# Chapter 6: Major salivary gland proteins from the tick *O. savignyi* function as toxic and granule biogenic proteins*

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## 6.1 PART 1: THE SECRETORY PATHWAY

### 6.1.1 Targeting of secretory proteins to the secretory pathway

All tick secretory proteins have to enter the secretory pathway. The conventional pathway of protein secretion starts with targeting to the endoplasmic reticulum (ER), vesicular transport to the Golgi-apparatus and shuttling through the Golgi compartments to the trans-Golgi network (TGN), where sorting of proteins to their various destinations takes place (Arvan and Castle, 1998). Secretory and plasma membrane proteins are sorted to vesicles that are continuously secreted (constitutive pathway). This anterograde movement up the secretory pathway is considered to occur by default for all proteins that do not contain any other targeting signals to other cellular compartments. Secretory proteins with the correct sorting signals may also be packaged into regulated secretory granules or sorted to endosomal organelles such as lysosomes (Kelly, 1985; Chidgey, 1993; Blazquez and Shennan, 2000).

### 6.1.2 Targeting to the ER and Golgi-apparatus

All proteins destined for secretion, sub-cellular organelles or the plasma membrane have an N-terminal signal peptide that targets it to the ER during protein translation (Nielsen *et al.* 1997). The common structure of signal peptides from eukaryotic proteins includes a positively charged n-region (only slightly arginine rich), followed by a hydrophobic h-region (short, very hydrophobic) and a neutral but polar c-region. The (-3, -1)-rule states that the residues at positions –3 and -1 (relative to the cleavage site) must be small and neutral for cleavage to occur correctly (von Heijne, 1990). The signal peptide ensures translocation across the ER-membrane and after translocation is cleaved off by a signal peptidase. In the ER, chaperone assisted folding occurs as well as the formation of disulphide bonds. While disulphide bonds are absent in cytoplasmic proteins, most of the

cysteines present in secretory proteins are disulphide bonded and function as a stabilizing force in the protein's structure (Fahey, Hunt and Windham, 1977). N-glycosylation occurs in the ER and is involved in correct protein conformation folding. A C-terminal retention signal (KDEL) is present in resident ER proteins and ensures retention in the ER and retrieval from the cis-Golgi, if ER-proteins escape the ER (Nilsson and Warren, 1994). Non-resident proteins are transported via COPII coated vesicles to the Golgi apparatus (Chidgey, 1993; Blasquez and Shennan, 2000). In the Golgi apparatus O-linked glycosylation can occur, while sorting of proteins to their different cellular destinations takes place in the TGN. There are three pathways by which proteins are targeted: constitutive, regulatory and lysosomal (Kelly, 1985).

### 6.1.3 The constitutive and regulated secretion pathway

Constitutive secretion is ubiquitous in all cells and considered to be the bulk-flow pathway of secretion, which secretes proteins without distinction. The secretory vesicles are normally small, electron lucent and characterized by uninterrupted secretion. Proteins secreted are normally plasma-membrane proteins or proteins secreted at a constant level. The regulatory pathway is limited to endocrine, exocrine and some neuronal cells. The vesicles that the proteins are packaged into are large, electron dense and are referred to as secretory granules. Secretion only takes place upon stimulation by a pharmacological agent (Chidgey, 1993: Urbé, Tooze and Barr, 1997; Arvan and Castle, 1998; Tooze, 1998; Glombik and Gerdes, 2000). As such, tick salivary glands can be described as exocrine glands that undergo regulated secretion during feeding. There is a selective sorting of regulated secretory proteins to granules and exclusion of those not involved, in contrast to the indiscriminate bulk-flow of the constitutive pathway (Urbé, Tooze and Barr, 1997). No specific signal sequence for regulatory secretion has been identified to date, due to the lack of sequence homology between secretory proteins. It has been suggested that targeting signals may be comprised of surface epitopes rather than being sequence specific (Chidgey, 1993). Currently two possible targeting mechanisms are considered.

### 6.1.4 "Sorting for entry" or "sorting by retention"

During "sorting for entry", proteins are selected in the TGN prior to immature secretory granule (ISG) formation, while "sorting by retention" indicate selective retention of granule specific proteins in the ISG and removal of mis-sorted proteins from the ISG (Arvan and Castle, 1998).

### 6.1.5 Aggregation as a means of sorting

The most characteristic feature of the regulated secretory pathway is the high concentration of secretory proteins and their aggregation into dense core granules. This process has been referred to as concentration, multimerization, polymerization and condensation. This part of the formation of granules (condensation of soluble secretory proteins into large aggregates) will henceforth be described as granule biogenesis. These interactions seem to reflect progressive protein insolubility that limits the ability of proteins to escape from the maturing granule (Arvan and Castle, 1998). A high local concentration of secretory proteins in the TGN is necessary for efficient aggregation, so that soluble secretory proteins are generally very abundant in cells with a regulated secretory pathway (Urbé, Tooze and Barr, 1997). Specific proteins are involved in granule biogenesis by sorting of other secretory proteins to the regulated secretory granules by co-aggregation (Arvan and Castle, 1998). These proteins have been identified in most endocrine and exocrine cells. They are present in high abundance, have acidic iso-electric points and can aggregate under conditions of mild acidity (pH5-6) and high calcium concentrations (50-100 mM), the conditions thought to be present in the TGN (Huttner, Gerdes and Rosa, 1991; Urbé, Tooze and Barr, 1997; Blasquez and Shennan, 2000).

### 6.1.6 Granule biogenesis in tick salivary glands

In the soft tick *Ornithodoros moubata*, dense core granules were shown to be secreted within five minutes after attachment (El Shoura, 1985) suggesting that these granules are intracellular stores of bioactive components. Such dense core granules are known to be universal among regulated secretory cell types (Arvan and Castle, 1998). One specific characteristic of granule biogenic proteins is their unusual abundance. In this study,

proteins from tick salivary glands were identified as potential proteins involved in granule biogenesis based on their high abundance and are referred to as tick salivary gland proteins (TSGPs). A study of granule contents could provide important information on proteins involved in granule biogenesis and could aid the understanding of the observed diversity in the number of different granular cell types observed for tick salivary glands. Disruption of granule formation and subsequent secretion of granule contents could be an efficient strategy to control tick feeding.

### 6.1.7 Granule biogenesis and tick toxicoses

During the investigation into the toxicity of savignygrin and the characterization of the TSGPs, the surprising discovery was made that some of the TSGPs are the toxins previously described for the tick *Ornithodoros savignyi*. Therefore, a brief review of tick toxicoses is imperative.

## 6.2 PART 2: TICK TOXINS

### 6.2.1 Tick toxicoses

Certain tick species can cause pathological changes in their host by inoculation of non-infectious components during feeding. Tick toxins have been identified in whole tick extracts, salivary gland secretions (SGS), SGE and tick eggs. Toxins can be a natural tick product that is either in itself toxic or is transformed into a toxic component in the host, or toxins are derived from breakdown of host tissue or are a product of a symbiotic organism in the tick (Gregson, 1973). Various other possibilities have also been reviewed (Neitz *et al.* 1983). While, arthropods such as spiders and scorpions are notoriously venomous organisms that utilize their toxins for protection as well as predation, the advantages of ticks being toxic is unclear. It has been suggested that tick paralysis may be a vestigial function conserved in ticks, when ticks evolved a parasitic lifestyle (Stone *et al.* 1989). Paralysis toxins have been attributed with functional significance during feeding of the tick, in that host mobility and grooming is impaired. Paralysis would also affect the respiratory system leading to heightened breathing rates and an increase in carbon dioxide expiration. This together with pheromone secretion could attract ticks to

the paralyzed animal, which accelerates the finding and feeding of ticks. It has been argued that this might be true for most ticks, even though no clinical symptoms can be observed in the majority of feeding events (Gothe, 1983). Toxins might also exert local anaesthesia, prevent blood coagulation or act as a general feeding stimulant (Stone *et al.* 1989). A role as a regulator of protein synthesis has also been suggested for the paralysis toxin from *R. evertsi evertsi*, based on localization to chromatin in the nuclei (Crause *et al.* 1993).

### 6.2.2 Toxicoses from an evolutionary perspective

Another consideration is whether tick toxins have a common ancestor shared with toxins from other toxic arthropods, as this would assign biological significance or function to tick toxins. Toxins might also have specific functions related to their toxicity that was specifically acquired during adaptation to a blood-feeding environment. Toxicity could also be a byproduct of proteins being in a novel environment and recognition of host targets, a chance event. To investigate these possibilities, it is important to clearly delineate various forms of toxicoses and find their shared properties or differences (mechanism of pathogenesis, homology), as this will give valuable information as to their origins. Such comparative studies are the one way in which a holistic view of tick toxicoses will be attained.

### 6.2.3 Tick paralysis

The most important tick toxicoses for veterinary and human medicine are tick paralysis (Gothe and Neitz, 1991). Of the approximately 869 tick species, paralysis has been described for 55 hard ticks and 14 soft ticks (Gothe, 1999). However, for many of these species only a few, often inadequate or dubious records regarding the actual toxicity exist in the literature. The most important include the hard ticks *Ixodes holocyclus* (Australia), *Dermacentor andersoni*, *D. variabilis* (North America), *I. rubicundus* and *Rhipicephalus evertsi evertsi* (South Africa). Soft ticks for which paralysis has been described, demonstrated or suspected include *Argas walkerae*, *O. capensis*, *O. savignyi*, *Otobius megnini* (South Africa) and *O. lahorensis* (Eurasia). In all ticks that induce paralysis the secretion of neurotoxin coincide with a definite repletion phase. For *R. evertsi evertsi*

paralysis, toxicity is associated with a short period between day 4 and 5 of feeding and a specific body mass of 15-21 mg (Gothe and Lämmler, 1982; Neitz and Gothe, 1986). Paralysis induced by the tick *I. holocyclus* only set in after 4-5 days of feeding, while paralysis for *D. variabilis* is only detected after approximately 6-8 days after attachment (Gregson, 1973; Masina and Broady, 1999). In the soft tick *A. walkerae*, it is only larvae that cause paralysis, which occurs after 5-6 days of feeding (Gothe, 1983; Gothe and Neitz, 1991). In all instances, paralysis coincides with the rapid engorgement phase that is marked by the production and secretion of numerous protein products by the salivary glands.

### 6.2.4 Pathogenic mechanisms of tick paralysis toxins

Paralysis is normally exhibited as an ascending flaccid tetraplegia, due to an impaired nervous system (Gothe and Neitz, 1991). While these are general observed symptoms, most neurotoxins have specific characteristics not necessarily shared with toxins from other tick species.

### 6.2.5 *Dermacentor andersoni*

*D. andersoni* affects motor neurons of the efferent pathway and not the afferent. The neuromuscular junction of peripheral nerves is targeted through inhibition of acetylcholine release from the synapse, suggesting a pre-synaptic target (Gothe and Neitz, 1991). Feeding ticks affect dogs, sheep, cattle, guinea-pigs, hamsters and man, but not cats, rabbits, rats and mice. In those animals affected the symptoms could be fairly rapidly reversed on removal of ticks, except for marmots that frequently do not recover (Emmons and McLennan, 1980). Gross symptoms of paralysis in marmots include the loss of the animals normal piercing cry (paralysis of the vocal cords) followed by an ataxia and weakness of the hind limbs. The condition progressess until the fore limbs are paralyzed and the animal is unable to move and lie on its side. There is retention of urine and faeces (Emmons and McLennan, 1980). No paralysis could be observed when SGE from fast feeding ticks were injected subcutaneously into mice and lambs and intraspinally into puppies (Gregson, 1943). Fractionated extracts also failed to produce any symptoms. However, paralysis was observed when saliva from fed females was

continuously injected subcutaneously into marmots and hamsters (Gregson, 1973). Variation in the ability of individual ticks to cause paralysis was also observed. This highlights the problems associated with paralysis from *D. andersoni*. It also points out why progress on this specific toxicoses has been so slow in recent years. No development of immunity has yet been reported for *D. andersoni*, while immunity to the holotoxin from *I. holocyclus* is well established. Hyper-immune serum against holotoxin also fails to relieve *D. andersoni* paralysis (Gregson, 1973).

### 6.2.6 *Ixodes holocyclus*

*I. holocyclus* can paralyze dogs, cats, cattle, horses and humans. Only adult ticks have been associated with paralysis, while larvae only causes local irritation. SGE affects dogs and mice, although only suckling mice (4-5g) are generally affected, while adult mice (20-25g) do not show paralysis symptoms (Stone and Binnington, 1986). An increase in blood pressure occurs during paralysis, in contrast to paralysis caused by *D. andersoni* where blood pressure stays normal. Purification attempts of the neurotoxin from *I. holocyclus* showed that the paralysis toxin was associated with high molecular mass complexes (40-80 kDa) (Masina and Broady, 1999). A toxic lethal fraction (Mr<20 kDa) without paralyzing activity was also identified in *I. holocyclus* and it was suggested that this might be the agent of cardiovascular failure previously attributed to holotoxin (Stone, Doube and Binnington, 1979). Recently this toxin was purified and cloned and was shown to have a molecular mass of ~5 kDa which binds to rat synaptosomes in a temperature dependent manner (Thurn, Gooley and Broady, 1992). This temperature dependence coincides with earlier observations that there is a temperature-dependent inhibition of evoked acetylcholine release during paralysis (Cooper and Spence, 1976).

### 6.2.7 *Rhipicephalus evertsi evertsi*

*R. evertsi evertsi* affects the peripheral nervous system by inducing a motor polyneuropathy in sheep and is appropriately known as spring lamb paralysis (Gothe and Kunze, 1982). Ticks fed on laboratory animals affected mice, rats, hamsters, guinea pigs and rabbits only slightly or not at all (Gothe and Lämmler, 1982). Injection of SGE into sheep, mice and chickens, failed to elicit a paralysis response (Viljoen *et al.* 1986). A

very sensitive *in vitro* assay using a sciatic nerve -gastrocnemius muscle preparation was developed to characterize this toxin (Viljoen *et al.* 1986). In this assay the dissected nerve was bathed with SGE or purified neurotoxin in a specially constructed nerve bath with a volume of 60 µl. In contrast to the *in vivo* tests, both SGE and neurotoxin preparations effectively paralyzed muscle contraction (Viljoen *et al.* 1986;. Crause *et al.* 1994). It could thus be argued that the neurotoxin affects the nerve and not the neuromuscular junction. Large quantities of SGE (400-900 mg protein) were however used to elicit a response and it could be argued whether this was a truly specific response. However, total inhibition of nerve impulse propagation was observed with purified preparations (74µg/ml), which is probably much closer to physiological conditions (Viljoen *et al.* 1986). The toxin from *R. evertsi evertsi* that purified as a ~68 kDa protein (Viljoen *et al.* 1986), was later shown to be the trimeric form of a ~20 kDa protein (Crause *et al.* 1994). A monoclonal antibody directed against this toxin showed cross-reactivity with both non-paralysis (*R. appendiculatus*, *Hyalomma marginatum rufipes*, *Boophilus decoloratus* and a non-paralysing strain from *R. evertsi evertsi*) as well as paralysis-inducing ticks (*I. rubicundus*, *A. walkerae* and a paralysis-inducing strain of R. *evertsi evertsi*). Significant was the fact that only paralysis-inducing ticks seem to posses a ~68 kDa reactive antigen (Crause *et al.* 1994).

### 6.2.8 *Argas walkerae*

Electromyographical studies indicated that the fast conducting nerve fibers of the peripheral nervous system were affected and the paralysis was classified as a motor polyneuropathy that does not affect the afferent paths (Gothe *et al.* 1971; Gothe and Kunze, 1971; Kunze and Gothe, 1971; Gothe and Neitz, 1991). The toxin seems to affect the liberation of acetylcholine as well as its receptor's sensitivity at the myoneural synapse (Gothe and Neitz, 1991). The monoclonal antibody directed against the toxin from *R. evertsi evertsi* also recognized protein complexes of molecular mass 60-70 kDa from crude *A. walkerae* extracts and prevented paresis of day old chicks (Crause *et al.* 1994). The toxic fraction was purified using a bioassay based on injection of day old chicks. The purified fraction showed two bands, with molecular masses of 32 and 60 kDa, using reducing SDS-PAGE, while one band (pI~4.5) was obtained with iso-electric

focusing. Macromolecular complexes (80-100 kDa) were observed using size exclusion chromatography (Viljoen *et al.* 1990). Recently, a monoclonal antibody directed against the toxin from *R. evertsi evertsi*, was used in an attempt to purify the neurotoxin from *A. walkerae* extracts and whilst a 68 kDa toxin was detected using western blot analysis, a 11 kDa protein was purified that showed cross-reactivity with the mAb using enzyme linked immunosorbent assay (ELISA), although not being detected during western blot analysis (Maritz *et al.* 2000). Crude *A. walkerae* larval extracts inhibited potassium stimulated and veratridine evoked release of [$^3$H] glycine from rat brain synaptosomes, suggesting that the toxin might be targeting ion channels involved in depolarization (Maritz *et al.* 2001). These conflicting results between purification by bio-assay and detection with a monoclonal antibody have not been resolved yet.

### 6.2.9 Common ancestors for paralysis toxins

There is no consensus yet whether the paralysis toxins from different species are homologs. Based on sequence similarity of holotoxin with scorpion toxins (data not yet published) it was speculated that other paralysis toxins from ticks might also be related (Masina and Broady, 1999). On the other hand, insect neurotoxins from predatory mites, *Pyemotes tritici* (Superorder: Acariformes) have been shown to be unique with molecular masses of ~30 kDa (Tomalski and Miller, 1991; Tomalski *et al.* 1993). Toxins from different arachnid subclasses (spiders and scorpions) also do not all fall into the same protein families (Mênez, 1998; Escoubas, Diochot and Corzo, 2000; Rash and Hodgson, 2002). Ticks are also more closely related to the ricinulei (tick-like spiders) and spiders, than scorpions (Lindquist, 1984; Schultz, 1990). The conclusion derived from this is that evolutionary relationships between toxins from the different arthropod classes cannot yet be inferred with certainty.

### 6.2.10 Other forms of toxicoses considered as non-paralytic

The fact that some forms of tick toxicoses are considered to be non-paralytic indicates that a study into the toxic mechanisms of ticks should take this into account. If toxicoses can be shown to clearly differ in their mechanisms of action, it would provide a specific reference point in cataloging the different toxicoses forms.

### 6.2.11 Toxins from tick eggs

It has been shown that tick egg extracts from 17 ixodid species are toxic, while extracts from 5 argasids species were not (Riek, 1957). Egg toxins are interesting due to their possible relationship with tick paralysis and other tick toxicoses. They have been studied in various species (*A. hebraeum, R. eversti evertsi, H. truncatum, B. microplus, B. decloratus*) (Neitz *et al.* 1981; Viljoen *et al.* 1985). The toxin from *A. hebraeum* exhibits protease inhibitory activity. After injection with crude extracts, guinea-pigs showed several symptoms: hyperaestasia, serous nasal and eye discharge accompanied by conjunctivitis and rhinitis, ascending paresis and a loss of voice over a 15-36 hour period. Pathology included necrosis of the liver and oedema of the urinary bladder, lungs and skin (at the site of injection). Lesions were probably all of vascular origin. The toxin had a molecular mass of ~10 kDa and a pI ~7.5-8.5 (Neitz *et al.* 1981). Egg extracts and purified fractions from *R. evertsi evertsi*, *H. truncatum*, *B. microplus* and *B. decloratus* all induced anorexia and severe hyperaestasia, hyperaemia of the skin, muco-purulent occulo-nasal discharge, mucoid diarrhea which prior to death become haemorrhagic. Only partial paresis was observed. The egg toxins differed in molecular mass: 27-40 kDa for *H. truncatum*, *B. microplus* and *B. decloratus* and ~5-6 kDa for *R. evertsi evertsi*. The *R. evertsi evertsi* toxin also had a lower pI (~6) compared to the other toxins (8.3-9.3) (Viljoen *et al.* 1985). This indicates that the toxic entities in *R. evertsi evertsi* salivary gland and egg extracts are different molecular entities.

### 6.2.12 Sweating sickness caused by *Hyalomma truncatum*

Sweating sickness affects especially young calves and this disease is widely distributed in central, eastern and southern Africa. Lethal, as well as two milder forms, Mhlosinga and Magudu occur and are associated with sweating sickness-inducing (SS+) strains of *H. truncatum*, while non-inducing strains (SS-) also exist (Neitz, 1962). Symptoms include fever, eczema and anorexia. Removal of ticks results in immediate recovery and salivary gland secretions have been implicated in the disease, suggesting the presence of a toxin (Neitz, 1959). Analysis of anti-sera raised against SS+ and SS- forms shows the presence of at least four novel antigens in SS+ salivary gland extracts, which might be associated with toxicity (Burger *et al.* 1991).

### 6.2.13 Sand tampan toxicoses

*O. savignyi* causes death of domestic animals, especially young calves and lambs. While death has been attributed to exsanguination (Howell, Neitz and Potgieter, 1975), the presence of a proteinaceous toxin in SGS, SGE and larval extracts of *O. savignyi* has been described. Toxic activity has not been found in coxal secretions or tick eggs, in contrast to eggs from hard ticks (Neitz, Howell and Potgieter, 1969; Neitz *et al.* 1983; Howell, Neitz and Potgieter, 1975). An acidic toxin has been purified and characterized in terms of molecular mass (15400 Da) and N-terminal sequence (Neitz, Howell and Potgieter, 1969; Neitz *et al.* 1983).

### 6.2.14 Pathogenesis of sand tampan toxicoses

A summary of the only report compiled yet on the symptoms produced by sand tampan toxicoses (C.J. Howell, unpublished research report, Onderstepoort Veterinary Research Institute, South Africa, 1969) follows:

(1) "A Fries bull of 14 months (300 kg) was confined to a tampan infested camp where it was tethered to a tree for two hours daily. The animal remained in a standing position while in the camp and died shortly after removal on day four (8 hours exposure). All bites were confined to the legs of the animal. No clinical signs of ill-health were observed during this period and only on day four, immediately after leaving the infested camp, did the animal appear in a state of shock prior to sudden collapse and death. Pathology showed edema in the myocardium, integument, intestinal tract, lungs, regional lymph glands and kidney. Eosinophilic granules were observed in the capillaries of the brain, myocardium, kidneys and adrenal glands. Degeneration of the myocardium and extensive necrosis, hemorrhage, congestion and edema of the epidermis and petechial hemorrhages in the lungs were also observed. The animal possibly died of heart failure, although the basic lesions are probably due to an increased vascular permeability.

(2) An adult merino wether (40 kg) was injected subcutaneous (2.5ml ~58 mg per subcutaneous site) with 12.5 ml of SGS over a period of 4 hours. Two and a half hours after the final injection the animal lied down, whereupon it died

immediately. No clinical symptoms were observed up to this time, although pathological and histological changes were extensive. Congestion and edema were observed in myocardium, spleen, kidney, lungs and lymph glands. An increased vascular permeability that possibly resulted in a severe degeneration and necrosis of the capillary vessel walls was observed in the integument and subcutaneous region."

(3) "Subcutaneous injection of SGS (100 µl~2.3 mg), into albino rats, caused facial irritation exhibited by rubbing of the face. A clear mucoid substance was discharged nasally after some hours, which soon became hemorrhagic. By this time there were signals of impaired breathing and the rats assumed an upright stance before becoming comatose, after which they never recover. Increasing the dosage rate leads to less symptoms and a more rapid mortality. Pathological changes include congestion, edema and emphysema of the lungs.

(4) The feeding of three ticks on a guinea pig were enough to cause mortality with no symptoms being observed before death (Howell, Neitz and Potgieter, 1975). Pathological changes are confined to the lungs and vascular system and include congestion, edema and hemorrhages.

(5) Subcutaneous injection of mice with SGS resulted in rapid death with few symptoms. Some animals appear completely normal until death, which proceeds swiftly. Some mice may jump in the air and immediately die with a few convulsive movements. Other mice appear slightly ruffled soon after injection, with accelerated breathing, which soon becomes labored, followed by animals reeling drunkenly about for a few seconds before dying after a few convulsive movements. Symptoms for the latter mice suggest respiratory failure, as the heart is still active after all respiratory movement has ceased. No pathological symptoms could be observed." Feeding of one tick on a 20-24g mouse is sufficient to cause death within 30 minutes (personal observation).

The present study characterizes these toxic activities in terms of cardio-toxicity and shows that tick paralysis is not associated with the pathology induced by *O. savignyi*.

Successful neutralization of toxic activities could relieve the burden that these ticks place on their domestic hosts.

## 6.3 Materials and methods

### 6.3.1 Two-dimensional polyacrylamide gel electrophoresis

SGE (~120 µg/ 10 µl) were prepared in water and diluted into 100 µl sample buffer (8M urea, 2% CHAPS, 2% IPG-buffer pH 3-10NL, 0.3% DTT) and applied to IPG strips, 3-10NL (non-linear) using the Immobiline® DryStrip Reswelling Tray (Sanchez *et al.* 1997). The Multiphor II system (500V for 10 min, 1500V for 4 hours at 10W) was used for iso-electric focusing (first dimension), under conditions recommended by the manufacturers (Amersham Pharmacia Biotech, Uppsala, Sweden). The second dimension was performed using a vertical 16% tricine SDS-PAGE system (Schägger and von Jagow, 1987). SDS-PAGE analysis without prior iso-electric focusing was performed with 16% tricine SDS-PAGE or 12% glycine SDS-PAGE (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue G250.

### 6.3.2 Isolation of salivary gland granules after dispase treatment

Due to the fact that no homogenous preparation of salivary gland granules could be obtained with collagenase digestion (Chapter 5), a new fractionation process was developed using dispase digestion. Salivary glands were dissected and a granule fraction was prepared by digestion with dispase (20 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.2, Roche Diagnostics) for 3 hours at 37 °C. After centrifugation (100xg, 10 min, room temperature), the pellet was layered onto a preformed Percoll density gradient (70% initial concentration). After density gradient centrifugation (180xg, 15 min, room temperature), visible bands (density ~ 1.14 g/ml) were processed for electron microscopy analysis.

### 6.3.3 Electron microscopy and X-ray microanalysis of granules

Granules were prepared for electron microscopy as described for platelets (chapter 2). For X-ray microanalysis, whole salivary glands were frozen in propanol, cooled with liquid nitrogen and lyophilized. Analysis was performed under low vacuum on uncoated

glands with a JEOL 5800LV SEM (0.3 Torr, 25 kV) by focusing (3 μm window) on visible granules beneath the cell membrane. Data were collected on a Noran Voyager ED System from four randomly selected granules with 7 μm diameters.

### 6.3.4 Reconstituting the conditions present in the *trans*-Golgi network (TGN)

Conditions thought to exist in the TGN include a mildly acidic pH and the presence of $Ca^{2+}$ (Arvan and Castle, 1998). It has been shown that proteins targeted to secretory granules can aggregate under these conditions (Colomer, Kickska and Rindler, 1996). Salivary gland extracts (1SG/10μl or ~6mg/ml protein), prepared in water, centrifuged (14000xg, 25 min) and filtered through 0.22 μm filters, were diluted in a high $Ca^{2+}$ buffer (20mM MES, 50 mM $CaCl_2$) and titrated to different pH values with HCl before incubation at 4 °C for 30 minutes. Extracts were centrifuged at 14000xg in a microcentrifuge for 25 minutes at room temperature. The supernatants were removed and precipitated protein washed twice in 1 ml of dilution buffer and recentrifuged before SDS-PAGE analysis.

### 6.3.5 Purification of the TSGPs

Tick salivary gland extract (60 glands, ~3600 μg) was prepared in 1 ml buffer A (20 mM Tris-HCl, pH 7.4) and were heat treated (60 °C, 5 minutes), as this is known to remove most proteins with molecular masses greater than 20 kDa (Fig. 6.4). Denatured protein was removed by centrifugation (10 min, microcentrifuge) and the supernatant was filtered through a 0.22 μm filter (Millipore Corporation, Bedford, Massachusetts) before application to AEHPLC as described in Chapter 2. Alternatively, supernatant was applied to a cation exchange column (CEHPLC) (SP-5PW, 7.5 mm x 7.5 cm, TosoHaas) using the same conditions as for AEHPLC. Fractions from AEHPLC were adjusted to 1.7 M ammonium chloride and applied to a hydrophobic interaction column (HIHPLC) (TSK-Phenyl-5PW, 7.5 mm x 7.5 cm, Bio-Rad, Richmond, California). Fractions were eluted with a gradient of buffer A (20 mM Tris-HCl, 1.7M $NH_4Cl$, pH 7.4) and buffer B (20 mM Tris-HCl, pH 7.4) from 0-100% over 15 minutes. Fractions from CEHPLC and HIHPLC were desalted using RPHPLC as described (Chapter 2). Elution was achieved with a gradient of buffer A (0.1% TFA, 0.1% acetonitrile) and buffer B (0.1% TFA, 60%

acetonitrile) from 0-100% over 5 minutes. SEHPLC was performed using isocratic conditions (SEHPLC buffer) with a flow speed of 0.5 ml/min (Mans *et al.* 1998b). In all cases 1ml fractions were collected and 200$\mu$l were injected subcutaneous to monitor toxic activity. Adult Balb/c mice (8-11 weeks old, ~20-24g) were used and survival was monitored over a period of 48 hours (Neitz, Howell and Potgieter, 1969). Injections were performed in duplicate.

### 6.3.6 Characterization of TSGPs

Proteins were quantitated with amino acid analysis, alkylated, N-terminally sequenced, molecular masses determined using tricine SDS-PAGE, MALDI-TOF-MS and ESMS as described in Chapter 2. Peptide mass fingerprinting was performed as described previously.

### 6.3.7 The effect of SGE on an isolated rat heart perfusion system

An adult Sprague-Dawley rat was anaesthetized with sodium pentobarbitone. The heart was dissected on ice using Tyrode buffer (5.4 mM KCl, 137.6 mM NaCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM Glucose, 11.6 mM HEPES, pH 7.4). The dissected heart was coupled to a reverse Langendorf perfusion system and was perfused at 37°C with Tyrode for 30 minutes until a stable heartbeat was obtained. SGE (10 glands diluted into 10 ml Tyrode buffer: 60 $\mu$g protein /ml) was then injected into the flow stream. Experiments were performed in duplicate with different salivary gland preparations.

### 6.3.8 The effect of toxic fractions on mouse ECG patterns

Female Balb/c mice were anaesthetized with sodium pentobarbitone (6%) solution and electric leads were connected to the left forepaw and right-hindpaw, with the earth lead to the left-hind paw. Mice were monitored until a stable ECG pattern was observed before toxic fractions (200 $\mu$l) were injected intra-peritoneal. The ECG pattern was monitored until cardiac seizure was observed.

## 6.4 Results

### 6.4.1 Identification of major salivary gland proteins

Putative proteins involved in granule biogenesis were identified by proteome analysis of SGE from *O. savignyi*. Two-dimensional analysis indicated the presence of at least 8 major proteins in salivary gland extracts from *O. savignyi* (Fig. 6.1). Four were identified as major tick salivary gland proteins (TSGPs), based on their high abundance and similar molecular mass (~20 kDa). It was hypothesized, that proteins with similar molecular masses might belong to the same protein family and would thus be suitable candidates for the study of gene duplication in tick salivary gland proteins. Analysis of the TSGPs shows molecular mass differences in decreasing order: TSGP1>TSGP4>TSGP3>TSGP2. This together with tricine SDS-PAGE and MALDI-MS were used to identify the respective purified proteins as TSGPs.

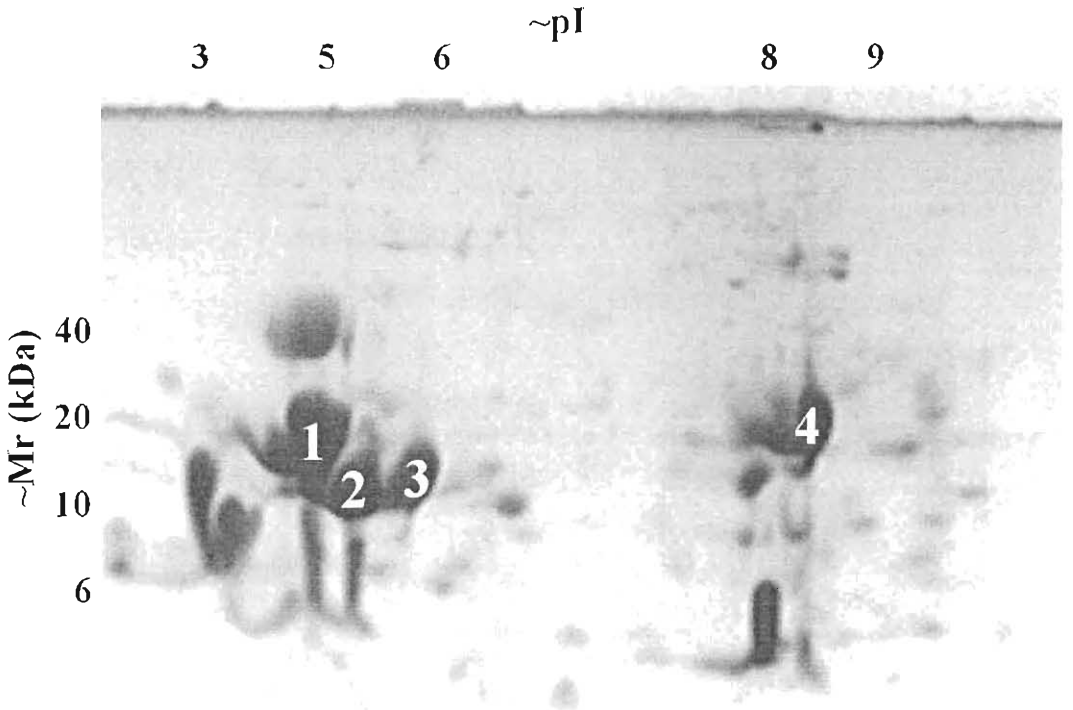


**Fig. 6.1:** The proteome of tick salivary gland extract. Highly abundant proteins characterized in this study are numbered (TSGP1, TSGP2, TSGP3, TSGP4). Approximate molecular masses and pI's are indicated.

### 6.4.2 Purification of dense core granules

While SGE can be considered to be representative of the proteins in the salivary glands, confirmation of the abundance of TSGPs in granule preparations was also required. Digestion of salivary glands with dispase and subsequent density centrifugation resulted in a homogenous preparation of dense core granules (Fig. 6.2a-c). Compared to density markers these individual granules had a density of ~1.14g/ml. Analysis of dense core granule showed enrichment of the TSGPs (Fig. 6.2d). The identity of the proteins present in the dense core granules were confirmed by two-dimensional electrophoresis, where they gave a similar electrophoretic pattern as observed in Fig. 6.1 (results not shown).

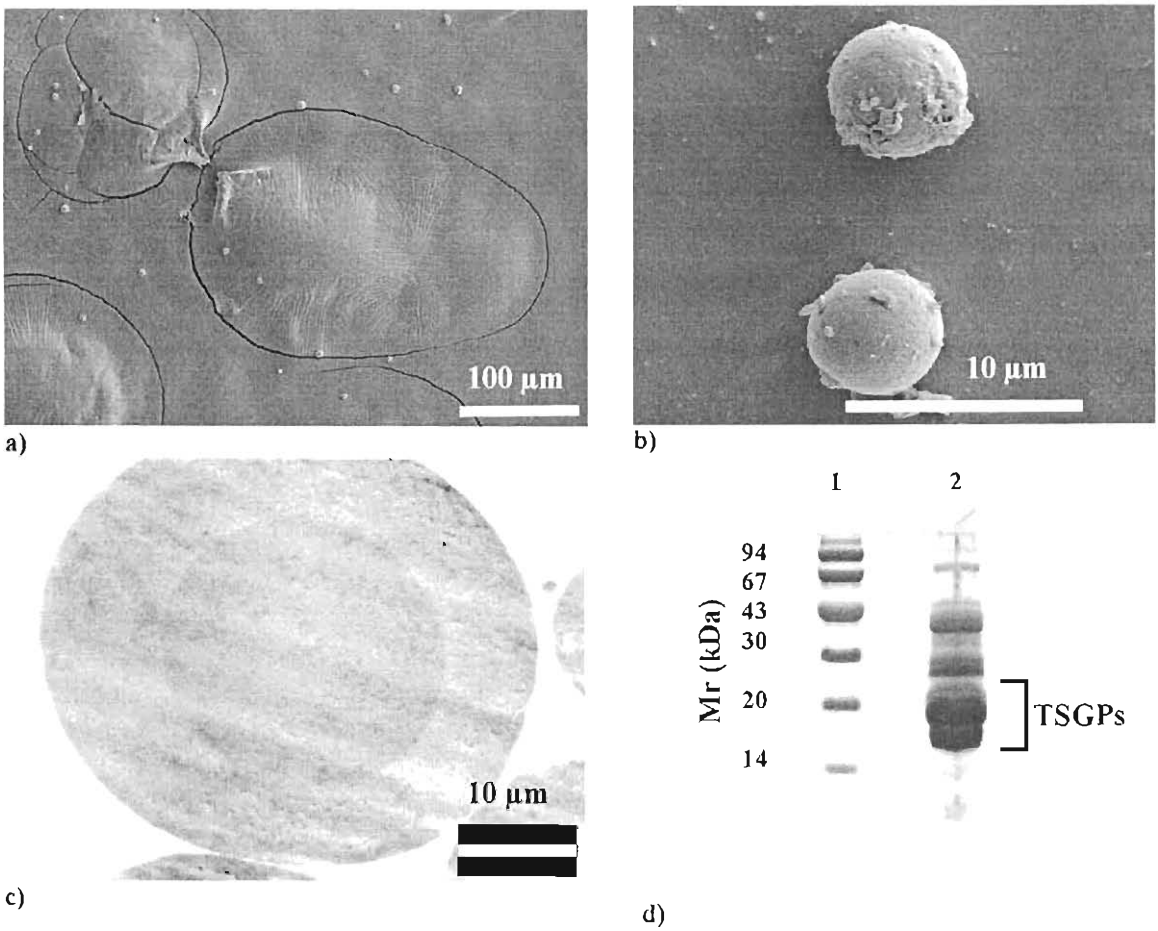


**Fig. 6.2:** Purification of dense core granules. (a) SEM of a granule preparation after dispase treatment and density gradient centrifugation. (b) An enlarged view of the granule preparation. (c) TEM of the dispase granule preparation, indicating a dense core granule. (d) Tricine SDS-PAGE analysis of a granule preparation.

### 6.4.3 Aggregation of proteins in acidic medium with high calcium concentration

High calcium concentrations (10-100mM) have been identified as a feature of secretory granules (Huttner, Gerdes and Rosa, 1991; Urbé, Tooze and Barr, 1997; Blasquez and Shennan, 2000). To confirm this in tick salivary glands, calcium was determined by X-ray microanalysis. Granules have calcium concentrations (0.3%-0.8%), which are similar to those of sodium (0.4-0.7%) and chloride (0.5-0.7%). The absence of potassium was notable (Fig. 6.3a). The granules thus have higher calcium concentrations than normally found in the cytoplasm (sodium-10 mM, chloride-8 mM, calcium-0.001 mM, potassium-155 mM), and are closer to what is predicted for the TGN (calcium-10 mM). With a granule density of 1.14g/ml and the elements expressed as a percentage weight basis, then calcium, sodium and chloride concentrations might be as high as 85-227 mM, 199-348 mM and 161-225 mM, respectively.

Secretory proteins aggregate under acidic conditions and high calcium concentrations normally associated with the TGN (Arvan and Castle, 1998). Under these conditions enrichment of specific proteins is evident in precipitated aggregates. Proteins that have equal densities are only found in the supernatant (Fig. 6.3b). The relatively low amounts of protein that precipitated during high calcium and acidic pH could be due to inadequate conditions that do not truly resemble that of the tick TGN or granule. The low concentration of the salivary gland extract (6mg/ml) could also be too dilute for adequate aggregation. Higher concentrations, on the other hand, could lead to non-specific association. Specific aggregation under these conditions was however, observed and it was decided to further investigate the most highly abundant proteins present in the SGE, by HPLC purification.

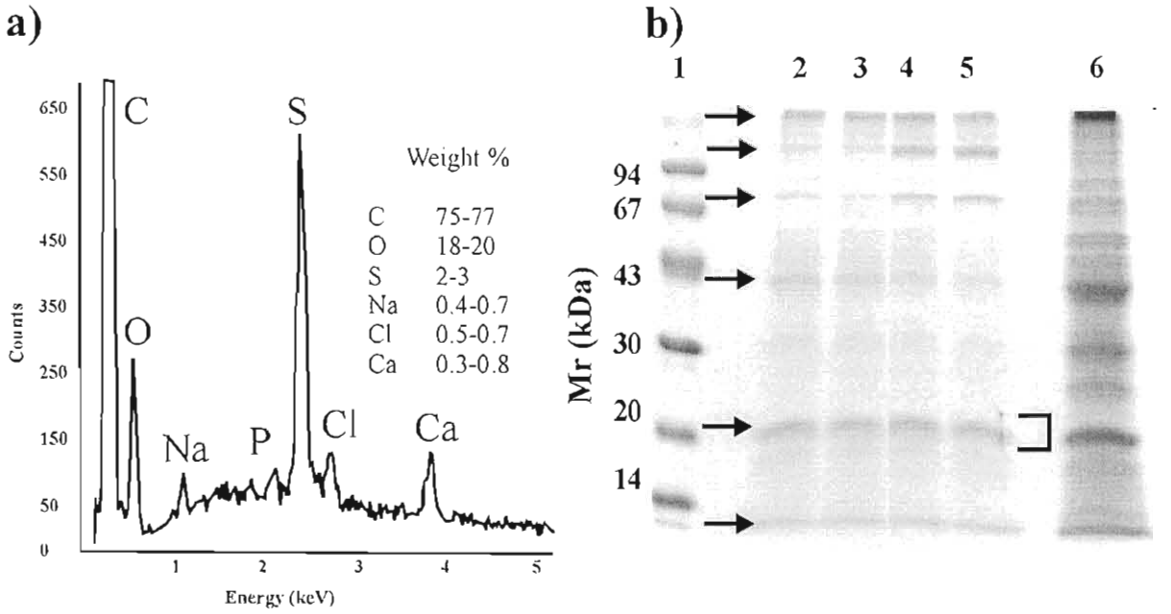


**Fig. 6.3:** Reconstitution of *in vitro* conditions present in the TGN. (a) X-ray microanalysis of salivary gland granules. Inserted are the % ranges obtained for 4 separate granules. (b) Aggregation of proteins under conditions of high calcium (50 mM) and different acidic pH. Lane 1 molecular mass markers, lane 2, 3, 4,5 at pH 4, 5, 6 and 7 respectively, while lane 6 indicates supernatant. Precipitated protein is indicated with arrows, while the TSGPs are bracketed.

### 6.4.4 A comparison of the toxicity of SGS and SGE

Some of the TSGPs were shown to be toxic during preliminary studies into the toxicity of the savignygrins (results not shown). For comparative purposes with results obtained for SGS, toxic activity in the SGE was investigated (Howell, Neitz and Potgieter, 1975). It should be noted that three-week old (10g) mice were used for the SGS study, while adult mice (20g) were used for the SGE study. With both SGE and SGS a concentration effect was noted, with survival of mice at gland equivalents of ~0.15 for both (Table 6.1). It was also found that 1 feeding tick caused mortality within 20 minutes after feeding. Previously, 200 $\mu$l of SGS was injected into a mouse, which died within 6 minutes (Howell, Neitz and Potgieter, 1975). Considering that a maximum of 10$\mu$l of SGS can be collected from a tick, then SGS from 20 ticks were injected (Howell, 1966b). Mice injected with SGE (2 glands) died within 20-30 minutes (Table 6.1). While this is 5X longer than that found for the SGS it is also shorter than the expected 10X. This could be due to the fact that more secretory products are sequestered inside the salivary gland granules than are secreted during stimulation with pilocarpine (Chapter 5).

**Table 6.1:** The effect of dilution on toxicity of SGE and SGS. Salivary gland or salivary gland secretion equivalents of a gland are indicated against the survival time. Values in parenthesis indicate the dilution factor, while the asterisk indicates times not recorded. SGS values have been reported previously (Howell, Neitz and Potgieter, 1975).

| SGE equivalents | Survival time | SGS equivalents | Survival time |
|---|---|---|---|
| 1 gland | 30-60 minutes | 5 glands (1/1) | 20 minutes |
| 0.5 glands | 8-9 hours | 0.6 glands (1/8) | Within 24 hours* |
| 0.25 glands | Within 48 hours* | 0.3 glands (1/16) | Within 24 hours* |
| 0.125 glands | Survived | 0.15 glands (1/32) | Survived |

### 6.4.5 Investigation into the possible neurotoxicity of SGE

Since it has been shown that toxic activity in SGS is stable to temperatures of ~80°C (Howell, Neitz and Potgieter, 1975), heat precipitation as a purification procedure was investigated. It was found that toxic activity was retained after treatment at 60°C (Table 6.1). Analysis of this preparation by tricine SDS-PAGE shows that the majority of proteins with Mr>20kDa were precipitated (fig. 6.4a). Proteins with Mr 6-20 kDa were fractionated using RPHPLC (Fig. 6.4a and 6.4b). Toxicity tests indicated a possible toxic activity eluting in fraction 3 (Fig. 6.4b). Paralysis like symptoms were however, only observed after ~48 hours. The equivalent of 4 salivary glands was injected, which contrasts with the toxicity observed for one gland and less after heat treatment (Table 6.1). Due to unpublished reports (C.J. Howell, 1969) of possible cardiac failure induced by the toxin, the effect of SGE on a Langendorf isolated rat heart perfusion system was tested.

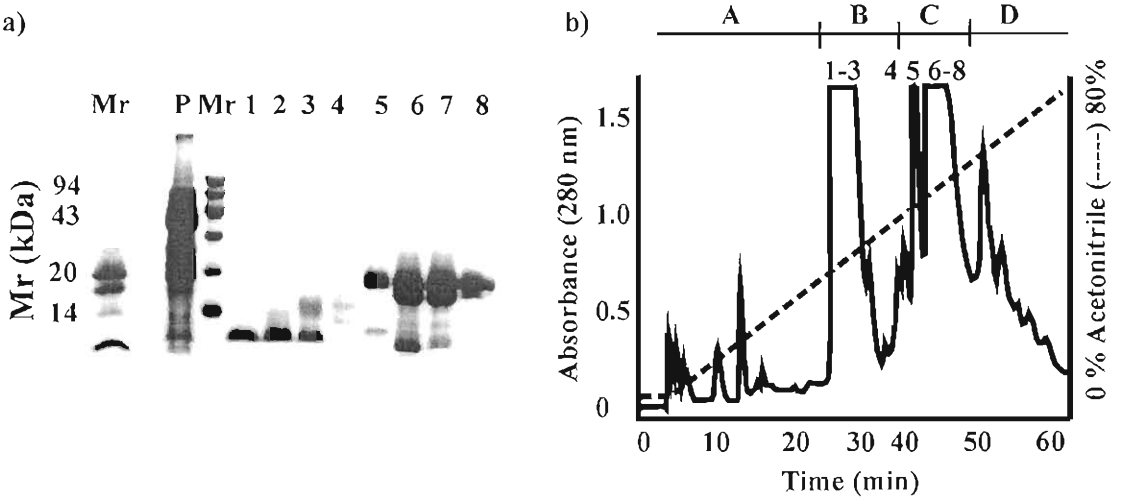


**Fig. 6.4:** Analysis of temperature inactivated SGE. (a) Tricine SDS-PAGE of heat precipitated fraction (P) and different fractions obtained after RPHPLC fractionation of the supernatant. (b) Preparative RPHPLC of temperature inactivated (60°C, 5 min) SGE (20 glands). Fractions collected, pooled and tested for toxicity is indicated (A-D), as well as those (1-8) analysed by tricine SDS-PAGE (Fig. 6.4a).

### 6.4.6 The effect of SGE on an isolated rat heart perfusion system

A rhythmic beat was observed that changed shortly after addition of SGE to an arrhythmic beat displaying bradycardia before final heart arrest (Fig. 6.5). This initial experiment was taken as evidence that the cardiac system rather than the nervous system is affected by the SGE from *O. savignyi* and toxic activities were purified before further analysis.

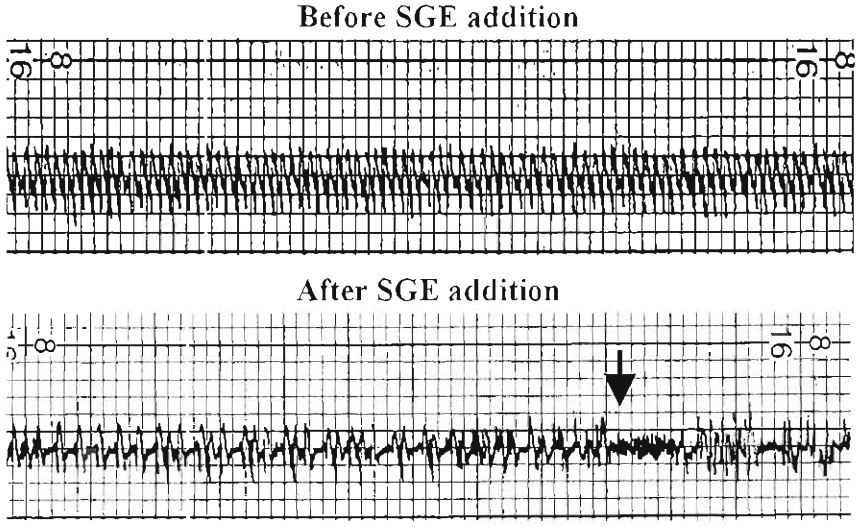


**Fig. 6.5:** The effect of SGE on a perfused rat heart system. The rhythm of the heartbeat is indicated before and after addition of SGE. The arrow indicates the onset of heart arrest.

### 6.4.7 Purification of TSGPs and toxins

HPLC purification of salivary gland extracts confirmed that the TSGPs are the main proteins present in tick salivary gland extracts (Fig. 6.6, Fig. 6.7 and Fig. 6.8). Yields of individual purified TSGPs were between 4% and 5% of the total initial salivary gland extract proteins (results not shown), giving the four proteins a cumulative concentration of 20% of the total protein in salivary gland extract. Assuming normal losses (20-50%) incurred after HPLC purification, the true concentration of the TSGP's would even be higher and closer to 40% of the total protein content, based on their densities after two-dimensional electrophoresis and their enrichment in the granule preparation. This is quite extraordinary considering normal expected values of proteins in tissues (0.01-0.1%). The abundant nature of these proteins was confirmed by the similar profiles obtained by two-dimensional electrophoresis and HPLC purification. This is quite important as it confirms the high abundance of specific proteins in the general proteome of the tick salivary gland by two different techniques.

### 6.4.8 Fractionation of toxic activities

Fractionation with AEHPLC showed that a basic (TSGP4) and acidic (TSGP2) toxic activity is present in SGE (Fig. 6.6a). While both fractions were lethal (survival times of 15-20 min) on the AEHPLC, only TSGP2 showed similar toxicity after CEHPLC. TSGP4 induced inactivity, shivering and muscle twitching that endured for two hours after which the mice recovered (Fig. 6.6b).

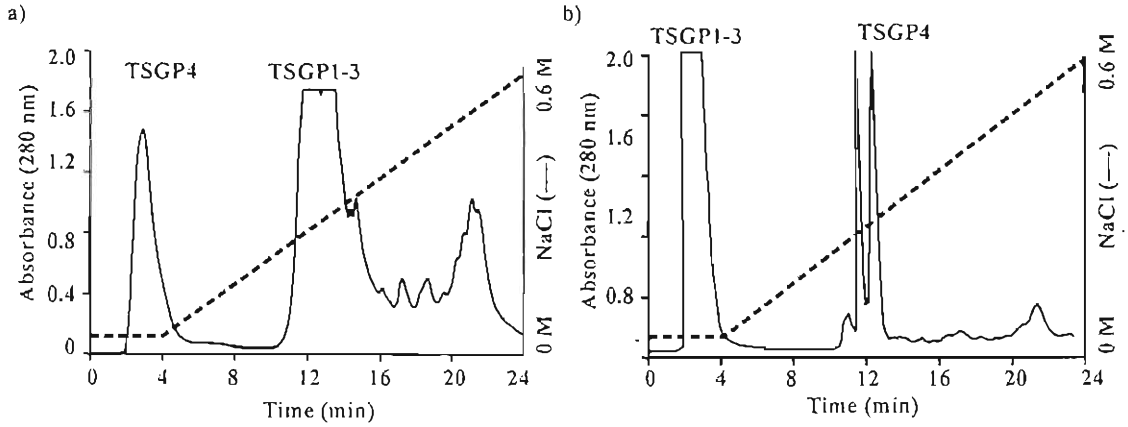


Fig. 6.6: Ion exchange HPLC of tick salivary gland extract. (a) AEHPLC with elution of TSGPs indicated. (b) CEHPLC with elution of TSGPs indicated.

TSGP4 fractionation of the AEHPLC fraction using SEHPLC, showed that toxic activity was associated with a ~17 kDa protein that corresponds with the toxic activity identified for CEHPLC (Fig. 6.7a). A non-toxic low molecular mass protein (LM: ~6 kDa) separated from TSGP4 during SEHPLC. The SEHPLC preparation of TSGP4 was lethal at 34$\mu$g within 30 minutes, although toxic activity was rapidly lost if stored. The lability of TSGP4 observed during this study probably accounts for its absence during previous studies into the toxic nature of *O. savignyi* (Neitz, Howell and Potgieter, 1969; Neitz *et al.* 1983; Howell, Neitz and Potgieter, 1975). Using the same SEHPLC conditions it was shown that the toxic activity of TSGP2 also eluted at a Mr~17kDa (results not shown). Rechromatography of the TSGP2 fraction from AEHPLC gave a protein with Mr~15kDa of which 24$\mu$g was lethal within 15-30 minutes (Fig. 6.7b). Dilution of this activity to 6 $\mu$g was still lethal over the course of one day, but 2$\mu$g failed to elicit any response.