zone	Proteolytic Activity (0.1 gut equivalents.μl^{-1})			
		Abs. (595nm)	Abs. (595nm)	Mean	%Rel.Comb.Activity
1(b)	Reservoir	0.000	0.001	0.0005	0.025%
2(b)	Opaque	2.069	1.964	2.0165	99.975%
Ctrl	Blank	0.000	0.000		

Tube No.	Temp. (°C)	Proteolytic Activity (Temperature Sensitivity) Assay			
		1:2 Abs.^{595}	1:4 Abs.^{595}	1:8 Abs.^{595}	1:16 Abs.^{595}
1(c)	30	1.354	0.786	0.384	0.171
2(c)	40	1.716	0.789	0.386	0.167
3(c)	50	0.906	0.439	0.221	0.115
4(c)	60	0.020	0.012	0.001	0.000
5(c)	70	0.001	0.000	0.000	0.000
6(c)	80	0.001	0.001	0.000	0.000
7(c)	90	0.000	0.001	0.001	0.001
8(c)	100	0.000	0.000	0.000	0.000
Ctrl	Blank	0.000	0.000	0.000	0.000

Tube No.	Temp (°C)	Proteolytic Activity of Frozen Samples							
		1:2	%	1:4	%	1:8	%	1:16	%
9(c)	30	1.264	93.4	0.701	89.2	0.346	90.1	0.157	91.8
10(c)	40	1.568	91.4	0.710	90.0	0.357	92.5	0.165	98.8
Ctrl	Blank	0.000		0.000		0.000		0.000	

% Standard Deviation (%SD) of the mean yield results was 3.28%, the mean post freeze-thaw yield was 92.15% of total fresh sample activity.

Fig 1.3.5: Temperature Sensitivity Profile: Opaque Zone Homogenate Proteolytic Activity



Having determined relative activities for the proteolytic and anticoagulant roles, investigations into suitable protease inhibitors were examined. The specific aim was to find suitable inhibitor to overcome the problems of proteolytic degradation, and allow further purification work. Ideally this strategy would utilise ground fly 'paste', provided in massive quantities, rather than the immensely tedious and time-consuming process of dissections. The disadvantage of 'paste' is that it contains everything, including the opaque zone, therefore effective proteolytic inhibition is a pre-requisite for any such procedure. The coagulation cascade is based upon a series of proteolytic reactions, carried out by serine protease enzymes (most of the coagulation factors), so it was also necessary to test the inhibitors against the assay to ensure compatibility. The following data demonstrate the results of these investigations :

Fig 1.3.6: Effect of Proteolytic Inhibitors on 50µl Trypsin (10mg.ml^{-1}) Standard

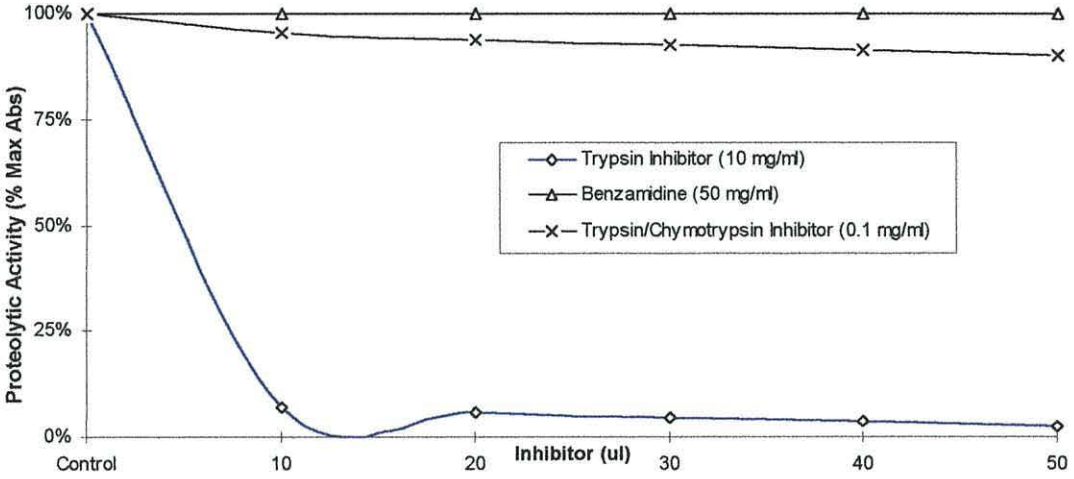
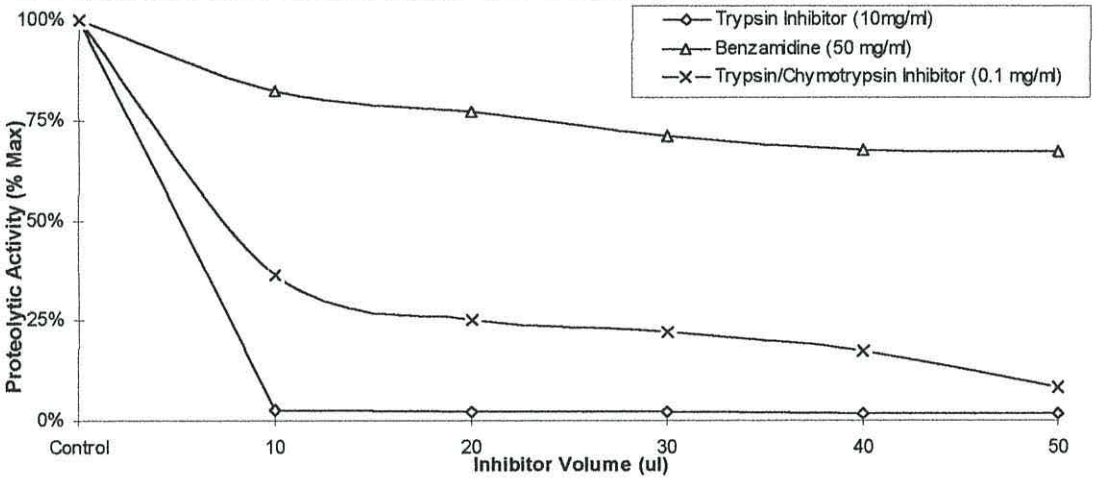
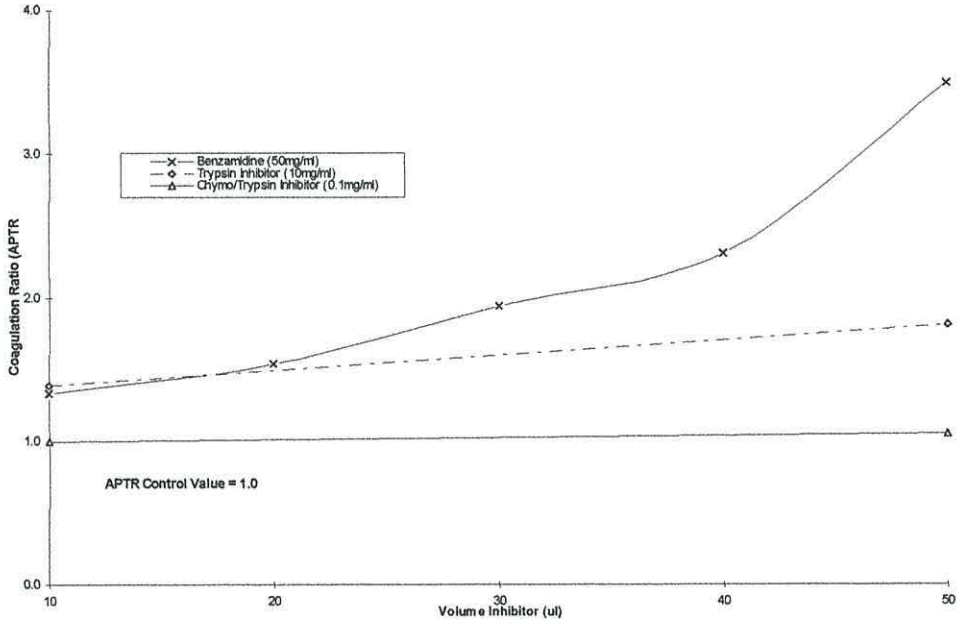


Fig 1.3.7: Effect of Proteolytic Inhibitors on 50µl of Opaque Zone (0.1 guts.µl^{-1}) Homogenate



The results demonstrate trypsin inhibitor is extremely effective against midgut proteolytic activity, and chymo/trypsin inhibitor also has a potent effect, with benzamidine showing limited inhibition.

Fig 1.3.8: Adverse Effects of Protease Inhibitors on the APTT Coagulation Assay



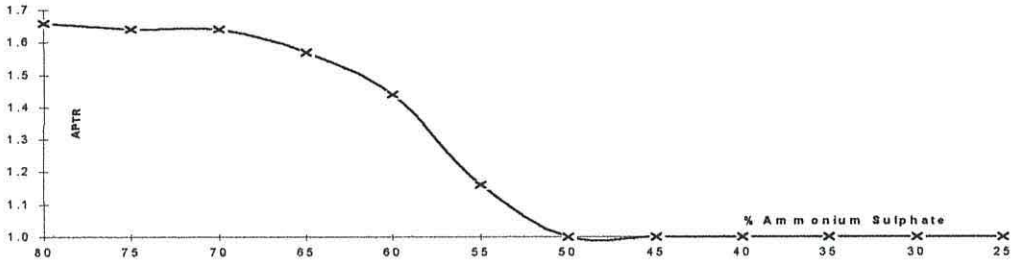
Ammonium Sulphate Protein Precipitation : Method 1 Results

The results from the preliminary sample analysis were designed to provide data so that the foundation of a purification strategy may be constructed. The principle is based upon the increasing precipitation of proteins from a gradient concentration of $(NH_4)_2SO_4$. The results of this preliminary assay are not quoted because the negative coagulation controls failed. It would appear that the coagulation assays are adversely affected in the presence of $(NH_4)_2SO_4$. Modification of the method was required to remove as much $(NH_4)_2SO_4$ as possible, to ensure coagulation assay compatibility. The initial removal strategy involved precipitation using $BaCl_2$.

Ammonium Sulphate Protein Precipitation : Method 2 ($BaCl_2$)

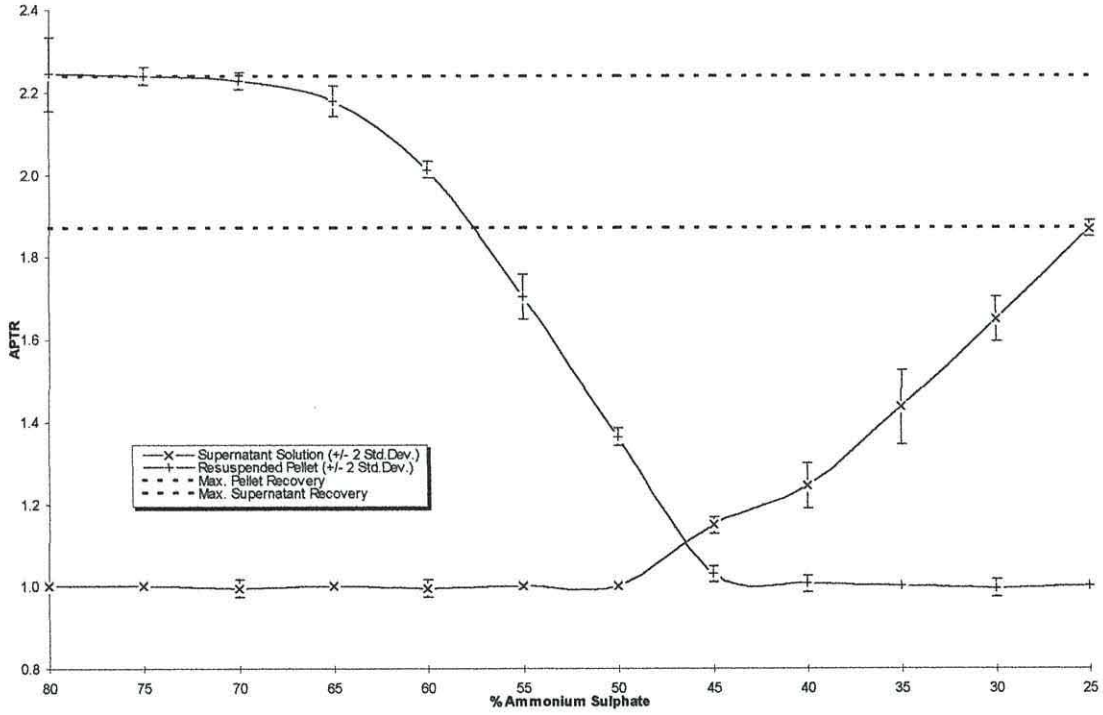
The results for this method demonstrated that reliable coagulation assay data could be obtained using the precipitated protein pellet in subsequent assays, but that the results remained unreliable due to $(NH_4)_2SO_4$ involvement with the supernatant coagulation assays, despite significant removal using $BaCl_2$. Further modifications were obviously needed, and an ultracentrifugation wash stage was added to aid in the clean-up process for the supernatant.

Fig 1.3.9: Anticoagulant Activity of Ammonium Sulphate-Precipitated Protein Pellets



The results (above) demonstrate precipitated anti-thrombin activity with varying concentration of $(NH_4)_2SO_4$ solution. The results (below) quantify the recovered activity which is obtained from the supernatant and resuspended protein ppt over a range of $(NH_4)_2SO_4$ concentrations. There remains an apparent loss in total yield using the supernatant method compared to the ppt method, as is demonstrated by the bi-level plateau's seen.

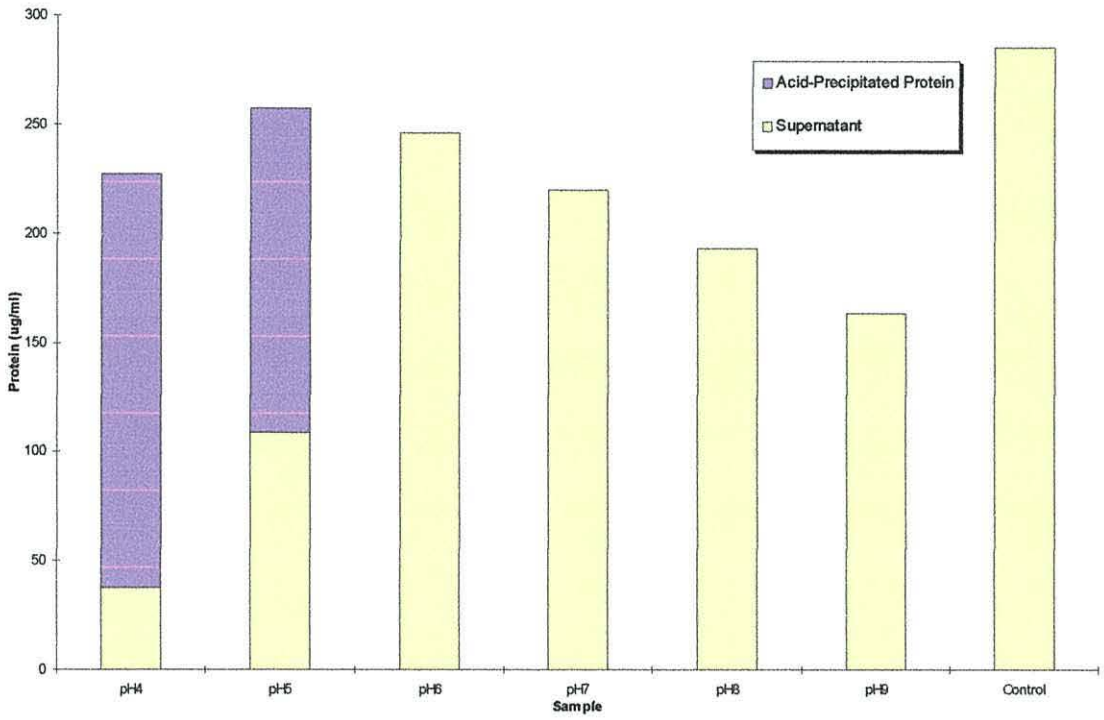
Fig 1.3.10: Coagulation Assay (APTR) Results from Ammonium Sulphate Method 3 Protein Precipitate and Supernatant Samples



Ion Exchange Chromatography Batch Method 1 Results

The control samples do not give APTT assay results within the anticipated activity range calculated in previous experiments and the results appeared to be somewhat erratic & unreliable. It was decided to carry out some preliminary investigations examining the stability of the anticoagulant in various pH solutions prior to modifying the ion-exchange methodology.

Fig 1.3.11: Total Supernatant Protein Results at Varying pH using Bio-Rad Protein Quantification Method



The results (above) demonstrate precipitation of protein with the pH 4.0 & pH 5.0 buffers, the blue segments indicating the recovered protein ppt. The specific activity values calculated for the samples are given below.

Table 1.3.14: Specific Activity and pH Stability Coagulation Assay Results

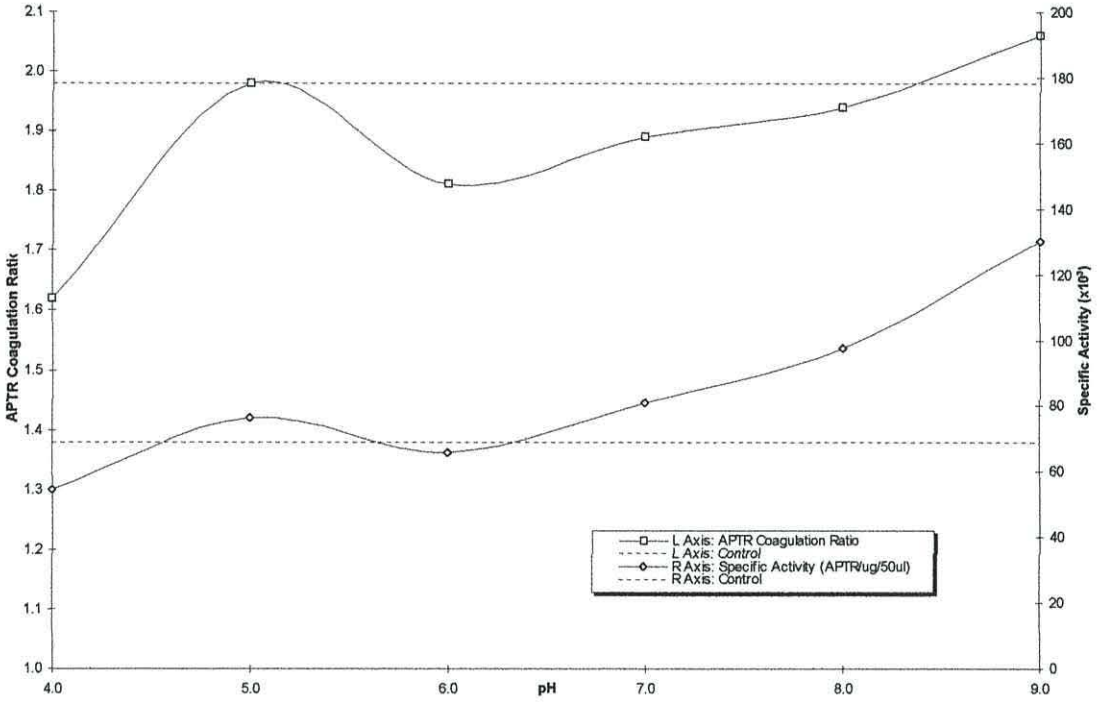
Sample Tube No.	Coagulation Results		Total Protein Assay Results		Calculated Activity Data	
	APTT (secs)	APTR (Ratio)	µg/ml	µg/assay	% Total Activity	**Specific Activity ($x10^{-3}$) ΔAPTR/µg/assay
Control	53	1.00	0	0.00	0.0	0.0
pH 4.0	64	1.21	37.6	1.88	162.4	111.7
pH 5.0	90	1.70	108.5	5.43	187.6	128.9
pH 6.0	96	1.81	246.0	12.30	95.76	65.9
pH 7.0	100	1.89	219.5	10.96	117.9	81.2
pH 8.0	103	1.94	192.9	9.65	141.7	97.4
pH 9.0	109	2.06	163.2	8.16	188.9	129.9
H_2O	105	1.98	285.0	14.25	100.0	68.8
4 ppt	75	1.42	189.7	9.49	64.4	44.3
5 ppt	68	1.28	148.7	7.44	54.8	37.6
CRA*	APTT	APTR	µg/ml	µg/assay	%	Specific Activity
pH 4.0	86	1.62	227.3	11.37	79.3	54.53
pH 5.0	105	1.98	257.2	12.86	110.8	76.21

*Calculated Recombined Activity (CRA) values were generated from the individual coagulation and protein assay values obtained for the acid-precipitate and supernatant of the pH4.0 and pH5.0 samples. The CRA corresponds to the combined assay values which would be expected if acid-precipitation of sample had not occurred, and allows a putative comparison of data with the other pH ranges tested.

**The Specific Activity of the sample is measured as the change in the Activated Partial Thromboplastin Ratio (APTR) per µg of protein per assay (50µl), and is calculated by the following equation :

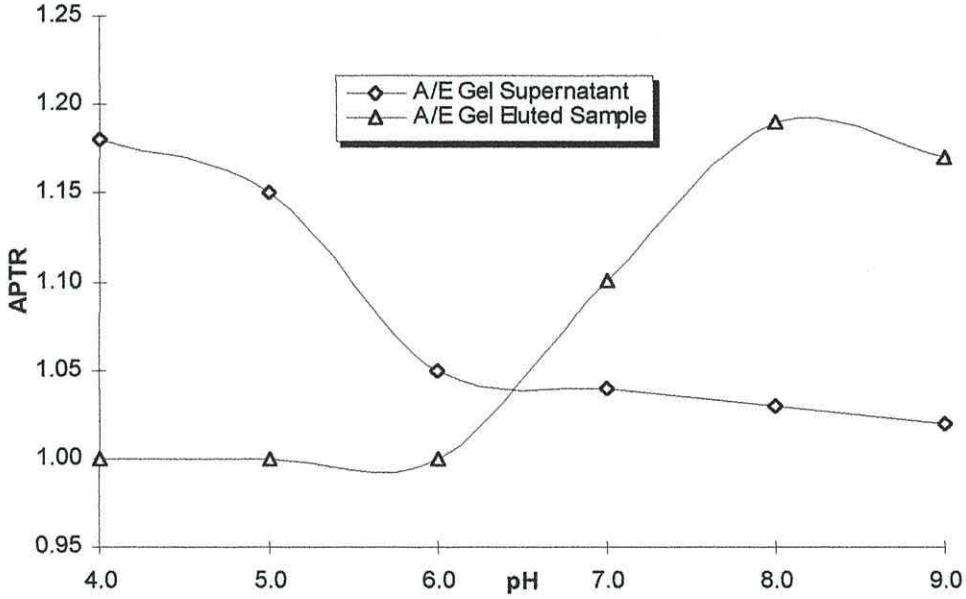
$$\Delta APTR/\mu g/assay\ (x10^{-3}) = ((APTR-1.00) / (\mu g\ Protein\ per\ assay)) \times 1000$$

Fig 1.3.12: Midgut Homogenate: APTR Coagulation Activity and Specific Anticoagulant Activity at Various pH Conditions



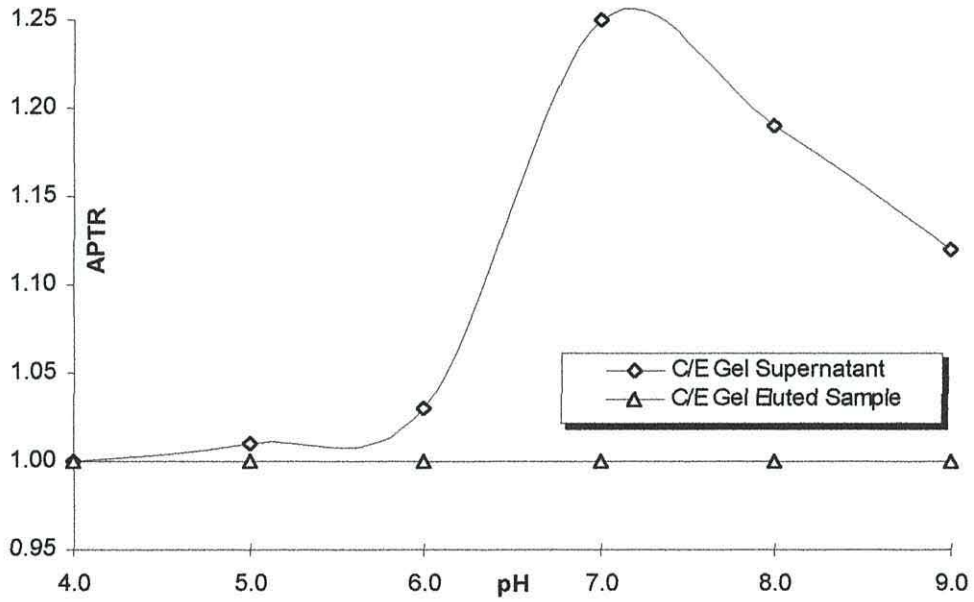
The results (above) clearly demonstrate that the anticoagulant activity and specific activity of the anti-thrombin remain closely linked, accross the whole pH range. Other than the general protein precipitation effect, which is observed at lower pH values (Fig.1.3.13), there is no evidence to suggest that the actual stability of the molecule is severely affected under these conditions. Taking into account these findings, and modifying the method accordingly, the ion-exchange chromatography investigations were repeated. The results of which are summarised :

Fig 1.3.13 - Supernatant and Eluted Anticoagulant (APTR) Activity of Anion Exchange Gel at Various pH Conditions



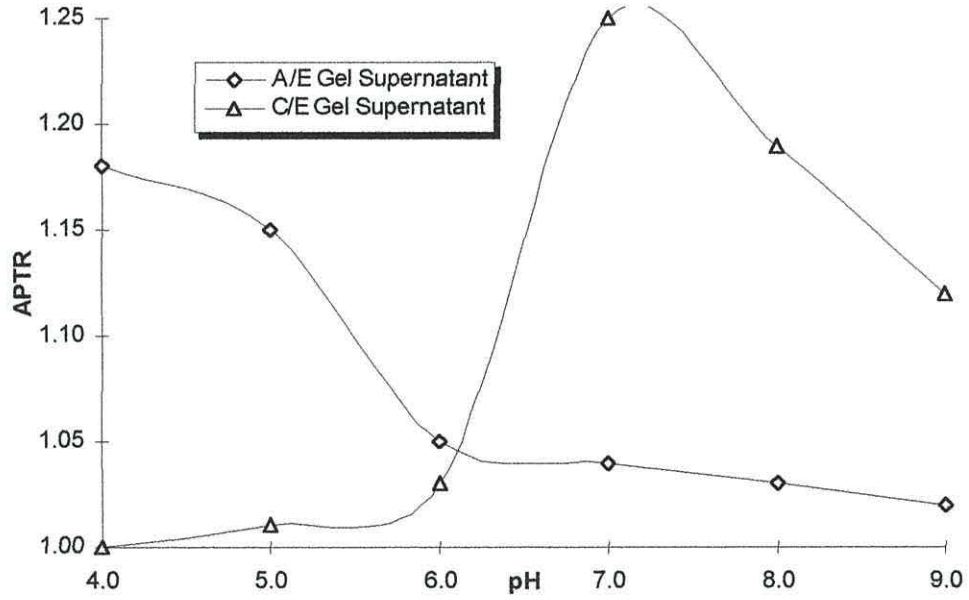
The anti-thrombin has been shown to bind anion exchange gel at ⇧pH & cation exchange gel at ⇩pH. The results demonstrate activity eluted from the anion exchange gel at high pH, and activity retained in the supernatant in the lower pH conditions.

Fig 1.3.14 - Supernatant and Eluted Anticoagulant (APTR) Activity of Cation Exchange Gel at Various pH Conditions



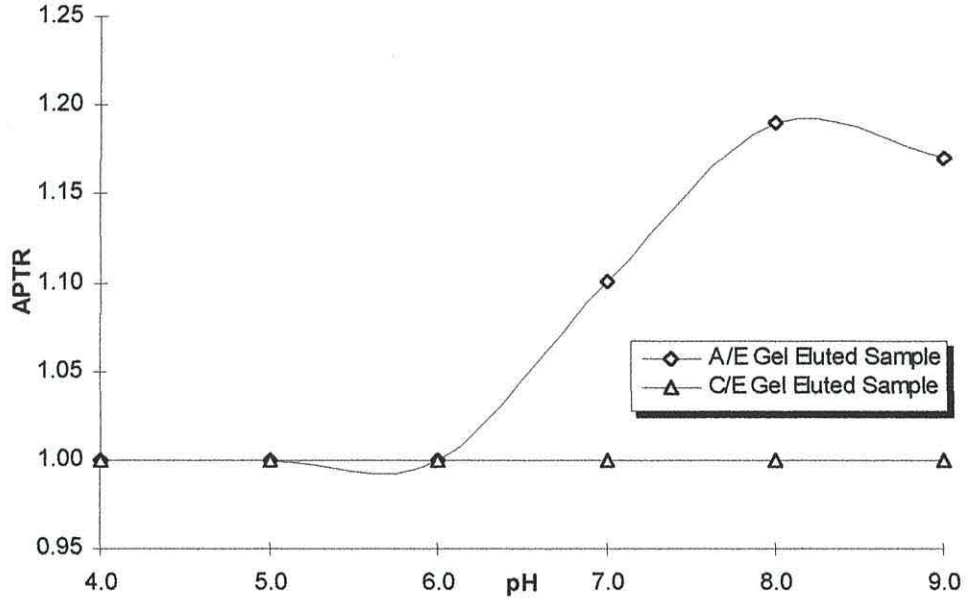
The results (above) demonstrate that the supernatant from the cation exchange gel would, as expected, show activity due to the lack of binding at this pH. Binding to the gel would have occurred at the lower pH range, but this is also the range in which precipitation has been shown to occur. Having bound to the gel, with precipitation on the surface of the gel extremely likely, the addition of higher salt conc. buffer at the same pH is not likely to coax the active back into solution and elute from the gel. Elution using a higher pH buffer with/without the extra salt would, most likely, retrieve the eluted activity in this case.

Fig 1.3.15 - Supernatant Anticoagulant (APTR) Activity of Anion and Cation Exchange Gels at Various pH Conditions



The supernatant activities would be expected to show mirror image activity of gel binding, demonstrating acvtivity with anion exchange gels at low pH, and cation exchange at higher pH. The results (above) confirm these expectations, and the slightly reduced activity seen with the lower pH supernatant is probably due to small losses from precipitation effects at these pH levels.

Fig 1.3.16 - Eluted Anticoagulant (APTR) Activity of Anion and Cation Exchange Gels at Various pH Conditions



The eluted activities relate to the binding of anti-thrombin to the gels at the particular pH. The active has been demonstrated to bind to anion exchange gel in ⇧pH conditions, as clearly demonstrated above. Similarly it would be expected to bind with cation exchange gels in ⇩pH conditions, however the precipitation effects seen at the lower pH levels, combined with the eluting conditions, meant that the eluted activity which was expected, was not recovered from these samples. The data obtained from these investigations provided the information which they set-out to do, allowing the pI value to be estimated in the pH 6.0 region, and determining that the best ion-exchange gel for use in further investigations would be anion exchange.

Table 1.3.15: Sephadex G100 Size Exclusion Chromatography Data

Sample	Collection Time (mins)			Volume Collection (ug & ul)			Coagulation Assay Results						Flow Rate
Aliquot	Start	End	Duration	Mass V+S	Mass S	Vol. S	#1	#2	#3	Mean APTT	APTR	Significant	(ml per min)
1	0.0	2.0	2.0	946.0	477.7	477.7	55	54	54	54	1.02	-	0.239
2	2.0	3.5	1.5	1061.2	592.9	592.9	55	54	55	55	1.04	-	0.395
3	3.5	4.5	1.0	871.8	403.5	403.5	96	95	95	95	1.79	Yes	0.403
4	4.5	5.5	1.0	841.6	373.3	373.3	115	115	116	115	2.17	Yes	0.373
5	5.5	6.5	1.0	867.1	398.8	398.8	100	100	100	100	1.89	Yes	0.399
6	6.5	7.5	1.0	829.2	360.9	360.9	95	94	95	95	1.79	Yes	0.361
7	7.5	8.5	1.0	857.4	389.1	389.1	61	60	60	60	1.13	Yes	0.389
8	8.5	9.5	1.0	829.2	360.9	360.9	53	52	52	52	0.98	-	0.361
9	9.5	10.5	1.0	848.4	380.1	380.1	54	54	54	54	1.02	-	0.380
10	10.5	11.5	1.0	842.5	374.2	374.2	56	56	55	56	1.06	-	0.374
11	11.5	12.5	1.0	857.9	389.6	389.6	52	52	52	52	0.98	-	0.390
12	12.5	13.5	1.0	880.1	411.8	411.8	53	52	52	52	0.98	-	0.412
13	13.5	14.5	1.0	827.1	358.8	358.8	54	54	53	54	1.02	-	0.359
14	14.5	15.5	1.0	832.4	364.1	364.1	53	53	53	53	1.00	-	0.364
15	15.5	16.5	1.0	866.7	398.4	398.4	54	54	53	54	1.02	-	0.398
16	16.5	17.5	1.0	842.8	374.5	374.5	52	52	51	52	0.98	-	0.374
17	17.5	18.5	1.0	851.5	383.2	383.2	54	54	54	54	1.02	-	0.383
18	18.5	19.5	1.0	853.1	384.8	384.8	54	53	52	53	1.00	-	0.385
19	19.5	20.5	1.0	853.5	385.2	385.2	53	53	52	53	1.00	-	0.385
20	20.5	21.5	1.0	839.7	371.4	371.4	52	52	52	52	0.98	-	0.371
21	21.5	22.5	1.0	838.0	369.7	369.7	53	54	53	53	1.00	-	0.370
22	22.5	23.5	1.0	849.4	381.1	381.1	52	52	52	52	0.98	-	0.381
23	23.5	24.5	1.0	863.6	395.3	395.3	53	53	53	53	1.00	-	0.395
24	24.5	25.5	1.0	846.0	377.7	377.7	55	54	55	55	1.04	-	0.378
25	25.5	26.5	1.0	866.9	398.6	398.6	53	53	53	53	1.00	-	0.399
26	26.5	27.5	1.0	854.1	385.8	385.8	52	53	53	53	1.00	-	0.386
27	27.5	28.5	1.0	874.6	406.3	406.3	52	52	52	52	0.98	-	0.406
28	28.5	29.5	1.0	849.3	381.0	381.0	54	53	53	53	1.00	-	0.381
29	29.5	30.5	1.0	880.0	411.7	411.7	53	53	52	53	1.00	-	0.412
30	30.5	31.5	1.0	791.8	323.5	323.5	53	52	51	52	0.98	-	0.323

The coagulation assay results from the Sephadex G100 size exclusion chromatography gel run are shown above. The data are represented graphically overleaf, together with the SDS-PAGE gels of the fractions obtained. Although in essence the fractions are still very 'dirty' and further purification is

obviously required, interestingly there are two bands which are present in all of the fractions showing anti-thrombin activity, but are absent in those which do not.

Fig 1.3.17: G100 Size Eclusion Chromatography Results: Anticoagulant (APTR) Activity from Eluted Fractions

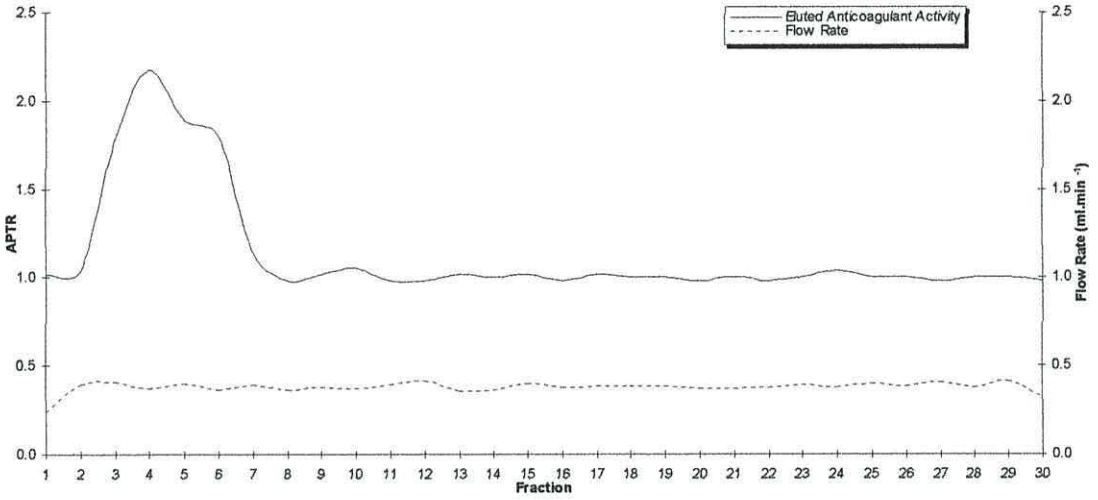
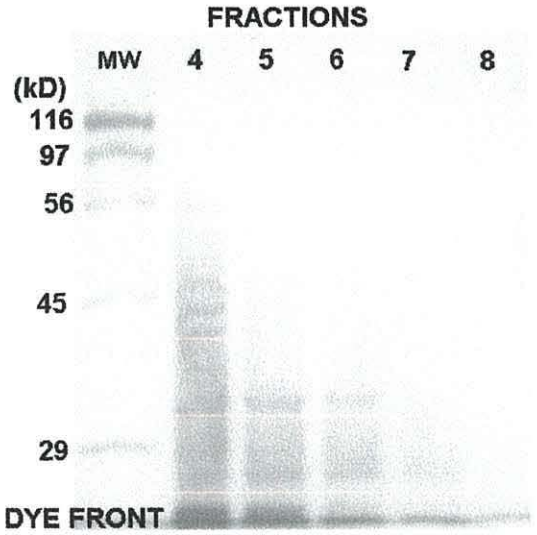


Table 1.3.16: Summary Data

100 Tubes (g)	46.83	Mean Flow Rate =	377.5	ul per minute	**Column Specifications**	
1 Tube (mg)	468.3		0.38	ml per minute	Molecular Wt. Range (kDa)	4 - 100
Norm APTT	53	"Active" Elution Duration =	5.0	mins	Length of Gel Column (mm)	55
1ml Buffer (g)	1.0	Time Until "Active" Elutes =	3.5	mins	Internal Diameter (mm)	10
Std.Dev. of	0.032	"Active" Elution Volume =	1925.4	ul	Volume of Column (mm^3)	4320
Flow Rate	8.48%	Volume Until "Active" Elutes =	1070.5	ul	Dry Bead Diameter (um)	20 - 50

Table 1.3.16 demonstrates that fractions 3, 4, 5 and 6 exhibit significant anti-thrombin activity, with the inhibition tailing-off in eluted fraction 7. Fraction 6 clearly shows the absence of virtually all other protein bands, and yet anticoagulant activity remains strong. These results strongly suggest that at least one, and probably both, of these two bands may be implicated in the anti-thrombin activity.

Fig 1.3.18: SDS-PAGE of Fractions from Sephadex G100 Size-Exclusion Column



Key

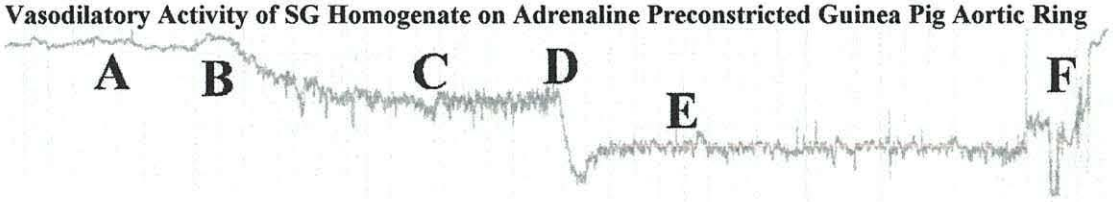
4 = Column Fraction 4
5 = Column Fraction 5
6 = Column Fraction 6
7 = Column Fraction 7
8 = Column Fraction 8

MW = Molecular Wt Markers (kDa)

All digital images are 1st-generation monochromatic computer scans, saved in the standard .JPG file format, and using a CanoScan N650U Colour Image Scanner.

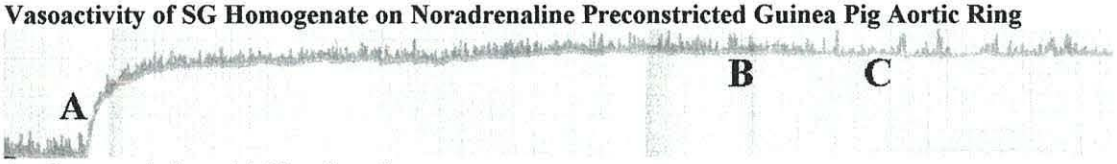
Results of The Determination and Characterisation of Vasoactive (Vasodilatory) Activity from the Salivary Glands of *Stomoxys calcitrans*

Fig 1.3.19:
Vasodilatory Activity of SG Homogenate on Adrenaline Preconstricted Guinea Pig Aortic Ring



A - Adrenaline Preconstricted Baseline
B - Salivary Gland Homogenate Introduced
C - Salivary Gland Homogenate Plateau
D - Acetyl Choline Introduced to demonstrate full vasodilation
E - Full vasodilation baseline
F - Adrenaline to demonstrate re-constriction possible

Fig 1.3.20:
Vasoactivity of SG Homogenate on Noradrenaline Preconstricted Guinea Pig Aortic Ring



A - Preconstriction with Noradrenaline
B - Plateau phase with NA - note background level of muscle twitching
C - Plateau with SG homogenate - note smoother signal, less twitchy

Summary:

- *Noradrenaline was used as the preconstrictor with the 50ml water bath, for both the endothelium* removed and endothelium intact aortic rings. No response was observed upon addition of the salivary gland homogenate.
- Noradrenaline was then used as the preconstrictor with the 20ml water bath, for both the endothelium removed and endothelium intact aortic rings. Again, no response was observed upon *addition of the salivary gland homogenate.*
- Noradrenaline was then used as the preconstrictor with the 5ml water bath, for both the endothelium removed and endothelium intact aortic rings. However, a significant relaxation of the natural smooth muscle "twitching" was observed, but this relaxation of the muscle was not sufficient to produce a 'measurable' vasodilation.
- *When phenylephrine was used as a preconstrictor, and using the 5ml water bath, the addition of* salivary gland homogenate showed no vasodilatory response.
- However, when Adrenaline was used as the preconstrictor, a small but significant vasodilation *was* measured on the addition of salivary gland homogenate. This vasodilation was observed with *both* the endothelium denuded and endothelium intact rings.

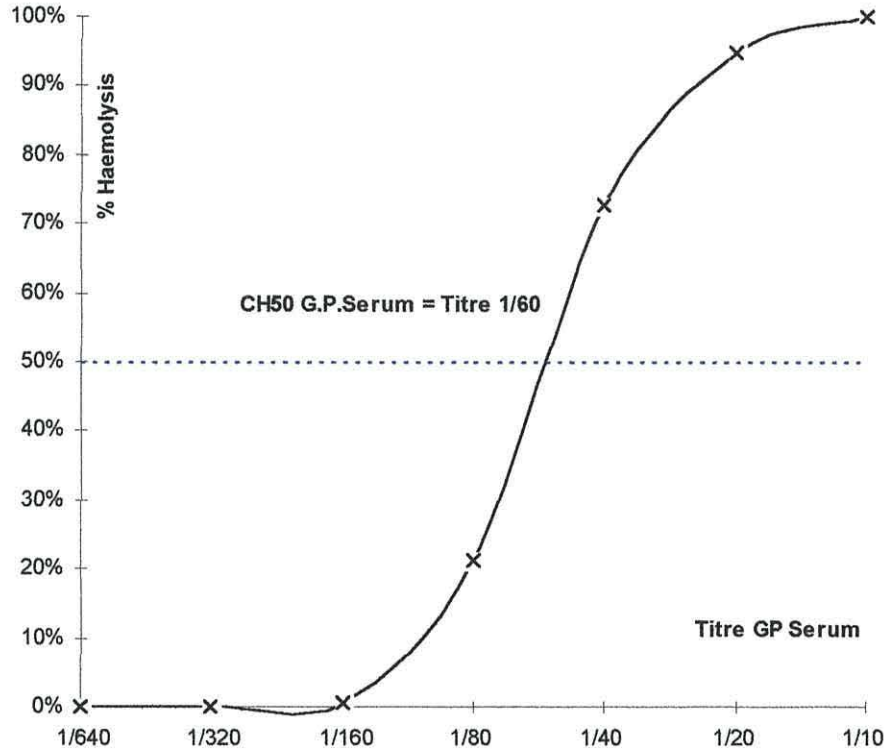
Following addition of salivary gland homogenate, the chambers were drained and washed a couple of times. Adenosine, a potent endothelium-dependent vasodilator, was then added. Only the aortic rings with intact endothelial cell linings demonstrated dilation, the denuded tissues did not. After further washings ACh, an endothelium-independent vasodilator, was added. Vasodilation was then observed in all tissue samples.

Chamber Volume	Status of Endothelium	Vasoactive Drugs Used			
		Noradrenaline	Phenylephrine	Adrenaline	Adenosine / ACh
50ml	Intact	-	-	-	+ / +
	Denuded	-	-	-	- / +
20ml	Intact	-	-	-	+ / +
	Denuded	-	-	-	- / +
5ml	Intact	*	-	+	+ / +
	Denuded	*	-	+	- / +

* Relaxation of smooth muscle twitching, although no vasodilation was observed.

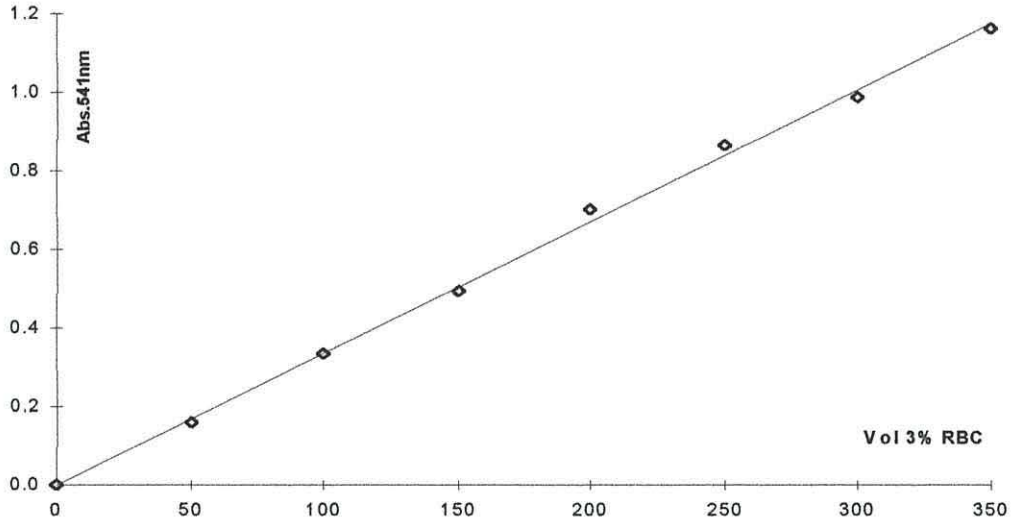
Results from the Determination and Characterisation of Anti-Complement Activity from the Salivary Glands & Midgut of *Stomoxys calcitrans*

Fig 1.3.21: CH_{50} of GuineaPig Serum Used



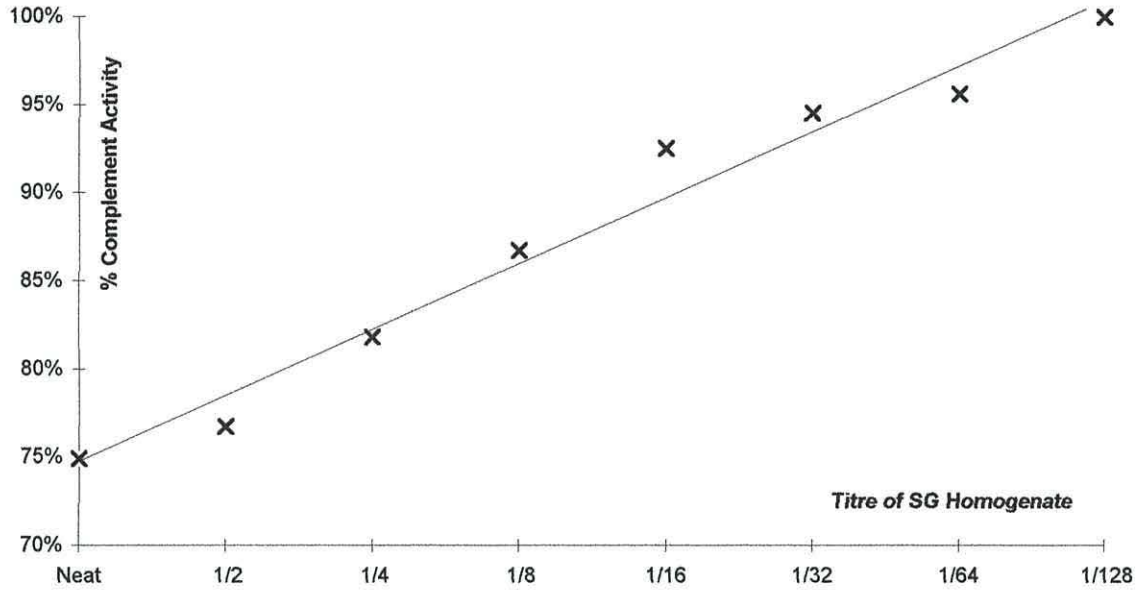
A CH_{50} assay was performed on the Guinea Pig Serum before any further testing was planned as a control, to ensure adequate complement activity was present, and to help determine a dilution which would be suitable for subsequent assays. The CH_{50} value was determined at a dilution of 1/60, indicating sufficient complement activity.

Fig 1.3.22: Erythrocyte Lysis Calibration Curve



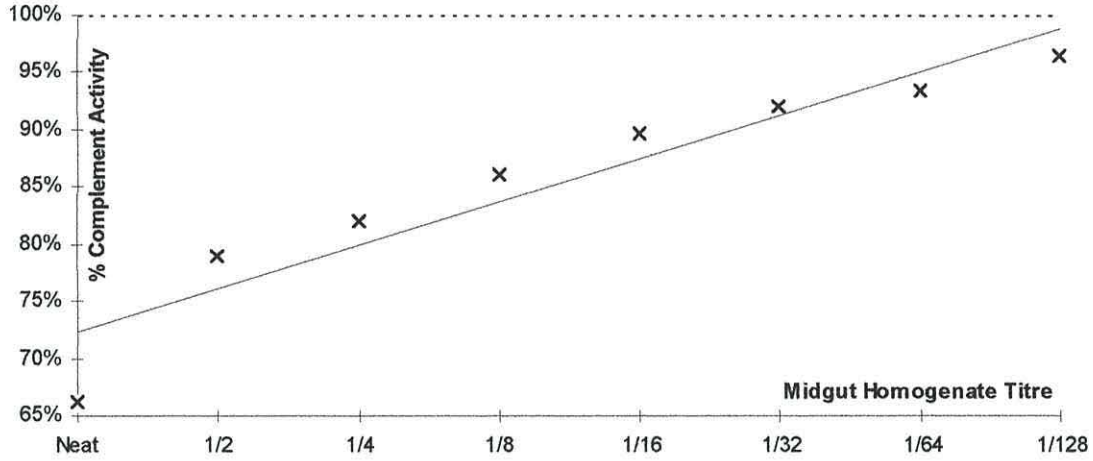
Following-on from the CH_{50} assay it was necessary to select a suitable volume of sensitised erythrocytes, a volume which would give maximal absorption at 541nm with 100% lysis. The graph (above) measures the absorbance of varying volumes of erythrocytes (3%), and from this a volume of 300µl was selected as giving a full scale absorbance with 100% lysis.

Fig 1.3.23: Salivary Gland Anti-Complement Activity



The graph (above) demonstrates anti-complement activity from salivary gland homogenate of the bloodsucking fly *Stomoxys calcitrans*. Neat homogenate gave maximal inhibition of 25% total observed complement activity, as measured by haemolysis (free haemoglobin at 541nm).

Fig 1.3.24: Midgut (Reservoir Zone) Anti-Complement Activity



The graph (above) demonstrates anti-complement activity from midgut reservoir zone homogenate of the bloodsucking fly *Stomoxys calcitrans*. Neat homogenate gave maximal inhibition of 33.8% total observed complement activity, as measured by haemolysis (free haemoglobin at 541nm).

Control reactions, involving heat-inactivated guinea pig serum (no complement activity), heat-inactivated homogenate samples (no inhibitory activity), and un-sensitised erythrocytes (no haemolysis), all gave satisfactory results. It is clear there is definately some form of complement inhibition demonstrated from both the salivary glands and midgut reservoir zone samples. What remains to be seen is whether there is some degree of cross-reactivity with the FXa / thrombin inhibitors, which are known to be present, or whether this inhibitory activity is specific for complement. Regardless of the outcome, this is the first conclusive demonstration of anti-complement activity observed in any bloodsucking animal known to-date, and as-such it is a milestone in antihaemostatic research.

1.4 Discussion

Preliminary investigations involving the modification of the manual APTT coagulation assay produced a tool, which could be used to demonstrate and monitor the activity of salivary and midgut-derived anticoagulant molecules, from the bloodsucking fly *Stomoxys calcitrans*. Further investigations revealed the mechanism by which these anticoagulants exerted their effect.

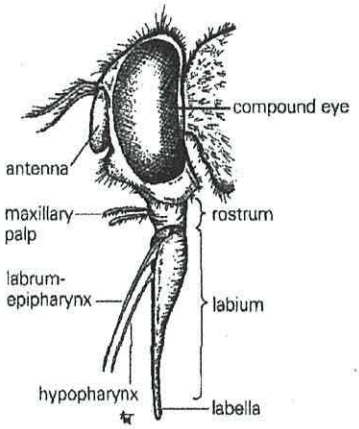
A salivary FXa inhibitor was demonstrated, together with a potent thrombin inhibitor from the reservoir zone of the insect midgut. Both act in the final (common) pathway of the blood coagulation cascade, preventing the cleavage of soluble fibrinogen to form a meshwork of covalently bound fibrin strands. These stabilise and consolidate the primary platelet plug, forming the main blood clot at the site of injury.[104]

In addition to the salivary FXa inhibitor, a potent salivary vasodilator was demonstrated, causing significant vasodilation of a fresh endothelium denuded adrenaline-preconstricted guinea pig aortic ring. Potent anti-complement activities were also detected in the salivary glands and midgut reservoir zone regions, measured using goat-anti-rabbit sensitised rabbit erythrocytes with fresh guinea pig serum as the source of complement. The implications of these activities for the process of haematophagy are extremely important.

S.calcitrans was demonstrated to have a potent FXa inhibitor in the salivary glands. This is perhaps not suprising, given that over 80% of FXa inhibitors from blood-sucking animals[2] are found in the salivary glands, and over 82% of all documented[2] salivary gland anticoagulant activities are directed at either FXa or thrombin.

usually take their bloodmeal either directly from a blood vessel, from a haematoma formed beneath the surface of the skin, or from a pool which forms on the surface. *Stomoxys calcitrans* has been shown to adopt the latter strategy,[73] and has a painful bite, which makes it even more important to make a quick and successful feed.

Fig 1.4.1: Mouthparts of S.calcitrans



Pool-feeding flies, such as *S.calcitrans*, do not pierce the skin in the same way as other bloodsucking diptera. Instead of using the maxillae and/or mandibles, they use specially adapted labium.[73] At the distal end of the labium are found 2 labellar lobes, these are everted during feeding to expose highly sclerotised teeth, which are thought to anchor the mouthparts in the tissues. They achieve a cutting motion with rapid & repeated motion of the labium in an upwards and outwards direction, forcing the tip of the labium deeper into the epidermis.[126] The effect of this action on the host vasculature and haemostatic response is significant.

The damage to extravascular tissue would cause the release of significant quantities of compartmentalised tissue factor.[104] In addition to this, subendothelial collagen layers would be exposed, these in addition to the foreign surfaces introduced to the wound in the form of mouthparts, would rapidly initiate the host haemostatic and immune responses.[104]

The release of tissue factor would cause the formation of the TF:VII complex, causing the increased activation of FVII, to produce FVIIa. The TF:VIIa complex would then

Additional FXa would be produced as FXII activates on the negatively charged surface of the exposed collagen layers. The FXIIa produced would cause the activation of FXI, which in-turn would activate more FIX, and then FXa as before. Factor Xa is then the pivotal molecule in the cleavage of prothrombin to generate thrombin,[104] it is therefore little suprise that many salivary-derived anticoagulants are FXa inhibitors.

Inhibiting the production of thrombin also prevents the thrombin-mediated activation of FXI, which would trigger a massive feedback amplification of the whole coagulation cascade. In addition thrombin is responsible for the direct activation of FXIII, and has been implicated in platelet activation.[104] All of these actions, if allowed to progress un-inhibited, would lead to rapid formation of blood clots at the site of the wound, around the mouthparts, in the cibarial pump, and in the digestive system of the insect. These clots would not only prevent further feeding at the site of the wound, but would pose a major obstacle in obtaining subsequent bloodmeals.[73]

The presence of a midgut-derived anticoagulant molecule would enhance the protective inhibition afforded by the salivary anti-FXa. As the anticoagulated bloodmeal is pumped through the mouthparts and proventriculus, into the reservoir zone, it encounters many foreign surfaces capable of causing prolonged contact activation of the coagulation and complement systems.[113,114,118]

Digestion of the bloodmeal can be achieved using either a 'batch' or 'continuous' system,[73] whereby digestion of the whole bloodmeal begins instantaneously, or it is stored and small aliquots are passed into the digestive system continually over a

bloodmeal may be stored in the reservoir zone for up to 6 hours prior to commencement of digestion. The initial anticoagulation, provided by the salivary FXa inhibitor, is unlikely to cope with the prolonged procoagulant conditions posed by the glycocalyx,[113,114] and peritrophic matrix,[118] especially over this time-frame. The fact that the bloodmeal remains intact, and in a fluid (anticoagulated) state during this time, strongly suggested a *secondary* inhibitory activity. The requirement for this '*secondary inhibition*' has never been described in detail, before now. It would appear that the midgut derived anticoagulants have been seen as a curiosity, which have traditionally taken second-place to the activities of the salivary glands.

The midgut-derived anticoagulant, described for the first time in this text, is derived from the reservoir zone of *Stomoxys calcitrans* and shows potent anti-thrombin activity. The putative structure comprises components 26kDa and 34kDa in size, with a combined (active) pI value of 6.0. The inhibitor demonstrates sensitivity to temperatures above 40°C, and its location - adjacent to the highly proteolytic opaque zone - posed many problems during the purification process.

The inhibition of thrombin, as the bloodmeal reaches the reservoir zone, ensures that any procoagulant activity of the midgut structures which may overcome the salivary FXa inhibitor, are adequately dealt with. The rationale for using an anti-thrombin at this stage, and not additional FXa inhibitors, is simple. Once the FXa threshold is overcome, and the cascade is able to activate even a few thrombin molecules, the positive feedback amplification loop kicks-in. Those few activated thrombin molecules could cause the activation of FXI, and (until now) the relatively inactive FV and FVIII molecules.[104] Extra FXa inhibitors at this stage would be of some help,

but it would not deal adequately with the developing response. The only effective strategy, once thrombin activation had occurred, would be anti-thrombin.

It is no suprise therefore that the midgut-derived anticoagulant response of *S.calcitrans* is shown to be an anti-thrombin. This is not an isolated case, indeed far from it. The fact that **every** gut-derived anticoagulant,[14,15,17,20] from all the haematophagous animals which have been described in the literature[2] are thrombin inhibitors, provides very strong support for the *secondary inhibition* hypothesis.

In addition to these observations, and in the case of *S.calcitrans*, there may be another aspect which must not be overlooked. The putative structure of the midgut-derived anticoagulant, comprising 2-subunits of 26kDa and 34kDa, whilst as-of-yet unproved, suggests the possibility of another, more subtle interaction.

The requirement for *secondary inhibition* has been demonstrated, and the most effective way this can be accomplished has been shown to be via anti-thrombin. Thrombin, by its nature, is an extremely active serine protease,[104] and any effective inhibitor would need to be equally potent. However, this very potent inhibition, directed against an extremely active serine protease enzyme, could conceivably have consequences for the digestion process further along the midgut.

The major digestive enzyme utilised by *S.calcitrans* has been demonstrated to have trypsin-like activity,[103] and trypsin is a known serine protease. Thrombin is also a potent serine protease.[104] Therefore the potential exists that, whilst the anti-thrombin may protect against bloodmeal coagulation in the reservoir zone, it may also interfere

protein in the bloodmeal, and the extreme danger that the acquisition of this bloodmeal involved, would imply that any digestive process would be configured to optimal efficiency. The protein resources, required for egg production, could not be squandered due to ineffective digestion.

It is possible that the opaque zone produces and secretes digestive enzymes far in excess of that which could be inhibited by the anti-thrombin activity. The fact that ultrastructurally this zone is packed with vesicles containing the digestive enzyme[73] may support this. However this would also appear to be a significant waste of resources if another solution could be found, one which involved selective activities and compartmentalisation.

One solution to this problem, and a very effective one, would be to produce a thrombin inhibitor which was significantly larger than anything else known so-far. For example a protein complex comprising 26kDa and 34kDa components. The large size of the inhibitor would make it particularly susceptible to proteolytic degradation (as was found to be the case in our purification regime). Once it enters the opaque zone, the high proteolytic activity would destroy the delicate tertiary and quarternary structure of the protein, and hence any cross-reactivity it may have against the serine proteases of the digestive system. The remnants of the anti-thrombin molecule(s) could then be digested, along with the mass of other proteins from the bloodmeal, conserving the fly's valuable stores of protein which are required for reproduction.

Although anticoagulant activities comprising 2-subunit protein complexes are already known in mammalian haemostasis,[104] (and are coincidentally linked with the actions

anithaemostatic systems, which has never before been described in the literature. It would also explain the many difficulties which were encountered during the purification process. These observations, whilst unable to provide unequivocal evidence for the existence of a 2-subunit thrombin inhibitor, from the midgut of *S.calcitrans*, do provide sufficient data to submit the hypothesis for future investigations. Scientific progress is, afterall, a journey of small foot steps.

However, anticoagulation is not the only requirement to obtaining a successful bloodmeal, and is not the only obstacle faced by haematophagous animals. Platelet inhibition and vasodilation are activities which are also commonly demonstrated in the saliva of haematophagous animals.[2] Preliminary (unpublished) data from this study, examining platelet aggregation responses to various activators in the presence of salivary gland homogenate from *S.calcitrans*, would suggest the presence of a salivary apyrase activity. The rupture of erythrocytes at the site of the wound, caused by shear stresses and complement activation, would release large quantities of intracellular ADP.[104] Increased levels of ADP are a potent activator of platelet aggregation and degranulation, forming an intitial platelet 'plug' and contributing to the overall haemostatic response. The apyrase enzyme is responsible for the removal of an inorganic phosphate group, hence ADP ⇨ AMP + Pi, ensuring that ADP-mediated platelet aggregation is effectively inhibited.[73]

Vasodilatory activity from the saliva of *S.calcitrans* has also been demonstrated for the first time in this text, a fact which is of little suprise given that a wide range of salivary vasodilators have been observed in haematophagous arthropods.[4]

Vasodilatory substances vary widely in their mode of action, having been identified as prostaglandin-like molecules in ticks, nitric oxide-binding proteins in triatomine bugs, and novel vasoactive peptides in sandflies and mosquitoes.[4] It was therefore a logical progression to investigate whether *S.calcitrans* also exhibited similar activities.

The apparatus used to make these investigations had originally been designed for vasoactive drug studies, and as-such it needed some small modifications.

The results demonstrate that, using noradrenaline as preconstrictor, the spontaneous smooth muscle 'twitching' which was observed prior to addition, was seen to be reduced significantly in the presence of salivary gland homogenate. The addition of salivary gland homogenate to the endothelium-intact and endothelium-denuded aortic rings, preconstricted with adrenaline, showed significant vasodilation in both cases. The fact that both the endothelium-intact and the denuded aortic rings showed vasodilation upon the addition of salivary gland homogenate demonstrates that the salivary vasodilator of *Stomoxys calcitrans* has an endothelium-independant mode of action. Therefore the mechanism of action cannot be based upon the nitrous oxide (NO) pathway, as is common in some haematophagous insects,[36,37,38] because NO-mediated vasodilation is endothelium-dependent.[103]

The fact that there was no measurable vasodilatory response to salivary homogenate with either the noradrenaline, or the phenylephrine preconstricted aortic rings, but there *was* a response to the adrenaline preconstricted aorta, is a significant observation in itself. All of the preconstrictor drugs used in this experiment are members of a group of drugs known as the direct-acting sympathomimetics.[110] In the host animal, adrenaline will activate the β_2-receptors causing relaxation of smooth muscle (hence increased blood flow) in the liver and gastro-intestinal systems.

muscle in arterioles supplying the skin, mucosal layers, kidneys and large vein capillary beds.[110]

The ability of adrenaline to shutdown the flow of blood to the capillary beds of the skin is a significant cause of concern for any blood feeding animal. When the host animal is stressed the adrenal glands will be pumping out adrenaline, a condition known as the *fight or flight response*. In the case of *Stomoxys calcitrans*, which has a very painful bite and is known to feed on cattle - sometimes in very large numbers - the effect of stress on the cattle would be quite significant.[65] In the absence of studies measuring adrenaline levels in cattle during such encounters, it would be safe to assume that adrenaline levels are high under such circumstances. Given that one of the effects of adrenaline is to shutdown the blood supply in the peripheral circulation, i.e. the skin,[110] the effect of this on blood feeding would be catastrophic. Any haematophage able to evolve a mechanism to inhibit this adrenaline-mediated vasoconstriction would be at a distinct advantage, and the natural route of delivery for any vasodilatory substance to aid in the process of haematophagy would be saliva.

The discovery of this novel salivary vasodilator, capable of relaxing blood vessels constricted by the actions of adrenaline, provides a strong evolutionary advantage to *S.calcitrans*. The implications of which are profoundly important to the process of haematophagy, and the fast & successful acquisition of a bloodmeal.

Last, but by no means least, was the discovery of anti-complement activity in the salivary glands and midgut reservoir zone of *S.calcitrans*. The Complement System

generate products with distinct inflammatory, opsonic, lytic and immunoregulatory activity.[104] A summary of the components and activation pathways are demonstrated:

Table 1.4.1: Components & Regulatory Proteins of the Complement System:

Protein Component	Serum Conc. (μg/ml)	Structural Motifs
Activation Pathways		
C1q	100	Collagen-type helix
C1r	50	Serine protease
C1s	40	Serine protease
C4	640	Internal thioester
C2	25	Serine protease
C3	1,200	Internal thioester
Factor B	200	Serine protease
Factor D	1	Serine protease
Factor I	35	Serine protease
Membrane Attack Complex		
C5	70	Homologous to C3, C4
C6	65	Pore-forming protein
C7	55	Pore-forming protein
C8	55	Pore-forming protein
C9	60	Pore-forming protein
Plasma Regulatory Proteins		
C1 Inhibitor	200	Serpin-type Inhibitor
Properdin	25	-
C4b-Binding Protein	250	Short consensus repeat
Factor H	500	Short consensus repeat
S-Protein (vitronectin)	500	Adhesive protein (RGD sequence)
Membrane Regulatory Proteins		
Homologous Restriction Factor (CD59)		GPI anchored
DAF (CD55)	n/a	GPI anchored
Membrane Co-Factor Protein (CD46)	n/a	Short consensus repeat
Membrane Receptors		
CR1 (CD35)	n/a	Short consensus repeat
CR2 (CD21)	n/a	Short consensus repeat
CR3 (CD11b/CD18)	n/a	Leucocyte β-2 Integrin
CR4 (CD11c/CD18)	n/a	Leucocyte β-2 Integrin
C1q Receptor	n/a	-
C3a Receptor	n/a	G-Protein coupling
C5a Receptor	n/a	G-Protein coupling

Adapted from Hoffman *et al.* Haematology, Principles & Practice. 3rd Ed. (2000)[104]

Complement is recognised as playing an increasingly important role in the pathology of disease states, and inflammation.[104] The pharmaceutical potential for a potent complement inhibitor is enormous, and this research provides the first documented evidence demonstrating complement inhibition from any haematophage. Saburo Minami *et al* (1998) make the observation that Chitin, a significant component of the peritrophic matrix, is capable of activating the Complement pathway.[118] In particular the Complement components C3 and C5. It is also a well known fact that the degradation products of Complement components C3 and C5 act as potent

anaphylotoxins and chemotactic agents, and play a significant role in the inflammatory response. (see table below)

Table 1.4.2: Specificity & Cellular Distribution of Major Complement Receptors:

Receptor	Ligand Specificity	Cellular Distribution (Humans)
C1q R	C1q (collagen)	Platelets, PMN, B-lymphs, monocytes, fibroblasts & endothelial cells
C3a R	C3a (C4a)	Mast cells, basophils, eosinophils, PMN, monocytes & many tissues
C5a R	C5a	Mast cells, basophils, eosinophils, PMN, monocytes & many tissues
CR1 (CD35)	C3b (C4b, iC3b, C3c)	Erythrocytes, B-cells, some T-cells, PMN, eosinophils, macrophages & FDCs (follicular dendritic cells)
CR2 (CD21)	C3d, C3dg (iC3b)	B-cells, T-cells & FDCs
CR3 (CD11b/CD18)	iC3b (C3d)	PMN, eosinophils, macrophages, K & (CD11b/CD18) NK Cells, FDCs
CR4 (CD11c/CD18)	iC3b	PMN, macrophages, monocytes, K & (CD11c/CD18) NK Cells

Adapted from Hoffman *et al.* Haematology, Principles & Practice. 3rd Ed. (2000)[104]

The advantage to the haematophagous insect of inhibiting the inflammatory response is an obvious one, particularly when you consider that the Complement response is very fast acting, taking just a few seconds, and would be one of the first lines of attack against any foreign body encountered by the host.[104] This advantage would be augmented by the additional anti-inflammatory and anticoagulant effect of the FXa inhibitor, which was also demonstrated in the saliva of *S.calcitrans*.

The implications of uncontrolled Complement activation within the midgut are intriguing, but the most likely outcome would be damage to the endothelial cell lining of the insect midgut. The fact that these cells require a structure such as the peritrophic matrix to protect them from bacterial contact, and/or general abrasion, would suggest they are particularly delicate in nature.[73] The membrane attack complex formed by the activated Complement components could potentially do a lot of damage to the endothelial cell lining, especially considering these membranes are the site of an extraordinary amount of activity during, and in the periods after, the ingestion of a bloodmeal.[73] *S.calcitrans* females have been estimated to ingest bloodmeals upto 110% of their unfed body weight, 80% of which is comprised of

water,[73] and this water must be rapidly and efficiently removed. Water movement accross the midgut lining of *S.calcitrans* is linked to an oubain-sensitive Na^+/K^+ATPase. This extremely efficient pump works by generating an osmotic gradient accross the epithelium, which water passively follows.[73] It must also be remembered there is a significant Na^+/K^+ gradient across the membrane of the erythrocytes, maintained by *Na^+/K^+ ATPase* ion-pumps involving active transport across the red cell membrane.[104] The effect of this is to maintain higher K^+ and lower Na^+ levels within the erythrocyte, compared to relatively higher Na^+ and lower K^+ in the plasma.[97]

The early lysis of erythrocytes in the bloodmeal, and the consequent release of large volumes of concentrated K^+ and proteins (mainly haemoglobin), would suddenly and significantly disrupt the osmotic balance of the bloodmeal. It is highly likely that this disruption would have a detrimental effect on the transport of water out of the midgut, placing an additional burden on the transport pumps, and is another very good reason to inhibit the actions of complement in the reservoir zone.

The fact that conditions exist in the midgut which would promote the activation of complement, and yet erythrocytes in the bloodmeal are observed to remain intact in these regions, strongly indicates the presence of complement inhibition. Also, the demonstration of complement inhibition in saliva, preventing the release of inflammatory mediators at the site of the wound, would be a compelling advantage.

These observations, combined with data demonstrating the presence of complement inhibition from salivary/midgut tissue homogenate, provides support for the hypothesis that protection against the lytic activities of Complement confers a distinct advantage to haematophagous insects, such as *S.calcitrans*.

Chapter 2

Construction and Immuno Screening of *S.calcitrans* Salivary Gland cDNA Library

2.1 Introduction

2.2 Materials and Methods

Salivary gland preparation and mRNA extraction

cDNA library construction

Production and confirmation of polyclonal antibodies in a rabbit, raised against secreted salivary antigens from *S.calcitrans*.

Purification of IgG antibodies from polyclonal rabbit serum.

Determination of *E.coli* background cross-reactivity in the IgG Serum.

Absorption of *E.coli* reactive immunoglobulins from the IgG fraction of polyclonal rabbit serum.

Screening of *S.calcitrans* salivary gland cDNA library using the double-absorbed IgG fraction from the polyclonal fly-fed-rabbit serum.

Determination of polyclonal serum and purified IgG activity against periodinated salivary gland homogenate.

2.3 Results

2.4 Discussion

2.1 Introduction

Having been unsuccessful in isolating the purified protein to a level where sequence information could be obtained, it was decided to tackle the problem from the other end of the spectrum. Instead of trying to isolate the protein of interest, i.e. the end-product, it may be possible instead to isolate the gene which codes for that protein. Although the information required to produce all the structural proteins and enzymes an organism needs, is stored within the genetic code of its DNA, the genomic DNA also contains a lot of junk code, long terminal repeats and spacer sequences, with no obvious translatable function. To translate the genomic DNA code into a protein, the relevant sections of genetic code from the genomic DNA, the gene, is copied in the form of messenger RNA (mRNA). The mRNA travels out of the nucleus, and carries with it all the information it needs to produce one particular protein. There may be hundreds of thousands of proteins required by the cell, being translated at any one moment in time, and hence a vast array of different mRNA species. A representation of all these different mRNA species, amplified in numbers and present in a stable form suitable for long term storage, is known as a cDNA library. The cDNA library represents a database which can be searched for molecules of interest, and the usefulness of this library is only as good as the methods used to search it.

Aims and Objectives

This chapter summarises the work involved in constructing a salivary gland cDNA library, and the methods which were employed to search it for potential antihaemostatic peptides, to obtain genetic sequence information for these molecules.

2.2 Materials and Methods

Salivary gland preparation and mRNA extraction

Salivary gland samples were dissected and prepared as outlined in **appendix (b)**, using DEPC-treated and sterilised dissection media, tweezers, slides and and reagents to minimise any RNAse activity. Total RNA extraction was carried out using the Chomsynski method, **appendix (i)**, and extracting the Poly(A)$^+$ mRNA from the total RNA sample using the Dynabeads™ magnetic particle method.
Following the Dynabeads™ extraction, there were some Li^+ salts still present in the mRNA preparation. MMLV-RT is sensitive to Li^+ salts, so to remove these and clean-up the mRNA sample, 5 volumes of 100% ethanol were added, quickly vortexed, and immediately placed in a -80°C freezer and allowed to precipitate for 5 mins. The tube was spun hard & fast at 20,000g in a refridgerated (4°C) centrifuge for 5 mins, and the supernatant carefully removed and discarded. The mRNA pellet was then placed immediately on ice, resuspended in 20µl of chilled sterile DEPC-treated water, and used immediately for the first strand synthesis reaction. The total mRNA extraction, and the subsequent Poly(A)$^+$ mRNA purification and clean-up from the total RNA, were all carried out in a cold room maintained at 4°C and using prechilled reagents and equipment.

cDNA Library - First Strand Synthesis

In an RNAse-free microcentrifuge tube, on ice, the following were added in order :

5 µl of 10x first strand buffer*
3 µl of first strand methly nucleotide mixture
2 µl of linker-primer (1.4µg/µl)
17.5 µl of DEPC-treated water
1 µl of RNAse Block Ribonuclease Inhibitor (40U/µl)

* 0.75M KCl, 0.03M MgCl, 0.5M Tris pH 8.3

The reaction was vortex mixed, and 20µl of the prepared Poly(A)$^+$RNA were added, and the primer was allowed to anneal for 10 minutes at room temperature.

1.5µl of Molony Murine Leukaemia Virus Reverse Transcriptase (MMLV-RT at 50U/µl) were added to the first strand reaction mix, taking the final reaction volume to 50µl. The sample was then mixed gently and quickly spun down in a centrifuge.

5µl of this mixture were then removed, and added to 0.5µl [α-^{32}P]dNTP (800 Ci/mmol) in a separate tube, as a control.

Both control and synthesis reactions were then incubated at 37°C for 1 hour.

Immediately following incubation, the control tube was stored at -20°C, and the products of the first strand synthesis reaction were placed on ice, ready for second strand synthesis.

cDNA Library - Second Strand Synthesis

Keeping on ice at all times, the following reagents were added to the tube containing the 45µl non-radioactive first strand synthesis reaction products :

20 µl of 10x second strand buffer
6 µl of second strand dNTP mixture
114 µl of sterile distilled water
2 µl of [α-^{32}P]dNTP (800 Ci/mmol)

Then the following enzymes were added :

2 µl of RNAse H (1.5 U/µl)
11 µl of DNA Polymerase I (9.0 U/µl)

The contents of the tube were then gently vortex-mixed, spun-down, and returned to ice for 5 mins. The tube was then transferred to a pre-chilled thermal cycler, and incubated at 16°C for 2.5 hours, then returned immediately to ice.

The cDNA termini were then blunted, by adding :

23µl of blunting dNTP mix
2µl of cloned Pfu DNA Polymerase (2.5 U/µl)

The mixture was quickly vortex-mixed, spun-down, and incubated in a thermal cycler at 72°C for 30 minutes, followed immediately by a 4°C hold. The tube was immediately put on ice, and 200µl of phenol-chloroform [1:1(v/v)] were added. The contents were vigorously vortex mixed.

The tube was spun in a Centaur centrifuge at maximum (13,000rpm) for 2 minutes at room temperature, and the upper (aqueous) layer containing the cDNA was transferred to a new tube. An equal volume of Chloroform was added, vortex-mixed, and centrifuged as before transferring the top aqueous phase to another new tube.

To the aqueous layer, the following were added :

20 µl of 3M sodium acetate
400 µl of 100% ethanol

This was vortexed and the cDNA was allowed to precipitate overnight at -20°C.

The tube was spun in a chilled centrifuge at 4°C for 1 hour at maximum speed, to pellet the precipitated cDNA. The radioactive supernatant was then removed, carefully, and the pellet was washed with 500µl of 80% (v/v) ethanol without mixing.
A further spin in a chilled centrifuge at 4°C for 2 minutes was performed, then the ethanol was aspirated and the pellet allowed to dry for 10 mins under vacuum.

cDNA Library - Ligating EcoR1 Adaptors

The cDNA pellet was resuspended in 9µl of EcoR1 adaptors, and incubated at 4°C for 45 minutes. To ensure the cDNA pellet was fully resuspended, the radioactive cDNA mixture was transferred to a new tube and the old tube was checked with a Geiger counter to determine its activity. 1µl of this mixture was then transferred to a new tube, and stored at -20°C as the second strand synthesis control.

The following reagents, kept on ice at all times, were then added :

1 µl of 10x ligase buffer
1 µl of 10mM rATP
1 µl of T4 DNA Ligase (4 U/µl)

The tubes were then spun-down and incubated at 4°C for 48 hours, and then the reaction was terminated by heating in a thermal cycler at 70°C for 30 minutes.

cDNA Library - Phosphorylating the *EcoR1* Ends

The tube is quickly spun-down and immediately placed on ice, the adaptor ends are then phosphorylated, by adding :

1 µl 10x ligase buffer
1 µl 10mM rATP
6 µl sterile water
1 µl of T4 Polynucleotide Kinase (10 U/µl)

The reaction was then incubated in a thermal cycler at 37°C for 30 minutes, and then immediately inactivated at 70°C for a further 30 minutes. The contents were then quickly spun-down and placed on ice.

cDNA Library - Digesting with *Xho1*

The following components, kept on ice, were then added :

28 µl of Xho1 buffer supplement
3 µl of Xho1 (40 U/µl)

The reaction was mixed, spun-down, and incubated at 37°C for 1.5 hours. The tube was then immediately placed on ice, 5 µl STE buffer were added, vortex-mixed & spun-down. Then 125µl of chilled ethanol were then added, mixed, and and the cDNA was allowed to precipitate at -20°C overnight.

The cDNA was then pelleted by spinning in a chilled (4°C)centrifuge at maximum (13,000rpm) for 60 minutes. The super-natant was then removed carefully, vacuum dried for 10 mins, and the pellet resuspended in 14µl of 1x STE buffer and 3.5µl of column loading dye.

The sample was now ready for size fractionation on the Sepharose CL-2B column.

Fig 2.2.1: Flow Chart of cDNA Library Construction

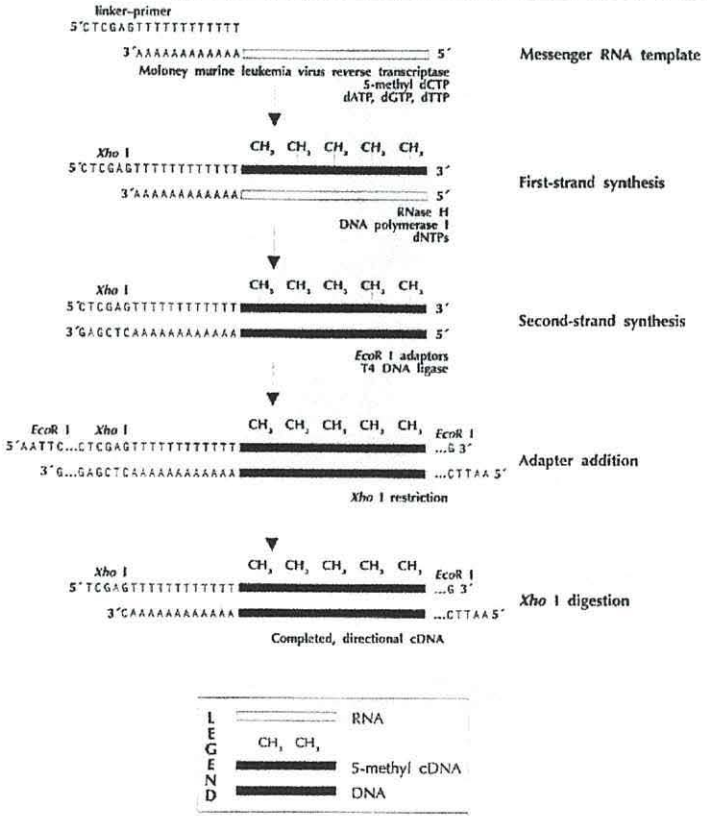


FIGURE 1 cDNA synthesis flow chart.

cDNA Library - Size Fractionation on Sepharose CL-2B Column

The Sepharose CL-2B column was prepared under sterile conditions, as outlined in the Stratagene cDNA Gigapack© III Gold Cloning Kit manual. All non-sterile materials had been soaked in 0.5% DEPC water for 2 hours prior to sterilisation by autoclave.

A sterile 10ml syringe reservoir was attached, using aseptic technique, to a sterile 1ml graduated pipette with a small sterile cotton wool plug at the bottom, using an 8mm length of sterile plastic connecting tubing. The column was held in a vertical position using a retort stand and claws. (See illustration)

The Sepharose CL-2B was mixed by inversion until the resin was evenly suspended, then the drip column was loaded with resin, using a sterile plastic pasteur pipette, and allowing it to settle about 0.5cm below the top of the glass pipette.

A minimum of 15ml STE buffer was then allowed to flow through the column, and when approximately 50µl of STE buffer remained above the surface of the column bed, the 17.5µl sample of cDNA in loading buffer was pipetted gently onto the surface of the column bed.

Once the sample had entered the column, the reservoir was gently refilled with STE buffer, and the sample allowed to run through the column. The progress of the sample was monitored using the Geiger counter, and by eye.

When the leading edge of the dye front reached the halfway mark on the column, 3-drop fractions were then collected using an HPLC automated fraction collector. These fractions were monitored for radioactivity, and collection continued until all of the radioactive nucleotides had eluted from the column, and the counts returned to background levels again.

8 µl from each fraction were removed and electrophoresed on a 5% nondenaturing acrylamide gel to select the fractions for use in the next step (see results).

The remainder of each fraction was stored at -20°C until it was known which were required.

cDNA Library - Processing the cDNA Fractions

The fractions to be used in the library construction were combined in a fresh tube, and an equal volume of phenol-chloroform [1:1(v/v)] was added, the sample was then vortexed vigorously, and spun-down at maximum (13,000rpm) for 2 minutes at room temperature.

The upper aqueous layer was then transferred to a new tube, and an equal volume of chloroform was added. This was vortexed and centrifuged as before, removing the upper aqueous layer to a new tube.

Two volumes of 100% ethanol were added to the aqueous phase, vortex-mixed, and allowed to precipitate overnight at -20°C.

The tube was then spun at maximum (13,000rpm) for 1 hour in a chilled centrifuge at 4°C. The supernatant was carefully removed and checked with a Geiger counter to ensure that all the radioactivity was present in the pellet.

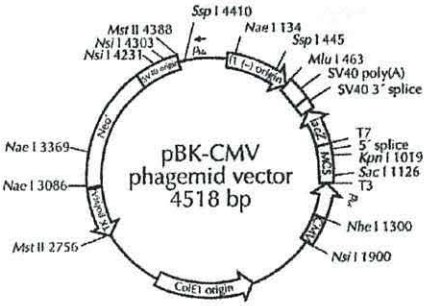
The pellet was then washed with 200µl of 80% (v/v) ethanol, and recentrifuged at 4°C for 2 minutes before carefully removing the supernatant, and vacuum drying the cDNA pellet.

The radioactivity of the pellet, i.e. the number of counts on the Geiger counter, was recorded.

The cDNA pellet was then resuspended in 3.5µl of sterile water, and the cDNA was quantified using the Ethidium Bromide plate method. (See **appendix g**)

Ligating cDNA into the ZAP Express Vector

Fig 2.2.2: pBK-CMV (ZAP Express) Vector



A control ligation was setup, on ice, as follows :

1.0 µl of ZAP Express Vector (1µg)
1.6 µl of test insert (0.4µg)
0.5 µl of 10x ligase buffer
0.5 µl of 10mM rATP (pH 7.5)
0.9 µl of sterile water

The cDNA sample ligation was also set up, on ice, as follows :

0.1 µl of resuspended cDNA (≈100ng)
0.5 µl of 10x ligase buffer
0.5 µl of 10mM rATP (pH 7.5)
1.0 µl of ZAP Express Vector (1µg)
2.4 µl of sterile water

Then 0.5µl of T4 DNA Ligase (4 U/µl) was added to each reaction, and the tubes were incubated at 4°C for 48 hours to allow ligation to occur.

Following ligation 1µl from each ligation reaction was packaged using the Gigapack III Gold packaging extract kit, according to the packaging instructions supplied.

The product of this packaging reaction (supernatant containing the phage) was then titred to determine the total number of plaque forming units (pfu) and the proportion which contained a cDNA insert. This was performed as follows :

Using a sterile cut yellow tip, 200µl of *E.coli* XL1-Blue MRF' cells (prepared as in **appendix f**) were added to 1µl of packaging reaction supernatant at dilutions of 1:1, 1:10, 1:100 and 1:1000 in SM buffer.

The tube was gently mixed, without vortexing, and incubated at 37°C for 20 minutes to allow the phage to attach to the cells.

The contents of the tube were then transferred, using a sterile 'cut' yellow tip, and added to 4ml of melted NZY Top Agar (**appendix h**), maintained at 48°C, and containing 15µl of IPTG (0.5M in sterile water) and 50µl of X-Gal (250mg/ml in DMF). The agar was gently mixed, by inversion, and then poured onto the surface of prewarmed (at 40°C) 100mm NZY agar plates.

Once set the plates were inverted and incubated at 37°C overnight. They were then transferred to a refrigerator and incubated for 2 hours at 4°C to aid the colour development.

Blue plaques indicate bacteriophage which does not have a cDNA insert, and white plaques are phage which contains a cDNA insert. The white plaques should outnumber the blue ones by a factor of 10-100 fold. The titre for the primary library was then recorded, and compared to the standard value of $1x10^6$ clones to determine if it was a good representational cDNA library.

Amplifying the Primary cDNA Library

Primary libraries can be unstable, therefore the amplification procedure was started immediately the 1° library had been constructed and quantified. Amplification was performed as follows :

To 600µl of host cells (OD_{600} of 0.5) in a 1.5ml Eppendorf tube, approx. $5x10^4$ pfu of primary library bacteriophage were added. The suspension was mixed gently, without vortexing, and incubated at 37°C for 20 minutes. The cells were then added, using a sterile 'cut' blue tip, to 30ml of molten NZY Top Agar maintained at 48°C in a waterbath. The tubes were then mixed, by inversion, and the agar was

poured onto the surface of prewarmed (45°C) 225mm x 225mm NZY agar plates. The top agar was allowed to solidify, and the plates were inverted and incubated for 6-8 hours at 37°C, so the plaques were just touching but not allowing them to grow larger than 1-2mm.

The plates were then overlaid with 10ml of SM buffer and then placed on a rocking plate in a cold room, and incubated (with gentle rocking) at 4°C overnight to allow the phage to diffuse into the buffer. The bacteriophage suspension was recovered from each plate and pooled into a sterile screw-cap polypropylene container. The plates were rinsed with a further 3ml of SM buffer, with gentle rocking for 5 minutes, and these washings were also pooled. Chloroform was added to the pooled buffer, to a final concentration of 5%(v/v), vortex-mixed vigorously and allowed to stand for 15 minutes at room temperature. The cell debris was removed by centrifuging at 500g for 10 minutes, and the aqueous supernatant was transferred to a fresh sterile polypropylene container. This process was repeated until the bacteriophage suspension was clear and free of debris or cloudiness.

The amplified library was then dispensed in 1ml aliquots, into sterile 1.5ml Eppendorf tubes, and stored at -80°C indefinately, or until required. Prior to use the library was quantified, and stored at 4°C for no longer than 2 weeks.

Production and confirmation of polyclonal antibodies in a rabbit, raised against secreted salivary antigens from *S.calcitrans*

Preparation of the flies for feeding

50 adult *S.calcitrans* flies from a colony, reared according to ***appendix (a)*** and sedated according to ***appendix (b)***, were placed a 1.5' x 1.5' x 1.0' square hollow container covered in fine mesh. The flies were then starved inside this container for 24hrs immediately prior to commencement of feeding.

Preparation of the rabbit for feeding

A previously 'unused' half-lop rabbit was bled from an ear vein to provide a background serum sample. The rabbit was returned to its hutch and the puncture wound was allowed to heal for a minimum of 1 week. On the day of feeding the rabbit was removed from the hutch, bound firmly in an old white laboratory coat so it could not struggle too much, and sat upon a metal examination table with its ears exposed.

Feeding procedure

The container of flies (prepared as above) was laid with the mesh against the designated ear, and the flies were allowed to feed for 10-15 mins or until the rabbit showed signs of discomfort.

Anaesthesia/Analgesia

A local anaesthetic cream (for the rabbits ear) was supplied by the veterinary inspector, this was available for use should the rabbit show increased or frequent signs of discomfort during the procedures. This was applied 1 hour prior to feeding, and held in place with a dressing attached with masking tape. During this time the rabbit was supervised constantly to ensure the dressing was not removed, or the cream was licked off the ear.

Post-Feeding

After the feeding had taken place, the container with the gorged flies was removed and placed in a -20°C freezer. The rabbit was unwrapped and placed in an exercise run for a minimum of 2 hours prior to returning to its hutch.

Bleeding

The rabbit was bled at frequent intervals after antibody production had began. The blood was drawn into a normal glass container from the major ear vein in accordance with the relevant legislation and by a person qualified to perform such a procedure. Details of the procedures carried out were recorded in the appropriate log book.

Serum Processing

The blood samples taken were allowed to clot for 60 mins at 37°C. The samples were then centrifuged at 3,600 rpm for 30 minutes, and the serum was removed and immediately frozen at -20°C.

Dot Blot Procedure

The dot blot test assesses the ability of the 1° antibody to detect antigen bound to a nitrocellulose membrane. It is also able to determine the appropriate antibody dilution (AG lane), checks the avidity of the 2° antibody conjugate for the 1° antibody (AB lane) and detects the presence of antibodies cross-reacting with E.coli phage antigens (EC lane).

The following solutions were prepared :

Blocking solution
20mM Tris-HCl (pH 7.5)
150mM NaCl
1% (w/v) bovine serum albumin

Colour Development Solution
100mM Tris-HCl (pH 9.5)
100mM NaCl
5 mM $MgCl_2$

Antibody Diluent
20mM Tris-HCl (pH 7.5)
150mM NaCl
1% (w/v) bovine serum albumin

Tri Buffered Saline Tween 20 (TBST)
20mM Tris-HCl (pH 7.5)
150mM NaCl
0.05% (v/v) Tween 20

Stop Solution
20mM Tris HCl (pH 2.9)
1mM EDTA

Then 18 strips of nitrocellulose membrane were cut out, ensuring that gloves were worn at all times, and the lanes were pipetted with 1µl dots, as follows :-

Fig 2.2.3: Dot Blot Layout - Concentrations

Row 1	Salivary Homogenate	1µg	100 ng	10 ng	1 ng	100 pg	10 pg
Row 2	Antibody (+ve Control)	1:50	1:100	1:150	1:200	1:250	1:300
Row 3	E.coli Lysate (10mg/ml)	Neat	1:10	1:100	1:1,000	1:10,000	1:100,000

The strips were air-dried for 5 minutes, before immersing in blocking solution for 1 hour at room temperature to block all of the remaining non-specific protein binding sites.

The test strips were then washed 5 times, each for 5 minutes, in 50ml of TBST soln.

Serial dilutions of 1° antibody, diluted in antibody diluent, were prepared as follows and the relevant strips were incubated in these for 2 hours at room temperature, with gentle agitation. Strips 1, 7 & 13 were the negative controls, and were incubated under the same conditions in BSA (blocking solution).

Table 2.2.1: Dot Blot Layout - Dilutions

Dot Blots	Bovine Serum	Titre (Rabbit serum)				
		1:50	1:100	1:200	1:300	1:500
1st Serial	1	2	3	4	5	6
2nd Serial	7	8	9	10	11	12
Pooled	13	14	15	16	17	18

The test strips were then washed 5 times, each for 5 minutes, in 50ml of TBST soln and all incubated in 2° antibody, the goat anti-rabbit alkaline phosphatase conjugate, (provided with the Stratagene PicoBlue Immunoscreening Kit, Cat.No. 200371) for 1 hour at room temperature with gentle agitation. During this time the colour substrate stock solutions were prepared, and immediately frozen ready for use :

150mg of Nitroblue tetrazolium (NBT) was diluted in 2.0ml 70% N,N-dimethylformamide (DMF) to produce a 75mg/ml stock solution.

75mg of 5-Bromo-4-chloro-3-indoyl phosphate (BCIP) was diluted in 1.5ml 100% N,N-dimethylformamide (DMF) to produce a 50mg/ml stock solution.

The test strips were then washed 5 times, each for 5 minutes, in 50ml of TBST soln, followed by two washes in TBS to remove any residual Tween 20. During the final wash step, the NBT-BCIP colour substrate solution was freshly prepared, as follows :

80µl of NBT Stock Solution was made up to 20ml with colour development solution, to give a final concentration of 0.3mg/ml, then 60µl of BCIP Stock Solution were added (dropwise) to give a final concentration of 0.15mg/ml.

The nitrocellulose membranes were removed from the final wash, excess moisture was blotted off with Whatman 3MM paper, and the membranes were immersed in the freshly prepared colour development NBT-BCIP substrate. The colour development reaction was allowed to proceed in the dark until the positive reactions were clearly visible, this should last no longer than 30 mins. The nitrocellulose membranes were then removed from the NBT-BCIP colour development solution, rinsed briefly in TBS, and immersed into Stop Solution. The membranes remained in stop solution for 5 minutes, and were removed and allowed to air-dry on Whatman 3MM filter paper prior to scanning their images.

Purification of IgG antibodies from polyclonal rabbit serum

It was decided that the best way to clean-up the serum, and hence reduce the background *E.coli* activity, was to extract the IgG from the rabbit serum and to use this fraction in the subsequent screening processes. This would remove any IgM present (including any *E.coli*-specific IgM) and other contaminating proteins or immunoglobulins, hence reducing the potential for background reactivity to *E.coli* phage lysate antigens. The procedure used was as follows:

500µl of 1M bicarbonate buffer (pH 8.0) were added to 4.5ml of polyclonal rabbit serum, mixed gently, and placed on ice. The column was then freshly packed with Protein-A resin, and equilibrated with 100mM bicarbonate buffer (pH 8.0) for 45 minutes. The 5.0ml of diluted polyclonal rabbit serum was then slowly loaded onto the column. The column was then washed using 3x column volume of 100mM bicarbonate buffer (pH 8.0) to remove unbound antibody, and the wash stage repeated a total of 3 times.

IgG was eluted using 6 column volumes of 200mM sodium citrate buffer (pH 3.0). 1M bicarbonate buffer (pH 8.0) was added dropwise to the eluate to return the pH to 8.0, checking the pH periodically with litmus paper. The IgG was then concentrated by centrifuging in a Centricon-100 ultrafiltration device, and resuspending the residue in 0.5ml of 150mM NaCl. A Biorad™ protein assay was performed on the purified IgG extract, **Appendix (j)**, and a small sample was run down an SDS-PAGE gel, **Appendix (k)**, to confirm the size and the presence of a single band.

Determination of E.coli background cross-reactivity in the IgG Serum

The following solutions were freshly prepared, as before :

Blocking solution
20mM Tris-HCl (pH 7.5)
150mM NaCl
1% (w/v) bovine serum albumin

Colour Development Solution
100mM Tris-HCl (pH 9.5)
100mM NaCl
5 mM $MgCl_2$

Antibody Diluent
20mM Tris-HCl (pH 7.5)
150mM NaCl
1% (w/v) bovine serum albumin

Tri Buffered Saline Tween 20 (TBST)
20mM Tris-HCl (pH 7.5)
150mM NaCl
0.05% (v/v) Tween 20

Stop Solution 1mM EDTA in 20mM Tris HCl (pH 2.9)

Then 6 strips of nitrocellulose membrane was cut out, ensuring that gloves were worn at all times, and the lanes were all pipetted with 1µl dots, as follows :-

Fig 2.2.4: Dot Blot Layout

Row 1	Salivary Homogenate	1µg	100 ng	10 ng	1 ng	100 pg	10 pg
Row 2	Antibody (+ve Control)	1:50	1:100	1:150	1:200	1:250	1:300
Row 3	E.coli Lysate (10mg/ml)	Neat	1:10	1:100	1:1,000	1:10,000	1:100,000

The strips were then air-dried for 5 minutes, before immersing in blocking solution for 1 hour at room temperature to block all of the remaining non-specific protein binding sites. The test strips were then washed 5 times, each for 5 minutes, in 50ml of TBST soln, and serial dilutions of 1° antibody in antibody diluent were prepared as before. The strips were incubated in these for 2 hours at room temperature, with gentle agitation.

The strips were then washed 5 times, each for 5 minutes, in 50ml of TBST soln, and then incubated in 2° antibody, the goat anti-rabbit alkaline phosphatase conjugate, (provided with the Stratagene PicoBlue Immunoscreening Kit, Cat.No. 200371) for 1 hour at room temperature with gentle agitation. During this time the colour substrate stock solutions were prepared, and the strips were developed (as before).

Absorption of E.coli reactive immunoglobulins from the IgG fraction of polyclonal rabbit serum.

Five 10cm x 10cm squares of nitrocellulose membranes were cut out and immersed in 50ml of a 1mg/ml solution of *E.coli* phage lysate in TBS, with occasional agitation. After 45 minutes the membranes were removed and air-dried on Whatman 3MM filter paper. The membranes were then washed five times, each for 5 minutes, with 50ml of TBS. They were then immersed in 50ml blocking solution and incubated for 30 minutes at room temperature, with gentle agitation as before.

The membranes were then removed from the blocking solution, rinsed briefly in TBS, and then washed 3 times in 50ml of TBST. The 1° antibody was then diluted to make a 1:5 concentration in TBS, this was then used to incubate one of the membranes for 15 minutes at room temperature, with gentle agitation. The membrane was then removed and discarded, and one of the remaining membranes was added to the 1° antibody solution and incubated for a further 15 minutes. This process was repeated until all 5 membranes had been used and discarded.

The 1° antibody solution was now ready to be tested for background activity to E.coli (as before). The entire process may be repeated again with a further 5 nitrocellulose membranes if background activity remains high. This was the case in this instance.

Screening of *S.calcitrans* salivary gland cDNA library using the double-absorbed IgG fraction from the polyclonal fly-fed-rabbit serum

E.coli XL1-Blue MRF' cells were prepared (as outlined in **appendix f**) and suspended in 10mM $MgSO_4$ to an OD_{600} reading of 0.50. 600µl of this suspension were then pipetted into 5 suitably labelled 1.5ml eppendorf tubes, to which is added a volume equivalent to 50,000 pfu of the quantified cDNA library. The cell suspensions were mixed gently, without vortexing, and incubated in a 37°C heating block for 15 minutes to allow phage attachment.

The incubated cell suspensions were then each added, using a sterile 'cut' blue tip, to 30ml of molten NZY Top Agar maintained at 48°C in a waterbath. The tubes were then mixed, by inversion, and the agar was immediately poured onto the surface of prewarmed (at 45°C for 60 mins) 225mm x 225mm NZY agar plates. The top agar was allowed to solidify, and the plates were inverted and incubated for approx. 6 hours at 37°C, so the plaques were just visible, but not touching. The plates were then immediately placed in the refrigerator at 4°C and cooled thoroughly.

A 200mm x 200mm square of nitrocellulose membrane was cut and carefully laid onto the surface of the agar/bacterial lawn. It was allowed to remain for 1 minute, and then carefully removed. During the 1 minute when the membrane was in contact with the surface of the agar, a mounting needle (heated until

it glowed orange/red in a bunsen flame) was plunged into the membrane at 3 different assymetrical points near to the margin, so that the membrane may be correctly & accurately orientated at a later date. The membrane is removed, washed 5 times, each for 5 minutes in TBST to remove bacterial cell debris, and placed in blocking solution and incubated for 1 hour, with gentle agitation.

During this time a 1:200 dilution of 1° antibody (double-treated IgG fraction from the polyclonal rabbit serum) in antibody diluent is prepared. The membrane is then incubated in this for 2 hours at room temperature, with gentle agitation. It was then removed and washed 5 times, each for 5 mins, in TBST soln. The membrane is then incubated in 2° antibody, the goat anti-rabbit alkaline phosphatase conjugate, (provided with the Stratagene PicoBlue Immunoscreening Kit, Cat.No. 200371) for 1 hour at room temperature with gentle agitation. During this time the colour substrate stock solutions were prepared, and the colour development reaction was performed (as before).

Examination of positive clones was achieved using a lightbox, comparing the various dots of colour on the nitrocellulose membranes with colony positions on the agar plates. The 3 asymmetric holes were used to align the membranes to the plates. Any dots which aligned with a colony were considered 'positive', and any dots which did not align with a colony were considered 'false positives'. All 'positive' clones were removed from the agar, using sterile tweezers and a scalpel, and transferred to 500µl of sterile SM buffer in a suitably labelled 1.5ml eppendorf tube. The procedure was repeated, and a further 3 sets of 5 membranes were prepared, as described, to give a total of 20 membranes - screening a total of $1x10^6$ clones.

Determination of polyclonal serum & purified IgG activity against periodinated salivary gland homogenate

Salivary gland homogenate extract was freshly prepared, as outlined in **appendix c**, and appropriate serial dilutions were made, and then kept on ice until required. This is the antigen. The serial dilutions are prepared for use in the control tubes, and will provide a standard curve against which a titre can be determined if there is any reduction in intensity with the periodinated samples. A 300µM stock solution of sodium periodate was prepared, and diluted accordingly to make the required concentrations :

Table 2.2.2: Periodate Solutions

A	Periodate	50µM	100µM	150µM	200µM	250µM	300µM
	Antigen	Neat	Neat	Neat	Neat	Neat	Neat
B	Periodate	Untreated	Untreated	Untreated	Untreated	Untreated	Untreated
	Antigen	Neat	1:2	1:4	1:8	1:16	1:32

In appropriately labelled 0.5ml Eppendorf tubes, 25µl of periodate solution (A tubes) or 25µl of water (B tubes) were added to 75µl of antigen. The samples were then vortex-mixed, incubated at 37°C for 1 hour, and the reaction stopped by instantly freezing the samples in liquid N_2 and storing at -80°C. During this time the nitrocellulose membrane for the dot blot procedure was cut-out, and the washing & blocking solutions were freshly prepared (as before). 1µl of each of the samples was dotted onto the membrane according to the layout described earlier. They are allowed to air-dry for 5 minutes, before placing the membrane in blocking solution and incubating at room temperature for 1 hour, with gentle agitation.

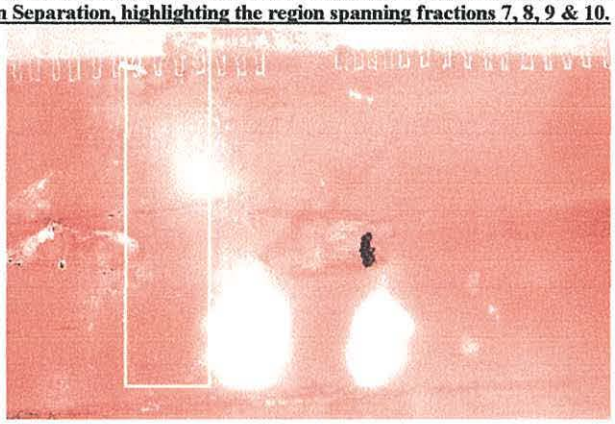
During this time a 1:200 dilution of 1° antibody (double-treated IgG fraction from the polyclonal rabbit serum) in antibody diluent is prepared. The membrane is then incubated in this for 2 hours at room temperature, with gentle agitation. It is then washed 5 times, each for 5 minutes, in TBST solution, followed by a further two washes in TBS to remove any residual Tween 20. The membrane is then incubated in 2° antibody, the goat anti-rabbit alkaline phosphatase conjugate, (provided with the Stratagene PicoBlue Immunoscreening Kit, Cat.No. 200371) for 1 hour at room temperature with gentle agitation. During this time the colour substrate stock solutions were prepared, and the colour development reaction is performed, as before.

2.3 Results

5μg of Poly(A)$^+$mRNA were successfully extracted for use in the first strand synthesis reaction. Due to the inherent instability of mRNA, and the abundance of RNAse enzymes, it was decided to extract total RNA from the salivary glands in the first instance using the Chomzinsky method. The reason for this was twofold. The moment the salivary glands are defrosted and homogenised the mRNA is exposed to attack from RNAse enzymes, but homogenising them immediately in Soln.D would protected the mRNA from degradation by RNAse. Secondly, to obtain the 5μg of Poly(A)$^+$ mRNA it required the sterile dissection and 'safe storage' of 3,500 pairs of salivary glands. This was a task which took several weeks to achieve, and during this time the samples needed to be stored and protected from RNAse activity. Storage of the tissue samples alone at -80°C for that period of time was not satisfactory.

The control reactions for the first and second strand synthesis reactions were satisfactory, indicating the 5μg of Poly(A)$^+$mRNA was successfully reverse-transcribed into cDNA.

Fig 2.3.1: 5% Nondenaturing PAGE Autoradiograph of Fractions from Salivary Gland cDNA Column Separation, highlighting the region spanning fractions 7, 8, 9 & 10.



The results of the 5% nondenaturing acrylamide gel indicated that fractions 7, 8, 9 and 10 (see fig 2.3.1) were suitable for use in constructing the cDNA library. The cDNA pellet obtained from these fractions gave a count of **12 cps** on the Geiger counter, the Ethidium Bromide plate quantification gave a total yield of 4,700ng of cDNA.

A mean 96% of plaques contained the insert (white), compared to 4% (blue) that didn't.

Table 2.3.1: cDNA Library - Plaque Count

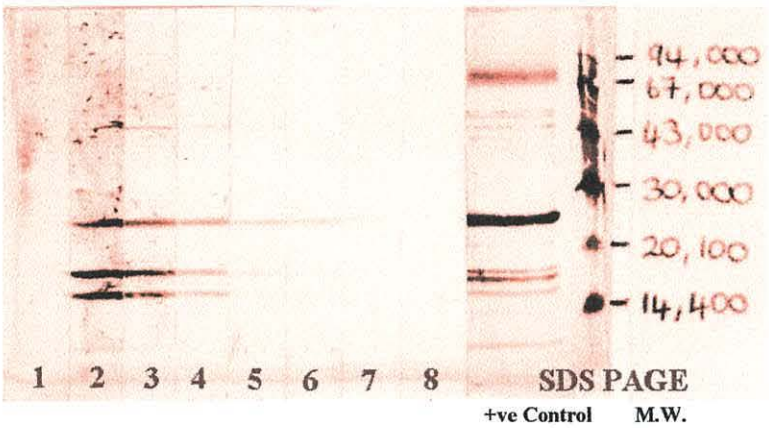
Tube No.	Plaques (1μl)	Tube Vol.(μl)	Total Plaques	cDNA Used	Plaques in library if all cDNA is packaged.
1	11	526	5,786	100ng	271,942
2	11	526	5,786	100ng	271,942
3	8	526	4,208	100ng	197,776
4	124	526	65,224	100ng	3,065,528
5	488	526	256,688	100ng	12,064,336
6	312	526	164,112	100ng	7,713,264
7	208	526	109,408	100ng	5,142,176
8	156	526	82,056	100ng	3,856,632
9	412	526	216,712	100ng	10,185,464
10	192	526	100,992	100ng	4,746,624
11	372	526	195,672	100ng	9,196,584
12	392	526	206,192	100ng	9,691,024
13	176	526	92,576	100ng	4,351,072
14	480	526	252,480	100ng	11,866,560
15	244	526	128,344	100ng	6,032,168
Ctl	344	526	180,944	100ng	8,504,368

Table 2.3.2: cDNA Library - Statistics

cDNA Library Statistics		Total cDNA Library Plaques	
Total cDNA Yield	4,700 ng		
		Mean Value	7,325,953
Mean No. Plaques	125,749 per 100ng cDNA	Control	8,504,368
Mean No. Plaques *(excluding outliers)*	**157,800 per 100ng cDNA**	High Value	12,064,336
		Low Value	3,065,528

A good representational library consists of no less than **1 x10^6** clones. This cDNA library contains on average **7.33 x10^6** clones, and can therefore be considered a "good" and fully representational cDNA library according to these criteria.

Fig 2.3.2: SDS-PAGE Western Blotting Results for the Specificity of Polyclonal Antibodies to Salivary Gland Antigens



Key:-

1. Pre-Feed Serum
2. 1:10 Polyclonal Serum
3. 1:20 P.C. Serum
4. 1:40 P.C. Serum
5. 1:80 P.C. Serum
6. 1:160 P.C. Serum
7. 1:320 P.C. Serum
8. 1:640 P.C. Serum
9. Control (SDS-Homogenate)
10. Mol.Wt.Markers

The western blots above were prepared from SDS-PAGE gels of salivary gland homogenate. The **test** samples (**lanes 1 to 8**) were exposed to the rabbit polyclonal antibody at various concentrations. The antigen bands which were recognised and bound by the polyclonal rabbit serum were then visualised using a standard preparation of goat anti-rabbit conjugate. The **positive control (lane 9)** was an identical SDS-PAGE Western Blot of the same salivary gland homogenate, which had been silver-stained directly (appendix k) without any treatment in the 1° or 2° antibody solutions. The positive control (lane 9) illustrates the total number of proteins bands which could be visualised from the sample homogenate, from which 3 bands are identified as antigens recognised by the polyclonal rabbit serum. Quantification (titre) values for the 1° polyclonal antibody can also be derived from these results, and hence these data also facilitate sample optimisation in subsequent dot-blot investigations.

Table 2.3.5: Summary of Dot-Blot Treatments at Various Dilutions of Antibody Solution

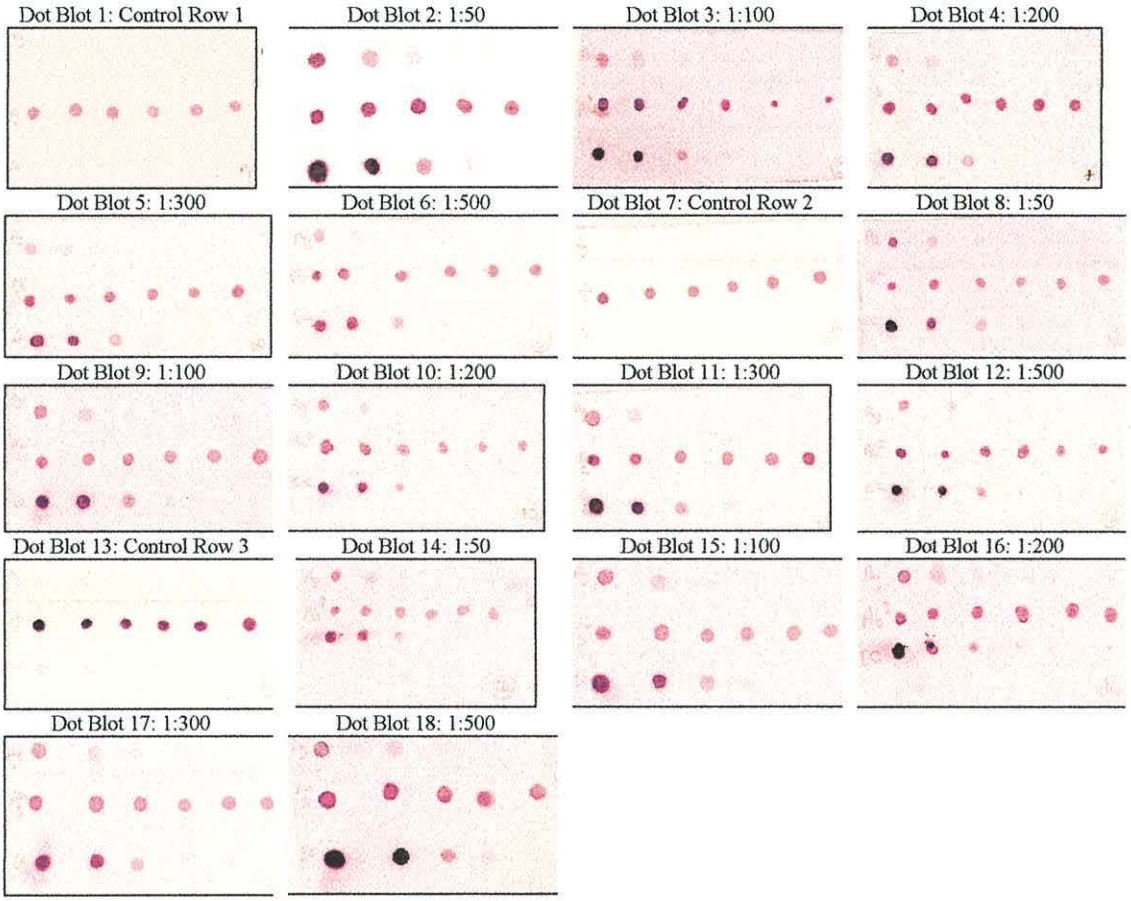
Dot Blots	Control (BSA)	Titre (Dilution of Rabbit serum)				
		1:50	1:100	1:200	1:300	1:500
1st Serial	Blot 1	Blot 2	Blot 3	Blot 4	Blot 5	Blot 6
2nd Serial	Blot 7	Blot 8	Blot 9	Blot 10	Blot 11	Blot 12
Pooled	Blot 13	Blot 14	Blot 15	Blot 16	Blot 17	Blot 18

Fig 2.3.3 Layout of Individual Dot-Blots Indicating the Various Dilutions and Antigens Used

Row 1	Salivary Homogenate	1μg	100 ng	10 ng	1 ng	100 pg	10 pg
Row 2	Antibody (+ve Control)	1:50	1:100	1:150	1:200	1:250	1:300
Row 3	E.coli Lysate (10mg/ml)	Neat	1:10	1:100	1:1,000	1:10,000	1:100,000

To summarise, there were 18 dot-blots sheets performed in this investigation. The general layout of antigens on the surface of *every* dot blot sheet, in a 6x3 pattern, is summarised in Figure 2.3.3. The varying concentrations of *rabbit serum* (test antibody) or *BSA* (negative control) to which *each* of these dot-blots is exposed, is summarised in Table 2.3.5. The antibody directly bound to the surface of *every* dot-blot (Row 2, Fig 2.3.3) acts as a **positive control**, wheras blots **1**, **7** and **13** which are exposed to bovine serum albumin (BSA) instead of polyclonal rabbit serum act as the **negative control** for all of the blots in that row.
Blots **2**, **8** and **14** were the test blots exposed to a **1:50** dilution of polyclonal rabbit serum.
Blots **3**, **9** and **15** were the test blots exposed to a **1:100** dilution of polyclonal rabbit serum.
Blots **4**, **10** and **16** were the test blots exposed to a **1:200** dilution of polyclonal rabbit serum.
Blots **5**, **11** and **17** were the test blots exposed to a **1:300** dilution of polyclonal rabbit serum.
Blots **6**, **12** and **18** were the test blots exposed to a **1:500** dilution of polyclonal rabbit serum.

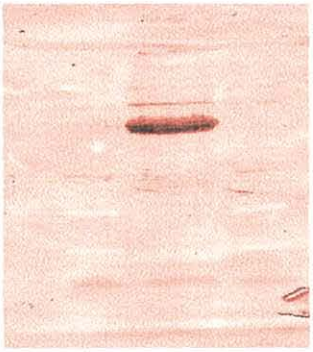
Figs 2.3.4 - 2.3.21: Dot Blot Results - Scans 1-18



The results clearly demonstrate that the polyclonal serum preparations from the 1st (blots 1-6) and 2nd (blots 7-12) serial bleeds, and the pooled samples (blots 13-18), all show similar reaction patterns. This provides valuable quantification data, and demonstrates that the serum samples can be pooled for the final screening, maximising the amount of polyclonal serum available for this investigation.

However, the peroxidase activity (blue colouration) on the top row (salivary antigens) of each blot is less intense than that of the bottom row (*E.coli* lysate) at that concentration. The need to pretreat the serum, to reduce/remove this background activity prior to screening, was therefore demonstrated.

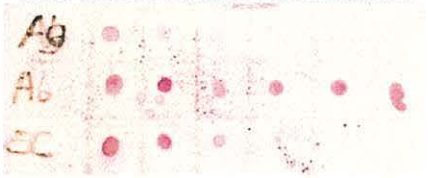
Fig 2.3.22: SDS-PAGE Gel Demonstrating Single Band of Extracted Polyclonal IgG



The results of the IgG extraction processes gave a total protein yield of 48.5mg IgG from the 4.5ml of polyclonal rabbit serum used. SDS-PAGE analysis (see fig 2.3.22) revealed a single major band, of approx 170 kDa, confirming a clean IgG extract. Therefore an IgG yield of 10.8mg/ml was obtained, and given that polyclonal serum is normally expected to contain between 8-15mg/ml of IgG, this was a good yield.

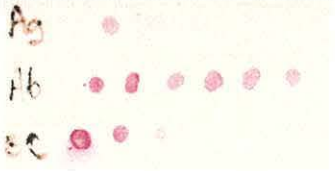
The IgG fraction, obtained above, was then treated (as before) to absorb-out the *E.coli* cross-reactivity, and the results of the first and second *E.coli* absorption treatments on the IgG fraction were as follows :-

Fig 2.3.23
Dot-Blot Results of *E.coli* Cross-Reactivity after 1st Pre-Treatment Procedure



Titre of *E.coli* Lysate Preparation (Bottom Row of Dot Blots)	NEAT	1:10	**1:100**	1:1k	1:10k	1:100k

Fig 2.3.24
Dot-Blot Results of *E.coli* Cross-Reactivity after 2nd Pre-Treatment Procedure



	NEAT	1:10	**1:100**	1:1k	1:10k	1:100k

It was decided that the results of the 2nd treatment met the general requirements of the cDNA library screening kit for *E.coli* background activity, which states that there must not be an intense colour visible at the **1:100 dilution of *E.coli* lysate**. The colour present after 2 treatments was visible, but by no means 'intense', and hence it was deemed to meet the requirements for the next stage. The double-absorbed IgG fraction of polyclonal rabbit serum was ready to be used for subsequent immunoscreening.

Reactivity of the extracted IgG fraction with E.coli lysate was as follows:

Fig 2.3.25: 1:50 Dilution of 1° Ab

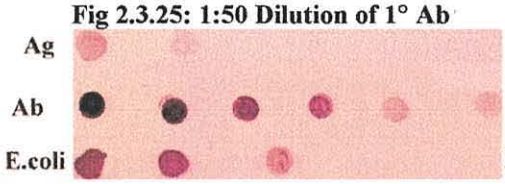


Fig 2.3.26: 1:100 Dilution of 1° Ab

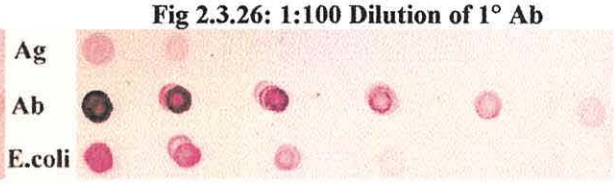


Fig 2.3.27: 1:200 Dilution of 1° Ab

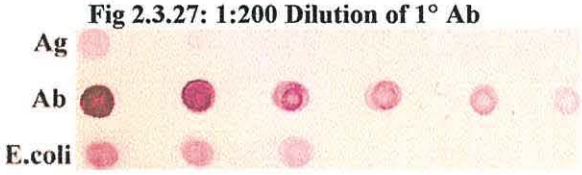


Fig 2.3.28: 1:400 Dilution of 1° Ab

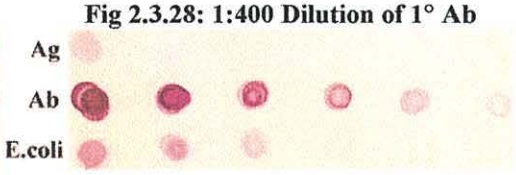


Fig 2.3.29: 1:800 Dilution of 1° Ab

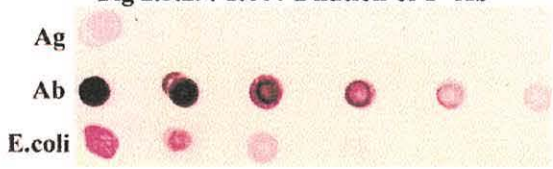
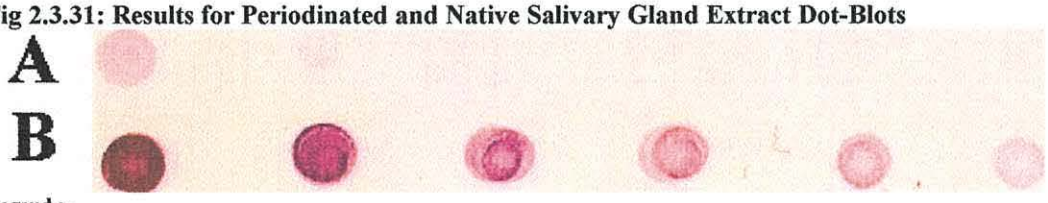


Fig 2.3.30: 1:1600 Dilution of 1° Ab

Fig 2.3.31: Results for Periodinated and Native Salivary Gland Extract Dot-Blots



Legend :-

A	50μM Periodate	100μM Periodate	150μM Periodate	200μM Periodate	250μM Periodate	300μM Periodate
	Neat Antigen	Neat Antigen	Neat Antigen	Neat Antigen	Neat Antigen	Neat Antigen
B	Untreated	Untreated	Untreated	Untreated	Untreated	Untreated
	Neat Antigen	1:2 Antigen	1:4 Antigen	1:8 Antigen	1:16 Antigen	1:32 Antigen

There is a significant decrease in specificity for the periodinated antigen compared to the native (untreated) antigen. The periodination process strips the protein of its carbohydrate groups, and hence the decrease in affinity of the antibody for the antigen after periodination would indicate that the antibody is dependent upon glycosylation of the antigen.

2.4 Discussion

Having successfully built the salivary gland cDNA library for *Stomoxys calcitrans* a method was required whereby the library could be screened for genes coding for products of interest. The gene products we most wanted to identify from the salivary glands were those which have antihaemostatic properties. These would naturally be secreted by the salivary glands into the wound during the feeding process, enhancing the chances of obtaining a successful blood meal. Since the cDNA library contained the gene sequences for the entire salivary gland, both the structural and housekeeping genes as well as those of interest, we needed a method whereby only the genes of secreted products (those of interest) were identified.

The laboratory had access to an animal facility, and had the necessary home office licence for such a procedure, so it was decided to identify the secretory products using polyclonal antibodies raised in a rabbit. Simply injecting whole salivary gland extract together with an adjuvant would not have allowed us to distinguish between secretory and non-secretory antigens, and the salivary exudate was insufficient to allow 'salivary milking' of the glands in order to artificially inoculate the rabbit with saliva. It was for these reasons that polyclonal antibodies, specific for the secretory products, were raised in the rabbit by allowing *S.calcitrans* to feed directly from the rabbit. The advantage of this method is that it would allow polyclonal antibodies to be raised against any antigens secreted during the normal feeding process, rather than salivary extract obtained artificially.

The rabbit was bled initially to provide normal background sera. Subsequent to this the rabbit was fed to the flies on a weekly basis, as outlined, where possible alternating the ears used in order to reduce the discomfort to the rabbit.

A succession of bleeds were then performed to obtain serum and to harvest the polyclonal antibodies therin. The sera samples containing antibodies were then pooled, and the pooled serum was tested against the pre-feed serum to ensure specificity to the antigen, and to determine the level of background activity which may also be present against *E.coli*.

The specificity of the polyclonal antibodies was demonstrated, with a minimum of 3 bands on the SDS-PAGE corresponding to antibody-antigen reactions from the post-feed serum. However, at this stage the general background activity in the serum against *E.coli* was demonstrated to be higher than the specific activity against the salivary antigens. Therefore the serum was going to require some treatment to absorb-out *E.coli* specific antibodies, and hence reduce the background activity before the screening process could continue.

The IgG component of the polyclonal rabbit serum was successfully extracted and *E.coli* specific activity was absorbed-out, ensuring its suitability for screening the cDNA library.

The lack of any positive results from this screening process was frustrating given the vast amount of work which had gone into the cDNA library construction, and the acquisition and processing of the polyclonal rabbit serum. However the background

level of staining in the membranes was not sufficient to suspect that this was the problem. The results from the treatment and clean-up processes were encouraging in this respect - bringing the background activity to within acceptable levels, therefore this possibility was ruled-out.

The controls in all of the experiments worked as expected, and the presence of a background staining in the screening process confirmed the activity of the enzyme and substrates involved. The main difference between that of the screening process, and those of the clean-up procedures in earlier sections, would appear to be the 'source' of the salivary antigens.

In the earlier procedures the salivary antigens were derived from a crude extract of salivary gland homogenate, and the antigens originated from *S.calcitrans* tissue. In this screening process, however, the antigen is supplied by the bacterium *E.coli* XL1-Blue MRF' which has the gene for the antigen inserted into its genome. The gene coding for the antigen has been taken directly from *S.calcitrans*, and so we can assume this is the same. However we do not know if any post-translational modifications of the gene product, such as the attachment of carbohydrate groups - which could be performed by *S.calcitrans*, could have had any effect. The *E.coli* XL1-Blue MRF' cells would be unable to perform such post-translational modifications given that the bacterium lacks a Golgi apparatus. Carbohydrate side-groups are certainly known to be highly immunogenic, and it is very likely that the antibodies raised against the salivary gland antigens secreted during the feeding process, were directed against such side-groups.

Results obtained from the periodination experiments, effectively stripping away the carbohydrate side-groups from the antigen, greatly decreased the affinity the antigen has for the antibody. From this we can conclude that the antibodies raised by the rabbit were predominantly directed at epitopes present on the carbohydrate side-groups, and not at the protein backbone itself. This poses major problems when we consider the potential usefulness, or otherwise, of the antibodies raised for the purpose of screening the cDNA library. Whilst this may have been a disappointing setback in the overal screening process, it does at least explain why the initial screening did not work, and that it was not a problem with the cDNA library itself. The direct result of these observations meant that another method of screening the salivary gland cDNA library had to be found.

Chapter 3

Abundancy & Degenerate Primer Screening of *S.calcitrans* Salivary Gland cDNA Library

3.1 Introduction

3.2 Materials and Methods

Single Clone Selection and PCR Protocol

Hae-III Digestion of Clone Insert PCR Products

Sau-3AI Digestion of Clone Insert PCR Products

Data Analysis, Clone Selection & Sequence Information

Degenerate PCR Screening of cDNA Library

3.3 Results

3.4 Discussion

3.1 Introduction

To effectively utilise, amplify and sequence any gene insert within the library it is essential to isolate the pure colony (clone) of the bacterium, which contains a specific insert, and that insert only. Colonies comprising a mixture of bacteria with different types of insert need to be excluded before further work can commence. The clones of potential interest are then identified, using a screening protocol.

The preliminary approach to this screening was immunological, raising polyclonal antibodies in a rabbit against antigens from the salivary secretions of *S.calcitrans*. The IgG fraction was then purified from the polyclonal serum, using an affinity column bound with Protein-A, and the IgG fraction was treated to absorb-out any cross-reactivity to *E.coli* antigens. Unfortunately this approach proved to be unsuccessful, with the polyclonal IgG fraction targetting epitopes which were mainly located on glycosylated side-groups, rather than the protein backbone itself. Repeating the experiment would have been costly & time-consuming, and given that carbohydrate groups are highly immunogenic there was little chance of success. It became obvious that, if any useful sequence information were to be gained from the cDNA library, another screening strategy had to be employed.

Aims and Objectives

Having successfully constructed a cDNA library in the previous chapter, but unable to identify molecules related to haematophagy using the immuno-screening methods. The following chapter utilises various other cDNA library screening processes in an attempt to identify these clones, and obtain the sequence information.

3.2 Method

Single Clone Selection and PCR Protocol

E.coli XL1-Blue MRF' cells were prepared (as outlined in **appendix f**) and suspended in 10mM $MgSO_4$ to an OD_{600} of 0.50. 600µl of this suspension were then pipetted into 5 suitably labelled 1.5ml Eppendorf tubes, to which is added a volume equivalent to 500 pfu of the quantified cDNA library. The cell suspensions were mixed gently, without vortexing, and incubated in a 37°C heating block for 20 minutes to allow phage attachment. The incubated cell suspensions were then each added, using a sterile 'cut' blue tip, to 30ml of molten NZY Top Agar maintained at 48°C in a waterbath. The tubes were then mixed, by inversion, and the agar was immediately poured onto the surface of prewarmed (at 45°C for 60 mins) 225mm x 225mm NZY agar plates. The top agar was allowed to solidify, and the plates were inverted and incubated overnight at 37°C. The plates were then placed in the refrigerator at 4°C and cooled thoroughly. During this time, two hundred suitably labelled 1.5ml Eppendorf tubes each containing 500µl of sterile SM buffer were prepared. Into each of these 10µl of chloroform was pipetted, aseptically, and the tubes were returned to the fridge until use.

The following morning the agar plates were removed from the fridge. A scalpel and mounting needle were sterilised, until glowing red/orange, in a hot bunsen flame. These were allowed to cool, and used to pick individual colonies of phage off the agar surface, and place them immediately into a tube containing SM buffer (prepared earlier). The scalpel and mounting needle were then re-sterilised in the bunsen flame, as before, and allowed to cool. The process was repeated until all 200 Eppendorf tubes were inoculated. The tubes containing the clones were then individually vortex-mixed for several seconds, and returned to the fridge for 72 hours. After the 72 hour incubation the tubes were removed from the fridge, centrifuged at 13,000 rpm for 5 minutes, and the supernatant phage solution used for the following PCR procedure.

PCR Selection of Pure Clones

Into 5 sterile 1.5ml Eppendorf tubes, and using sterile yellow tips, a PCR master mix each sufficient for a 40 sample PCR run was made, as follows :

450µl	dNTPs
450µl	10x PCR Buffer
90µl	25mM $MgCl_2$
90µl	T3 Primer
90µl	T7 Primer
180µl	Water
---	---
1,350µl in Total	
---	---

The tubes were vortex-mixed and immediately frozen at -20°C until required. When a batch of 40 PCR clone amplifications were ready to be performed, the master mix was removed from the freezer and allowed to thaw on ice. Into a sterile suitably labelled 0.5ml Eppendorf tube, kept on ice, 30µl of PCR master mix is added to 14.8µl of sterile distilled water. To this, 5µl of the appropriate clone supernatant (prepared earlier) are added, and the tube is returned to ice for a minimum of 5 mins. 0.2µl of Taq DNA Polymerase is then added, the tube is quickly vortex mixed, and immediately returned to ice. As soon as the last tube receives the Taq enzyme, the whole batch of 40 PCR tubes are immediately inserted into the thermal cycler, and the following cycle is run :

94°C for 2 minutes denaturation, then	***94°C***	***60 seconds***	***Seperation***
	55°C	***90 seconds***	***Annealing***
	72°C	***90 seconds***	***Extension***

For 35 cycles, followed by a final extension at 72°C for 10 minutes

Immediately after the PCR cycle had completed its run, the samples were removed and stored in the -20°C freezer awaiting further processing. Once all 200 clones had their inserts amplified by PCR, and were stored frozen ready for processing, the products could be run down an agarose gel for selection.

Selection of Pure Clones

In a 600ml conical flask, 8.0g of agarose powder was added to 400ml gel buffer to produce a 2%(w/v) gel. The conical flask was then placed inside a microwave oven, and heated on full power for 2 minutes. The flask was removed, mixed vigorously, and returned to the microwave on medium setting until the contents just began to boil. The flask was removed from the microwave, the neck covered with a piece of aluminium foil, and placed in a 50°C waterbath for 20 minutes. During this time the gel plate and combs were washed thoroughly, dried, and masking tape applied to each end to make the gel mould. Once the agarose gel was at 50°C, the foil lid was briefly removed and 20µl of 10mg/ml Ethidium Bromide solution was added. The agar was then carefully but thoroughly mixed, and poured immediately into the gel mould apparatus. The combs were inserted immediately, and the gel was allowed to stand and set for 45 minutes.

The PCR products were removed from the freezer and thawed on ice just prior to use. 5µl of loading buffer were pipetted into 40 sterile 0.5ml Eppendorf tubes. Into the relevant tube 10µl of PCR product was added, with mixing. 300µl of chilled 100% ethanol was added to the remaining 40µl of PCR product, vortex-mixed and immediately returned to the -20°C freezer. The 15µl of PCR product in loading buffer were then loaded onto the agarose gel and run at 150mV for 2/3 the length of the gel. During this time the tubes containing the ethanol, with the remaining DNA, are cold centrifuged at 15,000g for 10 minutes. The supernatant is carefully removed, the DNA pellet is dried under vacuum for 2 mins, and the pellet is redissolved in 40µl of sterile distilled water. The samples are the returned to the -80°C freezer. Once the samples had run two-thirds the length of the agarose gel, the gel plate was removed from the tank and the gel examined in a dark room over a U.V. light. Photographs of the bands in the gel were taken, and the samples with a single DNA band of reasonable size (corresponding to a pure clone) were selected for use in subsequent stages.

Hae-III Digestion of Clone Insert PCR Products

Into 5 sterile 1.5ml eppendorf tubes, and using sterile yellow tips, a *Hae-III* Digest master mix each sufficient for a 40 sample digest run was made, as follows :

450µl	10x *Hae-III* Digest Buffer
450µl	Bovine Serum Albumin
450µl	Water
---	---
1,350µl	Total
---	---

The tubes were vortex-mixed and immediately frozen at -20°C until required. When a batch of 40 PCR clone digests were ready to be performed, the master mix was removed from the freezer and allowed to thaw on ice. Into a sterile suitably labelled 0.5ml eppendorf tube, kept on ice, 30µl of *Hae-III* digest master mix were added to the 20µl of PCR product to be digested. 2µl of *Hae-III* restriction enzyme were then added, the tube is quickly vortex mixed, and immediately returned to ice. The tubes are then incubated at 37°C for 6 hours, to allow complete digestion to take place, then the reaction is stopped by fast-freezing the samples in liquid N_2 and then storing at -80°C. Once all the clones had been digested, and were stored frozen ready for processing, the digest products could be run down an agarose gel for selection, as before. The digest products were removed from the freezer and thawed on ice just prior to use. 10µl of loading buffer was pipetted into 40 sterile 0.5ml Eppendorf tubes. Into the relevant tube 25µl of PCR product were added, with mixing. The remaining 27µl of digest products were then immediately returned to the -80°C freezer. The 35µl of digest product in loading buffer were then loaded onto the agarose gel and run at 150mV for 2/3 the length of the gel. The agarose gel was then removed from the gel tank and examined in a dark room over a U.V. light. Photographs of the bands in the gel were taken, and the sizes of all the bands recorded.

Sau-3AI Digestion of Clone Insert PCR Products

As before, into 5 sterile 1.5ml Eppendorf tubes, and using sterile yellow tips, a *Sau-3AI* Digest master mix each sufficient for a 40 sample digest run was made :-

450µl 10x *Sau-3AI* Digest Buffer
450µl Bovine Serum Albumin
450µl Water

1,350µl in Total

The tubes were vortex-mixed and immediately frozen at -20°C until required. When a batch of 40 PCR clone digests were ready to be performed, the master mix was removed from the freezer and allowed to thaw on ice. The same procedure was then followed as for the *Hae-III* digests, the gels were then examined under U.V. light and photographs of the bands were taken.

Data Analysis, Clone Selection & Sequence Information

The size of each primary PCR product, and the sizes of all constituent bands for each restriction digest were entered onto a spreadsheet. The sizes of the bands were correlated to ensure that the sum of all bands equals the size of the original PCR product. In the absence of a suitable computer program to sort and analyse the data, this task was done manually, and the 25 clones which appeared to be most abundant were selected.

Degenerate PCR Screening of cDNA Library

Complete sequences for molecules with similar haemostatic or biological function were obtained from the international genomic database, and these were then grouped and compared on the DNA-Star MEGALIGN™ software.

The molecular sequences to be compared, were as follows :-

Table 3.2.1: Known Molecular Sequences Examined for Conserved Regions

Functional Activity	Details of Known Sequences Used			
	Peptide	Species	Name	Source
Disintegrin	Trigramin α	Trimeresus gramineus	Indian Green Tree Viper	Venom
Disintegrin	Cytotoxic Factor CF-II	Trimeresus flavoviridis	Habu Snake	Venom
Disintegrin	Disagregin	Ornithodorus moubata	Soft Tick	Saliva
Disintegrin	Trigramin γ	Trimeresurus albolabris	White-Lipped Pit Viper	Venom
Disintegrin	Halysin	Agkistrodon halys blomhoffi		Venom
Disintegrin	Applagin	Agkistrodon piscidorus piscidorus	Eastern Cottonmouth	Venom
Disintegrin	Kistrin	Agkistrodon rhodostoma	Malaysian Pit Viper	Venom
Disintegrin	Batroxostatin	Bothrops atrox		Venom
Disintegrin	Cotiarin	Bothrops cotiara		Venom
Disintegrin	Jararacin	Bothrops jararaca		Venom
Disintegrin	Crotatroxin	Crotalus atrox	Western Diamondback	Venom
Disintegrin	Durissin	Durissus durissus	Central American Rattlesnake	Venom
Disintegrin	Basilicin	Crotilus basilicus	Mexican Westcoast Rattlesnake	Venom
Disintegrin	Cerastin	Crotalus cerastes cerastes	Mohave Desert Sidewinder	Venom

Continued...

Functional Activity	Details of Known Sequences Used			
	Peptide	Species	Name	Source
Disintegrin	Molossin	Crotalus molossus molossus	Northern Black-Tailed Rattlesnake	Venom
Disintegrin	Cereberin	Crotalus viridis cereberus	Arizona Black Rattlesnake	Venom
Disintegrin	Lutosin	Crotalus viridis lutosus	Great Basin Rattlesnake	Venom
Disintegrin	Viridin	Crotalus viridis viridis	Prairie Rattlesnake	Venom
Disintegrin	Echistatin	Echis carinatus	Saw-Scaled Viper	Venom
Disintegrin	Eristicophin	Eristocophis macmahoni	Leaf-Nosed Viper	Venom
Disintegrin	Lachesin	Lachesis muta muta	Bushmaster Snake	Venom
Disintegrin	Barbourin	Sistrurus barbouri	SouthEast Pygmy Rattlesnake	Venom
Disintegrin	Flavostatin	Trimeresus flavoviridis	Habu Snake	Venom
Disintegrin	Rhodostomin	Agkistrodon rhodostoma	Malaysian Pit Viper	Venom
Disintegrin	Triflavin	Trimeresus flavoviridis	Habu Snake	Venom
Disintegrin	Haemorrhagic Metalloproteinase HR1B	Trimeresus flavoviridis	Habu Snake	Venom
Disintegrin	Atrolysin-E	Crotalus atrox	Western Diamondback	Venom
Disintegrin	LAPP (Leech Anti-Platelet Protein)	Haementeria officinalis	Mexican Leech	Saliva
Disintegrin	Mambin	Dendroaspis jamesoni kaimosae	Eastern Jameson's Mamba	Venom
Disintegrin	Toxin S5C1	Dendroaspis jamesoni kaimosae	Eastern Jameson's Mamba	Venom
RGD	Endoglin CD105	Homo sapiens	Humans	Membrane-linked
RGD	Endoglin CD105	Sus scrofula	Pig	Membrane-linked
RGD	Endoglin CD105	Galus galus	Chicken	Membrane-linked
TXA_2 Receptor	TXA_2 Receptor	Rattus norvegicus	Common Rat	Membrane-linked
TXA_2 Receptor	TXA_2 Receptor	Bos taurus	Bovine	Membrane-linked
TXA_2 Receptor	TXA_2 Receptor	Cercopithecus aethiops	African Green Monkey	Membrane-linked
TXA_2 Receptor	TXA_2 Receptor	Homo sapiens	Human	Membrane-linked
TXA_2 Receptor	TXA_2 Receptor	Mus musculus	Mouse	Membrane-linked
C4b-BP	C4b Binding Protein	Bos taurus	Bovine	Plasma
C4b-BP	C4b Binding Protein	Homo sapiens	Human	Plasma
C4b-BP	C4b Binding Protein	Mus musculus	Mouse	Plasma
C4b-BP	C4b Binding Protein	Rattus norvegicus	Common Rat	Plasma
C1-Inh	C1 Inhibitor	Homo sapiens	Human	Plasma
CLI	Complement Cytolysis Inhibitor	Homo sapiens	Human	Plasma
CLI	Complement Cytolysis Inhibitor	Sus scrofa	Pig	Plasma
Decay Accel. Factor	DAF CD55	Cavia porcellus	Guinea Pig	Membrane-linked
Decay Accel. Factor	DAF CD55	Homo sapiens	Human	Membrane-linked
Decay Accel. Factor	DAF CD55	Mus musculus	Mouse	Membrane-linked
Decay Accel. Factor	DAF CD55	Pongo pygmaeus	Orangutan	Membrane-linked
Decay Accel. Factor	Trypomastigote-DAF (T-DAF)	Trypanosoma cruzi	Trypanosoma cruzi	Surface Protein

Cont...

Functional Activity	Details of Known Sequences Used			
	Peptide	Species	Name	Source
MAC Inhibitory Factor (MACIF)	CD59	Aotus trivirgatus	Night Monkey	Membrane-linked
MAC Inhibitory Factor (MACIF)	CD59	Callithrix sp.	Marmoset	Membrane-linked
Complement Control Protein	VV-CCP	Vaccinia Virus	Vaccinia Virus	Surface Protein
Complement Control Protein	HVS-CCP	Herpesvirus Saimiri	Herpesvirus	Surface Protein
Plasminogen	Human Plasminogen		Rhesus Macaque	Plasma
Plasminogen	Human Plasminogen	Homo sapiens	Human	Plasma
Serpin	FXIIa Inhibitor	Bos taurus	Bovine	Plasma
Serpin	CPV-SPI 1	Cowpox Virus	Cowpox Virus	Surface Protein
Serpin	VaV-SPI 1	Variola Virus	Variola Virus	Surface Protein
Serpin	VV-SPI 1	Vaccinia Virus	Vaccinia Virus	Surface Protein
Serpin	VV-SPI 2	Vaccinia Virus	Vaccinia Virus	Surface Protein
Serpin	RPV-SPI 1	Rabbitpox Virus	Rabbitpox Virus	Surface Protein
Serpin	RPV-SPI 2	Rabbitpox Virus	Rabbitpox Virus	Surface Protein
Apyrase	Apyrase	Aedes aegypti	Sandfly	Saliva
Apyrase	Apyrase	Bos taurus	Bovine	Saliva
Apyrase	Apyrase		Electric Ray	?
Apyrase	Apyrase	H.influenzae	H.influenzae	Surface Protein
Apyrase	Apyrase	Homo sapiens	Human	Saliva
Apyrase	Apyrase		Potato	Cellular
Apyrase	Apyrase	Rattus norvegicus	Rat	Saliva
Apyrase	Apyrase	Vibrio cholerae	Vibrio cholerae	Surface Protein
Vasoactive	HMWK-1	Bos taurus	Bovine	Plasma
Vasoactive	HMWK-2	Bos taurus	Bovine	Plasma
Vasoactive	LMWK-1	Bos taurus	Bovine	Plasma
Vasoactive	LMWK-2	Bos taurus	Bovine	Plasma
Vasoactive	HMWK	Homo sapien	Human	Plasma
Vasoactive	LMWK	Homo sapien	Human	Plasma
Vasoactive	HMWK	Rattus norvegicus	Rat	Plasma
Vasoactive	LMWK	Rattus norvegicus	Rat	Plasma

The following degenerate primers were identified as potential candidates for cDNA library screening, however the more 'degenerate' the primer the smaller the probability of a positive PCR match. Due to the expense involved with synthesising the primers, only 3 degenerate primers were actually synthesised and used to screen the cDNA library, the remaining sequences remain to be investigated in further work.

Kazal-type Inhibitors

V	C	G	S	D
GUU	UGU	GGU	UCU	GAC
C	C	C	C	U
A		A	A	
G		G	G	
			AGU	
			C	
5'	-	-	-	3'

Disintegrin

L	C	C	D	Q	C
UUA	UGC	UGC	GAC	CAA	UGC
G	U	U	U	G	U
CUA					
C					
G					
U					
5'	-	-	-	-	3'

Disintegrin

E	D	H	C	Y	Y
GAA	GAC	CAC	UGC	UAC	UAC
G	U	U	U	U	U
5'	-	-	-	-	3'

Disintegrin

E	E	C	D	C	G
GAA	GAA	UGC	GAC	UGC	GGA
G	G	U	U	U	C
					G
					U
3'					5'

Thromboxane A_2

W	N	Q	I	L	D	P	W	V	Y
UGG	AAC	CAA	AUA	UUA	GAC	CCA	UGG	GUA	UAC
	U	G	C	C	U	C		C	U
			U	CUA		G		G	
				C		U		U	
				G					
				U					
3'	-	-	-	-	-	-	-	-	5'

Prostaglandin

Q	P	N	F	Q	Q	D	K	F	L
CAA	CCA	AAC	UUC	CAA	CAA	GAC	AAA	UUC	UUA
G	C	U	U	G	G	U	G	U	G
	G								CUA
	U								C
									G
									U
3'	-	-	-	-	-	-	-	-	5'

Platelet Factor 4

L	Y	K	K	I	I	K	K	L
UUA	UAC	AAA	AAA	AUA	AUA	AAA	AAA	UUA
G	U	G	G	C	C	G	G	G
CUA				U	U			CUA
C								C
G								G
U								U
3'	-	-	-	-	-	-	-	5'

FXa-Inhibitor Binding Site

K	P	G	N	Q	N
AAA	CCA	GGA	AAC	CAA	AAC
G	G	G	T	G	T
	C	C			
	T	T			
5'	-	-	-	-	3'

Apyrase

L	G	N	H	E	F	D
UUA	GGU	AAU	CAU	GAA	UUU	GAU
G	C	C	C	G	C	C
CUU	A					
C	G					
A						
G						

Disintegrin

G	E	E	C	D	C	G
GGA	GAA	GAA	UGC	GAC	UGC	GGA
C	G	G	U	U	U	C
G						G
U						U

Vasodilator

K	R	P	P	G	F
AAA	CGU	CCU	CCU	GGU	UUU
G	C	C	C	C	C
	A	A	A	A	
	G	G	G	G	
	AGA				
	G				

The sequence **LGNHEFD** was noticed to be totally conserved in 100% of ***apyrase*** amino-acid sequences examined, and **also** totally conserved in 100% of the ***5'-nucleotidase*** sequences. Given that apyrase and the 5'-nucleotidase enzymes have a similar role, that being the removal of phosphate groups from nucleotides (such as the reaction ADP → AMP + Pi). It would therefore seem very likely, given that this sequence is *absolutely conserved* from every sequence examined, that this corresponds closely to the activity of these enzymes. Despite the sequence being highly degenerate, and hence more unfavourable for the synthesis and utilisation of degenerate primers, the absolute conservance which was observed with this particular sequence combined with the observations that significant anti-platelet activity could be demonstrated from the salivary glands of *S.calcitrans*, made primer synthesis a worthwhile venture. Degenerate primer sequences for the *FXa Inhibitor*, and the *Vasodilator* were also synthesised, and these 3 degenerate primers were used in PCR screening of the cDNA library. The PCR conditions were as follows:

Table 3.2.2: PCR Reaction Tubes

#	Master Mix I	cDNA Template	H_2O (µl)	Primers (µl)					Final Volume
				T3	FXa	VD	Apy	T7	
1	20µl	1.0	27	1.0	1.0	-	-		50µl
2	20µl	1.0	27	1.0	-	1.0	-		50µl
3	20µl	1.0	27	1.0	-	-	1.0		50µl
4	20µl	1.0	27		1.0	-	-	1.0	50µl
5	20µl	1.0	27		-	1.0	-	1.0	50µl
6	20µl	1.0	27		-	-	1.0	1.0	50µl
7	20µl		28	1.0				1.0	50µl
8	20µl		28	1.0	1.0	-	-		50µl
9	20µl		28	1.0	-	1.0	-		50µl
10	20µl		28	1.0	-	-	1.0		50µl
11	20µl	1.0	27	1.0	-	-	1.0		50µl
12	20µl	1.0	27	1.0	-	1.0	-		50µl
13	20µl	1.0	27	1.0	1.0	-	-		50µl
14	20µl	1.0	27		-	-	1.0	1.0	50µl
15	20µl	1.0	27		-	1.0	-	1.0	50µl
16	20µl	1.0	27		1.0	-	-	1.0	50µl
17	20µl		30						50µl
18	20µl	1.0	27	1.0				1.0	50µl

Key
1-3 Test reactions with T3 primer
4-6 Test reactions with T7 primer
7-10 Negative control, primers no template
17 Negative control, no primers no template
11-13 Test reactions (duplicate) with T3 primer
14-16 Test reactions (duplicate) with T7 primer
18 Positive control T3 & T7 primers

The PCR Reaction conditions were as follows :-

Table 3.2.3: PCR Master Mix

Reagent	Master Mix I	Master Mix II
x10 Promega PCR Buffer	100µl	100µl
dNTPs	100µl	100µl
Apyrase Primer	-	20µl
T3 Primer	-	20µl
Taq Polymerase	4.0µl	-
50mM $MgCl_2$	40µl	-
H_2O	156µl	160µl

Table 3.2.4: Optimisation of Template/Mg^{2+} for the Apyrase Reaction

#	cDNA Neat	Volume (µl)				Total (µl)
		Master Mix II	H_2O	50mM $MgCl_2$	Taq Polymerase	
1	1µl	25µl	23.8	0.0	0.2	50
2	1µl	25µl	23.3	0.5	0.2	50
3	1µl	25µl	22.8	1.0	0.2	50
4	1µl	25µl	22.3	1.5	0.2	50
5	1µl	25µl	21.8	2.0	0.2	50
6	1µl	25µl	21.3	2.5	0.2	50
7	1µl	25µl	20.8	3.0	0.2	50
8	1µl	25µl	20.3	3.5	0.2	50
9	-	25µl	21.3	3.5	0.2	50
#	cDNA 1/100	Volume (µl)				Total (µl)
		Master Mix II	H_2O	50mM $MgCl_2$	Taq Polymerase	
10	1µl	25µl	23.8	0.0	0.2	50
11	1µl	25µl	23.3	0.5	0.2	50
12	1µl	25µl	22.8	1.0	0.2	50
13	1µl	25µl	22.3	1.5	0.2	50
14	1µl	25µl	21.8	2.0	0.2	50
15	1µl	25µl	21.3	2.5	0.2	50
16	1µl	25µl	20.8	3.0	0.2	50
17	1µl	25µl	20.3	3.5	0.2	50
18	-	25µl	21.3	3.5	0.2	50

3.3 Results

Of the 300 clone samples obtained from the plates, 160 were selected as suitable for further testing, and these were then digested with *Hae-III* and *Sau3AI* restriction enzymes. The results for the *Hae-III* and *Sau3AI* digests are best viewed together and in association with the complete (undigested) PCR product. In both cases the results of the preliminary digestion were satisfactory, complete digestion was achieved in all cases, and the bands produced were all accountable to the original template.

Table 3.3.1: The combined results of the *Hae-III* and *Sau3AI* digestions bands (b.p.)

cDNA Sample		PCR	*Hae-III* Digest of PCR Product								*Sau3A1* Digest of PCR Product							
No.	Clone	Product	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Unseen	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Unseen
1	FD	427.6	343	84						0	272.5	155.1						0
2	FE	705.3	552	153						0	408.2	253.7	43.4					0
3	FF	954.0	756	198						0	755.7	198.3						0
4	FG	229.0	178	52						0	183.0	46.0						0
5	FH	760.5	618	142						0	301.5	233.8	164.9	60.3				0
6	FI	618.3	510	108						0	510.4	107.9						0
7	FJ	650.0	618	32						0	389.9	224.0	36.1					0
8	FK	1085.2	957	129						0	314.6	233.8	536.8					0
9	FL	198.8	117	82						0	139.2	59.6						0
10	FM	386.7	299	88						0	311.9	74.8						0
11	FN	752.7	718	35						0	250.4	250.4	155.0	96.9				0
12	FO	737.1	648	90						0	411.1	249.1	76.9					0
13	FP	171.9	91	81						0	110.7	61.2						0
14	FQ	411.1	335	76						0	411.1							0
15	FR	481.0	177	101	71	71	62			0	432.4	48.6						0
16	FS	1345.5	1250	95						0	667.8	281.9	238.5	157.3				0
17	FT	349.7	282	68						0	281.9	67.8						0
18	FU	188.6	101	88						0	117.7	70.9						0
19	FV	328.0	255	73						0	268.7	59.3						0
20	FW	823.1	481	342						0	344.4	255.2	153.1	70.4				0
21	FX	379.2	312	67						0	312.3	66.9						0
22	FY	183.8	100	84						0	125.2	58.6						0
23	FZ	462.0	405	57						0	387.5	74.5						0
24	GA	270.0	191	79						0	212.0	58.0						0
25	GB	354.6	283	72						0	282.8	71.8						0
26	GC	179.1	96	83						0	121.7	57.4						0
27	GD	169.0	88	81						0	104.5	64.5						0
28	GE	466.7	393	74						0	410.1	56.6						0
29	GF	428.7	350	79						0	379.6	49.1						0
30	GG	694.6	615	80						0	395.4	258.6	40.6					0
31	GH	567.5	465	103						0	321.5	185.0	61.0					0
32	GI	752.7	641	112						0	436.6	244.6	71.5					0
33	GJ	527.8	437	91						0	198.4	150.8	89.3	89.3				0

Continued...

cDNA Sample		PCR	*Hae-III* Digest of PCR Product								*Sau3A1* Digest of PCR Product							
No.	Clone	Product	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Unseen	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Unseen
34	GK	298.3	216	82						0	234.8	63.5						0
35	GL	189.5	104	86						0	124.8	64.7						0
36	GM	705.6	465	147	94					0	411.1	260.1	34.4					0
37	GN	925.1	260	199	199	140	128			0	504.2	394.7	26.2					0
38	GO	2803.9	394	394	394	394	394	278	278	278	527.5	527.5	438.4	367.5	331.4	236.6	199.8	175
39	GP	199.8	131	69						0	145.7	54.1						0
40	GQ	199.8	131	69						0	145.7	54.1						0
41	GR	383.9	318	66						0	338.8	45.1						0
42	GS	604.0	464	140						0	435.4	168.6						0
43	GT	191.0	111	80						0	144.4	46.6						0
44	GU	408.7	160	160	88					0	318.4	90.3						0
45	GV	529.4	446	84						0	242.2	120.4	55.6	55.6	55.6			0
46	GW	445.5	353	93						0	275.7	78.1	91.7					0
47	GX	499.9	397	103						0	189.7	189.7	120.5					0
48	GY	374.0	259	115						0	293.6	80.4						0
49	GZ	903.1	853	51						0	903.1							0
50	HA	722.3	685	37						0	481.3	241.0						0
51	HB	244.9	161	84						0	179.8	65.1						0
52	HC	510.4	426	85						0	255.9	254.5						0
53	HD	588.7	487	101						0	407.4	181.3						0
54	HE	465.7	390	76						0	277.0	150.5	38.2					0
55	HF	925.9	844	82						0	499.8	426.1						0
56	HG	1125.9	1019	107						0	387.4	248.5	184.8	184.8	120.4			0
57	HH	421.9	249	173						0	272.6	149.3						0
58	HI	1578.2	841	737						0	466.8	367.5	367.5	313.1	63.3			0
59	HJ	769.9	313	277	180					0	339.3	244.6	186.0					0
60	HK	1400.4	1323	78						0	1186.5	213.9						0
61	HL	389.5	311	78						0	241.1	128.0	20.4					0
62	HM	647.7	325	222	101					0	448.9	158.7	40.1					0
63	HN	448.9	356	93						0	298.2	150.7						0
64	HO	728.8	356	241	132					0	298.2	298.2	132.4					0
65	HP	176.5	103	73						0	119.3	57.2						0
66	HQ	781.9	497	177	109					0	565.0	216.9						0

Continued...

cDNA Sample		PCR	*Hae-III* Digest of PCR Product								*Sau3A1* Digest of PCR Product							
No.	Clone	Product	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Unseen	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Unseen
67	HR	476.5	389	88						0	284.1	155.7	36.7					0
68	HS	224.7	156	69						0	176.5	48.2						0
69	HT									0								0
70	HU									0								0
71	HV	600.4	551	49						0	551.0	49.4						0
72	HW	903.6	775	129						0	539.2	121.5	121.5	121.4				0
73	HX	379.1	295	85						0	340.6	38.5						0
74	HY	1321.4	1321							0	1019.3	151.0	151.1					0
75	HZ	447.3	354	94						0	236.8	151.0	59.5					0
76	IA	977.2	836	141						0	343.7	297.8	250.8	84.9				0
77	IB	704.3	446	162	96					0	369.7	134.1	134.1	66.4				0
78	IC	418.8	316	103						0	336.7	82.1						0
79	ID									0								0
80	IE	194.0	109	85						0	128.8	65.2						0
81	IF	737.7	477	87	87	87				0	391.1	268.0	78.6					0
82	IG	212.0	136	76						0	150.8	61.2						0
83	IH	783.8	557	151	76					0	476.6	255.8	51.4					0
84	II	695.4	621	75						0	453.2	175.0	67.2					0
85	IJ	640.7	551	90						0	524.9	115.8						0
86	IK	334.7	249	86						0	270.6	64.1						0
87	IL	219.2	135	84						0	155.1	64.1						0
88	IM	1259.7	1260							0	477.6	294.5	238.5	184.8	64.3			0
89	IN	334.4	244	91						0	274.2	60.2						0
90	IO	813.9	529	156	130					0	464.3	263.5	86.1					0
91	IP	960.2	635	184	142					0	377.3	263.5	176.3	143.1				0
92	IQ	763.2	484	167	112					0	265.6	181.2	316.4					0
93	IR									0								0
94	IS	820.0	615	109	96					0	523.9	247.2	48.9					0
95	IT	439.8	355	85						0	370.5	69.3						0
96	IU	872.0	753	119						0	479.4	155.0	120.3	117.3				0
97	IV	685.1	299	299	88					0	685.1							0
98	IW	403.6	299	105						0	325.7	61.0	16.9					0
99	IX	789.9	501	150	139					0	459.1	239.4	91.4					0

Continued...

cDNA Sample		PCR	*Hae-III* Digest of PCR Product								*Sau3A1* Digest of PCR Product							
No.	Clone	Product	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Unseen	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Unseen
100	IY	597.4	501	97						0	421.3	101.7	74.4					0
101	IZ	267.9	189	79						0	208.7	59.2						0
102	JA	671.3	631	40						0	230.7	170.2	270.4					0
103	JB	817.7	818							0	528.8	170.2	118.7					0
104	JC	1104.5	1105							0	560.1	267.9	230.7	45.8				0
105	JD									0								0
106	JE	671.6	409	182	81					0	390.4	249.3	31.9					0
107	JF	449.0	356	93						0	373.0	76.0						0
108	JG	494.2	409	85						0	311.4	150.3	32.5					0
109	JH	203.4	121	83						0	133.7	69.7						0
110	JI	960.9	961							0	960.9							0
111	JJ	312.1	232	80						0	252.5	59.6						0
112	JK	311.9	218	94						0	250.4	61.5						0
113	JL	2685.9	752	570	437	366	281	280		0	1540.8	673.7	471.4					0
114	JM	182.6	91	91						0	116.4	66.2						0
115	JN	718.6	221	212	143	142				0	526.9	114.2	77.5					0
116	JO	916.7	790	127						0	382.0	382.0	76.4	76.3				0
117	JP	185.9	108	78						0	120.4	65.5						0
118	JQ	686.2	465	155	67					0	393.9	261.8	30.5					0
119	JR	526.9	428	99						0	427.6	99.3						0
120	JS	185.9	102	84						0	126.9	59.0						0
121	JT	681.8	461	151	70					0	409.6	272.2						0
122	JU	555.4	387	168						0	434.1	121.3						0
123	JV	934.8	461	346	128					0	934.8							0
124	JW									0								0
125	JX	538.0	456	83						0	481.2	56.8						0
126	JY	455.5	368	88						0	387.8	67.7						0
127	JZ	538.0	314	166	58					0	314.4	195.5	28.1					0
128	KA	217.8	139	79						0	156.3	61.5						0
129	KB	415.7	334	82						0	183.8	30.9	57.4	76.6	67.0			0
130	KC	415.7	321	95						0	283.3	132.4						0
131	KD	455.9	380	76						0	307.5	148.4						0
132	KE	524.9	456	69						0	219.2	141.7	101.0	63.0				0

Continued...

cDNA Sample		PCR	Hae-III Digest of PCR Product								Sau3A1 Digest of PCR Product							
No.	Clone	Product	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Unseen	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Unseen
133	KF	477.6	398	79						0	416.5	61.1						0
134	KG	675.2	609	67						0	398.4	238.5	38.3					0
135	KH	325.7	245	81						0	252.7	73.0						0
136	KI	806.2	682	125						0	410.0	396.2						0
137	KJ									0								0
138	KK	504.8	409	96						0	423.0	81.8						0
139	KL									0								0
140	KM	663.9	612	52						0	437.8	226.1						0
141	KN	817.7	594	224						0	344.6	344.6	128.5					0
142	KO	764.0	671	93						0	447.8	158.1	158.1					0
143	KP	715.4	631	84						0	382.0	254.9	78.5					0
144	KQ	560.1	473	87						0	402.5	157.6						0
145	KR	890.7	415	360	115					0	836.1	54.6						0
146	KS	481.5	396	86						0	415.4	66.1						0
147	KT									0								0
148	KU									0								0
149	KV	771.9	698	74						0	240.4	240.4	198.8	92.3				0
150	KW	698.3	606	92						0	380.8	231.5	86.0					0
151	KX	366.1	280	86						0	249.6	116.5						0
152	KY	595.1	504	91						0	595.1							0
153	KZ	447.3	357	91						0	118.2	117.3	86.8	63.2	61.8			0
154	LA	595.1	504	91						0	524.7	70.4						0
155	LB	963.1	525	240	99	99				0	515.8	447.3						0
156	LC	1018.4	622	277	120					0	865.8	152.6						0
157	LD	465.2	384	81						0	398.8	66.4						0
158	LE	347.4	269	78						0	286.8	60.6						0
159	LF	438.3	359	79						0	296.0	87.3	55.0					0
160	LG	453.6	371	83						0	305.6	90.6	57.4					0

Table 3.3.2: The clones selected for DNA-Sequencing :-

	Clones with Banding Patterns which are similar							
	BW CU CV CW DJ	CO DL	AB AC	AI AQ	CR CT BQ	EG EH	ET FB	BY BZ CB CI CN CM
Total # Bands								
Hae-III	2	2	2	2	2	2	3	2
Sau3AI	8	8	2	3	1	2	5	2
% Abundancy	**6.7**	**2.7**	**2.7**	**2.7**	**2.7**	**2.7**	**2.7**	**8.0**

The selected clones were DNA-Sequenced :-

Tube #	cDNA Clone	Sample Conc. (ng/ul)	Fragment Size
1	AB	10	657.5
2	AC	10	657.5
3	AI	5	725.5
4	AQ	5	725.5
5	BQ	5	797.9
6	BW	5	1040.9
7	BY	5	999
8	BZ	5	616.9
9	CB	10	441.9
10	CI	15	545.2
11	CM	10	475.3
12	CN	10	497.4
13	CO	5	627.1
14	DJ	5	750
15	DJ	5	750
16	DL	5	724.5
17	CR	7.5	780.5
18	CT	7.5	514.7
19	EG	10	189.3
20	EH	10	189.3
21	ET	7.5	1456.4
22	FB	7.5	1456.4
23	CU	7.5	716.4
24	CV	7.5	503.3
25	CW	15	503.3

The primers used for the DNA sequencing were specific for the T3 & T7 sites on the vector used for the cloning, hence the sequences obtained would include the whole of the insert (cloned) DNA, plus some flanking sequences of the vector phage. These flanking sequences of the vector are then identified, and removed, to leave the DNA sequence for the cloned insert.

The DNA sequences obtained were then submitted to the TBLASTX 2.2.1 database[127] to search & compare with known sequences, and obtain data on comparisons. The sequences, and the outcome of the database searches are listed overleaf.

The sequences for the cloned cDNA inserts, with the *forward* (T3) primer, were :-

Phil-01 T3 Primer
163 bases.
No matches found on BLASTx & SwissProt.
TTTGGAAACCCTTTGGANTTTCACNCCCTNTTACANGCCTACACTATTNGANNCNAATNATTCGGNTCCAGGCTTCATGGGCAATCCAGAGGAATGTTGTN
ATGTTNACNTATTCCGGGCNTTTANTGNGCGATTAANTCAAAGATAGANNAANGCNATATCT

Phil-01 T7 Primer
799 significant bases.
No matches found on BLASTx & SwissProt.
TCGTNATACCCTTTTGGNGAGCCGTANCTGNTNCCCCACCCGGGCGGAAAATCGATGGGCCCGCGGCCGCTCTATAAGTACTCTCTAGTTTTTTTTTTTTT
TTTTTTTTTTTTNAGGNNCCNAANNNAANTTTTTGNNCNNTTTNAAAAANCCCANTTAATTTTNNTNTTTNNNNNNNNNAANTNCCTTTTTTTNNCCNN
AAAAAANTAAAACCCTTTTNNGNNCCNNTTTTTTNNNNGGGGGGGNNCCNAANNGGGGNAANTTTTTTTTNNNCCCNGGGGGCCCCCCNATTTTTT
TTTTANANNNGGGGGGCATANNGGGGGGGNCCCCNNTTTNNGGGGGGGGCANANTNNCNNCCCCCCAAAAANNNTTTGNGGGGNTCCCCAGGNC
CCGGGGNGGGGGNNCACCCCCCCCCCCCCCCNGNTTTTTNNGNNTNCCCCCCNCTAANNNNGGGAANNTNNNANNTTNNGCNGGCCNCNTTNNACT
TTTTGGGGGGGTTTTCNNNAAANAAATTTNCCTTTNANCNTACCCCNGGNATNCNNACGGCCCCNCCATTTNGGGNNCGNGTTTTTTGGAANNAAN
NTNTCACNACCCCCCTTTNTTTTCGGCCCCNAAAAAAACCCCTGGGNCCCNAANTTTTTTTGNNNNCNCNCNNANGTGNGGCNCCCNGGGGGGGGG
NNCCNNACCNNNNANANTTTTTTTGTCCCNTTTTNNNGNGGGGGGTTNTTNCACCCCTCTCGCCNCCTCCTNCCGGANCTTCCCTCCCNNTANNCC
NNCTCTCTCCCGCATTATTTNTTNCANCNGCTTCNTTCCNGNTCNNNCTAACTTNGNTGCNNNTTNCNTNTTCNGCCCNCCNCGNTCNCNNNTTCTT
CCGATNCGNTTCNACNNNC

Phil-02 T3 Primer
474 significant bases.
Venom Allergen 5 (type 3) from Bald Faced Hornet.
NTGAAACCTTTGNTGGAGCTCGCGCGCNTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACGAGGGGCAATACCAAGGATGTTGACATGTTGAC
AAAAGCCGTCAATATGTGGTACGATGAAGTCAAGGATAGTAGAATGGAATATATTAACAAATACCCCAAAAGCTATAGTGGACCGGCAATTGGACAT
TTCACTGTTATGGTGGCTGATCGCAATATCCGTGTGGGTTGCGCTGCTGCCACCTACCCCGAACCTGGACAACCCTACAATGCTTATTTGGTTGCC
TGCAACTATGCCACCACCAACATGATGGACCATCCCATCTATGCCTCCTGTCCTAAAGCAACTACTGGTTGCACCACTGGATCAAACAAAAATTTCC
CCAACTTGTGCTCCCCCAACGAGAAATACGATGTCAACAAATGGTTTTAATTATTTTATGACTAAATATAGGATATTGTATGATGATATGAGAAATTG
AATTGTGTTTATTAAAATTGTCAAATAATTTANAAAAAAAAAAANNNNNNNNNNNNNNNNNCCTTNGNGNTNTTTTTAAGGGGNGNGGGGCCCCTTTTT
TTTCNCCCCGGGGGGGGGCCCCCGNAANGNCCCCCATTNCCCCTTTANGNGGGGNNNNNTNANNNNNNNNNNNNNNNNNNC

Phil-02 T7 Primer
857 significant bases.
No matches found on BLASTx & SwissProt.
TTTNGAAACCCTTTTGGNGTNTNATACCCTTTNGCCCCCCCGGCCGGTTNAATCAATGGGCCCGCGGCCGCTCTATAAGTACTCTCTAGTAATTNCTTTTT
TTTTTTTTTTTTTTTGGGGAAAAATTNGGGGGNAATTTTAAAAANNNNNTCTAATTNCNNTNNNTATTTAANAAANTNNCTTTTTTTNCCNNAAAAAN
AANAAANCCATTTTNGGCNNNCNNTTTTTCTNGGGGGGGGGNCNCNCCCGGGGNAAATTTTTTTTTTTCCANCGGGGGCNCCCCNNTNTTTTTTTT
NNNNNNGGGGNNNNANGNGGGGGGCCCCCNTTTTNNGNGGGGGGGNNTTTTNTCNCGCCCCCNNAAAAAAATTTTTTNGGGGNGNCNCNCCCNCC
NGGGGGGGGGGGGAANCCCCCCCCCCCCCCCTNNTTTTTTTTTNTTACCCCCCCCCCTNNTNAAGNAATTGGNNNNTNTTTCNNNNGGNNCCCAAATA
AANATTTNGGGGGGGGGGNGTNNGNNAAAAAAATNAANTCNTNNCAANTTCCTCGNCNNTTNNGCNGNCCCNCCCNANTATTTTTGAGNNGGGTTTT
TTTCACNCAACANACAAAACNCCNNTCNTNGTNNNGTCCCCCTCACANGCCCCCCNAATTATTNTAAATNNTTNCCCNCNAGNGANNCTCCTATATN
TGGNGGCGGNGNNCNCCCNCCANCTTTTTTCTCTCCTCCNCTCNNAGNGGGGGGNTAANNCANAANNCCGNTCGCGANANCCNCCNGACNNCATC
TCCTNATNACGNCGCCNCANACCCGNNTCGTTTTTCTATCTCCCNNTTANATGGNNNGGANCATCTTATGCNCCCTAATNNNCCNNCCNTGTNGTN
NTCCGTTTTNTCCGTCCTCNTTNCGTATTTCGNCANTTCCNNNATTGCNCTTTNCTGNATCNTCNTTCTANTNCATNTNAGCC

Phil-03 T3 Primer
557 significant bases.
Venom Allergen 5 (type 3) from Bald Faced Hornet.
TNTGAAACCNTTGNTGGTTCTCGCCCTCTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACGAGAAAATGGCGCATGATAAATGTCGCAACACCA
ACAAATTCAAGTACTCTGGGCAAAATTTGGCCTGGATGGGCTTCATGGGCAATACCAAGGATGTTGACATGTTGACAAAAGCCGTCAATATGTGGT
ACGATGAAGTCAAGGATAGTAGAATGGAATATATTAACAAATACCCCAAAAGCTATAGTGGACCGGCAATTGGACATTTCACTGTTATGGTGGCTGA
TCGCAATATCCGTGTGGGTTGCGCTGCTGCCACCTACCCCGAACCTGGACAACCCTACAATGCTTATTTGGTTGCCTGCAACTATGCCACCACCAA
CATGATGGACCATCCCATCTATGCCTCCTGTCCTAAAGCAACTACTGGTTGCACCACTGGACCAAACAAAAATTTCCCCAACTTGTGCTCCCCCAAC
GAGAAATACGATGTCAACAAATGGTTTTAATTATTTTATGACTAAATATAGGATATTGTATGATGATATGAGAAATTGAATTGTGTTTATTAAAATTG
TCAAATAATTAAAAAAAAAAAAAAAAAAACTCGAANGTTCTTTTAAAGCGGCGGGGGCCCATNTATTTTCCCCCCCGGNGGGGGNCCAGGTAAGTGTCCC
CAATTTCCCCTTTAAGGGGGNNNNNTNNANNNNNNNNNNNNNNC

Phil-03 T7 Primer
631 signficant bases.
No matches found on BLASTx & SwissProt.
AGAAGNNNNNNNNNNNGTTTGNNNCCNNNNNNNTACNNATACCTNTGAATACNCTTACCTGTTCCCCACCCGGGNGGAAAATCGATGGGCCCGCGGCCGC
TCTAGAAGTACTCTCGAGTTTTTTTTTTTTTTTTTTAAANAATTGGNNAATTTTAAAAAANNNAATTNAATTTNTNATNTNATNANANAANATCCTATNT
TTAGNCANAAAAAAATNAAAACCNTTNGTNGANATCGNNTTTNTNGTNGGGGGANCNNAANTNGGGNAAATTTTTNTTNGGCCCAGGGGGGCANCC
ANNATTTNTTTTAGNANAGGNGGCNNAAANGGGANGGCCCATNNTGTNGGGGGGGGGNANANTTGCNGGCANCCAAANAANCNTTGNGGGGTTNNC
CAGGTTCGGGGNAGGGGGNANNANCCCACCCCANNCGGNTTTTGGNATCACCCCCCNTAANAGGGAAANGTCCAATTGCNGGCCCACANTANNTT
TNGGGGNNTTTGTAAAAAAATNCCATTNNACNATCCTNGACTTAATNGGCCCANATATTGACGGTTTTTGTNAAAATGTAAANANCCTTGGTNTTGC
CCATNAANCCCCTCCGGCCAAATTTTGCCCAAAAAACTTNAATTTGNGGGGGGGGGNGAAAATTTATAATGCCCCCTTTTTTTTGGCCNAATTTTTTG
AACCCCAAAGGGGGAACCTGGANGGGCCCNANCCCCAACNTTTTGTCCCTTTTAANGGGGGGGGGTNATANNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNC

Phil-04 T3 Primer
568 significant bases.
Venom Allergen 5 (type 3) from Bald Faced Hornet.
NNNNNTANTGAAACCNTTGCTGGAGCTCGCGCGCNTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACGAGGTTCGCCAATGCAAAATGGCGCA
TGATAAATGTCGCAACACCAACAAATTCAAGTACTCTGGGCAAAATTTGGCCTGGATGGGCTTCATGGGCAATACCAAGGATGTTGACATGTTGACA
AAAGCCGTCAATATGTGGTACGATGAAGTCAAGGATAGTAGAATGGAATATATTAACAAATACCCCAAAAGCTATAGTGGACCGGCAATTGGACATT
TCACTGTTATGGTGGCTGATCGCAATATCCGTGTGGGTTGCGCTGCTGCCACCTACCCCGAACCTGGACAACCCTACAATGCTTATTTGGTTGCCT
GCAACTATGCCACCACCAACATGATGGACCATCCCATCTATGCCTCCTGTCCTAAAGCAACTACTGGTTGCACCACTGGACCAAACAAAAATTTCCC
CAACTTGTGCTCCCCCAACGAGAAATACGATGTCAACAAATGGTTTTAATTATTTTATGACTAAATATAGGATATTGTATGATGATATGAGAAATTGA
ATTGTGTTTATTAAAATTGTCAAATAATTAAAAAAAAAAAAAAAAAAACTCGANAGTCTTTTAAAGCGGCCGGGGGCCCATTGATTTTNCCCCCGGGNGGG
GTNCCCGGTAAGTGTCCCCAATTTTCCCCTTANNGGGNNNNNNNNTNANANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNN

Phil-04 T7 Primer
896 bases (total).
No matches found on BLASTx & SwissProt.
TNGNGATACCCTTTGGAATTTNNATACCCTTTTTNCCCACCCGGCCGNTTAAATNGATGGGCCCGCGGCCCGCTCTATAAGTACTCTCNNGTNNTTTCTTT
TTTTTTTTAANNNTTNGNNNATTTTAAAAAGGCNCAATTNAATTTCNNANTTCACCNCCCAAAANCCTNTNTTTAGCCCNAAAAAAANTAAAACCNTTTNN
TGACNTCNTTTTTNTCNTNGGGGGNCCNNAANTNGGGNAAATTTTTNNTNGGNCCNNGGGNGCANCCNNGANTTNNTTTANGANNGGNGGNNTAAANG
GGNNGGNCCCTNNNNNTGGGGGGGGGNTNTTTCGCNGNCNNCCNAAAAANCNTTTAAGGGNTTTCCANGNTNGGGGNAGGNGGGNAAATNCNNCCCNN
CCGGNTTTTGGGAACATCCCCCAAAANANGNAAATNNCCAATNGCNGGCCCCCTTTAANNTTTNGGGGGNTTTTNTTNAANANATCNCANTTTAAANNCN
CTTGNNCNTNTTCGGGNCCNNAATTTTGNGCNNCNTTTTTNCNCNNTTTTNAANCNCNCTTGGNAATTTCCCCCNNGANAAACCCCCCCCCGGGGCCCCA
ANTTTTNCCCCCAAAAATATCTTTNNAANNTTTTTTTGNGGGGTNCCNNACTTTTTTNATGCNCCCNTTTTTTTTTNNTTNTNGGCAAACCCTNTGGGCCCNA
AATTTTTTGGGNTCCNCNAATGGGGGCNCCCNTTGCAAGGCCCCCCNANNCCCCNACCNTTTTTTTTCCCTTTNTANAGGGGGGTGNAATTNTANACCCNC
CCCCCGCCTNTAACAACTCCTACNTAGCTCNTNGTCTCCCTNCNNCNNNTNTCCAAACCANNNTCTTCCATNGTCANTCNCTCNNNCNCCNCNCC

Phil-05 T3 Primer
507 *significant* bases.
CYTOCHROME C OXIDASE POLYPEPTIDE III from Drosophila.
TTTNNAAACCCTTTGGGAGGTTCCCGCCCTCTTGCAGGNCGACACTAGTGGATCCAAAGAATTCGGCACATTTTAACAGNTNATCNTTGATGACGAGAT
GTNTCNCGTGAAAGTACNTTTCAAGGACTNGATACTTTANCTGNNACTACAGGATTACCATGAGGAATAATTTTATTTATTCTATCTGAAGTTTTATN
TTTTGNTTCTTTNTTTTGAGCTTTTNTTCATAGAAGTTNATCTCCTTCAATTGAACTTGGAGCANTANGACCTCCNATNGGAATNACTCCNTTTAATC
CTTTTCAAATNCCTTTNNTANATNCAGTAATTCNATTAACATCANGAATTACCANCTACATGAGCTCATCATAGGCCNAATAAGAAAATAATCATTCA
CAANCTACTCAAGGATNATTTTTTACAGGGNTATTAGGGGTATATTTTACANTACTTCANGCANATGAATATATTGNANCTCCCTTTACTATTGCTGA
NTCNGTTTNTGGGGNCTCCATTNTNTTATAACCACAGGATTACATGGACTTNGTGTTTTAATGGNGAACAACNTN

Phil-05 T7 Primer
731 bases.
CYTOCHROME C OXIDASE POLYPEPTIDE III from Drosophila.
TTTGANACCCNTTGGAATAACAGTTCTCCNGGANACCCNTACCTGTTTCCCCACCCGGNNGGAAAATCGATGGGCCCGCGGCCGCTCTAGAAGTACTCTCT
AGTTTTTTTTTTTTTTTTTTTTAAAAAAAAAAANCCNCCNCNTNAANAAATNGNNCCNNAAGGGAANAANCAANCANTTTCANCAAANNGTCANTTTNAAN
CNGNNGTTTNAAATCCAANGGGNNNNTTTNTNGAAANGGGNTTTTTTAAGGGNCNANTTAANCNTNCTANNAAAAANGNNGTNCCANTTAANNCNT
NAAGCCCATNAAANCCTNTNGNTTTAAAAAANGTAACCCCNTAANCAAAATCNNCATTNNNAAANGGGNCTTCAANNTNTTNNNNTNCTNGAAGTTT
TGAAAAANNNCCCCCTAANAANNNNGNAAAAAANAANCCTTGAGNANTTNGGNAANNATTNTTTTNTTTTAAACTTTGNGGNNCNCNNGTNNCNGG
AATNCCNGNGGTNAAAAAAANNCCNGTTTTTAAAAANGGATTTNGAAAGGGTTTAAANGGGGTNTTTCNTTTNGGNGGNNNTTTNGNCCCNAGTCC
AATNGANGGAAANAANCTTTTTTGAAAAAANNCNCNAAAAAAAGAACCCNAANTTTAAAACTTCNNAAAAAAAAAAAAAAAAAATTTTCNTCANCCGNA
ACCCCNGNGGNAACCCNTAAAGGTNNGANGNCCCTTGAAAGGGCCTTTNCCGGGGAAACATTTNGGGNATTTTGGNAAACCNGTTAAAAAGGGGC
CCAAATTTTTTTGGAANCCCTANNGGGGACCCGGGNGGGNCCCGAACCCCNCTTTTTT

Phil-06 T3 Primer
61 bases.
No matches found on BLASTx & SwissProt.
TTAAANACTNTTGTAACAAACCCANNNNNTTTTGGGAAAAAGGNCAAAGGAANNAANNNANNNTATNAATGGGGTTTNAAGGGGGGGNTTTTAAAAAA
ANGNANGANAAG

Phil-07 T3 Primer
761 bases.
ATP SYNTHASE A CHAIN (PROTEIN 6) from Drosophila yakuba.
AANTNTGAAACNCTTGGANGACCCCGCGCGCTTGCAGGACGACCCTAGTGGATCCAAAGAATTCGGCACGAGGGTTGATTAAGCCTATTTATTATTTTC
TCAATCACTTTCATGATTTTTAATATAATAAACTATTATTCATATATTCCTTCTATACCTAAATCTAATTTAATTAACAAAACATACTCTACAAATTCA
TTAAATTGAAAATGATAACAAATTTATTTTCTGTATTTGACCCTTCATCTAGTATTTTTAATCTTTCATTAAATTGATTAAGAACATTTTTAGGAATTT
TAATAATTCCTTCTATATATTGATTAATACCTTCTCGATATCATATTTTCTGAAATAATATTCTTTTAACTCTTCACAAAGAATTTAAAAATTTATTAG
GACCTTTAGGAGCTAATGGTTCTACTTTTATTTTTGNTTCTTTATTTTCAATAATTCTTTTTAATAATTTTATAGGTTTATTTCCTTACATTTTCACTA
GTACTAGTCATTTAACTTTAACTTTAACATTAGCTTTACCTTTATGATTATGTTTTATATTATTTGGATGAATTAATAATACTCAACATATATTTGCTC
ATTTAGTTCCTCAAGGGACTCCTGCAATTCTTATCCATTTATAGTATGNATTGGAACTATTAGAAATATTATTCGCCAGGACCTTTAGCAGTCGATNA
CTGCTAAAATAAATTGCTGGACATTANTACTTACATTCTAGGAAAAACTGGGCCCTCATGGCACTATTTACCAGGATCNTATTATTNCCCAATGGCT
TATAGGTCTAAAATCACTGGGCTNTAATCAA

Phil-08 T3 Primer
421 bases.
No matches found on BLASTx & SwissProt. (Collagen)
ANNNTTTGNCCTTTCTAGNGCCCCTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACCAGGTTCGCACAAATACCTGTCTCTCACGCACTGGCG
TTGAATTGAGAAAGAAAACATGGCAAGAAGGCACCAATCCCAATGCCAAGGTTAGCGTACACATTGAAGGACTTCGCTATGTAGGATATACTCCTTA
CAAACCCGCTGCCAATGGCAAGCCCGCAAAACCAGGAAACCCAGCTAAGACAAAACCACAAAAACCGAATTCCAACTGGAACTTGCCAGATTCCAA
TGACGATGATAATGATGCATTTAATAGGGCTGTTGATGAACTTGCCAAGGATTTTGATTTTTAATACTTACACCTTAGCAAAAGAACCTTAAAGCTTT
AATGTTCCAATTGAAAAGAACTAACATCTTTGTAACAAAAGCTTAATAAAACAATTTTAAAATCCAAAAAAAAAAAAAAAAAAANCTCGNNNGTNNTTTT
ANNGNGGNCGGGGNCCCNTCGTTTTTCCCCCCGGGNGGGGCCCNGGTAAGNGTCCCCANTTCCCCCTTTAGGGGGGNNGTATNNANNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNC

Phil-09 T3 Primer
237 bases.
No matches found on BLASTx & SwissProt.
NNCGAAACCCTTGNAGGACCCCGCGCGCTTGCAGGTCGACNCTAGTGGATCCAAAGAATTCGGCACGAGGGCAATGATAAATGGCTTACTATGTTGCA
TACAAAAATGTTGTAAAAAATTTAAATATATCAGTAATTAATCAATATATTTTTTTATATTTTTGTGCCTTCTTCGTGAATTAATCTATTAGGTTGCCC
AAAAAGTAATTGCGGATTTTTTAAAAGAAAGTAAATGCATTTTTAATAAAACTTAGAATGAACTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACCC
CNGGGGGNNNTTTTANNGGGGNNNGGGNCCCCNTNTTTTTCCCCCCNGGGGGGGNCCCCGGNAAGGGCCCCCNNNNCCCCCNNNNGGGGGGGGNTTNA
NNANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

Phil-10 T3 Primer
509 bases.
No matches found on BLASTx & SwissProt.
TTGAANCCCTTGCNGTGCTCGCGCGCCTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACCAGGAGAGAGAGAGAGAGGAACTAGTCTCGGGTT
TTTTTTTTTTTTTTTTTTTTTTTTTTTNGGNAAANCCNNTTTTTTTTNNTNAAAAANCCCNAAANCCTTTNNAANAAATTTTTAAATTTTTNGGTTTNNN
TTTTTTTNNTNAAANAAAAAAAATTTTTNNTTTTNNTTNNNGCCCCCNNAAAAAAAAANCCTTTTTTNNCCNTGNANTTAATTTTTNNTTTTTTNGNNG
GGGGGGNAATTTAAAAAAAAAAAANCCCNAAGGGGGNATTTTTTTTCNNCCCCCTTTTTNAAANNNNNNNGGAAAAAAAAANANCCCNNCCCNCCCN
NAAAANTNCCCCCCCNTNNNNGGGGGNCCCCCCCCCCNNGGCCNNTTTTAAGGGGGGGGNNNNAAAAANNTTTTTTNAANNNNNNTTTTNANNNNN

NNNGGGNCCCCNTTTTTTTNCCCCCNGGGGGGGNCCCCGGGAANGGNCCCCCNNTCCCCCNTNNGGGGGGGNNTTACNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNC

Phil-11 T3 Primer
286 bases.
No matches found on BLASTx & SwissProt (MUCIN 2 PRECURSOR).
TCTGAANCCCNTTTGAANGACCCCGCGCGNTTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACGAGGGACAACCCTACAATGCTTATTTGGTTG
CCTGCAACTATGCCACCACCAACATGATGGACCATCCCATCTATGCCTCCTGTCCTAAAGCAACTACTGGTTGCACCACTGGACCAAACAAAAATTT
CCCCAACTTGTGCTCCCCCAACGAGAAATACGATGTCAACAAATGGTTTTAATTATTTTATGACTAAATATAGGATATTGTATGATGATATGAGAAAT
TGAATTGTGTTTATTAAAATTGTCAAATAATTAAAAAAAAAAAAAAAAAAANCTCGNNNGTNNTTTTAAAGNGGCCGGGGNCCCNTNNNTTTTCCNCCCG
GGGGGGGTCCCNGGNAAGNGTCCCCANTTCCCCCTNTAGGGNGNCGTNTTANANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNC

Phil-12 T3 Primer
326 bases.
FRUCTOSE-BISPHOSPHATE ALDOLASE from Drosophila melanogaster.
ANNTCTNAAACTTCTTTGGAAANCCCCGNGCGNTTGNAGGACGACNCTAGTGGATCCAAAGAATTCGGCACGAGAAAACGTTGCTGCTGGTCAAAACG
AACTACTCAAGCGTGCCAAGGGTAATGGTGAAGCAGCCGTTGGCAAATACGTTGCTGGTAGCGTTGTTGGTGCTGGTGCCGATGCTACACTGTTCG
TGGCTAACCACGCTTATTGAACGAAGCTCCTGCTATGGATTCACAAACGTTTGGAAGTTAATTGTTTGGCAATTTATTAATATATCAATTAAGTAACA
CATACATACACTCTAGGTTATTTTTTATTTTGATGGAGTCAAAATGCAATAGTAATAAAATAGACGAAGTTAAGAAATAAAAAAAAAAAAAAAAAAACCT
CGANAGTNNTTTTAAAGNGGCCGGGGNCCCNTNNNTTTTCCNCCCGGGGGGGGTCCCAGGNAAGNGTCCCCAATTCCCCCTATAGGGNGNNGTATTNAA
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNC

Phil-13 T3 Primer
484 bases.
No matches found on BLASTx & SwissProt (CBP-1 Protein).
NTNTGTTGACCTTNCNGGAGCTNGCGCGCTTGCAGNTCGACACTAGTGGATCCAAAGAATTCGGCACCAGGCGATATGAAGTATTTCGTTGTTATTTCT
TTGGGTATCATAGCCCTTGCAGTCTCAAGCTTTGCCCAAGGCGAAGGATTGGAAAGTTCAGATGACAAGGCCGCTGATAATGAACCATTATTGGGA
TCTGGGGGACCCTGGGGACCATTTTTTGGAACACCTCAACGTCAAGGTGAACAACAGGTCGATGAAGAATTTCAGCCAAATGATGACAATTTCTTC
ATTTTCCCAATTAATCCTTTACGTTTTCCATCGTTAAAGCCAGTTTTTTTTGATTTTGGATCAAATAATGACTTTGGTCTAGGAAATATATTCAAAGAT
AATAATGAACGCAAACGAACCACTACCACAACGGCTTCCCCATGATGGCAGCTGGTGGAGAGTAAATTATATTAAGTTGGTATTTGTTTTGGCGGCA
ATTTAAACATTTCGACAATAGAGTTCTTATTCTTGAAAAAAAAAAAAAAAAAAANCTCNANNGTNCTTTTAANGNGNCNGGGGCCCCTTTANTTTTCCCC
CCGGGGGGGGTNCCNGGTAAGGGCCCCAATTCCCCTTTTAGGGGGGGNNNNNTAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNC

Phil-14 T3 Primer
899 bases.
No matches found on BLASTx & SwissProt.
TCGAGGAAACTTNNTGCACAATCCCCCGNGCCNTTTGNAGGGNCGACCCTAGTGGATCCAAAGAATTCGGCACCAGGAGAGAGAGAGAGAGNNAGAGAGA
GAGAGAGAGAGAGAGAGAGAGAGAGAGAGNNTTGGCACATGANANATNTCCCCNNCNCCNANANATNGGANACNCTCANATGACANGGTCTNCNNGT
AATGGGCCATTNTNGGNATCCNNGGGACNCCNNCNCNCCAGNNNNANNCACACACTCNCANNNCACGGATANNTCCAGGNCTATGAANAATTTCN
NTCNAATGATGANCATTTNTTNATTTTCCCNANTANCNCTTTACGTTTTCCATCGGTTNAAGCCAGTTTTTTTTGATTTTTGGGATCAATATANTGAC
TTTGGTCTTGGAAATATATTNANNGATCATACTGAGCGCNNTCGACCNCNCTCCACTNNGNCTTCCCCCATNGATGGGCAGCCTCGTTGCGAAGAG
TANAANNTTTTTTTAAGTATGGGNNTTNTGNCTTTGGGCGGGCACTTTTNTAACCNTTTCCGNACAATTTNAGTTCTTTATTTTTTTCCCNAAANANG
ANAAATACCANNCTNNANNAATTTNCNTTTTNAAAANCGGCGCGGGGGGCCCCACTCTTATTTTTCCCCCCNCGNGGGGGGGGNTCCCCNGGGCT
GATNCNCCCNCCAAATATTNCCCCCTANTGNGNGNNCTTTGTCATTNTCCCCCCNNTGGCGCNCCGTTCTCTCTCCCCCCCGGNNTTNNNTCTCTCC
CCNGCGNGGGTNNTTCCCCNNCTTCTCTTCTTNTTTTTCNTCCCCTCTTTCTTGNGATGCNTNCNTCNTNNANNNNNTNTNGTNGCGCTCCCNCTC
CCNTCGNTTTATCCTTTTCACTATTTTNNTTGTCTNCTAGTNTCANCTCTATNCTTCNNNCTTCTCTCGNCTCTNGCTCTNTNNTNCCTCGNNCN

Phil-15 T3 Primer
623 bases.
No matches found on BLASTx & SwissProt.
ANNTCTTGAAACCCTTGCAGGACCTCGCGCGCNTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACCAGGAGAGAGAGAGAGAGAGAGAGAGAGAG
AGAGAGAGAGAGAGAGNGAGNNANANNNNCNCNTGCNNNNTNNCGCNNCNCCNNNNTNTNCACNCNCTCTGNNCCANANTCTCNCTGCNTGNNNT
TCTNNGNCTGNNCCNNGGGTGNTCNCNNGTNNANANACNCCNNCNCTCTCTCGCNCTCTGTNTGTCNCNCTATNANANTGNNNTNTATTNACATAT
ANCTATANATNTATNNCNCNCNCTCTATAGCGCNCNCCCCTGTTCTCGTNTCTGTTNTCTANATNCTTGTGNGTATCTCTGTGTGNATCTNNCTCGA
ACCTGCTCCCCCCNACNNGGCNTNTTTNNGTGTGTGTATGTGTGNCCCCGCCTNTGTGTNTGCCCCTCCGTATNTATNCCNCCNTCTCTATCGCTA
CTTCTGTATGCCCCTCTGTGTNNCNCACACTTTCTCCCACNTTGTTCTCCCCCNNCNGGNCTNNCCCTGTNAAAAAANGGGTGNAAANATNTGGTG
ACTAATTTTNTGACTTTGTNTGTGTGTTTTGTGNGNGANNNTNNGANAAAACCTTNNGNGGGTTTTTTTTTNNNNGNTTTTTNNNCCCCCGTTTTTC
CCCCCTGTNTNNNNCCCCCNGGGGGGGNCCCCCCNTTTTCCCNCNCNNNNNNNNNNNTTTTTAAANNNNNNNNNTNTNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCNNNNNNCNNNNNNTTNCCN

Phil-16 T3 Primer
562 bases.
Venom Allergen 5 (type 3) from Bald Faced Hornet.
TTGAANCCTTTGCCNTNCTCGCGCGCCTGCAGGTCGACACTAGTGGATCCAAAGATGTTCGCCAATGCAAAATGGCGCATGATAAATGTCGCAACACCA
ACAAATTCAAGTACTCTGGGCAAAATTTGGCCTGGATGGGCTTCATGGGCAATACCAAGGATGTTGACATGTTGACAAAAGCCGTCAATATGTGGT
ACGATGAAGTCAAGGATAGTAGAATGGAATATATTAACAAATACCCCAAAAGCTATAGTGGACCGGCAATTGGACATTTCACTGTTATGGTGGCTGA
TCGCAATATCCGTGTGGGTTGCGCTGCTGCCACCTACCCCGAACCTGGACAACCCTACAATGCTTATTTGGTTGCCTGCAACTATGCCACCACCAA
CATGATGGACCATCCCATCTATGCCTCCTGTCCTAAAGCAACTACTGGTTGCACCACTGGATCAAACAAAAATTTCCCCAACTTGTGCTCCCCCAAC
GAGAAATACGATGTCAACAAATGGTTTTAATTATTTTATGACTAAATATAGGATATTGTATGATGATATGAGAAATTGAATTGTGTTTATTAAAATTG
TCAAATAATTAAAAAAAAAAAAAAAAAAAAAAAACTNNAANATTNTTTTAAAACNGCCGGGGGCCCNTTTTTTTTCCCCCGGGGGGGGGNCCCGGGAANN
NCCCCAATTNCCCTTTANGGGGNNNNNNNNNANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNC

Phil-17 T3 Primer
569 bases.

Venom Allergen 5 (type 3) from Bald Faced Hornet.

GAANCCCTTTGAANNACCTCGNGCGCTTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACGAGCGCCAATGCAAAATGGCGCATGATAAATGTC
GCAACACCAACAAATTCAAGTACTCTGGGCAAAATTTGGCCTGGATGGGCTTCATGGGCAATACCAAGGATGTTGACATGTTGACAAAAGCCGTCA
ATATGTGGTACGATGAAGTCAAGGATAGTAGAATGGAATATATTAACAAATACCCCAAAAGCTATAGTGGACCGGCAATTGGACATTTCACTGTTAT
GGTGGCTGATCGCAATATCCGTGTGGGTTGCGCTGCTGCCACCTACCCCGAACCTGGACAACCCTACAATGCTTATTTGGTTGCCTGCAACTATGC
CACCACCAACATGATGGACCATCCCATCTATGCCTCCTGTCCTAAAGCAACTACTGGTTGCACCACTGGACCAAACAAAAATTTCCCCAACTTGTGC
TCCCCCAACGAGAAATACGATGTCAACAAATGGTTTTAATTATTTTATGACTAAATATAGGATATTGTATGATGATATGAGAAATTGAATTGTGTTTA
TTAAAATTGTCAAATAATTAAAAAAAAAAAAAAAAAAAAAAACTCGANAGTNNTTTTAAAGNGGGCGGGGGCCCATTNNTTTTNCNCCCGGGGGGGGGNC
CCGGGAAGGGTCCCCNTTTCCCCTTTANNGGNGNNNNNNNNAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

Phil-18 T3 Primer
303 bases.

No matches found on BLASTx & SwissProt.

GANACNTTTGCTGGAGCTCGCGCGCCTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACCAGGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTAAAANNTTNNNAAACCCCCTTTTTTTTTAAAAAAAAAANCNTTTTTTTNNAAAANGGGCNTTTNNNNNAA
AAAAAAAANGGGNNTNTNGNNNANAAAAAAAANGGGCNTTTNNNNNANNANCNNAANGGGGNGGNNAANAAAAAANGGGTTNNNTTTNNGNNNNNN
AAAANNNNNGAAAAAATTTAAATTTTTNCGGTTTTTTTNCCAAAAAAAAAAAAAAAAAAAAAAAANCCCCNGGGGGNTTTTTTNGGGGNNNNNGNNCC
CCNTTTTTTTNCCCCCNGGGGGGGGCCCCCNNNNGGNCCCCCCCCNCCCCCNTTNNGGGGGGNTTTNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNC

Phil-19 T3 Primer
128 bases.

No matches found on BLASTx & SwissProt. (HUMAN FORKHEAD-RELATED PROTEIN FKHL15)

TTGAANTTTTGNCCTTTGCTGGAGCTCGCGCGCCTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACCTGGTGCCGAATTCGGCACGAGGAGTA
CTTCTAGAGCGGCCGCGGGCCCATCGATTTTCCACCCGGGTGGGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTACANNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNN

Phil-20 T3 Primer
133 bases.

No matches found on BLASTx & SwissProt.

NNGAGCTCGCGCGCCTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACCAGGAGAGAGAGAGAACTAGTCTCGAGAGTACTTCTAGAGCGGCC
GCGGGCCCATCGATTTTCCACCCGGGTGGGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTACAANNNNNNNNNN

Phil-21 T3 Primer
818 bases total.

No matches found on BLASTx & SwissProt.

TTGAGCCCTTCCNGNCCCNGCCGCNTGCNCTCGCGCTCCTGGATCCCAGAACCCGGCNCGAGANANTACTTATCTCNGTGNACTTTANTANCGNCNCGCN
GACCCANNNATTTNCCNCCNGGTGGGGAGCCNTGTAAAAGAACCCNGAACTCCCTNGAAAAATTATTATTGCAAAAGAAAATTTAATTTATTGTACCTTGT
GTATCAGGGTTTATTAAATAAAAATTAAATAGTTAATTTTCTCGATTTTAAAAGAGTTAATATTTTATAAAAGTTAATGTGGTAAAATTATTTTAAATAAAA
TATTAGAAATGAAATGTTATTCGTTTTTAAAGGTATCTAGTTCTTTAAGAAATAAATTTAATTTAGAAATTTTATATTATTTAATTAAATAAATTAATTAAAT
AAATATTTAATTTTAATATAATATGGGATAAGCTGTATTATAAATTTTTAAAAATATTTAAATAAAATAATAAATATAGATTTAGAAATAGTTATTATTAAT
AAAAGTGGTATAATTTATTTTATAAAATAAATTATTTATTTAAATTTTAAATTTTTATTAAAGNATTAATTTTAATTTTAAAAATTAAAAAATAATGGGGAA
AATTAGTATATATTATTGGAATAATAAATAAATATGAAAATTTTATATTAAGGAACTCGGCCNAANTGNAATGGTCCCTGGTTACNAAAACNTGTCTTTTT
GNATTAAAATTAAAGNCTANCCNGNCCCCGGATTATTTAAATGGNCNCGGATCCTACCGNGCCANGGGGCCTAANCTTAGCCTTTAATGGAAGCNGGTTG
AATGGTC

Phil-22 T3 Primer
660 bases total.

No matches found on BLASTx & SwissProt.

CNNCCAANNGAACTNNTNGCCCTCCCCNNNCCTTTGGGTNTGGCCCCGCNCTANNGGATNCTAAGAATTCGGCNCCAGAAAAGACTTTTAAGNNCCTATA
AATCTTTATATTTAGANGGTTATGTTTTTTTAGATAAATNTTAATNTANTAANTAATAATATTTTATTGGGGNGATATTAAAATTTAATNAACTTTTAATTT
TATTAAATCATTAATNTATGAATANTTGATCCGNCNATAACGATTAAAAATTTAAGTTACTTTAGGGATAACAGCGTAATTTTTTTGGAGAGTTCATATCGA
TAAAAAAGATTGCGACCTCGATGTTGGATTAAGAAATATGTTTAGGTGTAGCCGCTTAAATAATAAGTCTGTTCGACTTTTAAATTCTTACATGATCTGAGT
TCAAACCGGCGTAAGCCAGGTTGGTTTCTATCTTTAAAAAATTAAAATATTTCAGTACGAAAGGACCTAATATTTGATAAATAATTATTTAATAAATTGAAT
ATTATTAATATGTATATCAAAANAGNCANAAATTAAANCNCCGNNNGACCNTTTANNNGGGGNNNGGGGCCCCTTTATTTTCCCCCCCCCGGGGGGGGGC
CCCCGGGANNGGNCCCCCCACTNGCCCCCTTNAGGGGGNGGGGGANTNTGCC

Phil-23 T3 Primer
479 bases.

No matches found on BLASTx & SwissProt.

TGANNCCCTTGCAGGANCNCGCGCGCNTGCGGTCGACACTAGTGGATCCAAAGAATTCGGCACCAGAAAAGACGAGAAGACCCTATAAATCTTTATAT
TTAGATTATTATATTTTTTTAGATAAATTTTAATATAATAATTAATAATATTTTATTGGGGTGATATTAAAATTTAATAAACTTTTAATTTTATTAAATC
ATTAATTTATGAATAATTGATCCGTTAATAACGATTAAAAATTTAAGTTACTTTAGGGATAACAGCGTAATTTTTTTGGAGAGTTCATATCGATAAAA
AAGATTGCGACCTCGATGTTGGATTAAGAAATATGTTTAGGTGTAGCCGCTTAAATAATAAGTCTGTTCGACTTTTAAATTCTTACATGATCTGAGTT
CAAACCGGCGTAAGCCAGGTTGGTTTCTATCTTTAAAAAATTAAAATATTTCAGTACGAAAGGACCTAATATTTGATAAATAATTATTTAATAAATTG
AATATTATTAATATGTATATAAAAAAAAAAAAAAAAAAAAACCNCGNNNGCNTTTNANNGGGGCNGGGGCCCCATTTNTTTTCCCCCCGGGGGGGGNCCC
CGGAAAGGGCCCCCATTTCCCCCTATAGGGGGGGNNNTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

Phil-24 T3 Primer
213 bases.

TGANNCCTTGCTNGNCTCGCGCGCCTGCAGGTCGACACTAGTGGATCCAAAGCCATCCCATCTATGCCTCCTGTCCTAAAGCAACTACTGGTTGCACC
ACTGGACCAAACAAAAATTTCCCCAACTTGTGCTCCCCCAACGAGAAATACGATGTCAACAAATGGTTTTAATTATTTTATGACTAAATATAGGATAT
TGTATGATGATATGAGAAATTGAATTGTGTTTATTAAAATTGTCAAATAATTAAAAAAAAAAAAAAAAAANCTCGANAGTNNTTTTANAGNGGCCGGGGN
CCCNTNNNTTTTCCNCCCGGGGGGGGGTNCCAGGNAAGNGTCCCCAATTCCCCCTNTAGGGNGTCGNTTANANNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

Phil-25 T3 Primer
169 bases.
No matches found on BLASTx & SwissProt.

```
TGAANCCCTTGCNGGACTCGCGCGCCTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACGAGGATCAAGGAGCTGCTGCAGGACAAGATAAAGG
AGCTGCTGCAGGACAAGCTAAGTAGGCTGGCAATGATAAATGGCTTACTATGTTGCATACAAAAATGTTGTAAAAAATTTAAATATATCAGTAATTA
ATCAATATAAAAAAAAAAAAAAAAAAAACCNCGNNNGTNCTTTTAAAGNGGCCGGGGNCCCNTNNNTTTTCCNCCCGGGGGGGGNCCCNGGNAAGNGTCC
CCAATTCCCCCTNNAGGGNGNCGTNTTANAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNC
```

Degenerate Sequences used for PCR Screening

Vasodilator Primer Sequence	Apyrase Primer Sequence	FXa Inhibitor Primer Sequence
AAG CGT CCX CCX GGX TT	**GGT AAY CAY GAR TTY GA**	**AAA CCX GGX AAC CAA AAC**

Fig 3.3.1 Degenerate PCR Screening 1 to 7

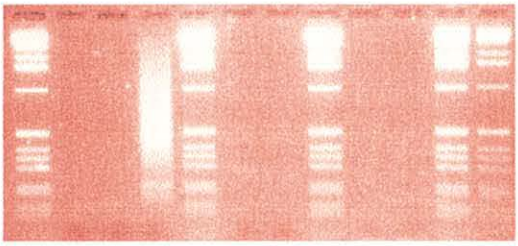
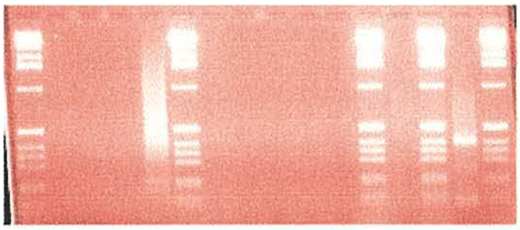
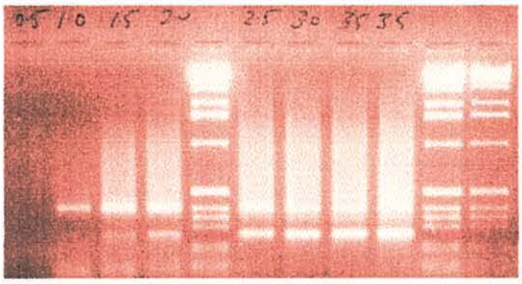


Fig 3.3.2 Degenerate PCR Screening 8 to 18



Samples 3 & 11 positive = 397 bp PCR product

Fig 3.3.3 PCR #2: Optimisation of Degenerate Apyrase Screen



PCR Markers	**Key:**	
2036	1 T3 / FXa	10 -ve Control
1636	2 T3 / VD	11 T3 / Apy
1018	3 T3 / Apy	12 T3 / VD
506	4 T7 / FXa	13 T3 / FXa
396	5 T7 / VD	14 T7 / Apy
344	6 T7 / Apy	15 T7 / VD
298	7 -ve Control	16 T7 / FXa
	8 -ve Control	17 -ve Control
	9 -ve Control	18 +ve Control

3.4 Discussion

Although a number of degenerate primers were identified as potential candidates for screening, the cost implications of having them all synthesised was the major limiting factor in this exercise. Hence the majority of the primers identified have not been used to probe the cDNA library, and their potential usefulness as screening tools is yet to be determined. However, the 3 degenerate primers which were tested, a FXa inhibitor, a vasodilator, and an apyrase, were selected to cover the 3 main antihaemostatic activities which are reported in most haematophagous animals.

The latter sequence, a highly conserved region of the *apyrase / 5'-nucleotidase*, was demonstrated to produce a PCR product in conjunction with the T3 primer. Optimisation of the PCR reaction, varying the concentrations of Mg^{2+} and cDNA template, gave a clear single band 397bp in size. This product, isolated using a primer specific for an absolutely conserved region from apyrase molecules, would suggest the presence of apyrase activity in the salivary glands. Observations from collaborative (unpublished) platelet studies,[128] using salivary gland homogenate from *S.calcitrans*, demonstrated inhibition of ADP-induced platelet aggregation. This would also be characteristic of apyrase activity, and whilst the preliminary data pointed strongly towards this, the time & cost implications of pursuing these investigations further were highly prohibitive.

Platelet aggregation can be triggered by exposure to collagen, ADP, thromboxane A_2, or thrombin, and therefore any of these mechanisms are suitable targets for the inhibition of platelet aggregation.

Most haematophagous arthropods have a salivary apyrase,[2,3] an enzyme which hydrolyses the pyrophosphate bonds of nucleoside tri- and di-phosphates, removing any free ADP at the site of injury. Normal intracellular concentrations of these nucleotides are 1,000-fold higher than extracellular levels.[104] Therefore rupture of cells at the site of injury releases these nucleotides and their presence acts as strong biological indicators of tissue damage.[104] Platelets which aggregate in the presence of ADP, degranulate, and release further ADP and the vasoconstrictor serotonin. Similarly neutrophils will aggregate in response to ATP and contribute to the developing inflammatory response.[104]

Apyrase may also inhibit the inflammatory response at the site of feeding through the indirect degradation of the apyrase end-product, adenosine monophosphate (AMP) to adenosine (a potent anti-inflammatory substance). This is final reaction carried out by the neutrophil enzyme ecto-5' nucleotidase.[42] Therefore apyrase may have a double-action, in that it can (in addition to inhibiting platelet aggregation) also act to "damp down" the inflammatory response. This has obvious benefits for haematophagous insects since the painful bite, in conjunction with the developing inflammatory response, would be the major factors in alerting the host organism to its presence. It is no suprise therefore that, given the central role ADP plays in aggregation, an apyrase of some form has been found in most haematophagous arthropods that have been investigated.[2,3]

The DNA sequence results, obtained from the abundancy screening of the cDNA library, are best discussed in conjunction with the results from the SSH screening performed in Chapter 4.

Chapter 4

Suppression Subtraction Hybridisation (SSH) cDNA Library Construction and Differential Screening

4.1 Introduction

4.2 Materials and Methods

Peparation, Construction and Hybridisation of the cDNA Library

PCR Amplification: Selection of Differentially Expressed cDNA's

Differential Screening of SSH cDNA Library

4.3 Results

4.4 Discussion

4.1 Introduction

Subtractive hybridisation is a powerful technique that enables two populations of mRNA to be compared, and to obtain clones of genes that are expressed in one population but not in the other. Although there are several different methods, the basic theory behind subtraction is simple.

First of all both mRNA populations are converted into cDNA. The cDNA population which contains the 'specific' differentially expressed genes is called the '*Tester*' and the cDNA which does not is called the '*Driver*'. *Tester* and *Driver* cDNA molecules are then hybridised, and the hybrid sequences (double-stranded cDNA) are then removed. Consequently, the remaining unhybridised (single-strand cDNA) represent genes which are expressed in the *Tester*, but are absent from the *Driver* mRNA. Although traditional subtractive hybridisation methods have been successful in some cases, they do tend to require several rounds of hybridisation, and they are not well suited for the identification of rare genes.[119-123]

The cDNA library construction kit used in this investigation, the *CLONTECH PCR-Select cDNA Subtraction Kit*, is a method combining the selective amplification of differentially expressed sequences with a suppression PCR technique, to prevent undesirable amplification whilst also allowing the enrichment of target molecules.

Having converted the Poly(A^+) mRNA to the more stable cDNA, digested it with the *Rsa-I* restriction enzyme, ligated it to an adaptor which allows PCR primer annealing to occur, and finally hybridised. The amplification and differential selection of the cDNA library can now commence. There are essentially two approaches to differentially screen a subtracted library. The first is to hybridise the subtracted library with ^{32}P-labelled probes, synthesised as first-strand cDNA from *Tester* and

Driver.[121,124] Clones corresponding to the differentially expressed mRNA's will hybridise only with the *Tester* probe, and not with the *Driver* probe. Although this approach is widely used, it has the drawback that, only cDNA from highly abundant mRNA will produce detectable hybridisation signals.[125] Hence clones corresponding to low-abundancy differentially expressed mRNAs will not be detected by this procedure. Given the wish to detect all differentially expressed mRNA sequences, whether or not they are in abundance, meant that this approach was not considered suitable. Instead a different strategy was pursued, bypassing the detection problem of low-abundancy sequences.

In this method, the subtracted cDNA library is hybridised with *forward-* and *reverse-*subtracted cDNA probes. The forward-subtracted probe is made from the same subtracted cDNA used to construct the subtracted library, and the reverse-subtracted probe is made by switching the Tester and Driver in the hybridisation reaction. Hence clones that represent mRNAs which are truly differentially expressed will hybridise only with the forward-subtracted probe, whereas those hybridising with the reverse-subtracted probe are background sequences, and of little value. However, the disadvantage of this method is a slightly higher incidence of false-positive results. This is overcome by screening 4 times, in effect combining the benefits of both methods.

Aims and Objectives

The aim of this chapter is to construct an SSH cDNA library and screen it accordingly. The sequence information can then be compared to that obtained using methods in the previous chapter, allowing the identification of cloned sequences transcribed in high numbers, which are also specific to salivary gland tissue.

4.2 Materials and Methods

RNA Preparation & Handling

The salivary gland samples (*Tester* mRNA) and the remaining fly tissues (*Driver* mRNA) were dissected and prepared as outlined in *appendix (b)*, using DEPC-treated sterilised dissection media, tweezers, slides and reagents to minimise RNAse activity. Approx. 2μg of Poly(A)$^+$ *Tester* and *Driver* mRNA were extracted, as per instructions using the Dynabeads™ magnetic particle method. A control Poly-A$^+$ mRNA was also used (human skeletal muscle) to ensure that the kit was performing as expected.

First Strand Synthesis Reaction

The Poly-(A$^+$) mRNA, for each of the *Tester* (salivary) & *Driver* (fly carcass) samples, was added to a sterile 0.5ml microcentrifuge tube and the contents were made-up to 5μl using sterile DEPC-treated water. The tubes were then mixed briefly, and the contents spun-down to the bottom of the tube.

The tubes were then incubated at 70°C for 2 minutes in a thermal cycler, and then immediately placed on ice. The tubes were centrifuged briefly to spin-down the condensated at the sides of the tube, and returned to ice. The following reagents were then added, in order, to each tube :

2μl	5x First-Strand Buffer
1μl	dNTP Mix (10mM each)
1μl	Sterile H_2O
1μl	AMV Reverse Transcriptase (20U/μl)

The tubes were then gently vortex-mixed, and spun down, and immediately placed into a thermal cycler at 42°C for 90 minutes, followed by a 4°C hold. After the 90 minutes first-strand synthesis incubation, the tubes were immediately removed from the cycler and placed on ice to terminate the reaction. Any condensation on the sides of the tube was then spun-down quickly prior to second-strand synthesis.

Second Strand Synthesis

To each of the tubes the following reagents were added :

48.4μl	Sterile H_2O
16.0μl	5x Second-Strand Buffer
1.6μl	dNTP Mix (10mM)
4.0μl	20x Second Strand Enzyme Cocktail

The contents were then briefly vortex-mixed, spun-down, and incubated in a thermal cycler at 16°C for 2 hours. After the incubation the tubes were removed from the cycler, and 2μl (6U) of *T4 DNA Polymerase* enzyme was added. The contents of the tube were thoroughly vortex-mixed, and briefly spun-down, then returned to the thermal cycler and incubated at 16°C for a further 30 minutes The second-strand synthesis reaction was then terminated by the addition of 4μl 20x EDTA/Glycogen mix.

The tubes were vortex-mixed thoroughly, spun-down, and 100μl of phenol:chloroform:isoamyl alcohol (25:24:1) were added. Each tube was then vigorously vortex-mixed for 30-45 seconds, then centrifuged at 14,000rpm for 10 minutes at room temperature. The upper (aqueous) layer was then carefully removed, and transferred to a sterile 0.5ml Eppendorf tube. The interphase and lower (non-aqueous) layers were then discarded. 100μl of chloroform:isoamyl alcohol (24:1) was then added to the upper (aqueous) layer, and each tube was then vigorously vortex-mixed for another 30-45 seconds, then centrifuged again at 14,000rpm for 10 minutes at room temperature. The upper (aqueous) layer was carefully removed again, and transferred to another sterile 0.5ml Eppendorf tube. The interphase and lower (non-aqueous) layers were then discarded, as before. 40μl of 4M Ammonium Acetate solution were then added to the saved aqueous layer, it was briefly vortex-mixed, and then 300μl of 100% ethanol were added.

The tubes were thoroughly vortex-mixed, and the precipitated cDNA was then pelleted by centrifugation at 14,000 rpm for 30 minutes at room temperature. The supernatant was carefully removed, and discarded, and the pellet was carefully overlayed with 500µl of 80% ethanol. The tube was then centrifuged for a further 10 minutes at 14,000rpm, and the supernatant was carefully removed, and discarded. The pellet was then air-dried, under vacuum, for approx. 10 minutes prior to resuspending and dissolving the pellet in 50µl of sterile H_2O. 6µl of this cDNA solution were then transferred to a sterile 0.5ml Eppendorf tube and stored at -20°C as a control. (ds cDNA synthesis control)

Rsa-I Digestion

The remaining ds-cDNA synthesised from the previous stage was then digested, using the *Rsa-I* restriction enzyme, to produce shorter blunt-ended ds cDNA fragments which are required for subtraction, and are necessary for the subsequent stages. To a fresh 0.5ml eppendorf tube, the following reagents were added:

43.5µl ds cDNA (from 2nd strand synthesis reaction)
5.0µl 10x *Rsa-I* Restriction Buffer
1.5µl *Rsa-I* (10U/µl)

The contents were then vortex-mixed, centrifuged briefly, and then incubated in a thermal cycler at 37°C for 90 minutes. The contents were then immediately placed on ice, and 5µl of the reaction mixture was removed as a control and stored at -20°C. To the remaining 45µl, 2.5µl of 20x EDTA/Glycogen Mix were added to terminate the reaction, the contents were mixed, and then 50µl phenol:chloroform:isoamyl alcohol (25:24:1) were added.

After vortex-mixing the tubes were centrifuged at 14,000rpm for 15 minutes, and the top aqueous layer was carefully transferred to a sterile 0.5ml Eppendorf tube. 25µl of 4M Ammonium Acetate solution was then added to the saved aqueous layer, it was briefly vortex-mixed, and then 187.5µl of 100% ethanol was added. The tubes were thoroughly vortex-mixed, and the precipitated cDNA was then pelleted by centrifugation at 14,000 rpm for 30 minutes at room temperature. The supernatant was carefully removed, and discarded, and the pellet was then air-dried under vacuum for approx. 10 minutes. The pellet was then gently resuspended by dissolving in 5.5µl of sterile H_2O, to make the *Driver* cDNA samples for each tissue, and these were then stored at -20°C until required.

Adaptor Ligation

To produce the *Tester* cDNA for each tissue, 1µl of the *Rsa-I* digested (*Driver*) sample was added to 5µl of sterile water.

A 'master mix' was prepared such that each reaction tube contained the following :

3µl Sterile H2O
2µl 5x Ligation Buffer
1µl T4 DNA Ligase (400U/µl)

The following reaction tubes were then prepared for each *Tester* sample:

Table 4.2.1: Ligation Reactions for *Tester* cDNA

Ligation Mix (Component Added)	Reaction Tube			
	***Tester* 1-1**	***Tester* 1-2**	***Tester* 2-1**	***Tester* 2-2**
Diluted cDNA	2µl	2µl	2µl	2µl
Adaptor 1 (10µM)	2µl	-	2µl	-
Adaptor 2R (10µM)	-	2µl	-	2µl
Master Mix	6µl	6µl	6µl	6µl
Final Volume	10µl	10µl	10µl	10µl

The tubes were then vortex-mixed, and briefly centrifuged to spin-down the contents.

In a fresh 0.5ml Eppendorf tube 2µl of *Tester* 1-1 was added to 2µl of *Tester* 1-2. Similarly 2µl of *Tester* 2-1 were added to 2µl of *Tester* 2-2. These tubes formed the control 'unsubtracted' samples -

Salivary(c) and *Carcass(c)* tubes. All tubes were then incubated in a thermal cycler at 16°C overnight. The reaction was stopped with 1µl EDTA/Glycogen, the tubes were quickly mixed, then returned to the thermal cycler for 5 minutes at 72°C. The unsubtracted control tubes were then removed from the -20°C freezer, thawed, and 1µl was transferred into 1ml of sterile H_2O, mixed thoroughly, and placed on ice. What remained of the control (unsubtracted) cDNA samples was returned to -20°C.

First Hybridisation

The 4x hybridisation buffer was removed from the -20°C freezer and thawed in a 37°C heating block for a minimum of 15 minutes prior to use. The following reaction tubes were then prepared:

Table 4.2.2: First Hybridisation Reaction:

Reaction Component	Hybridisation Sample			
	1s	2s	1c	2c
Rsa-I Digested *Driver* cDNA*	1.5µl	1.5µl	1.5µl	1.5µl
Adaptor-1 Ligated *Tester* 1-1	1.5µl	-	-	-
Adaptor-2R Ligated *Tester* 1-2	-	1.5µl	-	-
Adaptor-1 Ligated *Tester* 2-1	-	-	1.5µl	-
Adaptor-2R Ligated *Tester* 2-2	-	-	-	1.5µl
4x Hybridisation Buffer	1.0µl	1.0µl	1.0µl	1.0µl
Final Volume	**4.0µl**	**4.0µl**	**4.0µl**	**4.0µl**

* The *Rsa-I* digested *Driver* cDNA is the cDNA from the other tissue to that of the adaptor-ligated *Tester* cDNA. For example, *Rsa-I* digested *Driver* from the fly carcass was added to adaptor-1 and adaptor-2R ligated *Tester* cDNA (samples 1-1 & 1-2) from the salivary glands. Similarly salivary gland *Driver* was added to fly carcass *Tester* cDNA (samples 2-1 & 2-2).

The samples were then overlayed with 1 drop (≈50µl) mineral oil and centrifuged briefly to spin-down the contents. The samples were then placed immediately into a thermal cycler and heated to 98°C for 90 seconds, followed an incubation at 68°C for 8 hours.

Second Hybridisation

Immediately prior to the 8 hour incubation deadline, the remaining *Driver* cDNA sample for each tissue (stored at -20°C from earlier), was thawed on ice. The following reagents were then added to a clean sterile 0.5ml eppendorf.

1µl *Driver* cDNA (salivary or fly carcass, as before)
1µl 4x Hybridisation Buffer
2µl Sterile H_2O

The contents of the tube were vortex-mixed, and 1µl of this mixture was transferred to another sterile 0.5ml eppendorf and overlayed with 1 drop of mineral oil. This tube was then placed in a thermal cycler and heated to 98°C for 90 seconds. Following the 8 hour incubation of the hybridisation samples, and leaving them in the thermal cycler, the aqueous lower phase of hybridisation sample 1-2 was carefully drawn-up partway into the tip of a sterile pipette. The tip was then removed from the tube, a small amount of air was drawn into the tip to create an air space, and the appropriate freshly denatured *Driver* sample (carcass cDNA) was then drawn into the tip also. The pipette contents, comprising hybridisation sample 1-2 and freshly denaturated *Driver*, were then transferred to the hybridisation sample 1-1 tube, and mixed by gently pipetting up-and-down. This procedure was then repeated for hybridisation samples 2-1 and 2-2, using the salivary *Driver* sample. The tubes were then centrifuged briefly, and then returned to the thermal cycler and incubated at 68°C overnight.

Following incubation, 200µl of dilution buffer were added each tube and the contents were gently mixed by pipetting up-and-down. The tubes were then incubated in a thermal cycler for a further 7 minutes at 68°C, followed by an immediate 4°C hold.

The samples were then stored at -20°C until required for PCR Amplification.

PCR Amplification: Selection of Differentially Expressed cDNA's

A 'master mix' was prepared, as follows :

Table 4.2.3: PCR Amplification Master Mix:

PCR Reagent	Volume (µl)	
	Per Tube	7 Tube PCR Run
Sterile H_2O	19.5µl	156.0µl
10x PCR Reaction Buffer	2.5µl	20.0µl
dNTP Mix (10mM)	0.5µl	4.0µl
PCR Primer 1 (10µM)	1.0µl	8.0µl
50x Advantage cDNA Polymerase Mix	0.5µl	4.0µl
Total Volume	**24.0µl**	**192.0µl**

The cDNA samples to be tested were as follows:

Table 4.2.4: PCR Amplification Samples:

PCR Tube	cDNA Samples	
	Contents	Description
1	(1-1) & (1-2) hybridisation mixture	Forward Subtracted (Salivary) cDNA
2	Salivary (c)	Unsubtracted *Tester* (Salivary) Control
3	(2-1) & (2-2) hybridisation mixture	Reverse Subtracted (Carcass) cDNA
4	Carcass (c)	Unsubtracted *Tester* (Carcass) Control
5	Control cDNA	PCR Control Subtracted cDNA
6	Subtracted Control	Subtracted Control Skeletal Muscle cDNA
7	Unsubtracted *Tester* Control	Unsubtracted Skeletal Muscle *Tester* Control

24µl of master mix was added to 1µl of cDNA, and the mixture was overlayed with 50µl of mineral oil. The tubes were then incubated in a Perkin-Elmer 480 Thermal Cycler for 5 minutes at 75°C to extend the adaptors, followed immediately by :

27 cyles of	94°C	30 seconds
	66°C	30 seconds
	72°C	90 seconds

Immediately after the final incubation, the samples were cooled to 4°C on a holding cycle, and were transferred to ice. 8µl from each PCR product, were transferred to a sterile 0.5ml Eppendorf tube and stored at -20C. This is the primary PCR control. 3µl of the remaining PCR product were diluted in 27µl of sterile H2O to make a 1:10 dilution. This diluted primary PCR product was then stored at -20°C until required for the *PCR Select Differential Screening* procedure. The remaining PCR product was also stored at -20°C, for future use.

Differential Screening of SSH cDNA Library

A PCR master-mix was prepared, as below:

Table 4.2.5: PCR Master Mix

Master-Mix Component	Volume	
	1 Run (µl)	3x (µl)
Sterile H2O	18.5	55.5
Nested Primer 1 (10µM)	1.0	3.0
Nested Primer 2R (10µM)	1.0	3.0
10x PCR Buffer	2.5	7.5
dNTP Mix	0.5	1.5
50x PCR Enzyme Mix	0.5	1.5
Total Volume	**24.0 (µl)**	**72.0 (µl)**

The contents of the tube were then vortex-mixed, and the tube was centrifuged to collect the contents. 24µl of this master mix were then added to a sterile 0.5ml Eppendorf tube containing 1µl of the appropriate template. The negative controls contained 1µl of the mixture prepared earlier, and the forward- and reverse-subtracted probes uses 1µl of the diluted products from ***Chapter 4.2*** (cDNA from the *Primary PCR Amplification*). For the unsubtracted *Tester* and *Driver* probes, the Primary PCR Amplification products of the unsubtracted *Tester Control* were used, from the forward and reverse subtractions, respectively.

Thermal cycling was initiated immediately, as follows (see ⇨)
Immediately the final termination cycle ends, 8µl of the cDNA product from each reaction was removed, and stored in a sterile 0.5ml eppendorf tube at -20°C. The remaining cDNA product, from the *secondary PCR amplification*, is also stored at -20°C.

11 Cycles of:
• 30 secs at 94°C
• 30 secs at 68°C
• 90 secs at 72°C
Followed by a 5 minute termination at 72°C

Table 4.2.6: PCR for Topo-TA cDNA

PCR Reaction		PCR Conditions
Component	**Vol (µl)**	
DNA Template	5	35 Cycles of
10x PCR Buffer	5	
dNTP Mix	0.5	30 secs at 94°C
N.Primer 1 (10µM)	0.5	30 secs at 68°C
N.Primer 2R (10µM)	0.5	90 secs at 72°C
Sterile H_2O	37.5	
Taq Polymerase	1.0	Followed by a 10 minute
Total Volume	**50µl**	termination at 72°C

50ng of the cDNA from the secondary amplification were used as a template, for a further PCR, to produce cDNA to be cloned into the Topo-TA vector. The PCR conditions are summarised.

LB Agar plates were prepared, containing 50µg/ml ampicillin, and incubated at 37°C for 30 minutes. Onto the surface of each were spread 40µl of 40mg/ml X-Gal and 40µl of 100mM IPTG. The plates were then incubated at 37°C for a further 30 minutes before use.

Immediately, and on ice, 1 vial of chemically competent cells was then thawed, and to this was added 2µl of 0.5M β-mercaptoethanol. The cell suspension was carefully stirred, using a pipette tip, and incubated on ice for 30 minutes. During this time the following cloning reaction was setup, mixed gently by pipette, and incubated at room temperature (≈25°C) for 5 minutes. 1µl of 6x TOPO Cloning Stop Solution was then added, the solution was then mixed gently using a pipette, and placed on ice. 2µl of this vector solution were then added to the competent cells, and mixed gently by stirring. The cells were then incubated on ice for a further 30 minutes, followed by a 30 minute heat-shock at 42°C, before returning them to ice.

2µl	Fresh PCR Product
2µl	Sterile H_2O
1µl	pCR-TOPO® Vector
5µl	**Total Volume**

250µl of SOC medium (at 25°C) was then added, and the vial was incubated at 37°C for 30 minutes, with shaking. 50µl of cells were then plated-out onto the surface of the X-Gal/IPTG ampicillin selective agar plates (prepared earlier) and were incubated at 37°C overnight. Following this the plates were incubated at 4°C for 2 hours to aid with the colour development reaction, and the blue/white colonies were examined.

300 individual white colonies were then selected, and re-plated onto more X-Gal/IPTG treated ampicillin selective agar plates. These were incubated overnight, as before, and a single colony from each was used to inoculate 10ml LB medium containing 50µg/ml ampicillin, and incubated at 37°C overnight with shaking. The LB ampicillin culture was then pipetted, in 1ml aliquots, into sterile 1.5ml tubes. A 5µl aliquot of cells was then removed from each culture, and stored at -20°C. To the remaining cells 500µl of glycerol were added, briefly vortex-mixed, and then fast-frozen over liquid-N_2 before storage at -80°C.

Table 4.2.7: Clone Selection PCR Mix

PCR Master Mix		PCR Reaction Conditions
5.0µl	W-1	
10.0µl	10x PCR Buffer	30 secs at 94°C
4.0µl	25mM $MgCl_2$	then 35 Cycles of
10.0µl	dNTPs	30 secs at 94°C
2.5µl	Nested Primer 1 (10µM)	30 secs at 68°C
2.5µl	Nested Primer 2R (10µM)	90 secs at 72°C
0.4µl	*Taq* DNA Polymerase	
60.6µl	Sterile H_2O	Followed by a 15 minute
95µl	**Total Volume**	termination at 72°C

Each 5µl aliquot of cells was then used as the DNA template for a 100µl PCR reaction. A master mix was prepared on ice, as follows: 95µl of master-mix was then added to the frozen DNA template, briefly vortex-mixed, and the tubes were placed in a thermal cycler for amplification. 10µl of this PCR product was then electrophoresed on a 2% agarose/ethidium bromide gel in TAE buffer, and the remaining 90µl, for each of the 300 samples, was stored at -20°C until required. From the results of the 2% agarose gel electrophoresis, colonies which did not contain a single well-defined PCR product were discarded.

The DNA from the selected PCR products was then ethanol-precipitated using 4 volumes 100% EtOH, washed once in 80% EtOH, and then resuspended in 20µl sterile water. These DNA samples were then used to make a Dot-Blot Array, comprising over 200 colonies with single well-defined inserts. 5µl of resuspended PCR product from selected colonies were added to 5µl of freshly prepared 0.6N NaOH to denature the cDNA, the contents of the tube were then vortex-mixed, and briefly spun-down. 1µl of each sample was then dotted on a grid (in duplicate), onto the surface of an 18x14cm sheet of hydrabond nylon membrane. (See diagram below)

Table: Dot Blot Grid of Cloned cDNA Inserts

A	2	3	4	5	6	7	8	10	11	12	13	14	15	16	17	18
B	19	20	21	22	23	24	25	26	28	29	30	31	32	33	36	37
C	39	40	41	42	43	44	45	46	49	50	51	52	53	54	55	58
D	59	60	61	63	64	65	66	67	69	70	71	72	74	75	76	77
E	78	79	81	82	83	85	86	87	88	89	92	93	94	95	97	100
F	101	102	103	104	105	106	107	108	109	110	111	112	113	114	116	119
G	120	121	122	123	125	126	127	128	129	130	132	135	140	141	143	161
H	163	165	167	181	182	183	185	186	187	191	193	194	196	197	198	199
I	200	201	202	203	204	205	206	207	208	209	210	211	214	216	217	218
J	219	220	222	224	226	227	229	230	231	232	233	234	235	236	237	238
K	239	240	241	242	243	246	247	248	249	250	251	252	253	254	256	258
L	259	260	261	262	263	264	265	266	267	268	270	271	273	274	276	277
M	278	279	280	281	282	283	284	286	287	288	1R	2R				

The blots were then neutralised for 4 minutes in 0.5M Tris-HCl (pH7.5), washed in 0.3M NaCl with 0.03M NaCitrate (i.e. 2x SSC soln), and the cDNA was immobilised onto the membrane by heating at 80°C for 2 hours. The membranes were then wrapped in SARAN WRAP™ and stored at 4°C ready for use. Four probes, one for each of the forward- and reverse-subtracted samples from the *Tester* and *Driver* cDNA, were then made as follows :

30µl	Nuclease-free water
10µl	5x Labelling Buffer
2µl	Unlabelled dNTP Mix*
2µl	Denatured DNA Template (25ng)
2µl	Nuclease-free BSA
3µl	[α^{32}P] dCTP
1µl	DNA Polymerase I (Klenow) Fragment
50µl	Total Volume

* Unlabelled dNTP Mix comprised 1µl of each of unlabelled dGTP, dATP & dTTP making 3µl in total, of which 2µl was used.

The probe mixtures were mixed gently and incubated at room temperature for 1 hour, and the reaction was terminated by placing in a boiling water bath for 2 minutes, followed by the addition of 2µl 0.5M EDTA. The probe was then purified (according to manufacturers instructions) using an S400 spin-column. The final volume for each probe was 100µl.

Pre-Hybridisation

A solution of salmon-sperm DNA (10mg/ml) was prepared in sterile distilled water, and then sheared for a minimum of 15 minutes by repeatedly and forcibly squirting through a needle/syringe. The sheared salmon-sperm DNA solution was dispensed into sterile tubes in 1ml aliquots and stored at -20°C. Bottles containing 100ml of the pre-hybridisation solution were then heated to 70°C and transferred to the hybridisation bottles, already pre-heated to 65°C in the hybridisation oven. The hybridisation bottles, containing the pre-hybridisation solution, were then returned to the oven and maintained at 65°C while the salmon-sperm DNA was thawed at RT. The pre-prepared (thawed) salmon-sperm DNA was then placed in a boiling water bath for 10 minutes to denature the DNA, and then immediately chilled on ice for for a further 5 minutes. The contents of 1 tube (1ml) of salmon-sperm DNA was then added to the hybridisation bottle containing the pre-hybridisation solution at 65°C. This was then returned to the hybridisation oven and allowed to mix for a further 10-15 minutes.

The membrane was then spread onto the inside wall of each hybridisation bottle, orientated with the dot blot matrix on the inner (luminal) side. The membrane was carefully smoothed to ensure no air-bubbles, and the hybridisation bottle was returned to the hybridisation oven to prehybridise the membranes for 2 hours.

Hybridisation

In a similar manner as before, bottles containing 10ml of the hybridisation solution were heated to 70°C. Immediately prior to hybridisation the pre-hybridisation solution was poured out of the hybridisation bottle, and the contents of the pre-heated hybridisation solution were then transferred to the hybridisation bottles, and returned to the 65°C hybridisation oven. In a similar manner to before, 100µl of pre-prepared (thawed) salmon-sperm DNA were then placed in a boiling water bath for 10 minutes to denature the DNA, and then immediately chilled on ice for for a further 5 minutes. The 100µl of salmon-sperm DNA were then added to the hybridisation bottle containing the hybridisation solution at 65°C, and this was then returned to the hybridisation oven and allowed to mix for a further 10 minutes. The 100µl [α^{32}P]-labelled probe was then carefully added to the appropriate hybridisation bottle, returned to the oven, and allowed to hybridise overnight at 72°C.

Following hybridisation, the contents of each hybridisation tube were carefully collected into sealed glass bottles, wrapped in 3 layers of aluminium foil, and stored in the radioactive isotope freezer at -20°C in the event that they may be required later. Without allowing the membranes to dry, 80ml of pre-heated (70°C) 0.1% SDS soln in 2x SSC buffer was added to each hybridisation bottle, and they were returned to the hybridisation oven. After 10 minutes the washing solution was removed, and the washing process was repeated again a further 3 times. The membranes were washed twice, each of 30 minutes duration, with 0.1% SDS soln in 1x SSC buffer. The background activity of each membrane was then checked with the geiger counter, and membranes with an activity greater than 100 cps were washed again, for a period of 10 minutes, in 0.1% SDS soln in 0.1x SSC buffer.

The activity measurements and washings were then repeated until the background activity for each membrane fell below 100 cps. The membranes were then carefully removed from the hybridisation bottles, ensuring excess moisture was removed, and placed between 2 sheets of SARAN™ transparent plastic film. These were then placed inside an autoradiograph cassette for transport to the developing/dark room. In the dark room, and under safe-light conditions, the membranes were alligned with suitably sized & labelled photographic paper. These were sealed in the metal cassette and exposed overnight at -80°C.

Following exposure, the cassettes were removed from the -80°C freezer and allowed to reach room temperature for a minimum of 2 hours. The cassettes were then carefully opened, in a darkroom under safe-light conditions, and the autoradiographs were removed and developed (as shown right ⇨). According to the exposure observed on developing, where necessary the autoradiographs were re-exposed to new photographic film for a greater or lesser amount of time, and these were developed to produce images of the desired intensity.

Developing Autoradiograph

Place film in developer for 3 minutes
Wash in H_2O for 2 minutes
Immerse in Fixative for 2 minutes
Rinse under a tap for 30-45 seconds
Air dry for 2 hours

4.3 Results

The results of the developed autoradiographs from the cDNA Array were as follows:

Fig 4.3.1: cDNA Grid Array 1 (1c)

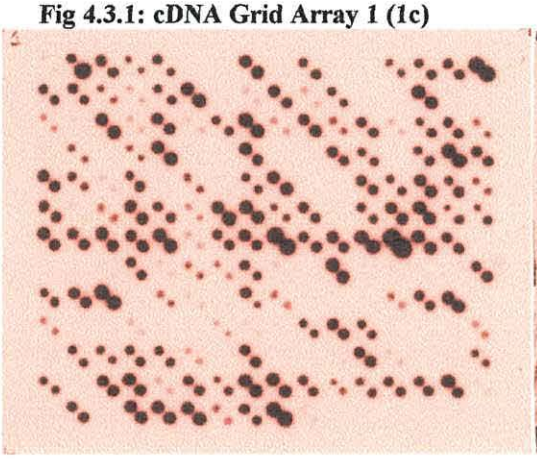


Fig 4.3.2: cDNA Grid Array 2 (2c)

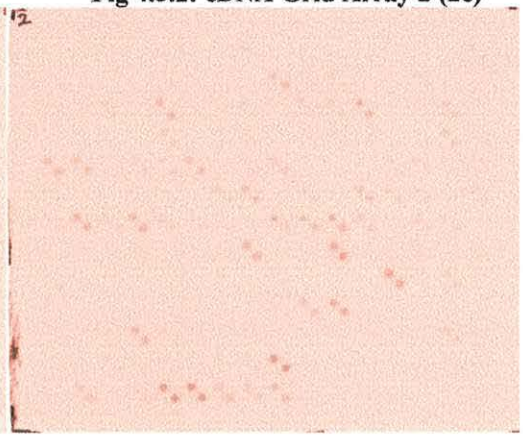


Fig 4.3.3: cDNA Grid Array 3 (3F)

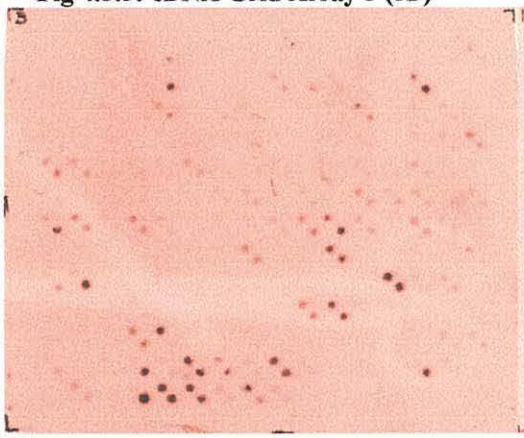
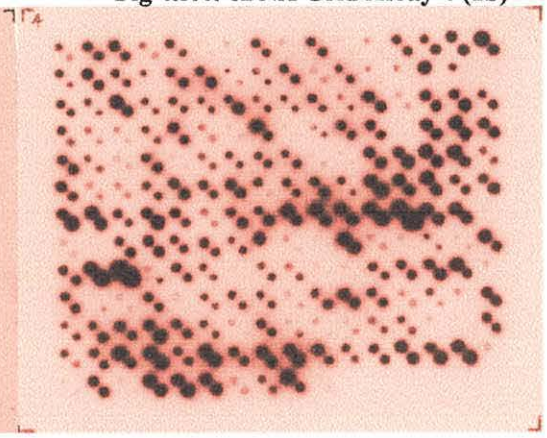


Fig 4.3.4: cDNA Grid Array 4 (1S)



NB: The shadow formed by the scissors on autoradiograph #3 indicated that the safe-light conditions when developing this film were slightly less than ideal.

DNA Array Analysis

The results of the DNA Arrays from the *forward-* and *reverse-subtracted* (1F & 3R) and *unsubtracted* (1c & 2c) probes were analysed as follows :-

Table 4.3.1: DNA Array Analysis Criteria

Type of Probe				Interpretation
FWD Sub	REV Sub	Unsub. *Tester*	Unsub. *Driver*	
+	-	+	-	(A) Clones that hybridise to the fwd-subtracted and unsubtracted Tester probes but not to the reverse-subtracted or unsubtracted Driver probes almost always (95% probability) correspond to differentially expressed genes.
+	-	-	-	(B) Clones that hybridise only to the fwd-subtracted probe are strong candidates for differential expression. These clones typically correspond to low-abundancy transcripts which have been enriched during the subtraction. In most cases the enrichment is the result of differential expression.
+	-	+	+	(C) Clones that hybridise to the fwd-subtracted probe and both the unsubtracted probes, but not the reverse-subtracted probe can be difficult to interpret. Many of these cDNAs have been artificially enriched. In some cases Northern Analysis shows that the clone hybridises to both the Tester and Driver RNA, but that either (a) the transcripts have different sizes and are probably alternatively spliced forms, or (b) several different transcripts are identified with one or more bands specific to the Tester RNA. The latter is often the case for genes that are members of multi-gene families.
++	+	+	+ / -	(D) Clones that hybridise to both subtracted probes, but with different intensities. When the difference is >5 fold the clone probably corresponds to a differentially expressed gene. However, when the difference in signal intensity is < 3 fold the explanation is most likel;y a random fluctuation in the efficiency of the fwd and rev-subtractions.
+	+	+	+	(E) Clones that hybridise equally to both subtracted probes and to both unsubtracted probes are almost never differentially expressed.
-	-	-	-	(F) Clones that do not have a detectable hybridisation signal for either of the subtracted probes usually represent nondifferentially expressed cDNAs. Some non-differentially expressed cDNA fragments are randomly present in the subtracted library as single copies. Because these molecules are present at such low levels in the subtracted probe, they do not hybridise to the corresponding clone.

Fig 4.3.5: Results from Analysis of DNA Array Patterns

Hybridisation Results					Analysis of Hybridisation Pattern					
Clone	1S	3F	1c	2c	A	B	C	D	E	F
2	1	0	0	0		✓				
3	2	0	3	0	✓					
4	2	0	2	0	✓					
5	1	0	1	0	✓					
6	2	0	2	0	✓					
7	0	0	0	0						✓
8	0	0	0	0						✓
10	0	0	3	0	✓					
11	0	0	0	0						✓
12	2	0	2	0	✓					
13	0	0	0	0						✓
14	0	0	0	0						✓
15	2	0	2	0	✓					
16	2	0	2	0	✓					
17	2	0	2	0	✓					
18	2	1	3	1				✓	✓	
19	1	0	2	0	✓					
20	2	0	2	0	✓					
21	1	0	1	0	✓					
22	1	0	1	0	✓					
23	2	0	2	0	✓					
24	2	0	3	0	✓					
25	1	0	0	0		✓				
26	1	0	0	0		✓				
28	2	1	2	1				✓	✓	
29	0	1	1	0						
30	2	0	2	0	✓					
31	0	0	0	0						✓
32	0	0	0	0						✓
33	2	2	3	0				✓		
36	1	0	0	0		✓				
37	0	0	0	0						✓
39	1	0	1	0	✓					
40	1	0	0	0		✓				
41	2	0	3	0	✓					
42	1	0	1	0	✓					
43	1	1	3	1					✓	
44	1	0	1	0	✓					
45	2	0	2	0	✓					
46	1	0	3	0	✓					
49	1	0	1	0	✓					
50	1	0	3	0	✓					
51	1	0	2	0	✓					
52	2	1	2	1				✓	✓	
53	1	0	1	0	✓					
54	2	0	2	0	✓					
55	2	1	2	1				✓	✓	
58	2	0	1	0	✓					
59	2	0	0	0		✓				
60	1	0	2	0	✓					
61	1	0	0	0		✓				
63	1	0	2	0	✓					
64	2	0	3	0	✓					
65	1	0	0	0		✓				
66	1	0	0	0		✓				
67	3	0	3	0	✓					
69	0	0	0	0						✓
70	1	0	0	0		✓				
71	2	0	3	0	✓					
72	2	0	2	0	✓					
74	0	0	0	0						✓
75	2	0	2	0	✓					
76	3	1	3	1				✓	✓	
77	2	1	2	0				✓		

Hybridisation Results					Analysis of Hybridisation Pattern					
Clone	1S	3F	1c	2c	A	B	C	D	E	F
78	2	1	3	1				✓	✓	
79	1	1	2	1					✓	
81	1	0	1	0	✓					
82	2	0	2	0	✓					
83	0	0	0	0						✓
85	1	1	2	1					✓	
86	1	0	0	0		✓				
87	1	0	2	0	✓					
88	1	1	3	1					✓	
89	0	0	0	0						✓
92	0	0	0	0						✓
93	2	0	2	0	✓					
94	2	0	2	0	✓					
95	3	1	3	1				✓	✓	
97	3	0	2	0	✓					
100	1	0	2	0	✓					
101	2	0	2	0	✓					
102	1	0	0	0		✓				
103	1	0	1	0	✓					
104	2	0	2	0	✓					
105	3	0	2	0	✓					
106	2	0	1	0	✓					
107	3	0	3	0	✓					
108	2	1	3	1				✓	✓	
109	2	0	2	0	✓					
110	3	1	2	1				✓	✓	
111	2	0	0	0		✓				
112	3	1	2	1				✓	✓	
113	3	1	2	1				✓	✓	
114	3	1	2	1				✓	✓	
116	2	1	2	0				✓		
119	2	1	2	0				✓		
120	3	1	2	0				✓		
121	3	1	2	1				✓	✓	
122	2	0	2	0	✓					
123	3	1	2	1				✓	✓	
125	2	0	3	0	✓					
126	1	0	1	0	✓					
127	2	0	2	0	✓					
128	2	0	2	0	✓					
129	3	1	3	1				✓	✓	
130	3	1	2	1				✓	✓	
132	3	1	2	1				✓	✓	
135	3	1	3	1				✓	✓	
140	3	0	3	0	✓					
141	2	1	3	1				✓	✓	
143	2	1	2	1				✓	✓	
161	0	0	0	0						✓
163	0	0	0	0						✓
165	0	0	0	0						✓
167	2	0	0	0		✓				
181	2	0	2	0	✓					
182	2	0	1	0	✓					
183	2	0	2	0	✓					
185	2	0	2	0	✓					
186	2	1	3	1				✓	✓	
187	1	0	0	0		✓				
191	0	0	0	0						✓
193	3	1	3	1				✓	✓	
194	1	0	0	0		✓				
196	0	0	0	0						✓
197	1	0	0	0		✓				
198	1	0	0	0		✓				
199	3	0	2	0	✓					

Hybridisation Results					Analysis of Hybridisation Pattern					
Clone	1S	3F	1c	2c	A	B	C	D	E	F
200	2	1	2	0				✓		
201	3	1	2	0				✓		
202	3	0	3	0	✓					
203	1	0	0	0		✓				
204	2	0	2	0	✓					
205	2	0	1	0	✓					
206	0	0	0	0						✓
207	2	0	2	0	✓					
208	2	0	1	0	✓					
209	0	0	0	0						✓
210	0	0	0	0						✓
211	2	0	0	0		✓				
214	2	2	1	1				✓	✓	
216	3	0	2	0	✓					
217	3	0	3	0	✓					
218	0	0	0	0						✓
219	2	0	1	0	✓					
220	0	0	0	0						✓
222	0	0	0	0						✓
224	2	0	1	0	✓					
226	0	0	0	0						✓
227	2	0	0	0		✓				
229	2	0	1	0	✓					
230	2	0	1	0	✓					
231	1	0	0	0		✓				
232	2	2	2	1				✓	✓	
233	3	2	3	1				✓	✓	
234	2	0	2	0	✓					
235	1	0	0	0		✓				
236	1	0	0	0		✓				
237	1	0	0	0		✓				
238	3	0	1	0	✓					
239	2	0	0	0		✓				
240	2	0	2	0	✓					
241	2	0	2	0	✓					
242	3	1	2	1				✓	✓	
243	2	1	2	0				✓		
246	2	0	1	0	✓					
247	1	0	0	0		✓				
248	2	0	3	0	✓					
249	1	0	0	0		✓				
250	1	0	0	0		✓				
251	1	0	0	0		✓				
252	2	0	3	0	✓					
253	2	0	1	0	✓					
254	1	0	0	0		✓				
256	0	0	0	0						✓
258	3	0	2	0	✓					
259	1	0	2	0	✓					
260	2	0	0	0		✓				
261	2	0	3	0	✓					
262	3	0	3	0	✓					
263	2	0	1	0	✓					
264	2	1	2	0				✓		
265	1	1	2	0						
266	2	0	3	0	✓					
267	1	1	3	1					✓	
268	1	0	2	0	✓					
270	1	0	1	0	✓					
271	2	0	2	0	✓					
273	2	0	2	0	✓					
274	1	0	2	0	✓					
276	0	0	3	0						

Hybridisation Results					Analysis of Hybridisation Pattern					
Clone	1S	3F	1c	2c	A	B	C	D	E	F
278	0	0	0	0						✓
279	2	1	2	1				✓	✓	
280	0	0	0	0						✓
281	3	1	3	0				✓		
282	3	1	3	1				✓	✓	
283	3	1	3	1				✓	✓	
284	1	1	1	1					✓	
286	2	1	2	1				✓	✓	
287	3	1	3	1				✓	✓	
288	0	0	0	0						✓
1R	0	0	0	0						✓
2R	0	0	0	0						✓

Summary :-

201 of the 204 patterns analysed (**98.5%**) match the A-F criteria

91	**matches for group A candidates**	**45.27 %**
32	**matches for group B candidates**	**15.92 %**
123	**matches for group A or B candidates**	**61.19 %**
0	matches for group C candidates	0.00 %
10	matches for group D only candidates	4.98 %
6	matches for group E only candidates	2.99 %
31	matches for group D & E candidates	15.42 %
31	matches for group F candidates	15.42 %

Using this data, suitable clones corresponding to differentially expressed species, were selected for DNA sequencing. The results are as follows :-

Table 4.3.2: *BLAST-X NR* Search Results

Subtractive Array Clone		BLAST X NR Analysis	
Clone Number	Sequencing PCR Probe	Degree of Uncertainty	Known Sequences on Database which match with the given degree of uncertainty
4	1	0.27	alpha / gamma adaptin
		0.27	delta-like subunit of yeast AP-3
		0.47	tight junction associated protein
		0.61	IDN3-B [Homo-spaiens]
		0.61	IDN3 [Homo-sapiens]
	2R	2×10^{-4}	Latent nuclear antigen
		7×10^{-4}	sex determining protein
		0.002	PV-100 [Cucurbita maxima]
		0.003	Herpesvirus saimiri ORF73 homolog
		0.004	Histidine-Rich Ca^{2+} Binding Protein
		0.008	Convicilin [Psium sativum]
16	1	5×10^{-48}	Antigen-5 Precursor *Glossina morsitans morsitans*
		4×10^{-45}	Antigen-5 related gene product *D. melanogaster*
		6×10^{-12}	Venom Allergen 5.02 precursor *Dolichovespula maculata*
		3×10^{-10}	Venom Allergen 5 (Antigen 5) *Dolichovespula arenaria*
	2R	5×10^{-48}	Antigen-5 Precursor *Glossina morsitans morsitans*
		4×10^{-45}	Antigen-5 related gene product *D. melanogaster*
		6×10^{-12}	Venom Allergen 5.02 precursor *Dolichovespula maculata*
		3×10^{-10}	Venom Allergen 5 (Antigen 5) *Dolichovespula arenaria*
21	1	5×10^{-48}	Antigen-5 Precursor *Glossina morsitans morsitans*
		4×10^{-45}	Antigen-5 related gene product *D. melanogaster*
		6×10^{-12}	Venom Allergen 5.02 precursor *Dolichovespula maculata*
		3×10^{-10}	Venom Allergen 5 (Antigen 5) *Dolichovespula arenaria*
	2R	5×10^{-48}	Antigen-5 Precursor *Glossina morsitans morsitans*
		4×10^{-45}	Antigen-5 related gene product *D. melanogaster*
		6×10^{-12}	Venom Allergen 5.02 precursor *Dolichovespula maculata*
		3×10^{-10}	Venom Allergen 5 (Antigen 5) *Dolichovespula arenaria*

Subtractive Array Clone		BLAST X NR Analysis	
Clone Number	Sequencing PCR Probe	Degree of Uncertainty	Known Sequences on Database which match with the given degree of uncertainty
67	1	0.004	Latent nuclear antigen
		0.005	sex determining protein
		0.011	*Legumin B - fava bean*
		0.014	Histidine-Rich Ca^{2+} Binding Protein
	2R	0.004	sex determining protein
		0.005	Latent nuclear antigen
		0.008	PV-100 [Cucurbita maxima]
		0.018	Histidine-Rich Ca^{2+} Binding Protein
97	1	0.18	delta-like subunit of yeast AP-3
		0.18	alpha / gamma adaptin [Saccharomyces]
		0.41	tight junction associated protein
	2R	2×10^{-4}	Latent nuclear antigen
		6×10^{-4}	sex determining protein
		0.002	PV-100 [Cucurbita maxima]
		0.002	Herpesvirus saimiri ORF73 homolog
105	1	1.8	tight junction associated protein
		2.4	conglutin alpha
		5.4	RNA Helicase [P.falciparum]
		9.3	Major allergenic storage protein
	2R	2×10^{-4}	Latent nuclear antigen
		7×10^{-4}	sex determining protein
		0.002	PV-100 [Cucurbita maxima]
		0.002	Herpesvirus saimiri ORF73 homolog
107	1	1.0	Beta-conglycinin alpha chain precursor
		1.0	hBRAVO/Nr-CAM precursor
		1.0	Neural cell adhesion molecule Nr-CAM precursor
		1.0	Ankyrin binding cell adhesion molecule
	2R	0.002	Latent nuclear antigen
		0.004	Sex determining protein
		0.008	PV100 Cucurbita maxima
199	1	5×10^{-4}	Convicilin [Pisum sativum]
		5×10^{-4}	Convicilin Precursor
		0.001	Latent nuclear antigen
		0.006	sex determining protein
		0.008	PV-100 Cucurbita maxima
	2R	5×10^{-4}	PV-100 Cucurbita maxima
		7×10^{-4}	sex determining protein
		0.002	Latent nuclear antigen - Kaposi Sarcoma Associated Herpesvirus
		0.004	Histidine-Rich Ca^{2+} Binding Protein
202	1	2×10^{-8}	Herpesvirus saimiri ORF73 homolog
		6×10^{-8}	ORF73
		7×10^{-8}	Latent nuclear antigen
		2×10^{-7}	Unknown [Kaposi's Sarcoma associated herpesvirus]
		3×10^{-7}	Mature-parasite-infected erythrocyte surface antigen*
			*Repeat structures in a Plasmodium falciparum protein (MESA) that binds human erythrocyte protein 4.1
	2R	2×10^{-8}	Herpesvirus saimiri ORF73 homolog
		6×10^{-8}	ORF73
		7×10^{-8}	Latent nuclear antigen
		2×10^{-7}	Unknown [Kaposi's Sarcoma associated herpesvirus]
		3×10^{-7}	Mature-parasite-infected erythrocyte surface antigen*
			*Repeat structures in a Plasmodium falciparum protein (MESA) that binds human erythrocyte protein 4.1
216	1	0.003	Sarcoplasmic reticulum histidine rich Ca-binding protein
		0.21	Sex determining protein
	2R	2×10^{-4}	Latent nuclear antigen
		7×10^{-4}	Sex determining protein
246	1	0.33	Conglutin alpha
		8.5	STPK1 [Leishmania major]
	2R	0.008	Legumin B -fava bean
		0.029	Latent nuclear antigen

Subtractive Array Clone		BLAST X NR Analysis	
Clone Number	Sequencing PCR Probe	Degree of Uncertainty	Known Sequences on Database which match with the given degree of uncertainty
262	1	$5x10^{-5}$	sex determining protein
		$2x10^{-4}$	latent nuclear antigen
		$2x10^{-4}$	Sarcoplasmic reticulum histidine rich Ca-binding protein
		$2x10^{-4}$	sex determining protein
	2R	$2 x10^{-4}$	latent nuclear antigen
		$7 x10^{-4}$	sex determining protein
		0.002	PV100 Cucurbita maxima
		0.002	sex determining protein
263	1	0.071	legumin B - fava bean
		0.075	choriogenin H minor
		0.093	conglutin alpha
	2R		*latent nuclear antigen protein*
271	1	$3 x10^{-4}$	sex determining protein
		$4 x10^{-4}$	latent nuclear antigen
		$8 x10^{-4}$	sex determing protein
	2R	$2 x10^{-4}$	latent nuclear antigen
		$6 x10^{-4}$	sex determining protein

4.4 Discussion

The DNA sequences obtained from both the abundancy screening and SSH screening of the salivary glands, from *Stomoxys calcitrans*, revealed a few very interesting sequence similarities to known genes. Perhaps what is more interesting is the fact that most of the sequences, obtained from the salivary glands, did not match any previously known genes. Whilst this latter observation is exciting in terms of the potential for novel molecules, and their activities, it does pose significantly more problems in the initial identification phase.

The most striking sequence similarities obtained from the salivary gland libraries were those which were isolated in both the abundancy screening and SSH techniques. The isolation of these same sequences, using both methods, demonstrates that they are not only unique to the target tissue (salivary glands) but that they are also produced in abundance and are therefore extremely likely to be associated with secretory (salivary) products. One set of sequences in particular demonstrated an extremely strong correlation to salivary antigens from the tsetse fly *Glossina morsitans morsitans*, and venom antigens found in the white-faced hornet *Dolichovespula maculata*, the bald-faced hornet *Vespula maculata*, and the imported fire ant *Solenopsis invicta* among others.[129-137]

The functional characteristics of these molecules have unfortunately not been identified in the literature.[129-137] However, the fact they were discovered immunologically, as the main allergens associated with the saliva of these animals, also provides firm support for the secretory (salivary) role of these antigens in

S.calcitrans. The sequences were identified and isolated from *S.calcitrans* cDNA libraries using 2 seperate screening methodologies, demonstrating both unique and abundant production in salivary gland tissue, and given the high correlation factor which was obtained (5×10^{-48}) from other salivary proteins of unknown function, isolated from haematophagous insect saliva and wasp venoms, it can be assumed that the sequences are derived from a secreted (salivary) molecule in *S.calcitrans* also.

In the absence of data[129-137] providing specific functional activity for the molecule in these cases, and without being able to provide a definitive relationship between the molcule and its activity, any conclusions would be purely speculative. However, given that this molecule has been identified in the venom of hornets & wasps,[130-137] and in the saliva of haematophagous insects,[117,129] - this would imply a functional role which is beneficial to both.

Previous experience tells us that the main categories of activities found in haematophagous insects fall into 3 groups - the anticoagulants, the platelet aggregation inhibitors, and the vasodilators. It is difficult to see how the former 2 categories, anticoagulants and platelet inhibitors, would have any beneficial effect when included in hornet/wasp venom. The main function of a venom is to cause pain and irritation, it is after all a defensive mechanism. The inhibition of the coagulation cascade and platelet aggregation would serve to damp-down any developing immune response, rather than aid in this process. The presence of these activities in a venom, especially given that many snake venoms have potent procoagulant effects, therefore seems highly unlikely.

However the latter of these activities, that of a potent vasodilator, would certainly benefit the hornet/wasp by its inclusion in venom, maintaining blood flow to the area of the sting and aiding in the systemic spread of the venom.

It may be a speculative conclusion, but the most likely activity of the venom-related peptide isolated from the salivary glands of *Stomoxys calcitrans*, is that of a potent vasodilator. The presence of a vasodilator, which is antagonistic to the peripheral vasoactive actions of adrenaline, has already been demonstrated from the salivary homogenate of *S.calcitrans* earlier in this project. It would seem highly likely that these sequences correspond to this activity, although further investigations would obviously be required to confirm these speculations.

Summary

Whilst the antihaemostatic repertoire of many haematophagous species had been painstakingly investigated, the apparent lack of activity[111] from the bloodsucking fly, *Stomoxys calcitrans,* was somewhat suprising, and conspicuous by its absence.

The midgut of *S.calcitrans* contains a type II peritrophic matrix, containing chitin.[113] The luminal surface of the cells lining the gut wall, the microvilli, have a glycocalyx - a carbohydrate-rich electron dense layer.[114] These structures create a perfect environment for the contact activation of the coagulation system and the complement cascade. Given that such an environment exists, one would expect to see the occurrence of clot formation in the midgut lumen, and the accompanied lysis of red blood cells due to membrane damage by complement, and to a lesser extent due to the secondary actions of platelets during the contraction phase, might also be expected. This, however, was not the case. Examination of the midgut regions post blood-meal demonstrates that erythrocytes from the blood-meal remained *intact* in the reservoir zone of *S.calcitrans*, while the contents of the lumen await digestion in the posterior midgut regions. These observations suggested that there must be some unknown protective mechanisms at work, and so began investigations into the antihaemostatic repertoire of *Stomoxys calcitrans*.

A wide range of salivary vasodilators have also been observed in haematophagous arthropods.[3] *Stomoxys calcitrans* is no exception to this, although no activity has been described in the literature to support this before now.[111, 73] This project describes, for

the very first time, an endothelium-independant vasodilator from the salivary glands of *S.calcitrans* with a proposed mode of action suggesting adrenaline receptor antagonism. The haematophagous implications for this mode of action are described, relating this activity to the natural habitat and host behavior observed during feeding, especially in large numbers. The sequence of a cloned gene fragment, putatively identified as the salivary vasodilator, and the high degree of sequence homology between this and other salivary / venom antigens, is also described.

The importance of the Complement system to mammalian defence mechanisms is recognised as playing an increasingly more important role in pathological and inflammatory disease states.[104] The speed of activation, and the interactions with the blood coagulation system, inflammatory response, and vasculature, made it another logical area for investigation. It came as no suprise, therefore, that inhibitory activity was demonstrated against complement-mediated lysis of erythrocytes from both midgut and salivary gland tissue homogenates.

The C1 esterase molecule is a serine protease, and the endogenous mammalian C1 esterase inhibitor (C1-INH) is a potent serpin.[104] The potential exists, therefore, that the midgut- and salivary gland-derived anti-complement activities are due to cross-reactivity of the coagulation inhibitors. Whilst this possibility cannot be excluded using samples of crude homogenate, the fact that complement inhibition has been demonstrated 'at all' has significant evolutionary and functional implications. This remains a significant and important observation, providing additional insight into the many potential hazards posed by haematophagy to those animals which rely upon it.

The hypothesis put forward in this chapter to explain the role of complement inhibition in haematophagy is straightforward, and yet it seems remarkable that no studies into complement inhibition have yet been published for any haematophagous insect.[2] The pharmaceutical potential for a potent complement inhibitor is enormous, and this research provides the first documented evidence demonstrating complement inhibition from any haematophage.

During the course of this project, it is estimated that in excess of 1.5 million flies were reared, from which 30,000 reservoir zone midgut regions and nearly 10,000 salivary glands were laboriously dissected, using extremely delicate micro-dissection procedures. A significant and extremely time consuming undertaking by itself. Whilst a lack of time, material and resources prevented anti-thrombin investigations being performed in parallel with anti-FXa samples, they did provide some very useful data, including a crude measure of the pI value, and other physical properties of the anti-thrombin molecules.

The outcome of these investigations provided data to suggest that the anti-thrombin activity most-likely corresponded to two peptide bands of 26kDa and 34kDa. These bands, visualised and quantified using silver-stained SDS-PAGE gels, always appeared together, and were quantitatively and qualitatively linked to the anti-thrombin activity measured in the column fractions. Data from size filtration experiments demonstrated that the 'active' anti-thrombin has a size range of between 50kDa and 100kDa. If correct, this suggests that the 26kDa and 34kDa peptides

observed in the SDS-PAGE gels may be sub-units, together forming the active thrombin inhibitor.

An anticoagulant comprised of 2 sub-units would not be unknown, since a situation very similar to this is already described in mammalian haemostasis, the Protein-C and Protein-S system.[104] It is particularly interesting to note that this system is activated and regulated by the actions of one enzyme - thrombin!!!

In evolutionary terms if a bloodsucking fly, such as *S.calcitrans*, needed to evolve a midgut-based coagulation inhibitor then it would be logical to direct the actions of this inhibitor late in the coagulation cascade. The bloodmeal has recently been acquired, and despite limited salivary anticoagulation with anti-FXa, all of the contact activation factors would, by now, have been exposed to numerous surfaces on which they can activate. The salivary FXa inhibitor has the inherent problem that any FXa inhibitor has: any FXa which is not totally inhibited is able to activate thrombin. Even a small amount of activated thrombin is sufficient to activate the FIIa:FXIa feedback loop, and the potential to produce a full coagulation response. Therefore the direct inhibition of thrombin would be, and is, a much more efficient strategy. The requirement for *secondary inhibition* then becomes significant, and midgut thrombin inhibitors were evolved.

However, thrombin is an extremely active serine protease, and as such some very potent inhibitors are required to overcome this activity. However, the presence of very potent inhibition, directed against a serine protease, could conceivably have

consequences for the digestion process further along the midgut. The major digestive enzyme used by *S.calcitrans*, which has been demonstrated to have trypsin-like activity, would also be a serine protease.

Therefore the potential exists that, whilst the antithrombin may maintain the bloodmeal in a fluid state in the reservoir zone, it may also interfere with the actual digestion of the bloodmeal. The high dietary value of the protein in the bloodmeal, and the extreme danger that the acquisition of this bloodmeal involved, would imply that any digestive process would need to be at optimal efficiency. The protein resources, required for egg production, could not be squandered due to ineffective digestion.

It is possible that the opaque zone produces and secretes digestive enzyme far in excess of that which could be inhibited by the antithrombin activity. The fact that ultrastructurally this zone is packed with vesicles containing the digestive enzyme may support this. However this would also appear to be a substantial waste of resources if another solution could be found, one which involved selective activities and compartmentalisation.

One solution, and a very effective one, would be to produce a thrombin inhibitor which was significantly larger than anything else known so far - for example a protein complex comprising 2 subunits, one 26 kDa and the other 34 kDa. The size of the inhibitor would make it susceptible to general proteolytic degradation once it enters the opaque zone, immediately destroying the delicate tertiary and quarternary

structure of the protein, and hence any inhibitory cross-reactivity it may have against the trypsin-like enzyme of the digestive system. The protein inhibitor would then be digested, along with the mass of other proteins from the bloodmeal, conserving the fly's valuable stores of protein, which are required for egg production.

This hypothesis would be a truly unique system, never before described in the literature, and it would also explain the many difficulties which were encountered during the purification process. These observations, whilst unable to provide unequivocal evidence for the existence of a 2-subunit thrombin inhibitor from the midgut of *S.calcitrans*, do provide sufficient data to submit the hypothesis for future investigations. Scientific progress is, afterall, a journey of small footsteps.

The decision to construct a salivary gland cDNA library was made, and screening methods were devised which would be used to obtain genetic sequence information for the inhibitors identified above. Construction of the primary cDNA library went very well, yielding 7.33 $x10^6$ clones, but the immuno-screening procedure encountered difficulties. The problems with this screening assay were finally resolved with the demonstration of glycosylation recognition sites by the polyclonal IgG fraction. Therefore an alternative screening protocol had to be devised.

The salivary glands are primarily a secretory organ. As such it was a fair assumption to conclude that the greatest proportion of individual genes, expressed at any point in time, would be secretory in nature. Any means by which these genes could be

compared, and those of similar sequences identified, would allow the identification of groupings which appear in relative abundance.

This *Abundancy Screening* was carried out using the technique known as RFLP (restriction fragment length polymorphism) on hundreds of pure clones, laboriously isolated and amplified to provide a single distinct band. The outcome of this screening process provided 25 potential gene candidates. The sequences obtained from these cloned DNA inserts were extremely interesting, not least because many were novel sequences which had not previously been described in the literature. With no known sequences from molecules with well-defined activities to compare these to, the drawback of this discovery also became apparent.

In the absence of useful information, helping to determine which sequences are related to known anticoagulant activities, it became impossible to distinguish which of these gene fragments (if any) coded for the salivary FXa inhibitor.

The potential benefit of combining the results from the abundancy screening, with those from another technique, was a logical step forwards. A selective subtractive hybridisation (SSH) technique, identifying short stretches of genetic sequence which *are* specific to the salivary tissues, would be the perfect complimentary method. Genetic sequences identified by both methods, i.e. those *specific* to salivary gland tissue *and* produced in greater *abundance*, are more likely to identify the genes and genetic sequences of relevance to the process of haematophagy.

The construction of the SSH library, though a much more complicated and intricate procedure than the cDNA library in some aspects, was a great success. The short gene

sequences in the library could then be used to screen an array of hundreds of pure clones. The hybridisation patterns of these arrays produced valuable data, data which allowed the identification of sequences unique to the salivary gland tissues.

The outcome of this combined screening process revealed sequences from one particular gene of interest, which was selected by both techniques. This sequence was also the only one to produce a strong match on the database, demonstrating extremely close correlation to molecules from the saliva of the tsetse fly, and the venoms of various hornet and wasp species. Whilst the specific function of the peptide coded by this gene is unknown, there is significant circumstantial evidence to suggest the activity would be that of a vasodilator. In addition, the presence of a vasodilator had been physically demonstrated in the salivary glands of *S.calcitrans*. This vasodilator was shown to be antagonistic to the effects of adrenaline, and to act independently of the endothelium. The implications of this mechanism for the process of haematophagy is significant, and the functional and evolutionary advantages of this particular strategy are discussed.

Platelet aggregation studies in response to ADP, and PCR screening of a salivary gland cDNA library, using degenerate primers for a highly conserved region of the apyrase/5'-nucleotidase gene, also suggest the possibility of an apyrase activity. The absence of both time and resources prevented the further investigations which would be required to confirm this activity. The haematological role of this molecule, and the advantage it confers to the process of haematophagy, were also discussed.

In summary, the perceived absence of any anticoagulant / anti-platelet / anti-complement / vasodilatory activity described in the literature for the bloodsucking fly *Stomoxys calcitrans*, led to various haematological and physiological investigations on the salivary gland and midgut tissue homogenates.

The presence of two distinct anticoagulants, targetted against the final (common) pathway of the coagulation cascade, were identified in *Stomoxys calcitrans*. One of these, a potent FXa inhibitor, was demonstrated in the salivary glands. In addition to this, significant vasodilatory activity was also documented, which was shown to be antagonistic to the actions of adrenaline. The putative genetic sequence for the vasodilator was determined, showing extremely strong correlation to antigens isolated from the saliva of tsetse flies, and the venom of wasps and hornets.

Another anticoagulant was identified and partially-purified from the midgut reservoir zone region of *S.calcitrans*. The activity was identified as an anti-thrombin. Physical data suggested the putative structure to be based upon a 2 subunit complex. The evolutionary implications of a midgut-derived inhibitor, in terms of the ultrastructural and haematological requirement for *secondary inhibition*, and digestive strategy in general, are also discussed.

The demonstration of putative salivary apyrase activity is suggested. This was identified using degenerate primers to screen a cDNA library, in conjunction with observations from a platelet aggregation study. Anti-complement activity was also demonstrated, from both salivary gland and midgut reservoir zone homogenates, and the evolutionary and functional implications of complement-mediated haemolysis of the bloodmeal on the digestive processes and midgut lining were also discussed.

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Appendices

(a) Culture methods for *Stomoxys calcitrans*

(b) Sedation and Dissection methods to obtain anterior midgut samples

(c) Homogenisation of anterior midgut samples

(d) Homogenisation of whole fly samples

(e) Transportation of samples to external laboratories

(f) Preparing host bacteria

(g) cDNA quantification using Ethidium Bromide plate method

(h) Media and Reagents

(i) Chomsynski Method of RNA Total RNA Extraction

(j) BioRad Protein Assay

(k) Silver Staining of SDS-PAGE Gels.

(l) SDS-PAGE (Standard Tris-Glycine)

(A) Culture Method for *Stomoxys calcitrans*

Environmental Conditions

Rel. Humidity	50-60% (not critical)
Temperature	26°C
Light Regime	12 hrs light, 12 hours dark

Equipment

Small fish tanks, approx. 13½" x 10" for larval development.
Tank covers, wooden framed lids (fit over top of tank) covered in cotton fabric.
Egg counter, perspex sheet with holes drilled, each hole capable of holding 1,000 eggs.
Fly cages, 12" cube wire cage covered in stockinette, tied-off closely on one side and loosely with a 10" sleeve on the other end for access.

Larval Development Medium

Per tank :
1 litre wood shavings
½ litre calf milk powder
5g dried yeast (15 yeast tablets, ground)
1 litre wheat bran
1¼ litre molassed chaff (e.g. Spillers Fibre Provider)
1 tablespoon malt extract
2 litres water

Procedure

Cages of stock flies for breeding are placed on sheets of coloured paper. The flies are fed daily on heparinised pig blood which is soaked onto a cotton wool pad and placed on top of the cage. After about 4 days of blood meals, female flies begin to lay eggs, which fall through the mesh at the bottom of the cage onto the coloured paper. The eggs are collected daily, and any unused eggs from that day are discarded. Using the egg counter, 3000 eggs are measured out which are then sprinkled into the tank of well-stirred larval medium. The following day the surface of the medium is stirred lightly to disperse any mould which may have grown, and a further 3000 eggs are added. This step is repeated again the next day, to give a total of 9000 eggs per tank. If the medium appears to be dry, and is "stodgy" to stir, then a small volume of water should be added to maintain the moisture content. After all of the 9000 eggs have been added the tank is then covered and left undisturbed for approx 3 weeks, after which time the flies will begin to emerge. Pupae may be floated-off the larval medium and placed directly inside the cages for emergence, or the spare end of stockinette from the cage may be placed around the lid of the open tank to contain the flies once hatched. The latter procedure is the preferred method, and the cages may be removed once a suitable number of flies are collected.

Removal of a cage is achieved by tying-off the stockinette above the tank whilst simultaneously covering the tank with the cotton fabric frame lid. The cage is removed and is replaced by an empty cage, in a similar manner, if further flies are required or expected to hatch. If the tank is depleted and no more flies are expected to hatch then the tank is removed, with its lid in place, and placed in the -20°C freezer for a couple of days. Any larvae still remaining in the tank will thus be killed, and the tank is then safe to be cleaned and re-used.

Mite infestation of the larval tanks is avoided by always making-up fresh medium and using clean equipment. Phorid fly infestation is restricted by sealing the lids on the tanks with double-sided sellotape.

(B) Method for the Sedation and Dissection of *Stomoxys calcitrans* to obtain Salivary Gland/Anterior Midgut samples

Dissection Equipment

Fibre-optic directional light source
Dissecting microscope
2 watchmakers tweezers
1 histology mounting needle
0.5 ml Eppendorf capped sample vials
Liquid nitrogen flask
Polystyrene lid for flask
0.05% Triton-X100 in 150mM NaCl
Glass microscopy/dissection slides
Scalpel
Ice bucket
Plastic tub
Disinfectant waste beaker

Fly Sedation Equipment

Dewar flask
Handheld vacuum
CO_2 Cylinder
Ice bucket
Plastic container
Plastic acquisition probe
Modified Dewar flask lid
Plastic tubing
Fly swat

Sedation Procedure

A modified lid with a long plastic probe is screwed onto a Dewar flask and attached to the handheld vacuum to provide suction. The stockinette of the cage is untied and the probe is inserted, ensuring that no flies are able to escape. The vacuum is switched on the the flies are sucked up and collected in the Dewar flask, the stockinette is re-tied to seal the cage and the vacuum is left on to ensure no flies escape. CO_2 is then pumped down the plastic probe into the flask to sedate the flies, the vacuum may now be switched off and the lid to the flask safely removed. While the flies are still sedated they are transferred into the plastic container which is sat partially submerged in the icebox containing ground ice. The low temperature of this sedation chamber ensures that, although the flies still remain alive, they are inactive once the effect of the CO_2 wears-off. Any flies which escape during these procedures are killed with the fly swat.

Dissection procedure

The flask is filled with liquid nitrogen and a circle of polystyrene is cut-out to form a lid, into which is punched a small hole large enough to hold the 0.5ml plastic Eppendorf sample vial. A drop of dissecting medium was placed in to the centre of the glass dissecting slide. Using the tweezers the flies were then removed from the chilled sedation chamber and placed, legs uppermost, on the dissecting slide so that the dissection medium "sticks" them to the slide and they are unable to fly away.

Holding the thorax firmly with one pair of tweezers, a small section of the lower abdomen along the midline is gripped by the other pair and firmly but slowly torn longitudinally in a direction away from the thorax to expose the contents of the abdomen. If it has not already seperated, the foregut should be seperated from the thorax by holding the gut firmly with the tweezers whilst slowly pulling the thorax longitudinally away in the opposite direction.

Anterior Midgut Samples

The head and thorax of the fly is then discarded in a beaker of bleach / disinfectant. Using the tweezers, the abdomen is now held firmly whilst the foregut is pulled slowly outwards revealing the midgut and hindgut regions. Using the scalpel the midgut is cut, just before the opaque zone, and the abdomen and the remainder of the midgut and hindgut is discarded into bleach / disinfectant.

Opaque zone samples may be collected in a similar manner by removing and freezing the remaining posterior section of the midgut and hindgut after the anterior sample is removed. These are also stored in batches of 50, and clearly labelled to avoid confusion.

Salivary Gland Samples

The abdomen, and its contents, are carefully teased away from the junction with the thorax to reveal the free ends of the salivary glands. These are held with the tweezers, and gently but firmly pulled out of the thoracic cavity. Sometimes, if the salivary glands are not readily visible, the head can be removed and they can be located and removed from this end instead. The sample is then transferred from the tweezers to the mounting needle, and placed in the 0.5ml Eppendorf vial, which is returned to the liguid nitrogen flask. After 50 samples have been collected and frozen in this manner, the Eppendorf vial is capped, labelled and transferred to a -80°C freezer for storage.

(C) Homogenisation of Salivary Gland & Midgut Samples

Equipment

Gilson 200µl pipette
MicroCentaur™ Centrifuge
Glass pasteur pipettes
JENCONS™ manual homogeniser (0.1ml)

Procedure

All manipulations of the sample took place in a cold room (4°C).
Using a Gilson 200 µl pipette, 50µl of 0.05% Triton X-100 150mM NaCl extraction solution (4°C) were added to the sample. The addition of the extraction solution at this stage allows the sample to thaw more uniformly, whilst at the same time providing some protection from overheating (>4°C) during operator handling of the specimen. The cap was replaced and the sample was centrifuged in a MicroCentaur™ for 1 min @ 13,000 rpm to produce a pellet at the bottom of the sample vial. This ensured minimal sample wastage due to adherance on the sides.
The sample was then transferred to a JENCONS™ 0.1ml manual homogeniser using a Gilson 200 µl pipette from which a 5mm section from the tip had been removed by a scalpel to create a wider bore. The sample was homogenised manually for 2 minutes, ensuring adequate rotational and suctional motion, and was then tapped sharply on the bench to dispel the detergent "froth". The homogenised sample was then transferred back into the original vial using a long small bore pasteur pipette, recapped and centrifuged in a MicroCentaur™ for 10 mins @ 13,000 rpm to produce a pellet as before. The supernatant was then removed, using a new long glass pasteur pipette as before, and transferred to a fresh 0.5ml Eppendorf sample vial. The sample volume was then made up to the required volume and the sample either be labelled and refrozen with liquid N_2 and stored at -80°C for future use, or kept on ice and used immediately.

(D) Homogenisation of Whole-Fly Sample

Equipment

Manual homogeniser
MicroCentaur™ Centrifuge
Fisons Chilspin Refrigerated Centrifuge
McCartney bottles
Gilson 5ml pipette
Ice bath

Procedure
A standard cage of flies were killed by placing in a -20°C freezer for 30 mins before they were transferred into a chilled plastic container in an ice bath. They were then transferred in 3 separate batches to the chilled homogeniser and were homogenised with 6ml of 0.05% Triton X-100 in 150mM NaCl soln. The apparatus was kept in the ice bath to maintain a reduced temperature throughout. The homogenated suspension was pooled into a McCartney glass bottle, kept on ice, and cold-centrifuged at 2,000 rpm for 20 minutes in the chilspin. The centrifuged sample exhibited several "bands" or regions (see Table 1) from which samples were carefully removed, using a disposable plastic pipette, into a 0.5 ml vial kept on ice.

Table A1: Centrifugation Zone Pattern of Whole Fly Extract

Position	Zone Colouration	Sample No's
Top	Brown/Dark Beige	1 - 3
↓	Cloudy Red	3 - 22
↓	Red	23 - 24
Bottom	Black	Not Sampled

The labelled vials were then centrifuged at 13,000 rpm for 3 minutes in the MicroCentaur™ centrifuge placed directly into the -20°C freezer, these were then transferred to the -80°C freezer when sample extractions were completed.

(E) Transportation of Samples to External Laboratories

The samples were all frozen in 0.5ml Eppendorf capped vials. These were transported inside a 250 ml plastic snap-shut container suspended in liquid N_2 contained within a vented stainless steel flask. Journey time from freezer to freezer was less than 8 hours, and at no stage during this time did the liquid N_2 boil-off completely, thus the sample arrived in a frozen condition.

(F) Preparing the Host Bacteria

The host strain used was *E.coli* XL1-Blue MRF' strain. It was streaked, from stock, onto LB-tetracycline (12.5μg/ml) agar plates and incubated overnight at 37°C. A single colony was isolated and used to innoculate 10ml of LB with 0.2%(w/v) maltose and 10mM $MgSO_4$ broth. This was grown at 37°C for 4-6 hours, to obtain an OD_{600} of approx. 0.8. The cells were the centrifuged at 500g for 10 minutes, and the supernatant was carefully discarded. The bacteria were then resuspended in 5ml of sterile 10mM $MgSO_4$, and diluted to obtain an OD_{600} of 0.5 using the same. The bacteria were then stored at 4°C and used within 2 hours of preparation.

(G) cDNA Quantification using Et.Bromide Plate Method

100 ml of 0.8%(w/v) agarose was prepared, using TAE buffer. It was allowed to cool to 50°C in a water bath, before adding 10μl of EtBr (10mg/ml) stock solution, and mixed thoroughly by swirling. The solution was immediately poured into 100mm petri-dishes, using approx. 10-12ml per plate, and allowed to set at room temperature. The plates were then inverted and dried in an incubator at 37°C for 45 mins. 1μl of DNA standards, of known concentration, were then applied to the plate covering the range 5-200ng. Then, immediately afterwards, serial dilutions of the sample to be quantified were also applied, in 1μl aliquots, and the plate was allowed to stand for 10 mins in the 37°C incubator to allow complete absorption. The plates were then examined over a UV lightbox, face-down without the lids. The dilutions of unknown were compared to the standards, and the point at which the spot is no longer visible was used to calculate the concentrations.

For example,

Unknown DNA Sample		**Standard (Known) Concentrations**	
Dilution	Visible	[DNA] (ng)	Visible
1:1	Yes	200	Yes
1:2	Yes	150	Yes
1:4	Yes	100	Yes
1:8	Yes	50	Yes
1:16	Yes	40	Yes
1:32	**No**	30	Yes
1:64	**No**	20	Yes
1:128	**No**	10	Yes
1:256	**No**	5	**No**

Lowest visible concentration from standards = 10ng

Lowest visible dilution from unknowns = 1:16

1:16 dilution of unknown = 10ng

∴ 16 x 10ng = **160ng per μl**

(H) Media & Reagents: Agar plates and top-agar

LB Agar

10g NaCl
10g Tryptone
5g Yeast Extract
20g Agar

Distilled water was added to 1 litre
Adjust pH to 7.0 with 5N NaOH
Autoclave

LB Broth

10g NaCl
10g Tryptone
5g Yeast Extract

Distilled water was added to 1 litre
Adjust pH to 7.0 with 5N NaOH
Autoclave

NZY Agar

5g NaCl
2g $MsSO_4.7H_2O$
5g Yeast Extract
10g NZY Amine
20g Agar

Distilled water was added to 1 litre
Adjust pH to 7.0 with 5N NaOH
Autoclave

NZY Top Agar

5g NaCl
2g $MsSO_4.7H_2O$
5g Yeast Extract
10g NZY Amine
7g Agarose

Distilled water was added to 1 litre
Adjust pH to 7.0 with 5N NaOH
Autoclave

(I): Chomczynski Method of RNA Total RNA Extraction

Method followed was exactly as described in :-

Analytical Biochemistry 162, 156-159 (1987):
Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Chomczynski P. & Sacchi N.

(J): BioRad Protein Assay

Standard Assay Procedure
(20-140μg Protein; 200-1400μg/ml)
Prepare several dilutions of protein standard, containing from 0.2 to 1.4 mg/ml. Prepare a standard curve each time the assay is performed.
1. Place 0.1ml of standards and appropriately diluted samples in clean dry test tubes.
2. Place 0.1ml buffer 'blank' in test tube.
3. Add 5.0ml diluted dye reagent.
4. Vortex mix and allow to stand for between 5 mins to 1 hour
5. The OD_{595} was then measured against the blank for the standards and samples.

Microassay Procedure
(1-20μg Protein; ≤25μg/ml)
Prepare several dilutions of protein standard, containing from 1 to 25 mg/ml. Prepare a standard curve each time the assay is performed.
1. Place 0.8ml of standards and appropriately diluted samples in clean dry test tubes.
2. Place 0.8ml buffer 'blank' in test tube.
3. Add 0.2ml dye reagent concentrate.
4. Vortex mix and allow to stand for between 5 mins to 1 hour
5. The OD_{595} was then measured against the blank for the standards and samples.

(K): Silver Staining of SDS-PAGE Gels

Fixing Solution
10% Acetic Acid
40% Ethanol

Staining Solution
0.2% $AgNO_3$ (aq)

Developer
10% Na_2CO_3
0.1% Formaldehyde

Stop Solution
5% Acetic Acid

DeStain Solution
1g Na Thiosulphate
0.3g Potassium Ferricyanide
0.1g Na_2CO_3

1. Soak in fixing solution for 30-60 mins at RT, or overnight at 4°C.
2. Wash 3 times each of 10 mins, gentle shaking in water.
3. Stain for 60 mins at RT with gentle shaking.
4. Brief wash 10 secs in water.
5. Add developer, shake vigorously until bands appear.
6. Discard developer and soak in stop solution for 5 mins.
7. Wash 3 times each of 10 mins, gentle shaking in water.
8. Destain until background is clear.
9. Wash 5 times each of 10 mins, gentle shaking in water.
10. Repeat staining process again if contrast is poor.

(L) Standard SDS-PAGE (Laemmli)[139] Gel Preparation

Buffers

Resolving Buffer pH 8.8
36.4g Tris Base
To 100ml w dH_2O

Stacking Buffer pH 6.8
3.0g Tris Base
To 100ml w dH_2O

Adjust pH of both with HCl and filter through a 0.22μm filter

Lower Reservoir Buffer (x10 Stock) pH 8.8
30.03g Tris Base
To 500ml w dH_2O
Adjust pH with HCl

Run Buffer (x5 Stock) pH 8.4
15.2g Tris Base
72.0g Glycine
5.00g SDS
To 1000ml w dH_2O
Adjust pH with Glycine not HCl

Separating Gel	7%	10%	12%	15%
distilled H_2O	5.1ml	4.1ml	3.4ml	2.4ml
1.5M Tris-HCl, pH 8.8	2.5ml	2.5ml	2.5ml	2.5ml
20% (w/v) SDS	0.05ml	0.05ml	0.05ml	0.05ml
Acrylamide/Bis-acrylamide (30% / 0.8% w/v)	2.3ml	3.3ml	4.0ml	5.0ml
10% (w/v) Ammonium Persulphate	0.05ml	0.05ml	0.05ml	0.05ml
TEMED	0.005ml	0.005ml	0.005ml	0.005ml

Stacking Gel	4%
distilled H_2O	3.075ml
1.5M Tris-HCl, pH 8.8	1.25ml
20% (w/v) SDS	0.025ml
Acrylamide/Bis-acrylamide (30% / 0.8% w/v)	0.67ml
10% (w/v) Ammonium Persulphate	0.025ml
TEMED	0.005ml