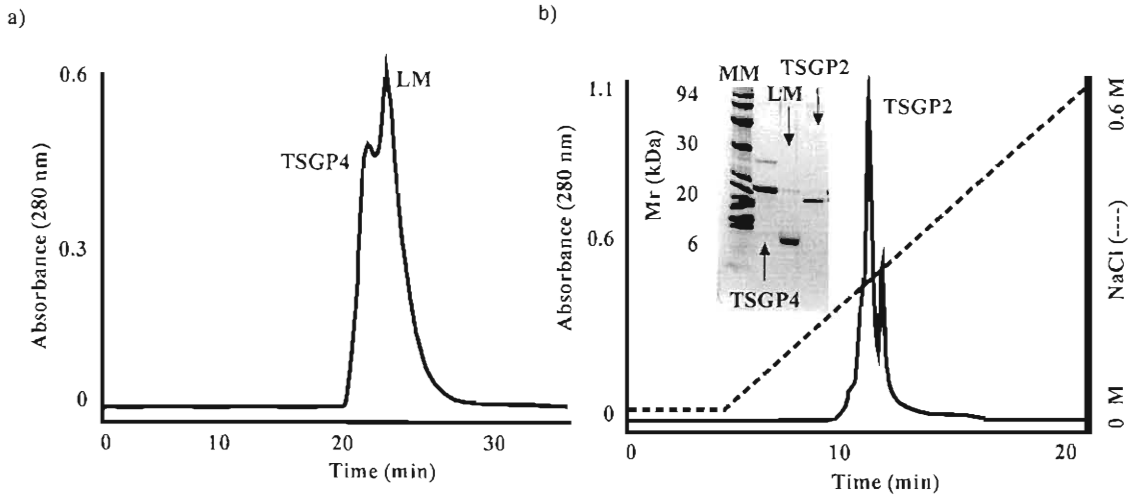

Fig. 6.7: Fractionation of toxic fractions from AEHPLCC. (a) SEHPLC of TSGP4 after AEHPLC. (b) Rechromatography of TSGP2 on AEHPLC. Inserted is a tricine SDS-PAGE gel that shows the TSGP4, LM and TSGP2 after their respective chromatography steps. LM is a low molecular highly abundant non-toxic protein with a similar pI as TSGP4.

Fractionation of TSGP2 from AEHPLC using HIHPLC resolved 3 main peaks of which two components were non-toxic, one a homolog (TSGP3) and the other a highly abundant protein (TSGP1) (Fig. 6.8a). After rechromatography on AEHPLC the toxic preparation (24μg) was also lethal within 30 minutes, supporting the observations of previous results (Fig. 6.8b). The toxicity of TSGP2 as observed during this study contrast sharply with the 400μg of toxin that was necessary to kill a mouse within 90 minutes (Neitz, Howell and Potgieter, 1969).

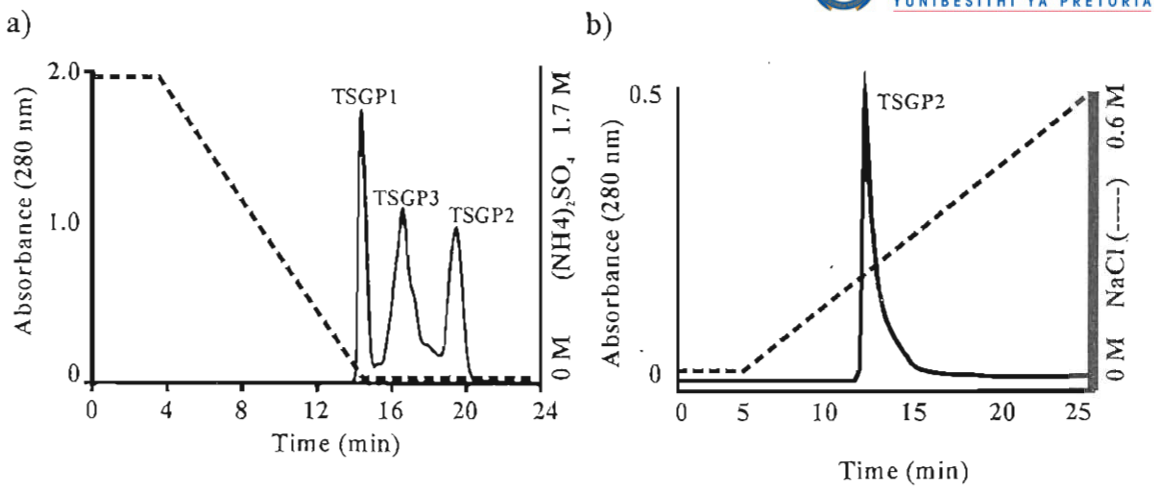

Fig. 6.8. Fractionation of the acidic TSGPs. (a) HIHPLC of the TSGP1-3 fraction after AEHPLC. (b) AEHPLC of TSGP2 after HIHPLC.

6.4.9 The effect of different toxins on the cardiovascular system

The effect of TSGP4 and TSGP2 on the cardiovascular system was investigated by recording ECG patterns of mice injected with purified toxic fractions. Preparations of TSGP4 after SEHPLC (Fig. 6.7) induced development of a hyperacute T-wave (A-I), with first tachycardia (B-C) followed by bradycardia (E-I) with a gradual increase in the P-R interval (F-I) (Fig. 6.9). This eventually manifested as Mobitz type 1 second degree AV block (H), followed by a Mobitz type 2 second degree AV block (I) (Goldberger and Goldberger, 1981).

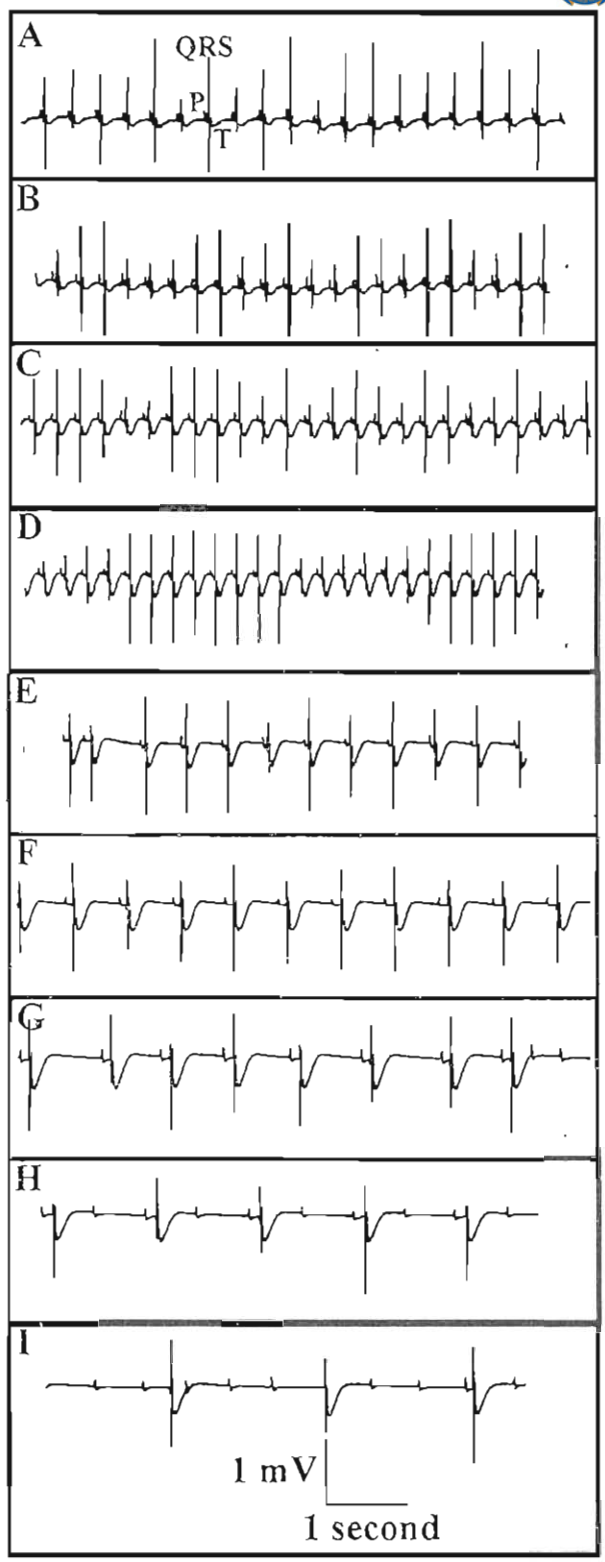

Fig. 6.9: The effect of TSGP4 on the mouse ECG patterns. The top panel indicates the ECG pattern before injection and the lower panels indicate different time periods after injection.

TSGP2 obtained from AEHPLC-HIHPLC-AEHPLC (Fig. 6.8) induced hyperacute T-waves (C) that diminished in amplitute (D), which was accompanied by a prolongation of the QT-interval (B-D) (Fig. 6.10). This was followed by a T-wave inversion and a complete heart block after which a normal pattern was regained (E). Severe ventricular tachycardia ensued with a QT prolongation, reminiscent of "torsades de pointes" (F) (Echardt *et al.* 1998).

Of interest is that non- anaesthetized control mice injected subcutaneous with TSGP2 died within 30 minutes, while mice injected with TSGP2 intra-peritoneal only succumbed after an hour and TSGP4 injected mice survived even longer. Only freshly prepared TSGP4 fractions showed toxicity and lost activity if left overnight at 4 °C. The delayed time of toxic activity could probably in both cases be assigned to the fact that intra-peritoneal injected material is effectively removed due to slow clearance from the intra-peritoneal cavity or sequestration in macrophages and organs. The results obtained together with that from the rat heart perfusion system and the nerve-muscle preparations, suggest that the pathology of these toxins are by targeting of the cardio-vascular system via disruption of electrical conductivity.

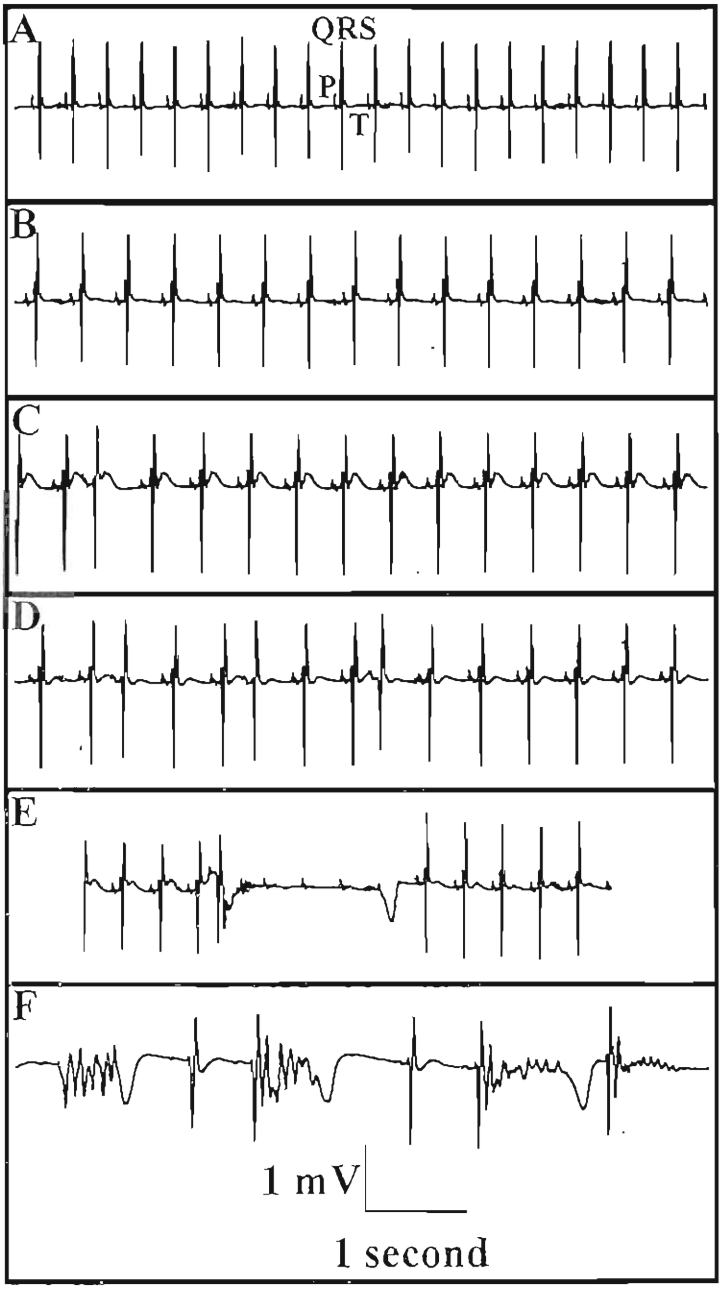

Fig. 6.10: The effect of TSGP2 on the mouse ECG patterns. The top panel indicates the ECG pattern before injection and the lower panels indicate different time periods after injection.

6.4.10 Desalting of TSGPs

Proteins were desalted for sequencing, ESMS and MALDI-TOF-MS purposes using RPHPLC (Fig. 6.11). All gave single peaks, although TSGP1 gave a broad tailing peak.

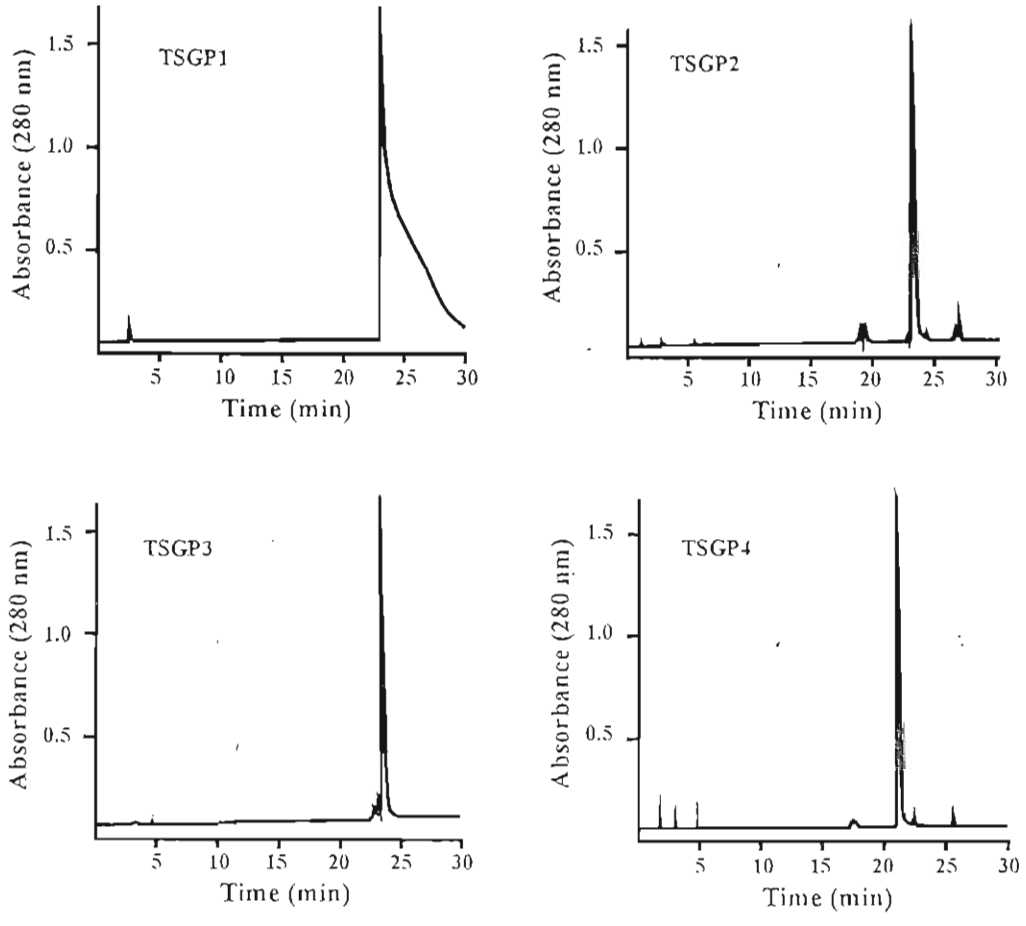

Fig. 6.11: RPHPLC of TSGPs. Fractions obtained of the acidic TSGPs after HIHPLC and fractions obtained of TSGP4 CECHPLC were desalted using RPHPLC.

6.4.11 Molecular mass analysis of TSGPs

Due to previously reported discrepancies on molecular mass of the acidic toxin (Neitz, Howell and Potgieter, 1969; Neitz *et al.* 1983), several different methods were used to determine the molecular masses of the toxins and TSGPs. Tricine SDS-PAGE analysis (Fig. 6.12) correlated well with masses obtained from MALDI-TOF-MS (Fig. 6.13) indicating that the molecular mass of the TSGPs ranged from 15800-18400 Da. MALDI-TOF-MS analysis of pyridylethylated TSGPs also indicated that these proteins have six cysteines.

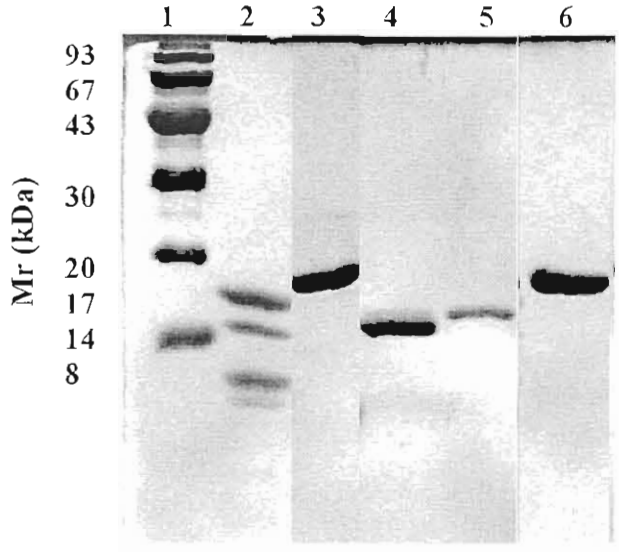

Fig. 6.12. Reducing tricine SDS-PAGE analysis of purified TSGPs. Lane 1 and 2 indicate low molecular mass markers and peptide mass markers respectively. Lane 3, 4, 5 and 6 indicate TSGP1-4, respectively.

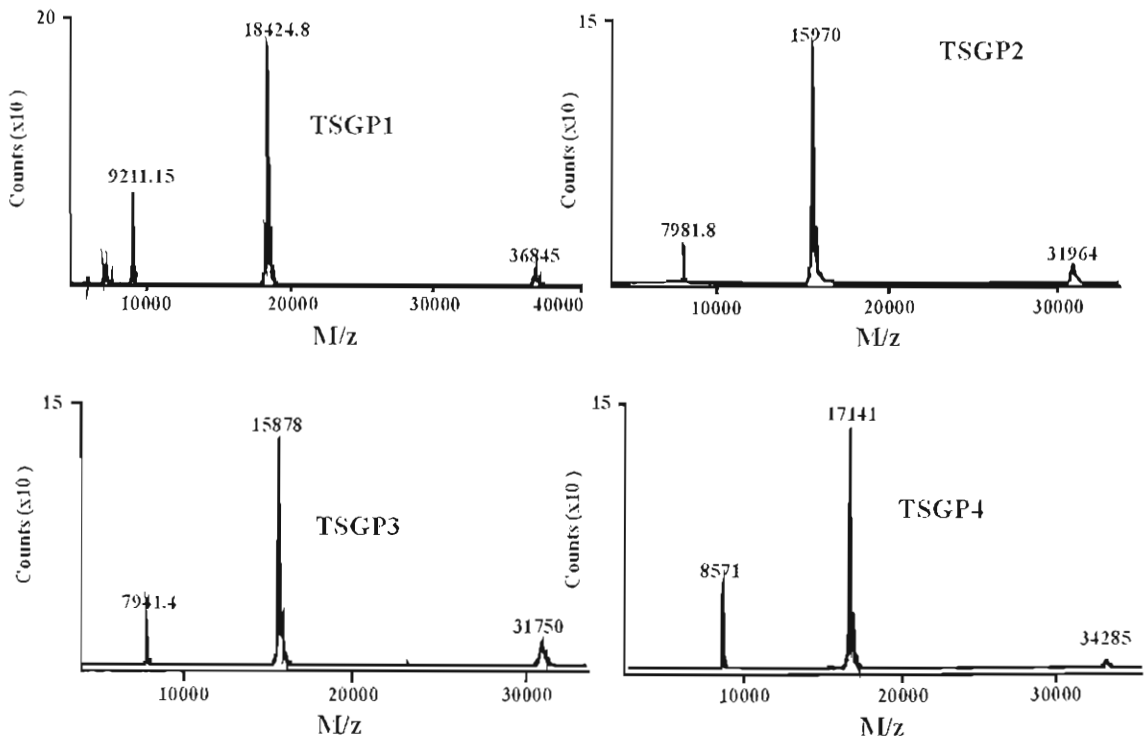

Fig. 6.13. MALDI-TOF-MS of TSGPs. Molecular masses are indicated for the respective proteins, as well as the $M+2H^+$ and $2M+H^+$ ion species.

ESMS analysis indicated masses comparable with those obtained from MALDI-MS and reducing tricine SDS-PAGE. TSGP1 gave multiple ions ranging from +10H to +17H, while TSGP2 and TSGP3 gave ions ranging from +10H to +15H and for TSGP4 multiple ions were obtained ranging from +10H to +19H (Fig. 6.14).

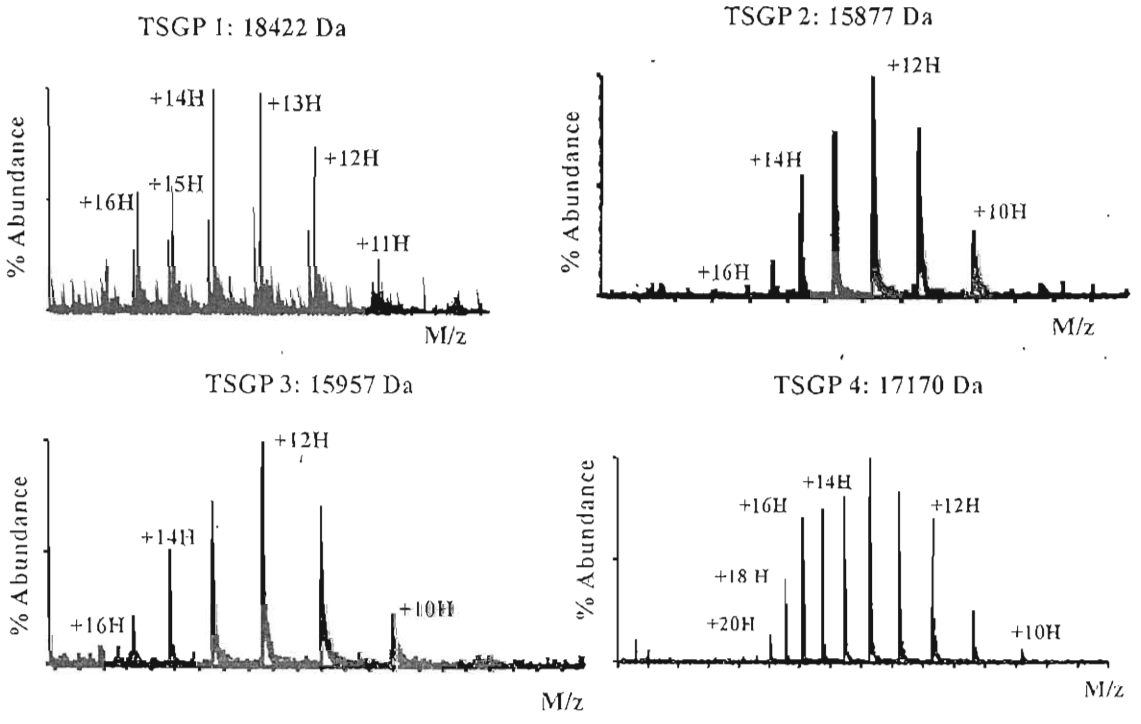

Fig. 6.14: ESMS spectra obtained for the TSGPs. Masses obtained after deconvolution are indicated, as well as multiple ion species.

6.4.12 Peptide mass fingerprinting of TSGPs

Peptide mass fingerprints obtained for the TSGPs indicated that TSGP1 and TSGP4 are unique proteins, while TSGP2 and TSGP3 share at least two peptide fragments and could thus be related (Fig. 6.15).

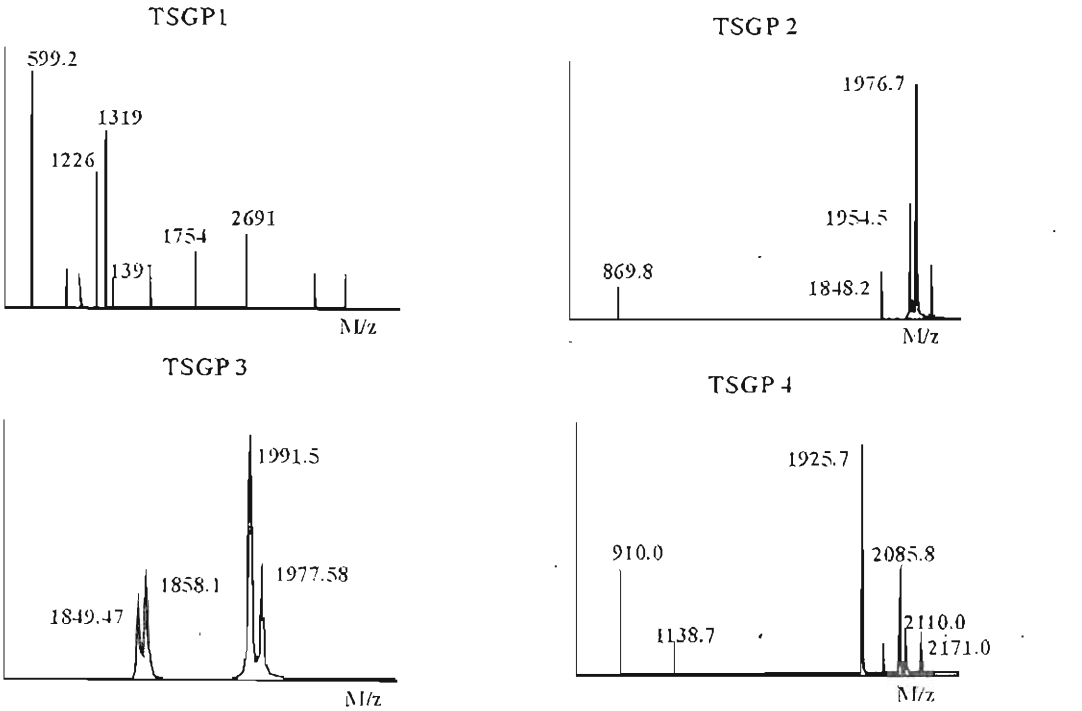

Fig. 6.15: Peptide mass fingerprints of TSGPs. Molecular masses are indicated for respective fragments.

6.4.13 Amino acid analysis of TSGPs

Amino acid analysis of the purified TSGPs indicated that the TSGPs have similar amino acid compositions (Fig. 6.16). Significant differences are the lower Asx and Glx and higher Arg in TSGP4, compared to TSGP1-3. This can explain differences in their iso-electric points. The higher arginine in TSGP4 explains the larger ion species observed for this protein with the ESMS results compared to the other TSGPs. TSGP4 also have significantly higher serine than TSGP1-3. The fact that TSGP1 gave slightly lower values for arginine and lysine compared to TSGP2 and 3, but still gave a higher number of charged species (+17H) compared to TSGP2/3 (+16H), is probably due to its higher mass compared to that of TSGP2 and TSGP3. Pyridylethylation in the presence or absence of a reducing agent also indicated that all cysteines (six according to MALDI-TOF-MS) are involved in disulphide bonds (results not shown).

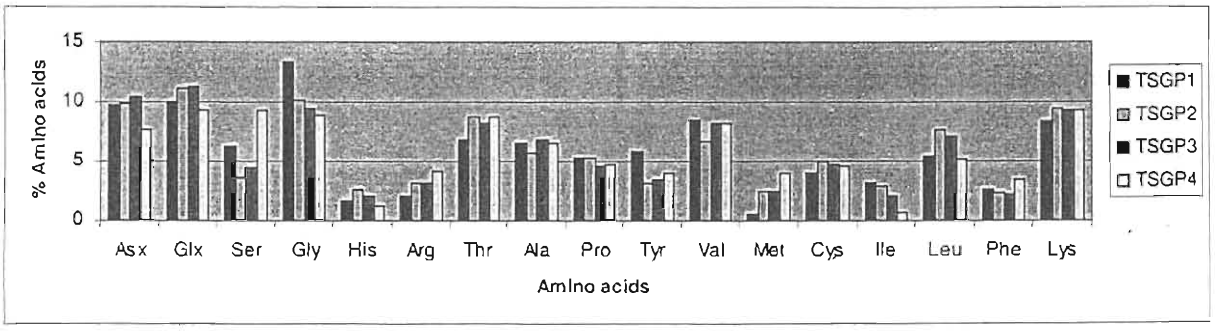

Fig. 6.16: Amino acid analysis of the TSGPs. Indicated is the cysteine content as determined by pyridylethylation.

6.4.14 N-terminal amino acid analysis of the TSGPs

N-terminal amino acid analysis indicated that TSGP1 and TSGP4 have novel sequences, while TSGP2 and TSGP3 were 95% identical and showed identity to previously described toxic and non-toxic homologs (Neitz, Howell and Potgieter, 1969, Neitz *et al.* 1983). No hits could be found using non-redundant BLAST analysis (Altschul *et al.* 1990), indicating that all sequences are unique. When using the Protein Prospector package (http://prospector.ucsf.edu/), which incorporates molecular mass, peptide mass fingerprint and N-terminal sequence data, no hits were found either.

```
TSGP1  :                    G-P-D-G-C-V-G-S-T-E-A-K-V-A-V      -15
20A1*  :          E-E-N-Q-R-G-K-G-M-L-G-S-T-A-A-S-V-A-V      -19

Toxin*:           G-C-P-P-G-V-P-T-R-A-Y-V-A-F-V-E-G-X-G-A    -20
TSGP2  :          D-C-P-T-G-K-P-T-D-A-Y-V-A-F-N-X-G-Q-G-A    -20
TSGP3  :          D-C-P-T-G-K-P-T-E-A-Y-V-A-F-N-X-G-K        -18
Non-toxin*:       D-C-P-P-T-K-P-T-R-A-Y-V-A-F-X-E-G-E        -18

TSGP4:            A-N-D-V-W-N-V-L-K-G-S-D-S-K-F              -15
```

Fig. 6.17: N-terminal sequence analysis of the TSGPs. N-terminal sequences obtained for the TSGPs are indicated. Cysteine residues were identified as their perethylated derivatives. The X in the sequences of TSGP2 and TSGP3 represent unidentified, modified amino acids. Identity between different sequences is indicated by black boxes. *The N-terminal sequences of toxin (Neitz *et al.* 1983), non-toxin (Neitz, 1976) and 20A1 (Baranda *et al.* 2000) were obtained from literature.

6.4.15 Specificity of polyclonal anti-sera for TSGPs

Polyclonal anti-sera were generated against the different TSGPs. Western blot analysis shows that all sera are specific for components in the ~20 kDa range in crude SGE (Fig. 6.18a). Anti-sera raised against TSGP1 and TSGP4 did not cross-react with other purified TSGPs. Anti-sera raised against TSGP2 did however cross-react with TSGP3, but not with TSGP1 or TSGP4 (Fig. 6.18c). This together with the shared peptide fingerprints and N-terminal sequences of TSGP2 and TSGP3 indicate sequence similarity and probably homology.

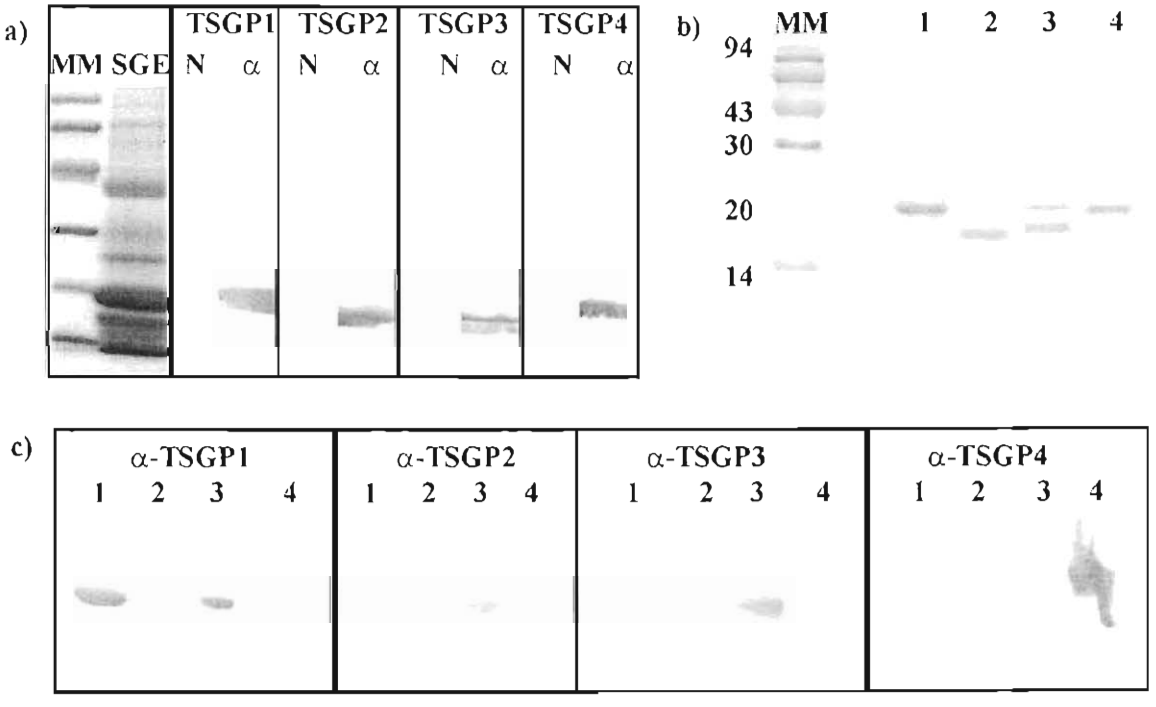

Fig. 6.18: Specificity of polyclonal anti-sera raised against different TSGPs. (a) Western blots of crude SGE with anti-sera against TSGP1-4, respectively. Also indicated is naïve serum (N) before immunization. (b) Purified fractions used for cross-reactivity studies between the various TSGPs. Indicated is TSGP1 (1), TSGP2 (2), TSGP3 (3) and TSGP4 (4). (c) Cross-reactivity of anti-TSGP1, anti-TSGP2, anti-TSGP3 and anti-TSGP4 with purified TSGP1 (1), TSGP2 (2), TSGP3 (3) and TSGP4 (4), respectively.

6.4.16 Localization of TSGPs to salivary gland granules

If the TSGPs are involved in granule biogenesis then this should be mirrored in their localization to different granule types. Localization of the TSGPs to all the granule types

identified in the salivary glands (Chapter 5) first came as a surprise (Fig. 6.19). It is however, logical that proteins involved in granule biogenesis should be present in all granule types if they assist in their formation.

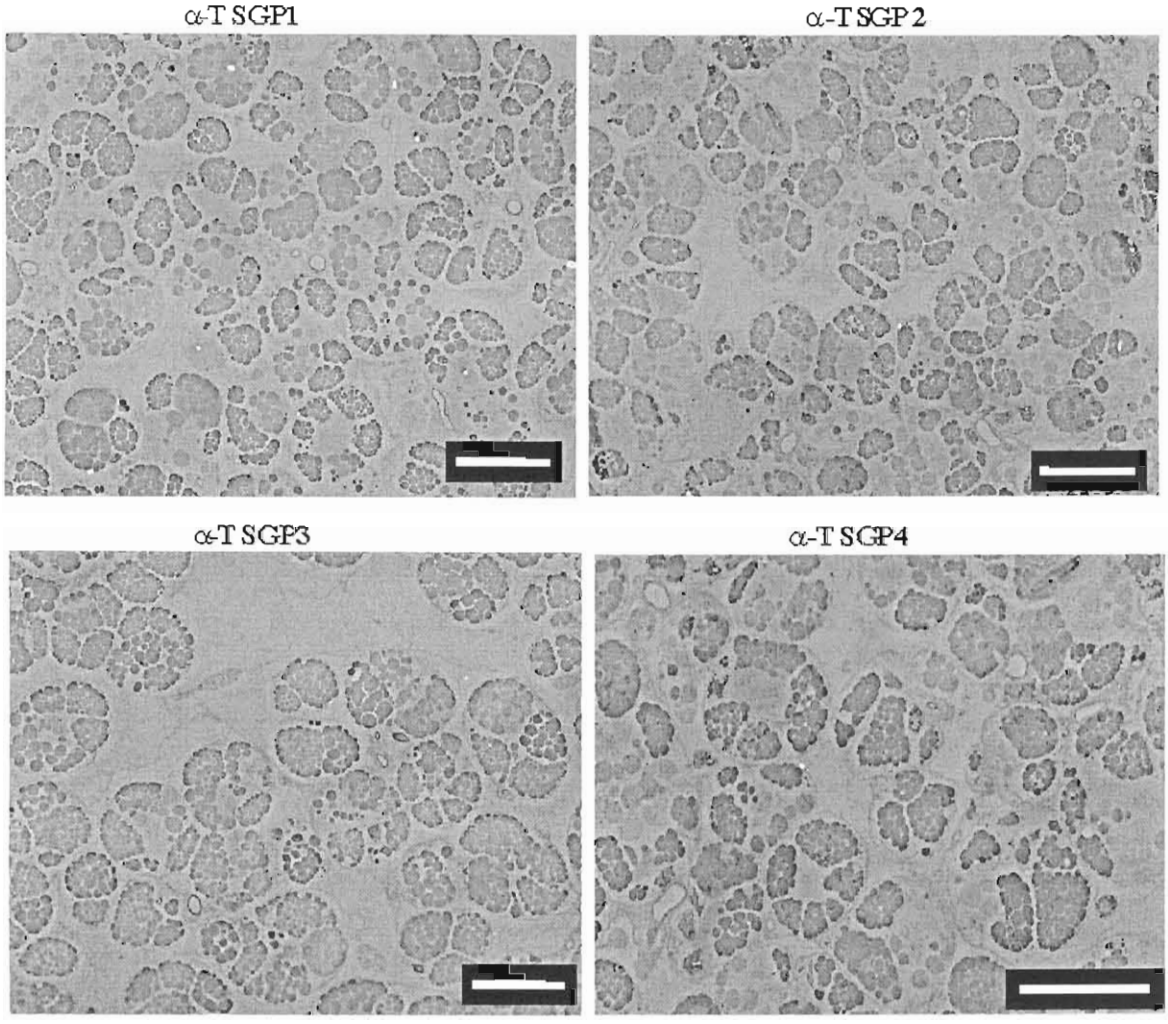

Fig. 6.19: Localization of the TSGPs to different salivary gland granules. Scale bar = 100 μm.

6.4.17 Protein concentration and its influence on granule biogenesis

Granule formation takes place at high protein concentrations in the TGN, resulting in high intra-granular protein concentrations (~0.1g/ml) (Urbé, Tooze and Barr, 1997; Arvan and Castle, 1998). The density of the isolated tick salivary gland granules (1.14 g/ml) is approximately 10X the reported intra-granular concentration. Expressed in terms of percentage protein concentration this amounts to 114%. If it is considered that the TSGPs represent between 20-40% of the total soluble SGE proteins, then their density in the granules is 0.228-0.57 g/ml. This is still higher than intra-granular concentrations of 0.1g/ml (Urbé, Tooze and Barr, 1997; Arvan and Castle, 1998).

Our first manuscript on the TSGPs commented on the relatively low amounts of protein that precipitated during conditions of high calcium concentration and acidic pH (Mans *et al.* 2001). It was speculated that the conditions used (Fig. 6.3) do not truly resemble that of the tick TGN or granule and that the concentration of the salivary gland extract (6mg/ml protein) used could also be too dilute for adequate aggregation. Using higher concentrations, could however, lead to non-specific association. However, after a consideration of the implications of the density of the salivary gland granules and further reading into the field of molecular crowding, we were forced to consider the effect that protein concentration might have on granule formation.

6.4.18 Molecular crowding in the cell

Molecular crowding is a phenomenon that would seem obvious, but for most part is ignored by the general biochemistry community when considering physiological cellular conditions. Cellular interiors have high total concentrations of macromolecules (20-30% of the total volume: compare this with the estimated 114% of the dense core granules!). Due to steric exclusion the volume occupied by these molecules are unavailable to other macromolecules: the excluded volume effect (Ralston, 1990; Zimmerman, 1993; Minton, 1997; Minton, 2000; Minton, 2001; Ellis, 2001a; Ellis, 2001b). The effect is adequately described by consideration of a macromolecule that occupies 30% of the available space. While the remaining 70% of volume is accessible to smaller molecules, it is inaccessible to any molecules as large or larger than the described macromolecule (Fig. 6.20).

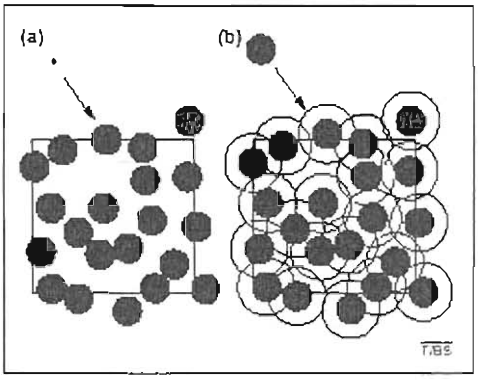

Fig. 6.20: The principle of volume exclusion. (a) The square indicates the volume of which 30% is occupied by a macromolecule. A small molecule has access to all of the remaining 70% volume. (b) A molecule similar in size is excluded from the remaining 70% as it cannot approach the molecules closer than the distance of indicated by the open circles. This is described as the excluded volume of a macromolecule. Figure adapted from Minton (2001).

The phenomenon of molecular crowding has several consequences. By excluding volume to one another, macromolecules reduce configurational entropy and as such increase the free energy of a solution. Volume exclusion in crowded media leads to destabilization of test species, so that the most favored state excludes the least volume. Compact structures exclude less volume than extended structures, while oligomeric or polymeric aggregates also exclude less volume than individual subunits. Macromolecular crowding thus provides a nonspecific force for macromolecular compaction and association in crowded solutions. Such macromolecular compaction or association can probably be equated with the concentration, multimerization, polymerization and condensation previously used to describe granule formation (Arvan and Castle, 1998). Molecular crowding effects could thus lead to condensation of secretory proteins into granules. A simple calculation of the volume that TSGPs encompass in a granule, indicate that they are optimally packed. This further support the possibility that molecular crowding leads to condensation of the TSGPs.

6.4.19 TSGPs as densely packed spheres

To demonstrate this, consider a granule with a diameter of 5 μm.

(A) The volume of a granule with a 5μm diameter is:

$$V = 4/3\Pi(r)^3$$

$$V = 4/3\Pi(2.5X10^{-6}\ m^3)$$

$$V = 6.545X10^{-17}\ m^3$$

(b) Subcellular fractionation and density centrifugation showed that the granule has a density of 1.14 g/ml.

i.e. 1.14g per $1X10^{-6}\ m^3$

From the above it follows that the mass of a granule with diameter of 5μm is:

$7.46X10^{-11}$ g.

(c) It has been shown that the TSGPs can take up to 20-40% of the total SGE protein.

For the granule this means that the TSGPs are:

$1.49X10^{-11}$ g – $2.98X10^{-11}$ g

of the total protein in granule.

(d) Assuming an average molecular mass of 17000 g/mol for the TSGPs, using the above masses we can calculate the amount of moles for the TSGPs in the granules.

This gives: $8.76X10^{-16}$ moles – $1.75X10^{-15}$ moles.

Using Avogadro's number ($6.022X10^{+23}$ molecules/mole) we can calculate the number of TSGP molecules in the granule:

$5.2X10^{+8}$ molecules – $1.05X10^{+9}$ molecules.

(e) Assuming a globular protein structure (this approximation is supported by the results presented in Chapter 7, that indicate that the TSGPs are part of the lipocalin family) and using the linear relationship between molecular mass and volume derived in Chapter 3 (Fig. 3.20), a volume of ~43Å^3 is obtained. As 1Å = $1X10^{-10}$ m, the dimensions can be converted to volume, which gives:

$7.95X10^{-26}\ m^3$.

The total volume for the TSGPs can be calculated:

$4.1X10^{-17}$ m^3 – $8.3X10^{-17}$ m^3.

(f) Using the volume determined for the granule, we find that this works out to 62%-126% of the total volume of the granule.

It would be impossible for the TSGPs to encompass 126% of the total volume of the granules. The estimation of 20%-40% is thus erroneous and the total TSGP concentrations are probably close to the determined 20%. Studies on the close packing of spheres have indicated that the maximum density that a large, random collection of spheres can attain is 63%-74% (Torquato, Truskett and Debenedetti, 2000). At 20% of the total protein concentration of the granule and 62% of the volume, the TSGPs approach the limits of their maximum density. This would have a profound influence on the packing of other macromolecules and the volume exclusion effect should play a significant role during granule formation.

To test whether volume exclusion might play a role during granule formation, SGE was incubated at pH 5.5 in the presence of 100 mM $CaCl_2$ at various concentrations of dextran. Dextran is a useful polymer for the investigation of volume exclusion effects (Ellis, 2001a). With increasing concentrations of dextran, specifically above 10%, more TSGPs and other salivary gland proteins are precipitated (Fig. 6.21). This suggests that volume exclusion (probably due to increasing concentrations of TSGPs in the TGN) could play a significant role during granule biogenesis.

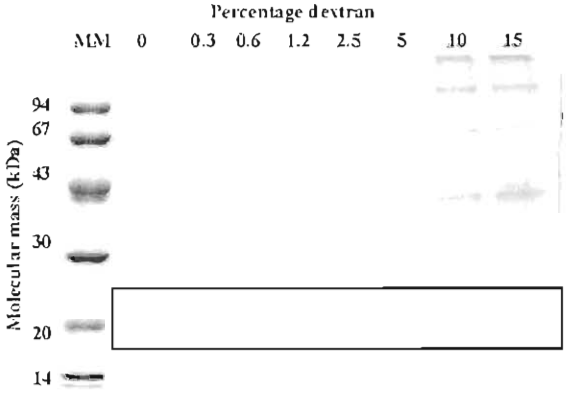

Fig. 6.21: Precipitation of TSGPs in the presence of increasing concentrations of dextran. TSGPs are boxed.

6.5 Discussion

6.5.1 Granule biogenesis in tick salivary glands

Ticks secrete bioactive compounds during feeding that are stored in salivary gland granules until released by a stimulus (Sauer *et al.* 1995). Granule formation plays an important part in this process as a mechanism by which secretory proteins are packaged. An adequate understanding of granule biogenesis can therefore aid in the elucidation of tick secretory mechanisms and defining a potential target for the development of tick control methods.

6.5.2 Evidence of TSGPs being granule biogenic protein's

Several different facts support a possible role for the TSGPs in granule biogenesis:

1. All proteins involved in granule biogenesis occur at very high concentrations in secretory cell types (Urbé, Tooze and Barr, 1997; Arvan and Castle, 1998). The TSGPs are the most abundant proteins in the SGE and granules as determined by two-dimensional electrophoresis, density gradient centrifugation and HPLC.
2. Proteins involved in granule biogenesis tend to aggregate under conditions of acidic pH and high calcium concentration (Huttner, Gerdes and Rosa, 1991; Urbé, Tooze and Barr, 1997; Blasquez and Shennan, 2000). The TSGPs showed aggregation under such conditions.
3. If the TSGPs are involved in the formation of all the different salivary gland granule types, then they should be present in all granules. The TSGPs were immuno-localized to all the different granular types in contrast to apyrase and savignygrin (Chapter 5).
4. The TSGPs aggregated in direct relation to the degree of molecular crowding. It was also indicated that the TSGPs are packed close to the optimum packing that can be attained by spherical particles. These two observations suggest that the TSGPs at high concentrations as observed in the granules would experience molecular crowding effects, i.e. aggregation (Ellis, 2001a). As was explained in the introduction, aggregation is synonymous with granule biogenesis (Huttner,

Gerdes and Rosa, 1991; Urbé, Tooze and Barr, 1997; Blasquez and Shennan, 2000).

5. If the TSGPs all exhibit the same function, then it could be expected that they should belong to the same protein family. Chapter 7 presents results that indicate that the TSGPs are indeed part of the same lipocalin protein family.

Secretory granules have to be formed in some way. Literature has presented several criteria for proteins involved in this process. So far, it would seem as if the TSGPs fit these criteria and are at this stage the best possible candidates for being granule biogenic proteins. The difficulties involved in finding an *in vitro* system that will simulate granule formation makes it difficult at present to give a more conclusive explanation.

6.5.3 Toxins from *O. savignyi*

Results obtained in this study confirmed the presence of at least two highly abundant and similar proteins. N-terminal sequence data, peptide mass profiles and western-blot analysis using polyclonal anti-sera indicate that TSGP2 and TSGP3 have similar amino acid sequences. At least one of these proteins (TSGP2) has been previously identified as a toxin (Neitz *et al.* 1983). A non-toxic homologue (TSGP3) that co-purified with the toxic component has also been identified (Neitz, Howell and Potgieter, 1969). This study reconfirmed the toxicity of TSGP2 and non-toxicity of TSGP3. The molecular masses obtained for TSGP2 (15877 Da) and TSGP3 (15957 Da) compare favorably with the masses obtained for the toxin (15400 Da) and non-toxin (16000 Da), respectively (Neitz, Howell and Potgieter, 1969). The iso-electric point of TSGP2 is more acidic than that of TSGP3 and compares favorably with the lower pI of the toxin (5.01) versus that of the non-toxin (5.1) previously described (Neitz, Howell and Potgieter, 1969). In addition it has been shown that a novel toxic activity is associated with TSGP4 that has a unique N-terminal sequence. TSGP1, showed 56% identity with the N-terminal amino acid sequence of the main antigen (20A1) present in the salivary gland extracts of *O. moubata* (Baranda *et al.* 2000).

6.5.4 Non-paralytic nature of sand tampan toxicoses

O. savignyi has been implicated in causing paralysis (Hoogstraal 1985; Gothe and Neitz 1991; Gothe 1999). All publications ultimately refer back to Kone (1948), Rousselot (1956), Howell (1966b) and Howell, Neitz and Potgieter (1975). Perusal of these publications show however, no direct reference to paralysis associated with *O. savignyi*. Kone (1948) describes severe symptoms and death that ensues after exposure of cattle to tick bites, but interprets this as possible anaphylactic shock. Rousselot (1956) states that several hundreds of cattle on the border of Lake Chad were affected and that after three weeks, high mortality from asphyxiation had resulted, presumably from the bite of *O. savignyi*. Howell (1966b) discussed paralysis in general in the introductory section, but only indicated that bovines died overnight as a result of exposure to *O. savignyi* (Howell, 1966). Perusal of the original literature dealing with toxicoses caused by the sand tampan and unpublished results on the clinical symptoms and pathology of various animals exposed to the tick secretions, gave no indication of characteristic paralysis symptoms (flaccid tetraplegia). Although the toxins could have an effect on the central nervous system, symptoms suggest that the hemostatic, respiratory or cardiac system of the various host's are affected. These findings are concurrent with the data of this present study. This report emphasizes the need for more stringent studies into tick toxicoses. As yet, it is not known how many reports of toxicoses are accurate, which hinders comparative studies that can shed light on the origins of tick toxicoses. Whether tick toxins have a similar origin is of crucial importance, as this would give answers to questions such as whether toxins from different ticks have similar protein structures and toxic mechanisms. A consideration of literature of paralysis toxins argue that the molecular properties differ from that of the sand tampan toxins:

(A) Paralysis toxins tend to form non-specific macro-molecular complexes so that molecular mass determination is complicated. Masses between 5-74 kDa have been reported for paralysis toxins from *I. holocyclus*, *R. evertsi evertsi* and *A. walkerae* (Viljoen *et al.* 1986; Crause *et al.* 1993; Maritz *et al.* 2000; Masina and Broady, 1999). In contrast, molecular mass determination of toxins from the present study, gave similar masses (~15-18kDa) using a variety of techniques,

which include sedimentation equilibrium ultra-centrifugation, MALDI-TOF-MS, ESMS, tricine SDS-PAGE and size exclusion HPLC (Neitz, Howell and Potgieter, 1969; Mans *et al.* 2001). These masses do not compare with any previously described for the paralysis toxins.

(B) A slow feeding period is necessary for toxin production in hard ticks, while subcutaneous injection of SGE of *R. evertsi evertsi* failed to produce paralysis in mice (2.5 mg) and ovines (3.5 mg), although ovines became paralyzed upon challenge with ticks, while *A. walkerae* only paralyze 1 day old chicks (Viljoen *et al.* 1986; Crause *et al.* 1993). In contrast the toxins from *O. savignyi* are present in all tick stages, SGS and SGE and are able to kill a variety of mammals when injected subcutaneously.

(C) A monoclonal antibody directed against the paralysis toxin from *R. evertsi evertsi* was shown to cross-react with epitopes from *A. walkerae* larvae and to protect day old chicks from paralysis (Crause *et al.* 1994). The same monoclonal was used as an assay method to isolate the toxin from *A. walkerae* (Maritz *et al.* 2000). No cross-reactivity was however, observed for salivary gland extracts of *O. savignyi* (Maritz, 1999).

(D) It was also shown that *A. walkerae* larval homogenates inhibit the release of [^{3}H] glycine from potassium-stimulated rat brain synaptosomes (Maritz *et al.* 2001). SGE from *O. savignyi* gave a lower percentage inhibition of this process than did SGE from adult *A. walkerae*, known to be non-paralytic (Maritz, 1999).

(E) Sinus arrhythmia has been shown to occur in marmots exposed to *D. andersoni*, but these effects on the cardiac system were only observed after complete paralysis of all four limbs (Emmons and McLennan, 1980). While paralysis toxins might thus also affect the cardiac system, paralysis symptoms would probably be observed first. In contrast, cardio-pathogenesis caused by *O. savignyi* is unaccompanied by paralysis.

6.5.5 Cardio-pathogenic properties of sand tampan toxin

The toxic effects during rat heart perfusion indicate that SGE can have a direct effect on the cardiac system. Animal toxins induce their specific pharmacological effects through

toxin-receptor interactions, while many interact specifically on different ion channels (Kordiš and Gubensek, 2000). Electrophysiological results suggest that cardiac ion currents are affected by the tick toxins, probably by blocking of specific ion channels. Potassium channel block is normally revealed as a prolongation of the cardiac action potential and its electrocardiac manifestation as a prolongation of the Q-T interval as observed for TSGP2 (Colatsky *et al.* 1994). AV-block as observed for TSGP4 is generally due to impaired ventricular depolarization and conduction through the AV-junction. The main ion channels involved in conduction through the AV-junction are calcium and sodium channels and could as such be the target of TSGP4 (Goldberger and Goldberger, 1981).

6.5.6 Toxicity of TSGP2 *versus* the non-toxicity of TSGP3

N-terminal sequences of TSGP2 and TSGP3 indicate 95% identity while amino acid analysis indicates similar compositions. TSGP2 and TSGP3 have identical elution times during RPHPLC that indicate similar global hydrophobicity. In contrast, TSGP2 and TSGP3 separate on HIHPLC, indicating differences in their surface hydrophobicity and hence surface conformation. This could account for the absence of apparent toxicity in TSGP3 and suggest that toxicity of TSGP2 is due to a local surface conformation difference. A mobile local surface conformation of TSGP4 and TSGP2 can also account for their lability observed during purification.

6.5.7 Biological functionality of toxins

The toxic nature of these proteins is probably of secondary importance, as is exemplified by the non-toxicity of TSGP1 and TSGP3. The environment also has a large effect on the stability of the purified toxins, which could indicate that the toxic nature of these proteins could be a secondary peripheral function. It could be that the toxins perform a very different function in the blood-feeding environment or even in the salivary glands themselves. *O. moubata* has been shown to be non-toxic in a study where 5 female, 10 male adults and 100 nymphs were fed on a mouse to induce an immune response (Astigarraga *et al.* 1997). This is in stark contrast to results obtained for one feeding tick in this study. *O. moubata* is a nidicolous tick that is probably in contact with only a few

individuals in its lifetime, so that any adverse effects could have serious implications for tick survival. *O. savignyi* on the other hand, is free-living and not restricted by the limited set of hosts encountered in a burrow and could thus afford to be more aggressive towards its host (Hoogstraal, 1956). The absence of toxic proteins in the SGE of *O. moubata* is concurrent with this view. While the killing of its host might not affect the survival of the tick population, to argue that toxicity might confer any evolutionary advantage to *O. savignyi* is a premature statement. It has been suggested that the toxins are part of a larger family involved in granule biogenesis of tick salivary gland granules. Possible functions in the regulation of the host's immune and hemostatic systems cannot be excluded either.

This study indicates unique cardio-toxic mechanisms of toxicoses induced by *O. savignyi* and conclude that reports of paralysis caused by this tick species are erroneous. It emphasizes the need for a more thorough investigation into all forms of tick-induced toxicoses, both to catalogue tick toxin variety but also to gain an understanding of toxin origins and possible biological relevance. The next chapter deals with the cloning and molecular characterization of the TSGPs and show that they are all part of the same lipocalin protein family.