Structure-Function Insights into the Biochemical Properties and Bilayer Interactions of the Saposin-like Domain of Plant Aspartic Proteases

by

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ABSTRACT

STRUCTURE-FUNCTION INSIGHTS INTO THE BIOCHEMICAL PROPERTIES AND BILAYER INTERACTION OF THE SAPONIN-LIKE DOMAIN OF PLANT ASPARTIC PROTEASES

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This thesis is an investigation of structure-function relationships of the saposin-like domain of plant aspartic proteases. Many plant aspartic proteases contain an additional sequence of approximately 100 amino acids termed the plant-specific insert which is involved in host defense and vacuolar targeting. Similar to all other saposin-like proteins, the plant-specific insert functions via protein-membrane interactions, however, the structural basis for such interactions have not been studied and the nature of plant-specific insert-mediated membrane disruption have not been characterized. This thesis presents the first comprehensive structure-function investigation of the less-understood arm of the saposin-like protein family, the so-called “swaposins”. Among the findings presented here are the quaternary, tertiary and secondary structures of the plant-specific insert from *Solanum tuberosum* (potato) aspartic proteinase both in terms of pH and lipid bilayer presence, the identification of a structure in potato saposin (Ile1-Leu20) that is a universal membrane penetrating motif based upon structural alignment, delineation of the structural basis for the acid pH requirement for bilayer interaction (pH-sensitive dimerization) and positively charged point of contact for anionic bilayers (Lys83) located at the C-terminal end of helix 3, a positively charged residue within an uncommon anti-bilayer motif found in some flocculant proteins and spider silk structural proteins, among others. Atomic force microscopy revealed that potato plant-specific insert destabilized (softened) bilayer whereas cryo-transmission electron microscopy showed several distinct shapes induced in LUV’s upon addition of potato saposin. Lastly, comparative characterizations of potato plant-specific insert along with three other plant saposin-like domains from barley (*Hordeum vulgare*), flowers of Cardoon thistle (*Cynara cardunculus* L.) and Rockcress (*Arabidopsis thaliana*) were carried out revealing that reduction of the disulfide bonds of potato swaposin caused a drastic increase in bilayer fusion rate and increase in typical fusion product sizes. *Arabidopsis* swaposin with reduced cystines showed relatively minor alterations to its fusion profile while essentially no difference to the fusogenic activities of barley and Cardoon swaposins were discernable upon reduction of their disulfide bonds. Taken together, implications for swaposin mechanism of action as well as future research directions are discussed.
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<tbody>
<tr>
<td>AP</td>
<td>Aspartic Protease</td>
</tr>
<tr>
<td>TRX</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>PSI</td>
<td>Plant Specific Insert</td>
</tr>
<tr>
<td>PSS</td>
<td>Plant-Specific Sequence</td>
</tr>
<tr>
<td>SAPLIP</td>
<td>Saposin-Like Protein</td>
</tr>
<tr>
<td>StAP</td>
<td><em>Solanum tuberosum</em> Aspartic Protease</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>LUV</td>
<td>Large Unilamellar Vesicle</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidyl-choline</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root mean square deviation</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally Regarded as Safe</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>POPE</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>POPS</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism Spectropolarimetry</td>
</tr>
<tr>
<td>TEM</td>
<td>Cryo-Transmission Electron Microscopy</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>LUV&lt;sub&gt;mix&lt;/sub&gt;</td>
<td>Large unilamellar vesicles composed of a vacuole-like phospholipid mixture</td>
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Chapter 1: Introduction

1.1 Background

Proteases are an important class of enzymes due to their relevance in both the physiological and commercial fields. Proteases are the most important type of industrial use enzymes because they represent approximately 60% of all commercialized enzymes in the world (Feijoo-Siota and Villa, 2011). The diverse fields of application include food science and technology, pharmaceutical industries, and detergent manufacturing. Novo Industries, Gist-Brocades, Genencor International, and Miles Laboratories are among the top producers in the world (Feijoo-Siota and Villa, 2011). Aspartic proteases (APs) constitute one of six classes of proteases (Seemuller, 1995). The studies herein consist of protein structure-function investigations of the saposin-like domain of plant APs, also known as the Plant Specific Insert (PSI) and the Plant Specific Sequence (PSS). This introduction will focus on specific functional and structural details of plant AP PSIs, however, the value of structure-function research in this area, as in the findings contained in the proceeding chapters, extends beyond the scope of the chosen focus models: Potato AP (StAP), Arabidopsis AP (AtAP), flowers of Cardoon AP (cardosin A) and barley AP (phytepsin) (Devaraj, 2008).

Although the individual roles of different PSIs within their respective environments, e.g. vacuoles, lysosomes, intercellular space, continue to be determined, there exists a clear universal role for PSIs; to interact with membranes (Egas et al., 2000). Such interactions can be with membranes of pathogenic invader species, e.g. potato leaf AP (Mendieta et al., 2006), or membranes of endogenous cellular compartments in targeting roles, e.g. barley
AP (Tormakangas et al., 2001). Research in this area to date has focused on specific cases involving PSIs of particular individual plants. There has been no apparent consensus on the specific reasons that PSIs interact with membranes beyond the factual assertion that PSIs are saposin-like protein structures. Lacking from the field of PSI structure-function investigations has been the more global structure-function question: Are there structural features common among PSIs that are universally responsible for their common function as membrane-interacting domains on behalf of their AP protein carriers? To answer this fundamental question, the present research will take a comprehensive approach that covers SFRs of four PSIs, focusing on potato PSI. To accomplish this goal, differences in their abilities to disrupt and/or fuse phospholipid bilayers, and structural comparisons at the secondary, tertiary and quaternary protein structure levels as well as liposome morphology will be exploited.

1.2 Literature Review

1.2.1 Plant aspartic proteinases - Throughout the plant kingdom, in angiosperms, gymnosperms, and green algae, APs have been detected (Simoes and Faro, 2004). Plant APs are distributed among various AP families; most plant APs are pepsin-like enzymes belonging to the A1 AP family (Simoes and Faro, 2004). Plant APs are not as well characterized as mammalian, microbial, and viral APs in terms of known structures and biological roles (Mutlu and Gal, 1999, Simoes and Faro, 2004). APs have a bilobal structure separated by an active site cleft, demonstrated well by the APs in in Figure 1.1 for distant members of the family (i.e., yeast vs. mammalian AP). Similar to pepsin, such plant APs are most active and stable under acidic conditions having two catalytic Asp
Figure 1.1: The aspartic protease bilobal structure. The crystal structures of secreted aspartic proteinase from *Candida albicans* (1ZAP) and human pepsin (1PSN) illustrate the bilobal tertiary structure of aspartic proteases.
residues in the active site responsible for hydrolytic cleavage of protein substrate (Dunn, 2002; Simoes and Faro, 2004). Typically, plant AP N-termini have a pre-region containing a signal peptide and a pro-region consisting of a peptide that binds to the active site usually rendering the AP catalytically inactive as is found with most APs. In most known plant APs the mature region contains an inserted sequence unique to plants called the plant-specific sequence (PSS), also interchangeably called the plant-specific insert (PSI). The term ‘insert’ is used to denote that this sequence is inserted into the C-terminal region of mature sequences in plant zymogens (Figure 1.2). Usually PSIs are partially, or completely, excised upon activation. The biological role(s) of this unique component of plant APs have been studied since the first description of a unique plant AP component in 1991 (Runeberg-Roos et al., 1991). The PSI is unlike any other AP component, but is highly similar to saposin-like proteins NK-lysin (Liepinsh et al., 1997) (see Figures 1.3 and 1.4) and granulysin (Anderson et al., 2003). Plant AP PSI primary structures are related to saposins by a swapping of the N- and C-termini hence the term swaposin was coined to describe the PSI (Ponting and Russell, 1995). Saposins are briefly described below in terms of saposin structure-function relationships relevant to this proposal. PSI functions in plant APs are still unclear; however, several roles have been suggested in the plant programmed cell death (Faro et al., 1999, Tormakangas et al., 2001, Vieira et al., 2001). Specifically, the presence of PSI in mature APs from potato (StAPs); tomato (LycoAP) (Schaller and Ryan, 1996) and Nepenthes alata (NaAP4; Philippine tropical pitcher plant) (An et al., 2002), has been considered as being a part of the defensive machinery against pathogens and/or an effector of cell death (An et al., 2002, Mendieta et al., 2006).
**Figure 1.2: Plant aspartic protease insert.** Schematic illustrating the position of the PSI within the C-terminal domain of plant aspartic proteases. Pro- is the prosegment, DTG/DSG are the catalytic consensus sequences, and heavy/light chain refers to the AP structure after post-translational processing and removal of the PSI, a process that occurs in many but not all plant APs.

**Figure 1.3: The crystal structure of prophytepsin (1QDM).** Note the bilobal main enzyme structure consisting of the N- and C-terminal domains, and the ~100 amino acid plant-specific insert. The catalytic Asp residues (magenta) are located in a deep cleft between the N- and C-terminal lobes, and the plant-specific insert (purple) is a separately folded domain whose sequence occurs within the C-terminal half of the aspartic protease overall primary structure.
Figure 1.4: The canonical saposin fold. The three saposin-like proteins shown share a common fold termed the “saposin fold”, first solved for NK-lys in (Liepins et al., 1997). The structures shown are NK-lys (1NKL), saposin C (2GTG) and phytepsin PSI (1QDM).
1.2.2 Solanum tuberosum aspartic proteinases (StAP) - In studying the effect of abiotic stress on potato tuber tissue protein content, including the nature of observed proteolytic activities, led to the isolation and partial characterization of an aspartic proteinases (StAP 1) (Guevara et al., 1999). Proteolytic activity was assayed for potato tuber disk extracts and an increase in activity of over 50% was observed after 24 h. Various inhibitors were tested and only pepstatin strongly inhibited proteolytic activity, thus an AP was suspected. The pepstatin inhibition was only prevalent for extracts of aerated tuber disks. Such extracts were subjected to anion-exchange, and pepstatin-affinity, chromatography yielding an approximately 40 kDa protein on reducing SDS-PAGE suggesting a monomer. The resulting protein had a pH optimum of 5 and activity was dependent on the presence of the reducing agent DTT (4mM). Glycosylation of potato tuber StAP was indicated by binding to concanavalin A (Guevara et al., 1999).

To further elucidate the biological function(s) of StAP, another AP was isolated from potato leaves (StAP 3) (Guevara et al., 2001). Extracts of wounded leaves were taken at 0 h, 24 h and 48 h. At 0 h and 24 h, 21% and 16% of proteolytic activity was inhibited by pepstatin, respectively, compared to 62% inhibition after 48h indicating a four-fold increase in the proportion of proteolytic activity due to APs and the induction of AP expression. The pH optimum of leaf StAP was shown to be 3, lower than that of tuber StAP, and activity was unaffected by reducing agent DTT. Also, leaf StAP did not bind to concanavalin A indicating that it is not a glycoprotein (Guevara et al., 2001). The wound response nature of AP expression in potato leaves was similar to the case of tomato leaf AP induction and cauliflower seed AP induction (Fujikura and Karssen, 1995; Schaller and Ryan, 1996), respectively, although APs are expressed in Arabidopsis constitutively.
(Mutlu and Gal, 1999), and pathogenesis-related APs are also constitutively expressed in tomato and tobacco (Rodrigo et al., 1991). Thus, wound or infection is not always necessary for plant AP induction.

Subsequent to the above studies on StAPs, further characterization of tuber StAP induction as it relates to pathogenic conditions was carried out for two field-tested potato cultivars; one resistant and one susceptible to Phytophthora infestans, the cause of late potato blight (Guevara et al., 2002). Interestingly, the resistant cultivar showed faster and higher StAP induction post-wound infection in intercellular fluid. Additionally, the isolated StAP showed \textit{in vitro} inhibition of Phytophthora infestans and Fusarium solani. Although the antimicrobial activity was absent for pure StAP samples inhibited by pepstatin, intercellular washing fluid (IWF) displayed substantial antimicrobial activity in the presence of pepstatin A. Apparently, the antimicrobial activity of StAP was at least in part proteolytic in nature under the conditions tested, and StAP accounted for approximately half of the antimicrobial activity of tuber IWF. However, neither pepsin nor trypsin inhibited either fungus in antimicrobial proteolytic activity control reactions tested at concentrations at the high end of the range used for StAP. These results suggested an antimicrobial functionality for potato AP (Guevara et al., 2002).

The cDNA for potato leaf StAP was later isolated yielding a gene sequence that included a PSI (Guevara et al., 2005a). Since isolated StAP from previous studies (Guevara et al., 1999, Guevara et al., 2001) yielded monomers then mature, active StAP must retain its PSI because the cDNA indicated that the putative PSI region is inserted into the C-terminal domain of prepro-StAP (Guevara et al., 2005b). In terms of predicted amino acid sequence, StAP PSI was shown to be similar to other plant PSIs with conservation of disulfide-bridge
Cys residues and saposin-like protein domain homologies consistent with StAP PSI being a swaposin (Guevara et al., 2005b, Ponting and Russell, 1995). Guevara et al. (2005) further tested for mRNA accumulation after *P. infestans* infection of one resistant and one non-resistant cultivar, and found that the resistant cultivar produced higher levels of StAP mRNA after infection further bolstering the role of StAP in the plant defense mechanism against potato blight (Guevara et al., 2005b). Further evidence of such a biological role was provided by showing direct membrane interaction for StAP with the fungal membranes of *F. solani* and *P. infestans* as well as membrane permeabilization (Mendieta et al., 2006). Both leaf and tuber StAPs displayed antifungal activities. Membrane binding was shown by fluorescence microscopy of fluorescein isothiocyanate-labelled StAP and membrane permeabilization was indicated by the uptake of a fluorescent dye by the test fungi (Mendieta et al., 2006). These results suggest a possible role of the StAP PSI in the light of saposin proteins’ functions of membrane interaction and permeabilization (outlined below). StAPs also display similar activities against bovine and human permatozoa in that motility was abolished and membrane permeabilization was shown by the same fluorescence-based method discussed above (Cesari et al., 2007).

*Solanum tuberosum* aspartic protease (StAP) PSI is able to kill spores of two potato pathogens in a dose-dependent manner without any deleterious effect on plant cells (Muñoz et al., 2010). The StAP-PSI ability to kill microbial pathogens is dependent on the direct interaction of the protein with the microbial cell wall/or membrane, leading to increased permeability and lysis. StAP-PSI and StAPs are cytotoxic to Gram-negative and Gram-positive bacteria in a dose dependent manner, and furthermore, StAP-PSI is able to kill human pathogenic bacteria in a dose dependent manner, but is not toxic to human red blood
cells at the concentrations and times assayed. MBC values determined for StAPs and StAP-PSI are in the same order of magnitude as those previously reported for NK-lysin and granulysin (Muñoz et al., 2010). Of interest is the structural basis for this selective toxicity among StAP-PSI and other SAPLIPs. More recently, Muñoz et al. (2011) proposed that the possible role of StAPs as pathogenesis related proteins into the plant defense response is to interact with the pathogen plasma membrane causing membrane destabilization and subsequent cell death and/or kill plant cells under stress conditions, thereby attenuating pathogen spread and colonization (Muñoz et al., 2011).

The structure-function role of StAP glycosylation has also been studied (Pagano et al., 2006, Pagano et al., 2007). De-glycosylation of StAPs did not alter pH-optimum for activity, temperature optimum for activity, nor index of surface hydrophobicity (Pagano et al., 2007). However, secretion into the intercellular space did not occur in response to wounding and infection as would normally be expected for StAPs. Additionally, in vitro antifungal activity was reduced substantially at various protein concentrations. It was thus concluded that StAP glycosylation was essential for in vivo plant defense, but structure and proteolytic activity was not affected (Pagano et al., 2007).

1.2.3 Saposins - The PSIs of APs are saposin-like proteins. Reviews of saposin-like proteins were most recently published in 2005 (Bruhn, 2005b; Kolter and Sandhoff, 2005). Saposins A, B, C, and D belong to the saposin-like protein family of proteins. Proteins of this family are membrane-interacting in three principal ways: Membrane binding, membrane perturbation without permeabilization, and membrane permeabilization (Bruhn, 2005b). The saposins are proteins that enhance the activities of lysosomal exohydrolases in glycosphingolipid degradation (Matsuda et al., 2001). Saposins A-C are implicated in
various disease states whereas no known deficiency of saposin D in humans has been documented, however, a saposin D mouse knockout resulted in deleterious effects (Matsuda, 2008). In general, defective saposin-disease states arise from the accumulation of ceramide derivatives in various tissues resulting in pathological states. Saposins are relatively small at 8 - 11 kD, they are heat stable, and they display non-enzymatic activities (Kolter and Sandhoff, 2005). Also, they contain three disulfide bonds holding together a five-helix, compact, globular structure that has been termed a ‘saposin-fold structure’ (You et al., 2003). The four saposins are all derived from the single precursor protein prosaposin, a protein sequence made up of the four saposins in tandem which are subsequently proteolytically processed in the lysosome thereby yielding the four individual products (Matsuda, 2008).

Atomic force microscopy revealed the effects of saposin C interactions on phospholipid-containing membranes (You et al., 2003). Two main effects were visualized: membrane regions were rearranged to form so-called patch-like domains (literally irregularly-shaped, circular regions of increased thickness) and membrane destabilization. Unlike the formation of patch-like domains, membrane destabilization was dependent on the presence and concentration of acidic phospholipids, e.g., phosphatidylserine (You et al., 2003). Of particular interest in this study, the first two helices of saposin C were synthesized and tested independently. Helix 1 was found to be incapable of membrane restructuring alone, however it destabilized membrane if it was added first followed by helix 2. By contrast, helix 2 was capable of patch-like domain formation only (You et al., 2003). Individual structural regions of saposins within the saposin fold are thus proven to play specific roles with respect to membrane interactions.
Study of saposin structure by X-ray crystallography suggested that human saposin B bound to phosphatidylethanolamine exists as a dimer (PDB 1N69) (Ahn et al., 2003b). The secondary structure of bound dimer was similar to that of the monomer, however, dimer contained rearranged helices at the tertiary structure level. Overall, a relatively large hydrophobic cavity (relative to the small saposin structure) is enclosed by a monolayer of helices. The large hydrophobic cavity could serve as an area of direct interaction with membrane surfaces (Ahn et al., 2003b). This “open” saposin fold was later observed again for saposin C (PDB 2QYP) such that two extended monomer form a homodimer. The closed and open saposin folds are shown for the same protein, saposin C, in Figure 1.5.

The existence of the open saposin fold was particularly important to understanding the mechanism by which saposin C induces bilayer fusion (Rossmann et al., 2008) in that it gave credence to the previously proposed “clip-on” mechanism. In this model, each of the saposin C monomer units bind adjacent bilayers thereby bringing them into proximity, and due to their having perturbed the bilayer structures, adjacent damaged lipid structure will have an increased likelihood of joining upon rearrangement of an energetically favourable state.

1.2.4 Structure-Function Studies of Saposin Disulfide Bonds - The saposin fold has been shown to interact with membranes (You et al., 2003). All four saposins contain six identically placed, conserved cysteine residues (Vaccaro et al., 1994). The highly conserved nature of disulfide bonds suggests an important biological role for the bonds’ effects on saposin structures. Establishing the location of saposin disulfide bonds was considered to be critical in assessing saposin conformations and their importance for saposin-lipid bilayer interactions (Vaccaro et al., 1995).
Figure 1.5: Comparison of the two known saposin folds. Top: The ‘closed’ saposin fold of saposin C (2GTG). This is the canonical fold for the saposin-like family. Bottom: The “open” saposin fold of saposin C (2QYP). The open fold is the only known fold for saposin dimerization.
1.2.5 Plant AP PSI-Membrane Interactions - Proteins belonging to the Saposin-Like Protein Family have various physiological functions that have been suggested: Saposins are critical to exohydrolase degradation of sphingolipids in the lysosome, NK-lysin is antibacterial and tumor-lysing, granulysin is antimicrobial, surfactant protein B lowers pulmonary surfactant surface tension, and amoebapores lyse bacteria and eukaryotic cells. Membrane interaction is the common theme among all of these functions (Egas et al., 2000). Beyond the direct study of saposin-like proteins’ primary functions, the understanding of structure-function relationships involving membrane-interacting AP surfaces could also extend to the membrane-bound APs, i.e. memapsins 1 and 2, implicated in Alzheimer’s disease, which do not contain PSIs. The interaction of procardosin A with membrane vesicles has been studied (Egas et al., 2000). The role of the PSI for membrane interactions of procardosin A with phospholipid vesicles was explored by recombinantly expressing procardosin A, a PSI-deletion mutant procardosin A, and PSI in E. coli. Full length procardosin caused membrane damage as evidenced by vesicle leakage whereas PSI-deletion mutant procardosin A was ineffective at disrupting lipid bilayers (Egas et al., 2000). The PSI and full-length enzyme leakage results suggested that the PSI is responsible for membrane interactions and hence may function as part of both a host defense mechanism as well as programmed cell death.

1.3 Scientific Investigation Plan

1.3.1 Research Questions - The present studies are not only important to improving the current state of knowledge and understanding of PSI structure-function relationships, but are prerequisite to future engineering of PSIs or PSI-templated interventions for disease resistance, e.g., potato blight resistance, or disease treatments including human medical
interventions. In conceptualizing an approach towards the above, several questions related to plant AP PSIs were formulated:

(1) Are plant PSI secondary and tertiary structures self-determined from their primary sequences?
(2) What structural characteristics determine observed bilayer interactions?
(3) How do swaposins (PSIs) compare with saposins in terms of bilayer effects?
(4) What is the mechanism by which PSI disrupts bilayers?
(5) Are there important functional differences between the PSIs of four representative plant species; potato, barley, flowers of Cardoon (thistle) and Arabidopsis? That is - is the primary sequence of a swaposin a universal determinant of bilayer disruption/interaction?

1.3.2 Objectives - The overarching objective of the present research projects are as follows:

1. To delineate the structural basis of potato PSI-PL bilayer interactions with respect to protein folding and structure, and bilayer PL composition.
2. To assess the universality of PSI structure-function relationships by comparing PSIs of four different plant species in terms of their bilayer interactions and protein structural features.
1.3.3 Hypotheses

1. The PSI (swaposin) domain of potato AP is an independently functional protein unit, independent of its parent AP for its bilayer disruption activity, and this bilayer perturbation occurs in a similar manner to saposins.

2. The quaternary structure of StAP PSI, i.e., dimerization, is critical to catalyzing bilayer fusion whereas bilayer disruption activity is dependent on the C-terminal portion of the PSI molecule, independent of tertiary or quaternary structure.

3. The bilayer effects of the PSIs of potato, barley, flowers of Cardoon and Arabidopsis are indistinguishable in terms of membrane disruption and bilayer fusion.
1.4 References


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2043-2057.
Chapter 2: Structure and Mechanism of the Saposin-Like Domain of a Plant Aspartic Protease

Note: The content of this chapter was published in *The Journal of Biological Chemistry* (Bryksa et al., 2011). The article includes reporting the crystal structure of StAP PSI (section 2.4.1), work done by Dr. Prasenjit Bhaumik, Eugenia Magracheva, Dr. Alexander Zdanov and Dr. Alexander Wlodawer (SAIC-Frederick, National Cancer Institute, Frederick, MD 21702, USA).

2.1 Abstract

Many plant aspartic proteases contain an additional sequence of approximately 100 amino acids termed the plant-specific insert which is involved in host defense and vacuolar targeting. Similar to all saposin-like proteins, the plant-specific insert functions via protein-membrane interactions, however, the structural basis for such interactions has not been studied and the nature of plant-specific insert mediated membrane disruption has not been characterized. In the present study, the crystal structure of the saposin-like domain of potato aspartic protease was resolved at a resolution of 1.9 Å, revealing an open V-shaped configuration similar to the open structure of human saposin C. Notably vesicle disruption activity followed Michaelis-Menten-like kinetics, a finding not previously reported for saposin-like proteins including plant-specific inserts. Circular dichroism data suggested that secondary structure was pH-dependent in a fashion similar to influenza A hemagglutinin fusion peptide. Membrane effects characterized by atomic force microscopy and light scattering indicated bilayer solubilization as well as fusogenic activity. Taken together, the present study is the first report to elucidate the membrane interaction mechanism of plant saposin-like domains whereby pH-dependent membrane interactions
resulted in bilayer fusogenic activity that likely arose from a viral-type pH-dependent helix-kink-helix motif at the plant-specific insert N-terminus.

2.2 Introduction

Aspartic proteases (APs) are characterized by a common bilobal tertiary structure containing two catalytic aspartic acid residues (Asp32 and Asp215 in pepsin) within an active site cleft (Blundell & Johnson, 1993; Davies, 1990). They are found in all higher organisms and their respective roles are well established, although structural and functional characteristics of APs in plants are least understood. Of practical interest among plant APs are their roles in plant pathogen resistance (Guevara et al, 2002) as well as in senescence and post-harvest physiology (Payie et al, 2000; Schaller & Ryan, 1996). Plant APs share the common AP bilobal structure; however, some contain an additional sequence of approximately 100 residues inserted within the C-terminal primary structure. These additional amino acids unique to plant APs (Glathe et al, 1998; Payie et al, 2003; Ramalho-Santos et al, 1998) create an extra domain protruding from the canonical AP molecule (Frazão et al, 1999; Kervinen et al, 1999; Mazorra-Manzano et al, 2010). This structural oddity among APs is called the plant-specific insert (PSI), also known as the plant-specific sequence (PSS), which belongs to the saposin-like protein (SAPLIP) family (Mutlu & Gal, 1999; Runeberg-Roos et al, 1991). Plant APs are found in either monomeric or heterodimeric forms (Egas et al, 2000; Kervinen et al, 1999); the latter result from post-
translational proteolysis which includes the removal of part or all of the PSI, whereas the PSI is retained in monomeric plant APs (Glathe et al, 1998; Ramalho-Santos et al, 1998).

In general, members of the SAPLIP family have various physiological functions all of which entail membrane interaction (Bruhn, 2005; Egas et al, 2000; Kolter & Sandhoff, 2005) manifested in three principal ways: membrane binding, membrane perturbation without permeabilization, and membrane permeabilization (Bruhn, 2005). Examples of SAPLIP functions include roles in exohydrolase degradation of sphingolipids in the lysosome (saposins) (Matsuda et al, 2001), antimicrobial activity (granulysin and NK-lysin) (Anderson et al, 2003), tumor lysis (NK-lysin) (Liepinsh et al, 1997), pulmonary surfactant surface tension regulation (surfactant protein B) (Gordon et al, 2000), and bacterial/eukaryotic cell lysis (amoebapores) (Zhai & Saier, 2000).

Fusion of cellular lipid membranes is an essential process in all forms of life (Stiasny et al, 2007) and the mechanism by which membrane fusion occurs, a process typically catalyzed by proteins, continues to be unraveled (Kasson et al, 2010). Merely bringing membranes in proximity to one another is insufficient for fusion (Kasson et al, 2010), and the nature of fusion peptide structures is critical to fusogenic function (Bissonnette et al, 2009; Lai et al, 2006; Qiao et al, 1999). Disordering of bilayers by fusion proteins, thought to be a critical first step in the catalysis of bilayer fusion (Lai et al, 2006), results in an increased rate of energetically unfavorable hydrophobic lipid tail protrusion (Kasson et al, 2010). The fusion transition state involves contact formation between lipid tails of opposite bilayers within the intervening hydrophilic region (Kasson et al, 2010) resulting in stalk formation(s) between the two disordered bilayer patches (Kasson et al, 2010; Lukatsky & Frenkel, 2004). Dimerization of helical structures is part of the saposin-mediated bilayer
fusion, transfer, and solubilizing mechanisms (Ciaffoni et al, 2006; Remmel et al, 2007; Wang et al, 2003) and these structural rearrangements take place after release from the parent molecule (prosaposin) (Kolter & Sandhoff, 2005).

The SAPLIP domains of plant APs display membrane permeabilizing activity independent of its “parent” protein (Egas et al, 2000; Simoes & Faro, 2004) and they likely act independently (post-proteolytic processing) as a part of the plant defense mechanism against fungal pathogens (Guevara et al, 2002; Muñoz et al, 2010). Like PSIs of heterodimeric plant APs, saposins are also expressed as a proprotein and are subsequently processed via proteolytic cleavage (Bruhn, 2005) resulting in distinct, active tertiary structures consisting of stable helical and coil secondary structures (Ahn et al, 2003; Ahn et al, 2006; Alattia et al, 2006; Bruhn, 2005; Ciaffoni et al, 2006).

Recently, recombinantly-produced PSI of *Solanum tuberosum* aspartic proteinase (StAP) was shown to kill human pathogens as well as inhibit fungal sporulation via interaction with, and permeabilization of, microbial plasma membranes (Muñoz et al, 2010). Understanding the structural basis for newly characterized antifungal activities is important in the development of novel therapeutic drugs for the treatment of fungal infections (Ghannoum & Rice, 1999) in immunocompromised patients (Meyer, 2008; Tseng & Perfect, 2011). Furthermore, we propose that understanding structure-function relationships involving PSI-membrane interactions may have relevance to non-plant membrane-bound APs, e.g. memapsins 1 and 2, implicated in Alzheimer’s disease, beyond the direct elucidation of SAPLIP primary functions. Using StAP PSI as a model system, the present study characterizes the structure of a plant AP PSI as it relates to membrane interactions. The observed saposin C-like tertiary structure and saposin B-like fusogenic
activity, and the apparent catalysis of energetically unfavorable membrane bilayer disruption and fusion via a pH-dependent helix fusion peptide motif at the PSI N-terminus are discussed.

2.3 Experimental Procedures

2.3.1 Materials - A PSI synthetic gene optimized for expression in *E. coli* was purchased from Mr. Gene GmbH (Regensburg, Germany). Plasmids pET19b(+) and pET32b(+), *E. coli* Rosetta-gami B (DE3)pLysS, and u-MAC™ columns were obtained from EMD Biosciences (San Diego, CA, USA). *E. coli* TOP10F’ was from Invitrogen (San Diego, CA, USA). GenElute™ Plasmid Miniprep Kit was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The QIAquick® PCR Purification Kit and QIAquick® Gel Extraction Kit were from Qiagen (Germantown, MD, USA). Restriction enzymes, T4 DNA ligase and *Pfu* DNA polymerase were obtained from Fermentas Life Sciences (Burlington, ON, Canada). Primers were synthesized by Sigma Genosys (Oakville, ON, Canada) and thrombin was purchased from Fisher Scientific Co. (Ottawa, ON, Canada). The RPC column was from GE Healthcare (Piscataway, NJ, USA). Phospholipids were from Avanti Polar Lipids (Alabaster, AL, USA).

2.3.2 Construction - of Expression Vector pET32z-PSI: Two constructs, a 6.0 kb construct named *pET19b*-PSI and a 6.2 kb construct named *pET32z*-PSI, were made for the expression of PSI and thioredoxin-PSI fusion protein (Trx-PSI), respectively, in *E. coli*. For *pET19b*-PSI, PSI insert was amplified using primers:

FwdPSINdeI 5’CATATGATTGTAAGCATGGAGTGTAAAACC and
RevPSIXhoI 5’ATCTCGAGTTACGGGATTTTTTCACACAGTTG,
followed by ligation between the \textit{NdeI} and \textit{XhoI} restriction sites. \textit{pET32z-PSI} construct was made using \textit{PSI} insert amplified using primers:

\textit{FwdPSINcoI} \quad 5’\text{ATCCATGGCGATTGAAGCATGGAGTAAAACC} \quad \text{and} \quad \textit{RevPSIXhoI},

followed by ligation between the \textit{NcoI} and \textit{XhoI} restriction sites of \textit{pET32z}, a modified version of \textit{pET32b} which contains a deletion between the thrombin cut site and the end of the enterokinase cut site. Each construct was transformed into \textit{E. coli} TOP10F’ using the method of Hanahan (Hanahan, 1983).

\textbf{2.3.3 Protein Expression} - Overnight cultures of \textit{E. coli} BL21 (DE3)pLysS or Rosetta-gami B (DE3)pLysS transformed with either \textit{pET19b-PSI} or \textit{pET32z-PSI} were used to express PSI as per the manufacturer’s instructions. Cells were harvested by centrifugation at 4,500 $\times$ g for 10 min at 4$^\circ$ C and stored at –20$^\circ$ C until further use. Frozen cells were thawed at room temperature (RT) and resuspended in 20 mL of 20 mM Tris-Cl pH 7.5. Suspensions were incubated at RT for 1 h with gentle shaking and the resulting cell lysates were centrifuged at 21,000 $\times$ g for 30 min at 4$^\circ$ C to remove insoluble matter.

\textbf{2.3.4 Protein Purification} - The following applies only to Trx-PSI fusion protein purification since \textit{pET19b}-derived PSI was expressed at far lower concentrations and thus was not pursued to purity. Protein purification was performed using an AKTA$^\text{TM}$ FPLC system (GE Healthcare, Piscataway, NJ, USA). Cell lysate soluble fractions were applied to five 1 mL u-MAC columns in series (EMD Biosciences, San Diego, CA, USA) equilibrated with 300 mM NaCl / 20 mM imidazole in 50 mM sodium phosphate pH 7.4 (binding buffer), followed by washing with the same buffer until a steady baseline was obtained. Samples were eluted with 300 mM NaCl / 250 mM imidazole in 50 mM sodium phosphate pH 7.4, then dialyzed in 20 mM Tris-Cl pH 7.4. Thrombin was added to the
dialysates at a 1:2000 mass ratio for incubation at RT for at least 12 h followed by re-
application of samples to u-MAC in binding buffer 3 times consecutively at 2 mL/min to
remove the Trx fusion tag. Flow-through was collected, dialyzed as above, then applied to
a 1 mL MonoQ column (GE Healthcare, Piscataway, NJ, USA) and separated using a 0-
500 mM NaCl gradient in 10 mM Tris-Cl pH 7.4. Eluent sample was further purified and
desalted on a 3 mL RPC column (GE Healthcare, Piscataway, NJ, USA), washed with 2%
acetonitrile/0.065% TFA and eluted with a 90 mL gradient (80% acetonitrile/0.05% TFA
elution buffer). The PSI peak, verified by SDS-PAGE and amino acid analysis (Advanced
Protein Analysis Center, The Hospital for Sick Children, Toronto, ON, Canada), was
collected and placed under vacuum in a Centrivap (Labconco Corp., Kansas City, MO,
USA) for 1 h at RT to remove the majority of the acetonitrile followed by dialysis against
4 x 1 L of 5 mM Tris-Cl pH 7.4 using 1 kDa MWCO dialysis tubing.

2.3.5 SDS-PAGE - Tris-glycine buffered SDS-PAGE was conducted according to the
method of Laemmli (Laemmli, 1970) in a Mini-Protean III electrophoresis cell (Bio-Rad,
Hercules, CA, USA). Gels were stained with GelCode Blue® (Pierce Biotechnology Inc.,
Rockford, IL, USA), and were analyzed for band size and relative intensities using a
ChemiGenius II system (Perkin Elmer, Waltham, MA, USA).

2.3.6 Crystallization - The purified StAP PSI protein sample was crystallized using
the sitting-drop vapor-diffusion method at 293 K using Qiagen PEG Suite screen solutions.
The best crystals appeared in the drop containing 0.4 µL protein solution / 0.2 µL reservoir
solution, equilibrated against 75 µL reservoir solution (0.2 M lithium sulfate/20%
PEG3350).
2.3.7 Diffraction Data Collection, Structure Solution and Refinement - X-ray diffraction data for StAP PSI crystals were collected to 1.9 Å resolution using a Rigaku MicroMax 007HF rotating anode and a MAR345dtb system at a wavelength of 1.5418 Å. A data set was collected at 100 K using 25% (v/v) glycerol added to the reservoir solution as cryo-protectant. All data sets were indexed and integrated using the program XDS (Kabsch, 1993). Integrated intensities were converted to structure factors with modules F2MTZ and CAD of CCP4 (Collaborative Computational Project, 1994). BUCCANEER (Cowtan, 2006) was used for initial automated model building. The structure was refined with REFMAC5 (Murshudov et al, 1997), rebuilt with COOT (Emsley & Cowtan, 2004), and analyzed using PROCHECK (Laskowski et al, 1993) and COOT. Structural superpositions were performed using SSM (Krissinel & Henrick, 2004) and ALIGN (Cohen, 1997). Figures were generated using PYMOL (DeLano, 2002) and UCSF Chimera (Pettersen et al, 2004).

2.3.8 Circular Dichroism (CD) Spectropolarimetry - CD analysis of PSI secondary structure was carried out using a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD, USA). 200 μL of 200 μg/mL PSI was loaded into a 1 mm pathlength quartz cell and scanned over 180-260 nm at 100 nm/min, 0.5 s response, standard sensitivity, and RT. Buffers 140 mM NaCl / 10 mM Tris-Cl pH 7.4 or 140 mM NaCl / 20 mM MES pH 4.5 were degassed under vacuum. For reducing condition effect determinations, DTT was added to final concentrations of 1.0, 2.5 and 5.0 mM, and heating was at 95º C in a standard heating block for 5 min in a fume hood, followed by a 30 min cooling period on the bench top.
2.3.9 Preparation of Large Unilamellar Vesicles (LUV) - LUVs were made of equimolar phosphatidylcholine (PC), phosphatidylethanolamine (PE) and/or phosphatidylinserine (PS). To obtain 4 mM phospholipid (PL) suspensions, aliquots of 12.5 mg/mL PL stocks were mixed in a tube and dried under N$_2$ flush for at least 30 min, then suspended in 500 µL of 80 mM calcein / 140 mM NaCl / 10 mM HEPES pH 7.4 by incubation at 37° C with periodic sonication and vortexing over a minimum of 30 min. LUVs were prepared using a standard mini-extruder (Avanti Polar Lipids Alabaster, AL, USA) containing a 100 nm pore membrane. The LUV prep was then desalted to remove untrapped calcein by gel filtration using a 5 mL HiTrap™ desalting column (GE Healthcare, Piscataway, NJ, USA) and visual detection of free calcein in column. To quantify PL post-desalting, the micro-Bartlett phosphorous assay (Bartlett, 1959; Fiske & Subbarow, 1925) was used to determine the concentration of PLs based on inorganic phosphate content (Sharom et al, 1995). Vesicle concentrations were calculated using PL concentrations, average vesicle diameter (140 nm), and the previously reported areas per lipid molecule; 59.7 Å$^2$ for PC (Petrache et al, 1998), 57.4 Å$^2$ for PS, and 59.2 Å$^2$ for PE (Huster et al, 2000).

2.3.10 LUV Disruption Assays - PSI-caused perturbation of LUVs was measured by calcein leakage (MacDonald et al, 1991; Matsuzaki et al, 1989) as detected using a Victor2 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA) at 25° C. 200 µL reactions were set up in 96-well microplates with varying concentrations of LUVs, 500 nM PSI and either 140 mM NaCl / 10 mM HEPES pH 7.4 or 140 mM NaCl / 20 mM MES pH 4.5. Leakage was detected using excitation at 385 nm and emission at 435 nm with 3 s shaking between readings. End points were measured by incubating LUVs in 0.5% triton for each
condition. Non-linear regression analyses were done using GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA).

2.3.11 Atomic Force Microscopy - A Veeco Picoforce Multimode Scanning Probe Microscope was used with a Nanoscope IV controller to image PE:PS membranes on the native oxide layer on silicon substrates. Images were collected in contact mode before and after the addition of PSI protein in situ. Suspensions of 3 mg/mL PE:PS vesicles were incubated on substrates for 60 min, rinsed with 100 μL 140 mM NaCl/20 mM MES pH 4.5 to remove unfused material, and inserted into the fluid cell under 50 μL of buffer. Soft triangular cantilevers were used with spring constants between 0.02 and 0.03 N/m, and the force applied during each scan was 1.5-2.0 nN. Scans of 5 μm × 5 μm were collected at a rate of 1.5 Hz, and 10 μm × 10 μm scans were collected at a rate of 0.75 Hz, corresponding to a tip velocity of 15.2 μm/s. After scanning the same region of substrate repeatedly over 30 min, 20 μL of 25 μM protein solution was injected directly into the buffer in the fluid cell, resulting in 7 μM PSI. Successive images were generated for a single region for time-lapse data, and for unscanned regions at the end of incubation, to assess changes to the membrane that were caused by repeated scanning of the AFM tip.

2.3.12 Particle Size Determination by Light Scattering - LUVs (100 μM) at pH 4.5 were incubated with PSI at RT and subjected to light scattering in a Malvern Zetasizer Nano-S (Malvern Instruments, Malvern, Worcestershire, UK). A standard 1 mL cuvette was used containing 0.6 mL sample that was allowed to equilibrate for a minimum of 15 min. Three consecutive measurements of five 30 s runs each were averaged using the refractive index for polystyrene, yielding the calculated average sizes and polydispersity indices.
2.4 Results

2.4.1 Structure Solution and Refinement - Recombinant StAP PSI was expressed and purified to >98% purity with a typical yield of 5 mg/L culture and its identity was verified by N-terminal sequencing (Advanced Protein Analysis Centre, Toronto, ON, Canada). Diffraction data collection statistics are presented in Table 2.1. Crystals were hexagonal in space group $P3_21$ with unit cell parameters of $a=b=56.47$, $c=55.34$ Å. The Matthews coefficient (Matthews, 1968) for the crystals was 2.24 Å$^3$ Da$^{-1}$, assuming the presence of one molecule in the asymmetric unit. StAP PSI exhibits a high level of sequence identity (53%) with the SAPLIP domain of prophytepsin PSI (PDB code 1QDM; residues 4S to 102S) which was used as a model for molecular replacement automated search by PHASER (Read, 2001). The starting model consisted of a compact molecule and produced a weak solution. Analysis of the initial map showed that PHASER had placed only half of the initial model in a proper orientation despite good quality of the resulting electron density; however, there was sufficient density to accommodate the properly oriented half. At the next step, BUCCANEER (Cowtan, 2006) was used for automated model building, thereby producing the model of the StAP PSI structure with proper side chains for residues 59-100 and assigning the other residues as polyalanine. Iterative refinement of the partial model using REFMAC5 (Murshudov et al, 1997) and rebuilding in the electron density maps using COOT (Emsley & Cowtan, 2004) produced the next model which corresponded to an elongated, boomerang-shaped molecule. When all the residues visible in the electron density were built, TLS parameters were introduced during the refinement. The overall anisotropy was modeled with TLS parameters by dividing the molecule into three groups comprising residues 0-26, 27-82, and 83-103. The final model lacks residues
Table 2.1: Data collection and refinement statistics

**A. Data collection statistics**

<table>
<thead>
<tr>
<th>Space group</th>
<th>$P3_21$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit cell parameters $a, b, c$ (Å)</td>
<td>56.47, 56.47, 55.34</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.5418</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>40.0-190 (2.00-1.90)</td>
</tr>
<tr>
<td>$R_{merge}$ (%)</td>
<td>5.9 (90.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 (100.0)</td>
</tr>
<tr>
<td>$I/\sigma(I)$</td>
<td>24.10 (2.87)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>8355 (1159)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>10.32 (10.27)</td>
</tr>
<tr>
<td>No. of molecules/asymmetric unit</td>
<td>1</td>
</tr>
</tbody>
</table>

**B. Refinement statistics**

| Resolution (Å) | 25.0-1.90 |
| Working set: number of reflections | 7936 |
| $R_{factor}$ (%) | 18.7 |
| Test set: number of reflections | 417 |
| $R_{free}$ (%) | 24.9 |
| Protein atoms | 633 |
| No. of water molecules | 63 |

**C. Geometry statistics**

| rmsd (bond distance) (Å) | 0.02 |
| rmsd (bond angle) (deg.) | 1.81 |
| Ramachandran plot$^c$ | |
| Most favored region (%) | 98.6 |
| Additionally allowed regions (%) | 1.4 |
| Generously allowed regions (%) | 0.0 |
| Disallowed regions (%) | 0.0 |

**D. PDB code**

| 3RF1 |

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$^a$ The values in parentheses are for the highest resolution shell

$^b$ $R_{merge} = \Sigma_h \Sigma_i |<I_h - I_{h,i}| / \Sigma_h \Sigma_i I_{h,i}.$

$^c$ As defined by PROCHECK.
40-63 which could not be built due to insufficient electron density in this region of the crystal. The statistics of refinement for the refined structure are presented in Table 2.1, and atomic coordinates and structure factors have been deposited in the PDB with the code 3RFI.

**2.4.2 Tertiary and Quaternary Structures of StAP PSI** - The overall fold of StAP PSI has a boomerang shape with an extended, open conformation (Fig. 2.1.A) composed of four helices labeled H1-H4; residues 1-24, 27-34, 66-82 and 85-99, respectively. H1 is connected to H4 via a disulfide linkage formed between Cys6 and Cys99, H3 is cross-linked to H2 via a disulfide bond between Cys31 and Cys71, and Cys37 forms a disulfide bond with Cys68. The tertiary structure is organized in such a way that one side (top) of the molecule is enriched with polar residues and the other side (bottom) is enriched with hydrophobic residues (Fig. 2.1B-C). Due to the crystallographic symmetry, two molecules form a very tight dimer (Fig. 2.1B-C) with a buried surface area of 1746 Å², with the residues involved in the formation of the dimer interface being predominantly hydrophobic.

**2.4.3 pH Dependence of Secondary Structure** - Since PSI-induced membrane disruption requires acidic conditions (Egas et al, 2000), the secondary structures of StAP PSI at neutral and acidic pH were compared. CD scans were done in the same buffers used for all other experiments, i.e. 140 mM NaCl buffered by either 10 mM Tris-Cl (pH 7.4) or 20 mM Na-MES (pH 4.5). Data below 195 nm was noisy so secondary structure content could not be quantified. Qualitatively, the scans revealed spectra typical for high helix proteins (Sreerama et al, 1999); distinct negative absorption peaks occurred in the 220 nm
Figure 2.1: The structure of StAP PSI. (A) Stereo view of the StAP PSI monomeric structure, shown in ribbon representation. (B) Crystallographic dimer of StAP PSI; two molecules are shown as ribbons inside the transparent surface of the dimer. (C) Electrostatic surface representation of the StAP PSI structure.
and 208 nm spectral regions (Fig. 2.2). Helix content was higher overall at pH 4.5, similar to the pH-dependence of influenza A hemagglutinin fusion peptide (Han et al, 2001).

2.4.4 Secondary Structure Dependence on Disulfide bonds - The structure of StAP PSI contains three disulfide bonds within its relatively small 12 kDa tertiary structure and it was recently suggested that these cross-links are critical to PSI antimicrobial function (Muñoz et al, 2010). Hence, the dependence of PSI secondary structure on the presence of cystines was investigated. Fig. 2.3 contains the spectra of PSI at three concentrations of the reducing agent DTT, each with and without heating. No changes were observed for PSI heated at 95º C under non-reducing conditions indicating that native PSI secondary structural elements were heat stable. DTT resulted in high interference at wavelengths below 200 nm so its levels were limited to 5 mM or lower. Qualitatively from Fig. 2.3, a relatively minor loss of helix structure (220 nm and 208 nm negative peaks) occurred with the presence of reducing agent in an apparent dose-dependent manner. By contrast, when PSI was heated under reducing conditions, a more pronounced loss of secondary structure was observed resulting in an approximate two-state structure change such that heating in 2.5 mM DTT resulted in a more dramatic loss of secondary structure. Further doubling of reducing agent concentration did not cause an equivalent effect suggesting differential susceptibility of the respective disulfide bonds; the more robust cystine(s) were apparently critical to secondary structure stability of this predominantly helical structure.

2.4.5 Membrane Disruption Activity – Vesicle Leakage - Large unilamellar vesicles (LUV) containing self-quenching fluorophore (80 mM calcein) were used as substrate for the characterization of StAP PSI phospholipid bilayer disruption activity. Four combinations of PLs were tested at varying concentrations. Neutral vesicles made of 1:1
Figure 2.2: Effect of acidification on StAP PSI secondary structure. Far-UV CD spectra in 140 mM NaCl at pH 4.5 (red squares) and pH 7.4 (shaded circles).
Figure 2.3: Importance of disulfide bonds on StAP PSI secondary structure. Far-UV CD spectra of StAP PSI at varying concentrations of reducing agent DTT with (dotted lines) and without (solid lines) heating. The locations of the various disulfide bonds are indicated (red) in the accompanying structure (bottom).
PC:PE were not affected by StAP PSI at any concentration tested and none of the PL combinations were disrupted at neutral pH. By contrast, equimolar preparations of PS combined with PC and/or PE resulted in readily detectable activity and leakage rates were calculated for 0.5 μM PSI over the PL concentration range 20-500 μM. Accurate LUV disruption rate determinations above 500 μM were prevented by excessive fluorescence signal and the quality of determinations below 20 μM were limited by excessive background noise levels due to untrapped calcein. Rate determinations were calculated relative to PL concentrations in units of μM/min (in terms of both PL and LUV concentration) and yielded initial rates with low relative errors. PSI-induced lipid bilayer disruption varied non-linearly with PL / LUV concentration and the order of leakage rates was PE:PS > PC:PS > PE:PC:PS within the concentration range tested (Fig. 2.4).

Non-linear regression analyses for both one-phase exponential association and the Michaelis-Menten equation were compared by F-tests using GraphPad Prism 4 and the independent data sets for all three PL combinations fit better to the Michaelis-Menten model. Table 2.2 summarizes the kinetic results including goodness of fit parameters for the regression analyses. Since the mechanism of action has not been characterized (see the Discussion section), apparent Michaelis constants (K_m) were not reported since their meaning in terms of substrate affinity would be undefined in the absence of understanding the relative dissociation rates of PSI and PL in original bilayer (reactant; k_1) and displaced PL in new environment (product; k_2). A lack of data at higher lipid concentrations (which produced excessive fluorescence signals) resulted in a relatively large turnover number standard error for PC:PS. LUV concentrations above 500 μM would allow for more precise
Figure 2.4: Kinetics of LUV disruption by 0.5 µM StAP PSI at 25º C. comparison of three acidic phospholipid mixtures (activity against non-acidic PE:PC was not detectable). Two vertical axes are presented for disruption rates in terms of both phospholipid concentration (left axis) and vesicle concentration (right axis).
Table 2.2: Turnover and goodness of fit to the Michaelis-Menten model for StAP PSI-induced vesicle leakage. Data are shown relative to both phospholipid and vesicle concentrations.

<table>
<thead>
<tr>
<th>Phospholipid Composition</th>
<th>$k_{\text{cat apparent}}$ (min$^{-1}$) (phospholipid)</th>
<th>$k_{\text{cat apparent}}$ ($\times 10^{-4}$ min$^{-1}$) (vesicle)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC:PS</td>
<td>71 ± 30</td>
<td>3.6 ± 2</td>
<td>0.992</td>
</tr>
<tr>
<td>PE:PS</td>
<td>45 ± 3</td>
<td>2.3 ± 0.2</td>
<td>0.999</td>
</tr>
<tr>
<td>PE:PC:PS</td>
<td>25 ± 4</td>
<td>1.3 ± 0.2</td>
<td>0.996</td>
</tr>
</tbody>
</table>
kinetic parameter determinations and will require further characterization. Nevertheless, the overall fit to the model was good for each data set including that for PC:PS ($R^2=0.99$).

2.4.6 Membrane Disruption Activity – Atomic Force Microscopy - AFM height images of PE:PS bilayers were collected using contact mode as described above in the Experimental Procedures. LUVs were incubated on the surface of the native oxide layer on a silicon wafer in the same buffer used for LUV disruption assays for 1 h resulting in bilayer fusion to the substrate surface. The fused membrane was organized as 5 nm high islands that were approximately 100 nm wide, dispersed across the substrate (Fig. 2.5.A; top). The bilayer height was within the expected range (Alattia et al, 2006; Egawa & Furusawa, 1999) and repeated scans in contact mode over a single region did not change the bilayer morphology significantly. Upon PSI injection, membrane patches fused to form larger regions of uniform membrane separated by larger membrane-free areas (Fig. 2.5.A; bottom). Additionally, 20-50 nm high lipid islands formed in regions unperturbed by the AFM tip (Fig. 2.5.B; right), thus PSI appeared to induce fusion of membrane patches as evidenced by bilayer rearrangement in areas that were not repeatedly scanned. Such formations did not occur on regions subjected to successive scanning; on these regions, considerable smoothing of the membrane occurred. PSI apparently softened or lubricated bilayers allowing them to be displaced by the AFM tip upon repeated scanning. Fig. 2.5.B (right) shows the distinct effects of PSI both with (centre square region) and without (surrounding region) repeated AFM tip scanning. Large regions of 5 nm high bilayer were observed after 8 scans on a single region whereas repeated scanning in the absence of PSI did not result in similar bilayer rearrangement.
Figure 2.5: AFM height images of PE:PS bilayer patches at pH 4.5 on the native oxide layer of a silicon wafer. (A) Top: Successive scans without PSI (left to right); the membrane is patchy with ~100 nm wide islands of height 5 nm. Bottom: Successive scans with PSI (left to right); the membrane is smooth, transforming from patchy islands to large continuous membrane. The white lines indicate height sections shown below each image. (B) AFM height images of the same region as (A); left: pre-injection, middle: 50 min (8th scan) post-injection, right: 60 min (9th scan) post-injection showing a larger region zoomed out to twice the scan width. Note the smoothing of the membrane over the region repeatedly scanned by the AFM tip post-injection and the appearance of islands much taller (white) than the original 5 nm bilayer height in the region not affected by repeated scanning of the AFM tip.
2.4.7 Membrane Disruption Activity – Light Scattering - LUVs (100 μM) at pH 4.5 were incubated with PSI at RT and particle size was measured by light scattering in a Malvern Zetasizer Nano-S (Malvern Instruments, Malvern, Worcestershire, UK); results are summarized in Table 2.3. After 60 min at a PSI concentration equivalent to LUV disruption assays (0.5 μM), average particle size slightly increased and a concomitant increase in the polydispersity index (mass distribution) was more pronounced (Table 2.3). By 100 min, average particle size had increased by nearly two thirds (65%), and the polydispersity index had more than tripled. Higher PSI concentration (4 μM) was assayed to further investigate the peak area tendency toward higher vesicle size. After just 15 min equilibration time post-PSI addition, average particle size and polydispersity index had not only increased more than for the entire 0.5 μM PSI assay, but a newly formed, larger vesicle size (1510 nm) accounting for one third of the total peak area was observed. Together with the above AFM results, vesicle size measurements indicated that PSI activity resulted in lipid bilayer fusion.
Table 2.3: Effect of StAP PSI on LUV size at pH 4.5.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Z-average size (nm)</th>
<th>Polydispersity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 µM 0</td>
<td>142 ± 0.85</td>
<td>0.0870 ± 0.020</td>
</tr>
<tr>
<td>60</td>
<td>146 ± 1.6</td>
<td>0.172 ± 0.018</td>
</tr>
<tr>
<td>100</td>
<td>235 ± 0.20</td>
<td>0.300 ± 0.0030</td>
</tr>
<tr>
<td>4 µM 0</td>
<td>137 ± 1.2</td>
<td>0.0737 ± 0.0098</td>
</tr>
<tr>
<td>15</td>
<td>257 ± 4.4</td>
<td>0.529 ± 0.064</td>
</tr>
<tr>
<td>(overall)</td>
<td>167 ± 11</td>
<td>* 65% ± 1 of total area</td>
</tr>
<tr>
<td>15 (peak 1)</td>
<td>1510 ±300</td>
<td>* 34% ± 1 of total area</td>
</tr>
</tbody>
</table>
2.5 Discussion

2.5.1 Structural Comparison - The overall fold of StAP PSI is similar to the open, extended form of human saposin C (Fig. 2.6.A) as observed in the tetragonal crystal structure (Rossmann et al, 2008). The structure of StAP PSI has been compared with the PSI domain of plant phytepsin (Kervinen et al, 1999) revealing that the RMSD for 38 aligned Cα atoms is 0.9 Å. Overall, superposition of a pseudo-monomer StAP PSI structure (constructed from its dimer crystal structure) onto the PSI of phytepsin produced an RMSD of 1.4 Å (71 Cα pairs). Helices H1 and H4 of StAP PSI were superimposable with the equivalent helices of phytepsin PSI (Fig. 2.6.A) while helices H2 and H3 from the second StAP PSI molecule in the crystallographic dimer superimpose onto helices H2 and H3 of phytepsin PSI. This result clearly shows that the crystallographic dimer structure of StAP PSI is composed of two extended, domain-swapped monomers. The StAP PSI structure has also been compared with three different crystal structures of human saposin C (Fig. 2.6.A). The Cα atoms of residues 6-19 from saposin C were superimposed (LSQ) to the Cα atoms of the first 13 residues of the StAP PSI structure yielding an RMSD of 0.6 Å (tetragonal crystal form, 2Z9A), 0.6 Å (orthorhombic crystal form, 2QYP) and 0.9 Å (hexagonal crystal form, 2GTG). This “open” conformation for SAPLIPs has also been observed for saposin B (1N69), and all three structures are shown side-by-side in Fig. 2.6.B.

2.5.2 Saposin-like Activity - StAP PSI-liposome disruption rates varied with PL/LUV concentration in a non-linear fashion and, to our knowledge, the present study is the first to characterize a SAPLIP’s kinetic activity for varying PL/LUV concentrations of multiple PL compositions, revealing Michaelis-Menten-like kinetics. In attempting to elucidate the
Figure 2.6: Tertiary structural features of StAP PSI. (A) Structural superposition of StAP PSI (green) with the plant-specific insert domain of prophysetpsin (cyan), tetragonal (yellow), hexagonal (grey) and orthorhombic (red) crystal forms of saposin C. (B) Side by side comparison of the “open” saposin boomerang fold of saposin B (1N69), saposin C (2QYP) and StAP PSI (3RFI). (C) Pairwise structural alignment of influenza A hemagglutinin fusion peptide (1IBN; purple) superimposed over StAP PSI (3RFI; green) using UCSF Chimera version 1.5.2 implementing the Needleman-Wunsch algorithm. The overall RMSD was 2.013 Å for 19 residues that aligned, ignoring gaps.
nature of PSI activity, AFM results suggested that StAP PSI caused pronounced rearrangement of acidic PL bilayers under the same conditions used for liposome disruption. The type of lipid structures that formed were similar to those previously characterized for saposin C which showed that saposin C interactions with PL bilayers (You et al, 2003) resulted in rearrangement of membrane patches of increased thickness and membrane destabilization (Alattia et al, 2006; You et al, 2003). Saposin C has also been shown to cause vesicle fusion (Vaccaro et al, 1994) which involves insertion of the terminal helices into the membrane where saposin–membrane and saposin–saposin interactions carry out a ‘clip-on’ mechanism at neutral and acidic pH. Saposin C causes fusion when present at as little as 0.05 µM and produced fused vesicles up to 3000 nm (Wang et al, 2003).

By contrast, saposin B displays fusogenic activity only against anionic vesicles and exclusively at acidic pH, and induces only minor vesicle size increases at protein concentrations of 1 µM or below. However, at 2 µM saposin B induces more significant vesicle fusion yielding an average product ~1800 nm in diameter (Wang et al, 2003). The fusogenic results for StAP PSI in the present study were strikingly similar in that 0.5 µM PSI induced only minor average diameter increases whereas 4 µM PSI induced dramatic changes resulting in new lipid structures averaging 1510 nm (Table 2.3). Also similar to StAP PSI disruption and AFM observations, saposins B and D disrupt anionic membranes in a pH-dependent process where they solubilize (Ciaffoni et al, 2001; Remmel et al, 2007) and mobilize (Remmel et al, 2007) lipids. Furthermore, saposin B binds and transfers PLs of anionic membranes such that it has a preference for PC transfer (Ciaffoni et al, 2006). Although StAP PSI-mediated vesicle disruption rates were all higher for PE:PS within the
concentration range used in the present study, kinetic analysis suggested a higher maximum velocity for PC:PS disruption (P=0.06), indicating a PL preference similar to saposin B. Perhaps the bulkier choline substituent, relative to ethanolamine, results in more favorable PSI interactions with the bilayer surface and/or results in different bilayer packing density. A study of the nature of SAPLIP PL preference is underway.

The crystal structure of saposin B with bound lipid indicated that PLs interact with the dimeric form of the protein. Dimerization occurs via clasping together two V-shaped protein monomers, thereby forming a shell-like monolayer of α-helices with a long interface that buries a relatively large hydrophobic cavity (Ahn et al, 2003). A similar V-shape and quaternary structural arrangement was observed in the present study for the StAP PSI crystal structure, although PSI and open saposin C contain a more obtuse angle in their boomerang shape relative to saposin B (see Fig. 2.6.B), according to structural alignments. Furthermore, PSI membrane effects as detected by AFM were in partial agreement with saposin B in that new lipid structures formed that were higher than the surrounding bilayers; structures observed for saposin B were smaller in size however (Alattia et al, 2006). These newly formed ‘granules’ could be dislodged by the scanning AFM tip indicating that they were loosely bound upon saposin B action (Alattia et al, 2006). Similarly, raised lipid bilayers acted upon by StAP PSI became more fluid in regions repeatedly scanned by the AFM tip and were spread out into smooth continuous lipid regions (Fig. 2.5.B) indicating solubilising and/or mobilizing activity.

2.5.3 Plant AP PSI-Activity - Recombinant StAP PSI is toxic to plant (P. infestans and F. solani) and human (S. aureus, B. cereus and E. coli) pathogens, and it permeabilizes their plasma membranes (Muñoz et al, 2010). In both procardosin A and StAP, the PSI has
been confirmed to be responsible for membrane interactions (Egas et al., 2000; Muñoz et al., 2010). The interaction of procardosin A PSI with membrane vesicles is pH-dependent and varies with lipid composition (Egas et al., 2000), in agreement with the present study. Interestingly, the only other crystal structure for a plant AP PSI, prophytepsin, was shown to have a tertiary structure consisting of the “closed” saposin fold (Kervinen et al., 1999). That crystal structure dealt with a PSI attached to its parent zymogen molecule and contrasts with the “open” saposin fold observed in the present study for a PSI independent of its zymogen source. By definition, fusogenic activity via the “clip-on” mechanism (Wang et al., 2003) is dependent upon open structure dimerization (interfacing of the respective hydrophobic surfaces). Thus, we propose that plant AP PSIs likely change from closed to open fold upon release from the parent AP molecule thereby facilitating bilayer interaction and subsequent protein quaternary structure dimerization, yielding fusion of neighboring bilayer structures.

The cytotoxicity and plasma membrane interactions of StAP PSI were previously shown to be dependent on its secondary and/or tertiary structure, as evidenced by loss of activity upon DTT treatment (Muñoz et al., 2010). In the present study, CD scans at 1 mM DTT resulted in no apparent spectral change, and only minor changes were indicated at DTT concentrations up to 5 mM (without heating). This suggested that disulfide bonds are not critical to PSI secondary structure under normal temperature conditions. When synthetic peptides equivalent to the individual helices of saposin C (i.e., no native tertiary structure) were studied, bilayer fusogenic activity was not observed (Wang et al., 2003), suggesting a critical role of tertiary structure in SAPLIP-catalyzed bilayer fusion. Since cystines are
critical to antimicrobial function (Muñoz et al, 2010), their role must be to maintain tertiary structure required for fusogenic activity.

Additionally, the DTT titration CD experiment indicated that the disulfide bonds conferred stability to PSI secondary structures as evidenced by a much more pronounced CD spectral change for heat-treated PSI. The latter was superimposable with that for the non-reducing, unheated sample (Fig. 2.3) indicating that the PSI disulfide bonds apparently protected the individual helices from heat denaturation. Since both open and closed forms of SAPLIPs contain the same disulfide bonds (Bruhn, 2005), the primary role of PSI cystines is not likely the maintenance of the overall fold, but perhaps to confer rigidity. Fusion of adjacent membranes consists of the displacement of lipid from its stable, energetically favorable bilayer environment to an aqueous, high energy intermediate state and such a transition requires enzymatic action (Baker & Agard, 1994). Perhaps the energy required to catalyze this event is related to multimer formation (Markovic et al, 1998) and conformation changes (Chernomordik et al, 1998; Kingsley et al, 1999) related to PSI rigidity/stability dependent on disulfide bonds.

2.5.4 Fusogenic Mechanism - There is a correlation between the ability of a fusion peptide to adopt a helical configuration and its ability to promote membrane fusion (Hernandez et al, 1996). In addition to saposins, the formation of dimers via complexing of stable, predominantly helical structures is a protein structure scheme common to yeast SNARE-mediated membrane fusion (Paumet et al, 2005; Paumet et al, 2004) as well as viral fusases which are derived from larger precursors that require proteolytic processing to potentiate their fusion activity (Hernandez et al, 1996). These associations of α-helices contain one hydrophobic face, an arrangement similar to StAP PSI with its 5 helices,
hydrophobic inner cavity and N-terminus. An important fusase fusion peptide for virus-cell fusion is the N-terminal portion of influenza A hemagglutinin which adopts an \( \alpha \)-helical conformation in lipid bilayers (Han & Tamm, 2000), constitutes an autonomous folding unit in the membrane and catalyzes lipid exchange between juxtaposed membranes (Han et al, 2001).

In some plant APs, the release of PSI occurs \textit{via} proteolytic cleavage upon acid-induced autoactivation of the precursor protein and subsequent processing, albeit \textit{via} self-cleavage (Faro et al, 1999; Glathe et al, 1998; Ramalho-Santos et al, 1998). Interestingly, influenza A hemagglutinin fusion peptide is inaccessible to membranes at neutral pH; however, a drop of the pH inside the endosome below a critical threshold induces a large conformational change in the parent protein and is subsequently activated by a protease (plasmin) that cleaves the precursor polypeptide (Lazarowitz et al, 1973) into two disulfide-linked polypeptides and a fusion peptide (Gray et al, 1996). The hemagglutinin fusion peptide has a slightly higher helix content at pH 5 than at pH 7.4 as revealed by comparison of CD spectra (Han et al, 2001; Han & Tamm, 2000) which are remarkably similar to those for StAP PSI in the present study (Fig. 2.2) in terms of overall shape (dominant helix content), as well as their x-intercepts and superimposed relative spectra (gain of helical structure upon acidification). In addition, the fold of the hemagglutinin fusion peptide (Han et al, 2001) is similar to the StAP PSI structure reported here as well as to saposins (Vaccaro et al, 1995): Structural alignment of the N-terminus of hemagglutinin fusion peptide (1IBN) (Han et al, 2001) with the N-terminus of StAP PSI revealed similar (RMSD 1.43 Å for 9 \( C_\alpha \) carbons) helix-kink-helix folds (Fig. 2.6.C). The tryptophan within the hemagglutinin fusion peptide has been shown to induce its characteristic boomerang shape
(Lai et al, 2006) which is critical to the fusogenic and membrane disrupting activities of
this fold (Lai & Tamm, 2010). Alignment of StAP PSI with hemagglutinin fusion peptide
suggested a similar role for the critical tryptophan residue (Fig. 2.6.C). In this context, the
likely protein structural reason for the acidic pH requirement of AP PSI-membrane
interactions is the existence of acid-induced helical structure critical for membrane
interaction.

2.5.5 Fusase Within a Protease - The apparent sharing of a common hydrophobic
region fold that is subject to similar pH-dependent secondary structure changes required
for membrane fusion suggests a common mode of action. Perhaps this helix-kink-helix fold
is universal (i.e., animal; saposins, virus; hemagglutinin, plant; APs) in its membrane
fusogenic nature extending functionality across various species and kingdoms.
Collectively, the findings that a plant AP domain displays fusase-like activity (liposome
disruption, bilayer solubilization/lubrication and bilayer fusion) as well as fusase-like
structure-function character (inter- and intramolecular helix oligomer association,
hemagglutinin fusion peptide-like fold, hydrophobic helix end region, and pH-dependence
of secondary structure-activity) lead to the conclusion that they are indeed fusase-like
proteins, acting as discrete entities.

Considering the myriad proteins that have more than one function, the idea of one gene
- one protein - one function is insufficient in the study of proteins (Jeffery, 2009). Recently,
a bifunctional AP has been engineered (Bryksa et al, 2010), however, no reports have
characterized cases of AP “moonlighting” (Moore, 2004), i.e., to serve an additional
function beyond the main enzymatic reaction (Copley, 2003), and only one moonlighting
plant peptidase (mitochondrial processing peptidase) has been reported (Braun & Schmitz,
1999; Moore, 2004). In this case, the multiple functions arise from a singular structural fold (Braun & Schmitz, 1995) whereas PSIs are structurally unrelated to their AP “hosts”. That is, the PSI domain of a plant AP has apparent enzymatic activity and is independent of its “parent” proenzyme which has its own distinct class of enzymatic activity. Thus, the saposin-like domains of plant APs present a unique case: A distinct, functionally unrelated domain within the primary structure of another domain (the C-terminal domain) of its enclosing protein. Fusase activity from within a protease sequence presents, to our knowledge, the first confirmation and characterization of an independently acting “enzyme within an enzyme”.

2.6 References


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results in a late-onset, chronic form of globoid cell leukodystrophy in the mouse. *Human Molecular Genetics*, 10(11), 1191-1199.


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Chapter 3: Protein Structure Insights into the Bilayer Interactions of the Saposin-Like Domain of *Solanum tuberosum* Aspartic Protease

*Note:* The content of this chapter was submitted (December 2016) to *The Journal of Biological Science* for review.

### 3.1 Abstract

The present study sought to clarify the nature of plant saposin membrane-active features through a detailed examination of the *Solanum tuberosum* aspartic protease saposin-like domain structure. Cryo-transmission electron microscopy revealed phospholipid bilayer vesicle morphologies having prominent flat surfaces. Furthermore, the plant saposin was active exclusively as an acid pH-dependent dimer. Secondary structure changes occurred between inactive and active pH conditions, and consisted of a 7% gain in helix and concomitant 7% loss in strand/turn. Upon mixing with bilayer vesicles, increases in helix structure and tryptophan fluorescence emission occurred on a similar timescale. Reduction of disulfide bonds did not appear to alter secondary structure at low pH, however, it did result in faster apparent vesicle fusion. Individual PSI segments corresponding to its secondary structural elements (four helices and an unstructured mid-sequence region) were subsequently characterized. Although inactive, helix-1 (the N-terminal 24 residues) was the only segment which experienced pH-dependent secondary structure changes, becoming disordered at neutral pH. Helix-3 (equivalent to saposin helix-1) was the only peptide segment that showed activity, causing both vesicle leakage and apparent fusion. Helix-3 secondary structure was pH-insensitive and predominantly β-strand/turn. Furthermore, mutation of the sole positively charged residue (Lys83Gln) on helix-3, disordered its
secondary structure and eliminated bilayer interaction. Taken together, the helical character of the component segments of the plant saposin domain are not intrinsically determined and are instead dependent upon overall tertiary and quaternary structures. Implications of the findings for the bilayer disruption mechanism(s) are discussed for both the saposin domains as well as helix-3.

3.2 Introduction

Unlike their non-plant counterparts, many plant aspartic proteases (APs) contain a ~100-residue sequence insert that results in an ‘extra’ domain attached to the C-terminal lobe. This unique additional segment, aptly referred to as the plant-specific insert (PSI), is a member of the saposin-like protein (SAPLIP) superfamily (Bruhn, 2005; Bryksa et al, 2011; Kervinen et al, 1999; Mutlu & Gal, 1999; Runeberg-Roos et al, 1991). Delineation of the PSI primary sequence revealed its N- and C-terminal sequence portions to occur in the opposite order, or swapped, relative to other SAPLIPs, hence the term swaposin (Runeberg-Roos et al, 1991). Upon post-translational processing and activation, plant AP saposin domains are either removed (heterodimeric plant APs) or retained (monomeric plant APs) (Egas et al, 2000; Glathe et al, 1998; Kervinen et al, 1999; Ramalho-Santos et al, 1998). Even in the latter scenario, the PSI and AP moieties exist as structurally distinct features of a shared overall tertiary structure (Frazão et al, 1999; Kervinen et al, 1999), and function independently (Bryksa et al, 2011; Frazão et al, 1999; Muñoz et al, 2010; Pereira et al, 2013; Tormakangas et al, 2001).

SAPLIPs have diverse biological functions (Anderson et al, 2003; Bruhn, 2005; Liepinsh et al, 1997; Matsuda et al, 2001; Zhai & Saier, 2000) all involving lipid
interactions (Bruhn, 2005; Egas et al, 2000; Kolter & Sandhoff, 2005). As such, plant AP saposin-like domains display bilayer perturbation (Bryksa et al, 2011; Egas et al, 2000; Muñoz et al, 2011) and membrane permeabilizing (Mendieta et al, 2006; Muñoz et al, 2014) activities. Mechanisms by which membrane-SAPLIP contacts occur have been proposed spanning a variety of modes (Alattia et al, 2007; Andreu et al, 1999; Miteva et al, 1999; Qi & Grabowski, 2001), with PSI being poorly understood relative to the more studied members of the SAPLIP superfamily such as helminth amoebapores, mammalian saposins A-D, surfactant protein B, NK-lysin and granulysin. The roles of PSIs in vivo have been proven to not only include vacuolar targeting and Golgi/endosomal sorting (Glathe et al, 1998; Kervinen et al, 1999; Mutlu & Gal, 1999; Runeberg-Roos et al, 1991), but also a clear association with the ability of some plants to stave off phytopathogen invasion (Guevara et al, 2002). Globally, the negative impacts on agricultural products by plant diseases cause annual losses in the tens of billions (USD) (Almgren et al, 2000; Edwards et al, 1993; Montesinos & Bardají, 2008). Due to their roles in plant pathogen resistance (Guevara et al, 2002), and senescence and post-harvest physiology (Payie et al, 2000; Schaller & Ryan, 1996), plant APs are important for the development of novel biocontrol technologies (Curto et al, 2014) as well as understanding biochemical and biological phenomena of natural plant disease resistance.

Biochemical insights into the antimicrobial etiology of Solanum tuberosum aspartic protease (StAP) PSI have been revealed (Mendieta et al, 2006; Muñoz et al, 2011; Pagano et al, 2007) including its effectiveness against human pathogens and inhibition of phytopathogen sporulation via interaction with, and permeabilization of, microbial plasma membranes (Muñoz et al, 2010). The PSI domain of cirsin, from Cirsium vulgare (Lufrano
et al, 2012), has also been shown to have activity against different phytopathogens in vitro (Curto et al, 2014). Furthermore, cirsin has been produced in a biologically active form using a generally regarded as safe yeast recombinant expression system (Curto et al, 2014), a promising advance towards a potential role in pest and/or pathogen control. In order to develop novel plant biocontrol agents as well as therapeutic drugs for the treatment of fungal infections (Ghannoum & Rice, 1999) in immunocompromised patients (Meyer, 2008; Tseng & Perfect, 2011), characterizing the structural features that modulate lipid bilayer interactions is critical for rational design approaches.

Our group previously reported on StAP PSI structure and function including the findings that the domain-swapped plant saposin adopted the less commonly observed “open” saposin fold consisting of a dimer of extended V-shaped monomers (Bryksa et al, 2011). It was also revealed by direct contact imaging that PSI rendered anionic phospholipid bilayer more prone to deformation, and that PSI secondary structure is pH-dependent, but independent of its disulfide bonds. The PSI was also shown to cause apparent fusion of anionic phospholipid (PL) bilayer liposomes (Bryksa et al, 2011). Our findings led us to conclude that, due to multiple structure-function commonalities with certain fusases, PSI may possess a fusion peptide-type mechanism of action that is common to influenza A hemagglutinin fusion peptide. Furthermore, we predicted the existence of an acid pH-induced helical structure within PSI as being the structural basis for the low pH requirement of PSI-membrane interactions (Bryksa et al, 2011).

The present investigation sought to better understand the structural underpinnings for PSI-mediated bilayer interactions and structure changes. Specifically, what significance, if any, do the tertiary (open V-shape) and quaternary (homodimer) structures observed in the
PSI crystal structure (3RFI) have for the pH-dependence of PSI-bilayer interactions? This knowledge is required for ultimately assessing the applicability of the clip-on mechanism originally postulated for saposin C (Wang et al, 2003) as well as aiding in identifying what region of the PSI structure mediates bilayer contact and selectivity for anionic targets? To answer these questions, secondary, tertiary and quaternary structures were characterized for intact PSI as well as its individual component regions (based upon 3RFI; see Figure 3.1) in terms of bilayer disruption, and vesicle fusion and morphological changes.

3.3 Materials and Methods

3.3.1 Materials - A PSI synthetic gene optimized for expression in *E. coli* was purchased from Mr. Gene GmbH (Regensburg, Germany). Plasmid pET32b(+), *E. coli* Rosetta-gami B (DE3)pLysS, and u-MAC™ columns were obtained from EMD Biosciences (San Diego, CA, USA). *E. coli* TOP10F’ was from Invitrogen (San Diego, CA, USA). GenElute™ Plasmid Miniprep Kit was obtained from Sigma-Aldrich (St. Louis, MO, USA). The QIAquick® PCR Purification Kit and QIAquick® Gel Extraction Kit were from Qiagen (Germantown, MD, USA). Restriction enzymes, T4 DNA ligase and *Pfu* DNA polymerase were obtained from Fermentas (Burlington, ON, Canada). Primers were synthesized by Sigma Genosys (Oakville, ON, Canada) and thrombin was purchased from Fisher Scientific (Ottawa, ON, Canada). The RPC column was from GE Healthcare (Piscataway, NJ, USA). Phospholipids were from Avanti Polar Lipids (Alabaster, AL, USA).

3.3.2 PSI Expression and Purification – PSI was recombinantly expressed and purified as per (Bryksa et al, 2011).
Figure 3.1: Crystal structure of StAP PSI (3RFI). The figure indicates the positions of the three disulfide bonds in red, the two Trp residues in blue, the sequences of the five chosen segments (H1, H2, X, H3, H4), and the three ionizable residues of H3 (shown in purple). Note that “H” stands for helix, however “X” is an unknown structure that was not resolvable in the 3RFI crystal structure.
3.3.3 Preparation of Large Unilamellar Vesicles - LUV stocks were prepared as per (Bryksa et al, 2011) with the exception that 80 mM calcein / 140 mM NaCl / 25 mM Na-acetate pH 4.5 was used in order to suspend dried PL mixtures at 42º C after removal of storage solvent under nitrogen flush. Phospholipids used were 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS). Note that information on vesicle stability is summarized in Appendix 3.A.

3.3.4 Circular Dichroism Spectropolarimetry - CD analysis of PSI secondary structure was carried out using a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD, USA). Samples (200 µL of 100 µg/mL PSI or 200 µg/mL peptide) were scanned over 180-260 nm at 100 nm/min, 0.5 s response, standard sensitivity, and at ambient temperature using a 1 mm pathlength quartz cell. Iso-ionic (164 mM) Na-phosphate or Na-acetate saline buffers were degassed under vacuum. Secondary structure contents were calculated using DICHROWEB (Whitmore & Wallace, 2008) with SELCON3 (Sreerama et al, 1999) and CDSSTR (Sreerama & Woody, 2000; 2004) algorithms. For CD time trial scans of PSI mixed with LUVs, 4 µM (47 µg/mL) or 10 µM PSI (118 µg/mL) and 100 nm LUVs (100 µM total PL; 1:1:1 molar ratio of POPC:POPE:POPS) were scanned over 196 nm -260 nm at 200 nm/min every 2 min.

3.3.5 LUV Disruption Assays - PSI-caused perturbation of LUVs was measured by calcein leakage (56, 57) as detected using a Victor2 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA) at 25º C. 200 µL reactions were set up in 96-well microplates with varying concentrations of LUVs, 0.5-2.0 µM StAP PSI, or 4-10 µM peptides (peptide concentrations up to 40 µM were screened to verify lack of activity for H1, H2, H1H2 and...
H4) in either Na-phosphate or Na-acetate buffered saline (constant ionic strength 164 mM). Leakage was detected using excitation at 385 nm and emission at 435 nm with 3 s shaking between readings. Leakage was monitored until increase in fluorescence ceased and/or for a minimum of 15 min / maximum 120 min. End points were measured by incubating LUVs in 0.5% triton for each condition. Non-linear regression analyses were done using GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA).

3.3.6 Bilayer Fusion Assays - Ten μM PSI or peptide was incubated with 100 nm LUVs (100 μM total PL; 1:1 molar ratio of POPE:POPS) at 22° C in either 25 mM Na-acetate / 140 mM NaCl pH 4.5 or 5 mM Na-phosphate / 140 mM NaCl pH 7.4 (control). Mixtures were then monitored for changes in average LUV size by dynamic light scattering in a Malvern Zetasizer Nano-S (Malvern Instruments, Malvern, Worcestershire, UK) using a disposable polystyrene 1.5 mL semi-microcuvette. Three consecutive measurements of five runs (30 s per run) were averaged using the refractive index for polystyrene. Considerations for using DLS to characterize PSI/peptide-induced vesicle effects are discussed in Appendix B.

3.3.7 Sedimentation Equilibrium Analytical Centrifugation - Sedimentation equilibrium studies were carried out using a Beckman Optima XL-A Analytical Ultracentrifuge (Biomolecular Interactions and Conformations Facility, University of Western Ontario, Canada). An An-60Ti rotor and six-channel cells with Eponcharcoal were used at 22,000 rpm, 28,000 rpm and 35,000 rpm, 20° C. To achieve adequate detectability, 26-28 μM PSI were compared at iso-ionic strength in 27 mM sodium phosphate pH 3.0, 20 mM sodium phosphate pH 6.2 or 10 mM sodium phosphate pH 7.4, each containing 140 mM NaCl. Absorbance measurements at 280 nm were collected in 0.002 cm radial
steps and averaged over 10 readings. Solvent densities (ρ) were calculated using SEDNTERP software. Partial specific volume (v) of the protein was calculated from its amino acid sequence to be 0.13337 mL/g. Data were analyzed using a single ideal species model in GraphPad Prism.

3.3.8 Tryptophan Intrinsic Fluorescence Emission Spectrometry - Fluorescence emission spectra were recorded using a Shimadzu RF-540 spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan) with a 1-cm quartz three-sided ultra-micro cuvette in a water-circulating, temperature-controlled cell holder at 25°C. The settings used were $\lambda_{\text{excitation}}$ 295 nm with 3 nm slit width and $\lambda_{\text{emission}}$ scan 300–400 nm with 3 nm slit width. PSI samples were diluted to a working stock concentration of 80 µM and allowed to incubate in a water bath for 15 min at 25°C as was done for a working stock of anionic LUVs (1000 µM total PLs) to pre-equilibrate. Ten µM PSI and 100 µM PL (1:1:1 molar ratio of POPC:POPE:POPS as 100 nm LUVs) were mixed in buffered saline pH 4.5 and monitoring began at 20 s. Scans were completed at 500 nm/min allowing for a scan every 15 s.

3.3.9 Cryo-Transmission Electron Microscopy - Liposome imaging was done at The Microscopy Imaging Facility, University of Guelph Advanced Analysis Centre (Guelph, Canada) on an FEI Tecnai G2 F20 TEM (FEI Co., Hillsboro, Oregon, USA) with a bottom mount Gatan 4k CCD camera and 200kV field emission. Samples consisted of 1000 µM or 500 µM PL (as 100 nm LUV; 1:1 molar ratio of POPE:POPS) with or without 10 µM or 16 µM PSI in 25 mM Na-Acetate pH 4.5 / 140 mM NaCl incubated for 15 min at 22°C. All were loaded onto sample grids and plunge flash frozen in a dust-free moisture-controlled work space, and subsequently maintained at or below -170°C.
3.3.10 PSI Structure Component Peptides - Synthetic peptides corresponding to the PSI structure regions (outlined in Figure 3.1) were purchased from GenicBio Ltd. (Shanghai, China). The sequences were as follows (positively charged (bold underlined) and aromatic (underlined italics) residues are indicated for easy reference):

H1: IVSMECKTIVSQYGEMIWDLLVSG
H2: VRPDQVCSQAGLCFV
H1H2: IVSMECKTIVSQYGEMIWDLLVSGVRPDQVCSQAGLCFV
X: DGAQHVSSNIKTVVERETEGSSVG
H3: EAPLCTACEMAVVWMQNQLKQ
H4: EGTKKEKVLEYVNQLCEKIP

Peptides were delivered lyophilized in known quantities and were initially resuspended in 20 mM Na-phosphate pH 7.2 / 10 mM DTT (with volumes targeting 1 mg/mL peptide concentration), and gently rocked at room temperature for 15 min followed by centrifugation at 10,000 x g for 5 min. Only peptide H4 was stably soluble at this point. All peptides were then further diluted 5-fold with 20 mM Na-phosphate pH 7.2 / 10 mM followed by 2 h gentle rocking and clarification. Due to low solubility, H1 was prepared alternatively by suspending directly into 6 mM NaOH followed by neutralization with 1 M Na-phosphate pH 7.2 to a final concentration of 50 mM Na-phosphate. DTT was then added to 10 mM final concentration. H1, H1H2, H3 and H4 were quantified by A_{280} and stored at -30° C after flash freezing in liquid nitrogen as 50 µM stocks (except H1 which was 20 µM). H2 and X could not be quantified accurately due to their negligible UV absorptivities. Since neither peptide was used for quantitative purposes, and neither presented solubility problems, their concentrations were based upon the weighed lyophilized mass prior to solubilisation. Due to their respective solubility and quantitation
problems, H1H2 was also studied. Lastly, three H3 mutants (Glu63Gln, Glu74Gln and Lys83Gln) were also purchased and prepared as above.

3.3.11 Statistical Analyses - Statistical significance of differences within and between data sets were calculated with Kruskal-Wallis 1-way ANOVA and Dunn’s Multiple Comparison Test using GraphPad Prism 6.07.

3.4 Results

3.4.1 StAP PSI Quaternary Structure in Solution - Although StAP PSI (3RFI) crystalized as a dimer at neutral pH (Bryksa et al, 2011), its tertiary/quaternary structure status in solution at or near its active pH remained unknown. With a predicted pI 4.6, solubility of PSI close to optimal pH for bilayer disruption (Egas et al, 2000) is insufficient for analytical centrifugation at the required concentration range. Our experience working with PSI at pH 4.5 has been to work at or below 80 µM avoiding elevated centrifugal force whereas it is soluble at >34 mg/mL at neutral pH. As a compromise, PSI was analyzed below and above pH 4.5, at pH 3.0 and 6.2 as well as inactive conditions at pH 7.4. Recombinant PSI was determined to have apparent masses of 12.5 and 13.6 kDa at pH 7.4 and 6.2, respectively, and approximately double, 21.7 kDa, at pH 3.0 (Figure 3.2). These results indicated that recombinant PSI (11,771 Da) exists exclusively as a dimer in the active pH range, and as a monomer near neutral, inactive pH. To determine the PSI monomer-dimer status at pH 4.5, intrinsic tryptophan fluorescence emission was used (Figure 3.3.A) which indicated it to be distinct from pH 6.2 and 7.4 (monomer), but similar to pH 3.0 (dimer). Although the pH 3.0 dimer had stronger fluorescence emission, optimal emission wavelength remained unchanged (Figure 3.3.B).
Figure 3.2: Sedimentation equilibrium analytical centrifugation analyses in iso-ionic saline buffers at pH 3.0, 6.2 and 7.4. (A) Absorbance measurements at 280 nm collected in 0.002 cm radial steps and averaged over 10 readings; measurements were determined in triplicate. (B) Summary of the calculated masses from matching tests (mean +/- one standard deviation, n=6).
Figure 3.3: Intrinsic Trp Fluorescence Emission. (A) Solution Intrinsic Trp Fluorescence of StAP PSI at pH 3.0, 4.5, 6.2 and 7.4. (B) $\lambda_{\text{max}}$ at the indicated pH values were not significantly different comparing all respective pairings (P>0.05). (C) Relative maximum fluorescence emission were not significantly different (P>0.05) between pH 3.0 and 4.5, nor between pH 6.2 and 7.4 (P>0.05), whereas the former were each significantly different (P≤0.05) from the latter, respectively, suggesting distinct electronic environments for one or both Trp residues at active and inactive pH conditions. (D) Relative areas under the emission spectra were significantly different (P≤0.05) for all data set pairings (mean +/- one standard deviation, n=3).
3.4.2 Secondary Structure Dependence on pH and Disulfide Bonds - PSI secondary structure content was compared as a function of pH by far-UV circular dichroism (CD) in iso-ionic buffers for non-saline, saline and reducing saline conditions (Figures 3.4.A-C, respectively). From the non-saline spectra, a trend of increasing helix content with decreasing pH (P≤0.05 for each paired comparison) and an accompanying increase in strand content with increasing pH such that pH 3.0 < 4.5 < 6.2 (P≤0.05 for each paired comparison) while pH 6.2 and 7.4 PSI were not significantly different (P>0.05). Unordered secondary structure did not appear to follow clear patterns based on pH (Figure 3.4.D).

Spectra collected for reducing and non-reducing saline conditions were suitable only for qualitative comparisons due to CD signal interference by DTT and NaCl at wavelengths below 205 nm and 196 nm, respectively, as well as increasing UV absorbance by DTT below 225 nm. The presence of saline conditions did not change the pattern of higher helical character at lower pH values and increasing β-structure character with increasing pH. However, a difference was noted in the saline spectra in that PSI appears to exist in two discreet secondary structure states as evidenced by indistinguishable spectra pairings at pH 3.0 and 4.5, and pH 6.2 and 7.4, respectively (Figures 3.4.A-B). This contrasted with an overall pattern of continuous decreasing CD signal magnitudes over the whole spectra for PSI in the absence of NaCl.

In addition to pH-dependence of secondary structure, differences in saline low pH spectra relative to their non-saline counterparts (Figures 3.4.A-B) suggest that secondary structure sensitivity across the pH range is mitigated by saline conditions. This is evidenced by two observations: First, the magnitudes of the overall spectra are greater at pH 3.0 and
Figure 3.4: Far-UV CD spectra of StAP PSI. The spectra at pH 3.0 (blue), 4.5 (red), 6.2 (green) and 7.4 (purple) are for 100 µg/mL PSI and are averaged from five scans at RT. To maintain consistent ionic strength between conditions, samples were prepared in either 12 mM sodium phosphate pH 3.0, 25 mM sodium acetate pH 4.5, 10 mM sodium phosphate pH 6.2 or 5 mM sodium phosphate pH 7.4 buffers. Spectra were collected for PSI in (A) buffer; (B) buffer containing 140 mM NaCl; (C) Buffer containing 140 mM NaCl and 10 mM DTT, overlaid with spectra from (B); and (D) Calculated secondary structure contents of StAP PSI at the indicated pH values calculated using DICHROWEB (Whitmore and Wallace, 2008) and the SELCON3 (Sreerama et al., 1999) and CDSSTR (Sreerama and Woody, 2000) algorithms (calculated values were averaged).
4.5; and second, there appears to be a distinct shift in the relative ellipticities for key important local minima and maxima (see Figure 3.4.A,B,E). Dominant features particularly evident in the pH 3.0 and 4.5 spectra include a large positive peak centred below 195 nm and two narrow large negative peaks at 222 and 208 nm, indicators for high helix content (Kelly & Price, 2000). Increasing pH resulted in reduced intensities of these peaks and a broadening of the local minima in the 222-208 nm region (Figure 3.4.A), as reflected in the calculated secondary structure contents (Figure 3.4.D) where helix structure is lost at the expense of β-structure. The NaCl-containing samples’ spectra reveal qualitative differences in that they have an overall broader and lower magnitude 222-208 nm region at pH 3.0 and 4.5 (Figure 3.4.B). Although noise is limiting below 215 nm, the CD spectra for reducing conditions suggest similar overall secondary structure (Figure 3.4.C).

3.4.3 pH-Dependence of PSI-induced Phospholipid Bilayer Disruption - Bilayer disruption was compared at pH 3.0, 4.5, 6.2 and 7.4 using 1:1 POPE:POPS large unilamellar vesicles (LUVs; 100 µM total PL) mixed with 0.5-10 µM PSI. Leakage was monitored until increase in fluorescence ceased (15-120 min). Within roughly 15 min, PSI-induced leakage consistently plateaued at approximately 25% of maximum fluorescence (i.e., upon addition of 0.1% w/v Triton X-100). Results are summarized in Figure 3.5 showing that PSI caused vesicle leakage at pH 3.0 and 4.5, with the latter having a 3-fold higher maximum leakage rate (significantly different, \( P \leq 0.05 \)), while leakage was not detected at pH 6.2 and 7.4 (not significantly different, \( P > 0.05 \)).
Figure 3.5: pH-dependence of PSI-induced LUV leakage. Maximum leakage rates in iso-ionic saline buffers at 25° C are expressed as a function of fluorescence increase relative to fully released fluorophore controls, i.e., Triton X-100-lysed LUVs.
3.4.4 Intrinsic Tryptophan Fluorescence for PSI in the Presence of Anionic Bilayer

The intrinsic tryptophan fluorescence of PSI 1:1:1 POPC:POPE:POPS LUVs was measured over a wide range of emission wavelengths (305–380 nm) and monitored over time. Figure 3.6A shows the emission scans monitoring PSI fluorescence for emission wavelengths between 305-380 nm over 285 s. Note that the indicated times refer to scan start times, each requiring 12 s to complete. Maximum fluorescence emission followed a single phase non-linear association (R²=0.99), increasing steadily for approximately 1 min followed by a deceleration period that was near static after 5 min (see Figure 3.6B). However, λmax did not trend up or down (no significant departure from zero slope; P>0.05), an unexpected finding as it was hypothesized that the N-terminal helix Trp would embed in bilayer resulting in a blue shift in the emission spectrum.

3.4.5 PSI Secondary Structure in the Presence of Anionic Bilayer LUVs

PSI secondary structure upon encountering anionic bilayer was monitored by collecting far-UV CD spectra for 10 µM PSI with or without 1:1:1 POPC:POPE:POPS LUVs (100 µM PL) in buffered saline pH 4.5 at 22°C. Continuous monitoring resulted in excessive UV exposure such that coagulation of the LUV mixture into a gel-like state occurred within minutes, therefore, spectra were collected at a relatively rapid scan rate. Scans were normalized, respectively, to control spectra for buffered-saline with or without LUVs scanned in the same manner as samples in terms of UV exposure and timing in attempts to account for any hidden UV-induced changes to PLs. PL-containing controls and samples were also verified by dynamic light scattering (DLS) which confirmed that the respective expected vesicle sizes were present after the elapsed time of the assays. Figure 3.7 summarizes the CD spectral changes with overlapping spectra beyond 8.5 min omitted for
Figure 3.6: Intrinsic Trp fluorescence emission of StAP PSI. (A) Spectra with anionic bilayer vesicles - Times indicated in the legend correspond to scan start times (seconds) after initial mixing of 10 µM PSI and 1:1:1 POPC:POPE:POPS LUVs (100 µM PL). (B) Maximum fluorescence at $\lambda_{\text{max}}$ and $\lambda_{\text{max}}$ are summarized, indicating that no shift in $\lambda_{\text{max}}$ for Trp emission occurs despite the increased emission over the period observed (mean +/- one standard deviation, n=3).
Figure 3.7: Comparison of timed far-UV CD spectra of PSI with 1:1:1 POPC:POPE:POPS LUVs (100 µM PL) in buffered saline pH 4.5 at 22° C. Spectra were collected through 30 min, however, overlapping scans are omitted for clarity. Each data set is an average of 5 scans at 500 nm/min. The comparison is made to test the hypothesis that the PSI would gain helix structure upon interacting with anionic bilayers.
clarity. The principal difference revealed by the scans was an increase in magnitude across the 222-208 nm region at both PSI concentrations. At 222 and 208 nm, ellipticity increased by approximately 26% and 16% for 10 µM PSI, and 13% and 4% for 4 µM PSI, respectively, relative to the control signals at the respective wavelengths. Signal-noise was only modestly increased by the presence of vesicles and increased absorbance of incident light was only marginally higher below 215 nm, gradually increasing to ~130% of buffered saline by 200 nm (data not shown).

3.4.6 Characterization of PSI-induced Bilayer Effects: Cryo-Transmission Electron Microscopy - Imaging was used to characterize liposome morphology. Shown in Figure 3.8 are images representative of the observed phenomena acquired for two concentrations of PSI+PL (10+500 µM and 16+1000 µM) having similar PSI:PL molar ratios (1:50 and 2:125, respectively) using 1:1 POPE:POPS LUVs, incubated at 22° C for 15 min prior to cryo-plunging. Control LUVs were in agreement with LUV quality control DLS measurements for stock LUV preparations, consistently yielding stable vesicles that measured 125-140 nm. Note that vesicles substantially contacting the sample support grid were excluded from analyses due to typical artefactual vesicle morphologies (Goodwin & Khant). The images were collected from three separate experiments using different PSI preparations and freshly made (<24 h) LUV preparations, verified for size by DLS. Drastic changes were noted for PSI-treated LUVs which were categorized into five distinct morphologies: (i) Less narrow oblong structures that appear to be collapsed vesicles (blue arrows); (ii) Individual semi-circular liposomes with a flattened or straight edge (pink arrows); (iii) Vesicles interfacing at respective flat edges (orange arrows); (iv) Wedge-type vesicles having two flat edges meeting at their ends (yellow arrows); and (v) Narrow
Figure 3.8: Cryo-Transmission Electron Microscopy images of 1:1 POPE:POPS LUVs in buffered saline at pH 4.5. Incubations were in 25 mM Na-Acetate / 140 mM NaCl pH 4.5 for (A) untreated LUVs; (B) LUVs (500 µM PL) treated with 10 µM PSI; and (C) LUVs (1000 µM PL) treated with 16 µM PSI. Assays were 15 min in length at 22°C at which time all were cryo-plunged. Images are representative of the observed phenomena from 9 control and 8 test images. Different observed morphologies are identified by arrows as follows: Oblong structures that appear to be partially-collapsed vesicles (blue arrows); individual liposomes with a flattened or straight edge (pink arrows); vesicles interfacing at respective flat edges (orange arrows); and wedge-type vesicles having two flat edges meeting at an end (yellow arrows); and narrow elongated rod-like structures approximately 100–200 nm in length (red arrows).
elongated rod-like structures approximately 100–200 nm in length (red arrows), which appear to show evidence of bilayer by way of a thin, white, low contrast layer completely surrounding a high density thin centre region, suggesting a collapsed liposome.

The various vesicle morphologies were enumerated and analyzed from all suitable (9 control and 8 test) cryo-TEM images (Figure 3.9). The occurrence and diameter of each observed vesicle are summarized in Figure 3.9.A. Histograms of the vesicle diameter distributions for circular vesicles exclusively as well as for overall vesicle populations are summarized in Figure 3.9.B-C, respectively. Analyses of variance indicated that the respective vesicle diameter distributions are significantly different (P≤0.05) for both comparisons, respectively (Figures 3.9.B-C). Comparing the circular vesicle size distributions (Figure 3.9.B), both 10 µM PSI/500 µM PL and 16 µM PSI/1000 µM PL tests are significantly different (P<0.05) from control, respectively, as well as to each other (2-tailed Kolmogorov–Smirnov tests). Comparing the vesicle diameter distributions of the overall populations (i.e. all morphologies; Figure 3.9.C), the 10 µM PSI/500 µM PL distribution is not significantly different (P>0.05) from control while the 16 µM PSI/1000 µM PL distribution is significantly different (P≤0.05) from control.

The differences in diameter distributions among circular vesicles consist of a broadening of sizes, both larger and smaller (Figure 3.9.A). Although both test PSI:PL concentrations resulted in several larger circular vesicles, the 16 µM PSI/1000 µM PL incubations resulted in a significantly lower average diameter compared to both control and the 10 µM PSI/500 µM PL test (P≤0.05). The average diameter (not to be confused with size distribution, above) for the latter group was not significantly different from control (P≥0.05).
Figure 3.9: Summary of phenomena from cryo-TEM images. (A) Enumeration and comparison by size of vesicle morphologies as identified in the legend; (B) histogram comparison of circular vesicle size distributions; and (C) histogram comparison of all vesicle morphologies’ size distributions. Note that results are derived from images not limited to the selected examples in Figure 8, and exclude vesicles in contact with the sample grid.
Nevertheless, it is clear that increased diameter vesicles appear in test images irrespective of diameter averages within a broadened size population.

With respect to imaging, a challenge that was encountered concerned vesicles’ tendency to preferentially adhere to the sample support grid instead of becoming entrapped within the pores. As reflected in the relatively low number of vesicle measurements for the control group in Figure 3.9, vesicle adherence was particularly problematic for non-treated vesicles. This was even more pronounced for 1:1:1 POPC:POPE:POPS vesicles where insufficient quality of the control images resulted in exclusion of test images (i.e., virtually no vesicles were observed within the sample support grid pores while countless adhered to the grid material). Images for the excluded PSI-POPC:POPE:POPS tests showed similar morphologies to those described above (data not shown). Further characterizations as part of a more comprehensive TEM-based study will address the adherence problem.

3.4.7 Characterization of Bilayer Fusion Activity: Dynamic Light Scattering - DLS was used to measure apparent size changes in 1:1 POPE:POPS LUVs over time as a means of monitoring fusogenic activity as has been used previously (Bryksa et al, 2011; Michalek & Leippe, 2015; Yang et al, 2015). Assays were carried out at room temperature until polydispersity or aggregation of samples became excessive for DLS measurements, up to 26 h. LUV size was monitored in both non-reducing and reducing buffered saline (Figure 3.10.A-B, respectively). Native PSI induced vesicle changes within 5 min, causing peak broadening. By 30 min, a population of large particles (4000±1000 nm) constituted ~2% of the vesicle population and the remainder constituted a size range averaging almost double their original apparent size (220±100 nm). Beyond 60 min, aggregates began to form thus precluding further DLS measurements. Fusion assays for PSI in reducing
Figure 3.10: PSI-treated vesicle size changes over time monitored by DLS. 1:1 POPE:POPS LUVs (100 µM PL) were incubated with 10 µM PSI in either (A) non-reducing or (B) reducing buffered saline at pH 4.5.
conditions also initially shifted the vesicle population to higher average diameter, however, a broader and larger size population formed more quickly compared to native PSI. This effect was also reflected in excessively high polydispersity prior to the formation of aggregates. In the absence of its disulfide bonds, PSI appeared to produce intermediate size products, shifting the entire population of LUVs, unlike native PSI which tended to increase the size range (broadening of the initial peak in Figure 3.10) via more modest and gradual size increases.

3.4.8 PSI Component Peptide-Induced Bilayer Disruption - Peptides corresponding to the secondary structure subdivisions in PSI (3RFI), as outlined in Figure 3.1, were screened for bilayer disruption activity by LUV leakage assays at concentrations up to 20 µM using 1:1 POPE:POPS LUVs (100 µM PL) in buffered saline pH 4.5. The results are summarized in Figure 3.11A where H3 was the only peptide that induced leakage, significantly different from each of the other groups including negative control tests (P≤0.05). Like PSI, H3 leakage activity plateaued at approximately 25% of positive control, however, rates were slower than the full length protein (Figure 3.5) such that calcein leakage proceeded for approximately twice as long (75-90 min). Leakage assays were also done for combinations of peptides, none of which yielded activity beyond that of H3 alone (data not shown). Although H3 was subsequently confirmed to induce leakage in both reducing and non-reducing conditions, all peptide leakage data analyzed below were restricted to rates measured in buffered saline containing 10 mM DTT. This was done to prevent oligomerization of peptides via unpaired Cys residues which would normally be contained in one of three disulfide bonds within native PSI (see Figure 3.1).
Figure 3.11: Leakage assays for peptide-treated 1:1 POPE:POPS LUVs at 25° C in buffered saline pH 4.5. (A) Peptide screening assays for LUV leakage activity; (B) Leakage rate-dependence on [PL]:[H3] ratio; and (C-D) Extent of leakage induced by 4-20 µM H3 (mean +/- one standard deviation, n=3). Leakage was non-linearly proportional to H3 concentration, and semi log curves were significantly different (P≤0.05). Error bars indicate +/- one standard deviation, n=3.
H3-induced bilayer disruption was further characterized in terms of concentration dependence of leakage. First, maximum leakage rate was confirmed to be non-linearly dependent on [PL]:[H3] ratio (Figure 3.11.B). Second, insight into the mode of H3-induced bilayer disruption was investigated by relating peptide concentration to the extent of leakage at 10, 20 and 30 min (Figure 3.11.C-D). The semi-log analyses (Figure 3.11.D) revealed distinct (P≤0.05) linear curves having increasing slopes (P≤0.05) and similar intercepts (P>0.05) (see inset table in Figure 3.11.D). Solving for the x-intercepts, there appeared to be a critical peptide concentration (average 2.9±0.5 µM) necessary for leakage/disruption activity.

3.4.9 H3 Intrinsic Tryptophan Fluorescence in the presence of Anionic Bilayer Vesicles - Since H3 contains one of StAP PSI’s two Trp residues, and H3 shows fusion and leakage activities, Trp emission was measured upon incubation with 1:1 POPE:POPS LUVs to determine whether its bilayer interactions involve bilayer penetration by its hydrophobic mid sequence MAVVWM (see Figure 3.1). Assays were carried out in the same manner as for PSI and the resulting spectra are presented in Figure 3.12. Like PSI, λmax did not shift, and initially, H3 fluorescence emission gradually increased up to the 95 s scan. However, emission subsequently trended down, overshooting the start scan level and then returned to near the initial 20 s scan. Although changes between consecutive scans seemed to indicate a coherent progression, scans were all within error of one another, leading to the conclusion that H3 bilayer penetration is unlikely.

3.4.10 Secondary Structure of PSI Component Peptides - Each of the PSI secondary structure component peptides (Figure 3.1), as well as a H3 Lys83Gln mutant, were characterized in terms of secondary structure by far-UV CD (Figure 3.13.A-F). Since the
Figure 3.12: Intrinsic Trp fluorescence emission spectra upon incubation of 10 µM peptide H3 with 1:1:1 POPC:POPE:POPS LUVs (100 µM PL) in buffered saline pH 4.5 at 25°C. Times indicated correspond to scan start times (seconds) after initial mixing. Although peak emission increased over the initial 95 s, signal subsequently decreased until 225 s at which point it remained constant over scans collected through 405 s (overlapping scans omitted for clarity). No shift in $\lambda_{\text{max}}$ was detected. Note that each spectrum is the average of three scans, error bars were omitted for clarity in distinguishing the spectra, and differences in $\lambda_{\text{max}}$ and Emission$_{\text{max}}$ were not significantly different ($P>0.05$).
Figure 3.13: Far-UV CD spectra of peptides in buffered saline containing 10 mM DTT at the indicated pH values. Spectra were averaged from three scans: (A) H1; (B) H2; (C) H1H2; (D) X; (E) H3 (solid lines), H3 with 1:1:1 POPC:POPE:POPS LUVs (100 µM PL; broken lines) and Lys83Gln H3 (dotted lines); and (F) H4.
peptides (except X) contain Cys, spectra were collected in reducing conditions. Firstly, in the absence of the PSI structure, peptides H2, X and H4 (Figure 3.13.B,C,F, respectively) have secondary structures that are predominantly disordered/random coil (large negative peak centred at 200 nm) possibly mixed with helix or β-structure sufficient to cause an overall negative ellipticity across most of the spectra, a region that would have near zero CD signal if these peptides were entirely disordered (Kelly & Price, 2000; Whitmore & Wallace, 2008). The high coil character of these spectra was present irrespective of reducing or non-reducing conditions.

The most notable findings among the peptides’ spectra concerned the PSI N-terminal helix, H1 (Figure 3.13.A,C), and H3, the peptide having apparent bilayer fusion and disruption activities (Figure 3.13.E). The spectra for H1 and H1H2 (Figures 3.13.A,C) indicate a distinct structural transition delineated by the activity pH profile for PSI such that pH 3.0 and 4.5 have similar spectra, distinct from those at pH 6.2 and 7.4. It is apparent that as pH transitions to inactive pH, the PSI N-terminus becomes disordered. In terms of active pH conditions, H1 and H1H2 appear to have substantial β-structure. With respect to H3, secondary structure content appears to be dominated by β-structure across the four pH values tested as all four of the spectra contain minima near 218 nm. H3 Lys83Gln, which eliminated fusion activity, also had an accompanying drastic effect on H3 secondary structure in that it became largely disordered across all pH values tested (Figure 3.13.E).

3.4.11 Peptide-induced vesicle fusion – Peptides were screened for fusogenic activity at up to 20 µM. As was the case for leakage activity, only peptide H3 induced fusion (Figure 3.14). Similar to PSI (Figure 3.10), H3 caused broadening of the original population comparatively slower (e.g., Peak 1: 154±50 nm vs. 256±120 nm, respectively,
Figure 3.14: H3-treated vesicle size monitored over time by DLS. 1:1 POPE:POPS LUVs (100 µM PL) were incubated in buffered saline pH 4.5 containing 10 mM DTT with either (A) 10 µM H3 or (B) 10 µM Lys83Gln H3.
at 60 min). H3 caused an immediate appearance of very large particles by 5 min at an abundance of 4%, whereas PSI did not produce any large particles as of the 15 min reading. Since Lys83 provides the sole positive charge within H3, mutant Lys83Gln H3 was also assayed to test its suspected role in PSI interaction with anionic bilayers. Lys83Gln H3 induced no discernible change in vesicle size through 26 h indicating that Lys83 is essential for PSI-bilayer interaction.

### 3.5 Discussion

Dimer formation has been implicated in saposin-membrane interactions in several structure-function studies (Ahn et al, 2003; Hawkins et al, 2005; John et al, 2006; Rossmann et al, 2008). Although the crystal structure of StAP PSI (3RFI) revealed a dimer (Bryksa et al, 2011), PSI quaternary structure in solution remained unclear. Determining the pH-dependence of PSI dimerization was thus a priority for understanding its mechanism of action. Sedimentation equilibrium (Figure 3.2) and Trp fluorescence emission (Figure 3.3) experiments together indicated that PSI exists as a dimer at low pH and as a monomer at neutral pH. At pH 6.2, there appeared to be a small proportion of dimer mixed into the predominantly monomer population since the calculated apparent mass (Figure 3.2) was slightly higher compared to pH 7.4 (P≤0.05). Considering that pH 6.2 is within a unit of endosomal/vacuolar pH in some circumstances (Nozue et al, 1997), it seems reasonable for a pH-induced structure transition in PSI to begin near this point. Since reduced solubility precluded analytical centrifugation at pH 4.5, comparison of Trp fluorescence measurements at four pH values (Figure 3.3) was used to infer the quaternary structural state. Indistinguishable spectra at pH 3.0 and 4.5 indicated that PSI is a dimer at
pH 4.5. In this context, the findings that bilayer disruption was strong at pH 3.0 and 4.5, negligible at pH 6.2 and inactive at pH 7.4 (Figure 3.5), and that apparent vesicle fusion occurs exclusively in acidic conditions (Figure 3.10.A), together indicate that the PSI dimer is the initial protein structural state in the swaposin-bilayer interaction mechanism.

Monitoring Trp fluorescence emission upon mixing PSI with bilayer vesicles (Figure 3.6) indicated a time-dependent emission increase. After 5 min, slowing spectral changes suggested a system approaching equilibrium, presumably reflective of Trp stabilizing in a new environment (Figure 3.8.B). Unexpectedly, there was no change in $\lambda_{\text{max}}$, a counterintuitive result in consideration of the increasing emission signal for a system having a tight, very hydrophobic core PSI dimer (Bryksa et al, 2011) as the start structure. The overall net increase in Trp emission would presumably derive from the development of an even more hydrophobic environment accompanied change in $\lambda_{\text{max}}$. Possibly, the lack of blue shift was caused by phenomena unrelated to PSI Trp residues’ bilayer environment.

An internal Stark effect (Vivian & Callis, 2001) can be manifested if a positively charged residue becomes positioned near the Trp benzene ring, or a negative charge near the pyrrole ring, causing a $\lambda_{\text{max}}$ red-shift via a process outside of the intended experimental design. H1 and H3 contain Trp18 and Trp77 (non-recombinant StAP PSI numbering), respectively:

(H1) IVSMECKTIVSQQGM\underline{E}MI\underline{D}LLVSG
(H3) EAPLCTACEMAVVW\underline{M}QNQLKQ

Trp18 (underlined italics) is neighbored by an ionisable residue Glu19 (underlined) that could reasonably undergo a change in charge induced by an altered pK$\alpha$ upon protein structure change and/or contact or even penetration of PL bilayer. Further contributing charge in the vicinity in question is the negatively charged PL bilayer itself, again
potentially directly impacting the electric field near the Trp pyrrole ring (Vivian & Callis, 2001). In addition to these factors, another potentially confounding situation could be the formation of oligomers such that Trp becomes buried in a hydrophobic protein environment (Chen & Barkley, 1998; Shai, 1999) other than its dimer start structure.

Insights into PSI-induced effects on bilayer vesicle size and morphologies were gained from analysis of DLS and cryo-TEM data. DLS experiments showed that PSI caused broadening of vesicle size range and shifting to larger vesicle diameters (Figure 3.10). Cryo-TEM images revealed several different structures (Figure 3.8.B-C) whose characteristics are summarized in Figure 3.9. Anionic vesicle interaction with sample grids and limited access constrained our choice of concentration and lipid:protein ratio combinations. Cryo-TEM imaging of 15-minute vesicle incubations with PSI showed structures that spanned a significantly broadened size distribution compared to untreated control vesicles (see Figure 3.9). Comparison to the DLS size distribution at 15 min suggests overall agreement on moderately broadened size ranges, the appearance of both smaller and larger vesicles compared to controls, and an absence of particles exceeding ~600 nm. Furthermore, the appearance of individual spheroid vesicles having larger diameters than control samples in cryo-TEM images, taken together with the DLS and TEM size distributions’ similarities, give direct evidence of PSI-mediated vesicle fusion.

The varied vesicle sizes and morphologies detailed in Figure 3.9 may represent different stages of vesicle collapse when viewed in the context of TEM image interpretation discussed previously (Almgren et al, 2000; Edwards et al, 1993). The array of shapes appears to follow a progression from initial flattening/creation of a liposome edge (pink arrows), followed by expansion of edging such that wedge type shapes may form (yellow
arrows), followed by elongated narrow vesicles (blue arrows), and finally a fully flattened dense elongated structure (red arrows). The semi-circular single edge morphology is a known phenomenon regarding vesicle-fusase initial interactions (Gui et al, 2016), and it has also been reported for melittin-induced vesicle effects (Strömstedt et al, 2007).

Regarding secondary structure, the present study confirms the pH-activity-helix structure increase relationship not only by the solution CD determinations, but also via evidence produced from the PSI-vesicle CD analyses (Figure 3.7) and intrinsic Trp fluorescence measurements (Figure 3.6). Qualitatively, spectra for PSI with LUVs and NaCl indicated increased ellipticity peak magnitudes at 222 and 208 nm, and were distinctive and consistent at the two concentrations of PSI tested. This structure change is solely dependent upon interaction with PL bilayer as the PSI samples themselves had already been equilibrated in CD buffer prior to scans. These findings lead to the conclusion that PSI-PL bilayer induces secondary structure changes resulting in an increase in helix structure.

Furthermore, it is worth noting that native and reducing conditions yielded very similar CD spectra for respective pH values. Disulfide bonds, it would appear, have little role in the overall secondary structure content of the PSI in saline conditions. PSI DLS profiles (Figure 3.10) for native and reducing conditions also indicated that disulfide bonds were non-essential for vesicle fusion. In contrast to vesicle size range broadening and increasing diameters by native PSI, elimination of PSI disulfide bonds in reducing conditions uniquely resulted in a relatively rapid and complete shift to discreet populations of larger vesicles. We reasoned that a disulfide bond-free PSI structure would allow increased accessibility of protein structural features, perhaps resulting in the altered vesicle size profiles in Figure
3.10. In this context, altering overall PSI structure appears to change the kinds of fusion products, but does not prevent it from occurring. It would thus appear that at least basic PSI-bilayer interaction must be embedded in sequence(s) or structural feature(s) independent of quaternary and tertiary structure considerations.

Secondary structure comparisons over different pH conditions indicated important relationships between structure and environmental changes not only for PSI, but also among its component helices. The clear trend of increasing helix and decreasing strand structure content upon acidification was expected based on previous work (Bryksa et al, 2011) which only considered pH 7.4 and 4.5. With respect to peptide secondary structures, it was hypothesized that the pH-induced increase in helix content would originate from some portion of the overall PSI structure. The reasoning for this expectation was rooted in that the PSI is a domain-swapped saposin in terms of the arrangement of the N- and C-terminal halves of the respective monomers. That is, the PSI N- and C-terminal halves occur in the opposite order relative to saposins (i.e., H1-H2-H3-H4 becomes H3-H4-H2-H1). Despite this drastic difference at the tertiary structure level, PSI and saposin C (and to a lesser extent saposin D) share highly similar pH dependencies as well as anionic PL requirement for activity (Ahn et al, 2003; Ahn et al, 2006; Alattia et al, 2006; Ciaffoni et al, 2001; Ciaffoni et al, 2006; de Alba et al, 2003; Hawkins et al, 2005; Mendieta et al, 2006; Vaccaro et al, 1995a; Vaccaro et al, 1995b; Vaccaro et al, 1994), and PSI and saposins B and C share highly similar tertiary structures (Ahn et al, 2003; Bryksa et al, 2011; Rossmann et al, 2008). Hence, we hypothesized that the structure-function “controls” must be intrinsic features originating from a lower structural level than the backwards PSI “swaposin” tertiary structure.
The activities of the component peptides, measured in reducing conditions to prevent inter- and intramolecular cross-linking, produced some unexpected results. The bilayer disruption activity of H3 (Figure 3.11) disproved the hypothesis that the C-terminus of PSI (equivalent to the N-terminus of saposins) is the source of PSI bilayer disruption. Furthermore, H3 caused bilayer fusion in PL vesicles (Figure 3.14) while none of the other peptides resulted in leakage or fusion. The fusion profiles for H3 and PSI are similar overall, however, H3 caused apparent fusion faster and was seemingly more efficient in creating a higher proportion of LUVs having large (~4000 nm) particle sizes. Component regions of saposin C have been studied previously (Wang et al, 2003) with notably different results. For saposin C, H1 and H2 (equivalent to PSI H3 and H4, respectively) did not cause bilayer fusion. Comparing sequences, PSI H3 is more hydrophobic than either of the saposin C peptides, and although the positive charge content is inferior for PSI H3, its amphipathic charge distribution, together with the hydrophobic AVVW middle patch, may explain the different fusogenic abilities for the respective regions of these two otherwise similar proteins:

PSI H3 - EAPLCTACEMAVWMQNLKQ
SapC H1 - YCEFCEFLVKEVTKLID
SapC H2 - EKEIADFJKLMCSKLPK

Upon finding that H3 had both leakage and fusion activities, three mutants were designed each eliminating the respective charges: Glu63Gln, Glu74Gln and Lys83Gln. While the sole Lys was predicted to be essential for anionic bilayer interaction, the other two charges were suspected of potentially having a role in governing pH-dependence of structure. Unfortunately, low solubility for both Glu mutants prevented their use, at least under the conditions used in the present study. H3 Lys83Gln was soluble and was shown
to lack both bilayer disruption and vesicle fusion activities, thus confirming that Lys83 is critical for H3-bilayer interaction. As a result of this finding, its role is being investigated as part of a larger mutational study on PSI charge features.

From analysis of H3 bilayer disruption activity (Figure 3.11), the apparent requirement for attaining a critical peptide concentration to initiate leakage suggests a cooperative mechanism of action. The findings of the present study regarding H3 share certain characteristics with other membrane-active peptide types, including magainins (Matsuzaki et al, 1989; Matsuzaki et al, 1996; Matsuzaki et al, 1998) and amyloid peptides (Burke et al, 2013; Butterfield & Lashuel, 2010). In common with the magainins is that they yield abnormally slow rates of vesicle leakage (Matsuzaki et al, 1991; Matsuzaki et al, 1989), that leakage increases dramatically with peptide concentration once the concentration threshold is met, that leakage rate slows with time for all peptide concentrations (Matsuzaki et al, 1989; Vandenburg et al, 2002), and that leakage stops prior to attaining full leakage (Matsuzaki et al, 1991). The above kinetic features, all in common with the findings of the present study, indicate that H3 may operate by a carpet-like mechanism (Vandenburg et al, 2002) wherein a cooperative bilayer perturbing process results in leakage of vesicle contents, functionality which subsequently dissipates due to peptide equilibration across the bilayer (Matsuzaki et al, 1989; Vandenburg et al, 2002). In the case of amyloid-β peptide, calcein leakage from peptide-treated LUVs also proceeds only to partial leakage (McLaurin & Chakrabartty, 1997; Williams et al, 2010). More importantly perhaps, certain amyloid peptides, proposed to employ the carpet mechanism in some situations (Engel et al, 2008; Hebda & Miranker, 2009), produce similar distortions in anionic LUV shape (i.e., the appearance of flat surfaces and elongated vesicles) to those of the present study.
observed by cryo-TEM (Engel et al, 2008; Williams et al, 2010; Yip & McLaurin, 2001). The implications of these similarities remain to be elucidated in future investigations.

Although unexpected, the possibility of shared mechanistic bilayer disruption features with amyloid peptides seems reasonable in the context of the predominantly β-strand/turn secondary structure (Figure 3.13.E). While it was found that PSI undergoes a secondary structure transition such that helix is gained at the expense of strand upon acidification (Figure 3.4.D) and concomitant dimerization (Figure 3.2), and this gain in helix modestly increases upon interaction with bilayer (Figure 3.7), H3 alone seemingly has no substantial helix content nor does its structure appear to transition to helix upon bilayer interaction or have any obvious pH-sensitivity at all (Figure 3.13). These key differences indicate that PSI and H3 may have two different mechanisms of action, a possibility that is further supported by the different trends in Trp emission spectral changes upon mixing with bilayer vesicles; PSI Trp gain hydrophobicity reaching a new and stable state (Figure 3.6) whereas H3 appeared to experience short-lived changes in hydrophobicity only to return to the start spectrum within approximately 5 min and remaining static thereafter (Figure 3.12). The lack an increase in H3 Trp hydrophobicity indicates a non-penetrating mechanism of action, consistent with the aforementioned carpet model wherein peptides interact electrostatically with PL headgroups covering regions of the bilayer surface (Bobone, 2014). By contrast, increased PSI Trp hydrophobicity reaching equilibrium within ~5 min of encountering bilayer, with a half time of ~30 s (Figure 3.6.B), agreed with the approximate time required for detectable secondary structure changes to reach their apparent end point (Figure 3.7); where obvious spectral shifting was underway by 30 s and essentially completed by ~4.5 min. The increased hydrophobicity suggests insertion into
the bilayer such that Trp interact with PL tail moieties. These features, together with the finding of a critical [H3] for commencing leakage, are consistent with the previously proposed mechanism of StAP PSI bilayer disruption as involving pore formation as per the Barrel-Stave model (Muñoz et al, 2014), which consists of pore formers oligomerizing with hydrophobic moieties facing away from the pore opening and interacting with hydrophobic inner bilayer (Phoenix et al, 2013). This model may also account for incomplete leakage noted in the present study in that pore formers may be situated in a fraction of vesicles thereby causing leakage in just some targets (i.e., all-or-none leakage) while others remain intact (Last et al, 2013).

H3, while apparently membrane-active irrespective of PSI, is particularly interesting in regards to its sequence features. H3 has desirable elements characteristic of particular antimicrobial sequences:

\[
\text{EAPLCTACEMAVVWMQNQLKQ}
\]

First, there is a single positive charge (bold underlined) that was confirmed in the present study to interact with anionic PL bilayer; second, the peptide is relatively small at 21 residues yet is amphiphilic (Glu63 and Glu74 underlined; and Lys83 bold underlined); third, it contains a hydrophobic four-residue patch centred within the sequence (underlined italics); and fourth, it contains multiple Gln residues in the vicinity of a positive charge (bold), common for antimicrobial cationic peptides (Patrzykat & Douglas, 2005), although typically in conjunction with more positive charge than present in H3 (Sharifahmadian et al, 2013). The C-terminus of H3 corresponds to a membrane-associated protein motif
typically found in the plant defense-related 2S storage proteins and chitin binding proteins, in addition to some saposins:

\[ [N/Q]-[N/Q]-[A/L/I/V]-[K/R]-[N/Q]. \]

This motif is present in many flocculating/coagulating proteins (see Table 3.1 for examples) where Asn and Gln, and Lys and Arg are interchangeable, respectively, and the third position from the C-terminus can be any aliphatic residue. Recently, bacterial membrane damage by *Moringa oleifera* seed flocculating cationic peptide (see Table 3.1) was shown to be caused by a membrane fusion mechanism (Shebek et al, 2015). Also, it was reported recently that the antibacterial activity of a *Ricinus communis* 2S albumin involves leakage of cell contents postulated to be the result of magainin-like (noted above) pore formation (Souza et al, 2016). A Motif Query of the RCSB Protein Data Bank (avoiding redundancy) for the full C-terminal 6-residue motif \([N/Q]-[N/Q]-[N/Q]-[A/L/I/V]-[K/R]-[N/Q]\) yielded just 28 PDB structures in total (Table 3.2), all of which are lipid-interacting or surface active proteins.

A structure alignment search of the RCSB PDB structure database based on protein domains (as opposed to whole protein chains) yielded matches for diverse surface-active/membrane-interacting proteins. High-scoring alignments are presented in Figure 3.15. Interestingly, among these are endosomal/vacuolar factors (Figure 3.15.A,C), a seed storage albumin protein (Figure 3.15.F) and a calcium sensing protein (Figure 3.15.E). The latter is particularly noteworthy due to its utilization of the calcium binding protein EF-hand motif, i.e., a helix-loop-helix segment usually occurring in adjacent pairs (Lewit-Bentley & Réty, 2000), reminiscent of a similar N-terminal kinked helix motif within the side-by-side boomerang-shaped open saposin fold of PSI (Bryksa et al, 2011).
Table 3.1: Examples of antimicrobial/membrane-interacting proteins that contain the [N/Q]-[N/Q]-[A/L/I/V]-[R/K]-[N/Q] motif. Residues/sequences are indicated as: hydrophobic (bold black underlined), the motif in question (bold red underlined) or similar sequence to that motif (bold red).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAP PSI H3</td>
<td>EAPLCTACEM\textbf{AVVWM}\textbf{QNLKO}</td>
<td>(Guevara et al., 2005)</td>
</tr>
<tr>
<td>Mabinlin I</td>
<td>EPLCRQFQQHQLRACQRYIRQRAQ RGPLVDEQRGPALRLCC\textbf{QQLRQ} VKNP CVCPVLRQAHH\textbf{QQLYQQ} QIEGPR\textbf{QVR QL}FRAARNLPPNI\textbf{CKIPA} VRCQFTWR</td>
<td>(Nirasawa et al., 1994)</td>
</tr>
<tr>
<td>Mabinlin II</td>
<td>QPRRPALRQCC\textbf{QQLRQ} VDRPCVCP Vil RQA AQVLFQRQ IIGF\textbf{PQQRL} LRFDAA RNLPNCIPNIGACPFRAW</td>
<td>(Li et al., 2008; Liu et al., 1993)</td>
</tr>
<tr>
<td>Flocculent-active protein MO2.1 and MO2.2</td>
<td>QGPGRQP\textbf{DFQRCQGQQ} LNPQIP\textbf{QVRQ} MYR VASNPST</td>
<td>(Broin et al., 2002)</td>
</tr>
<tr>
<td>Moringa oleifera CBP$_3$</td>
<td>CPAI\textbf{QRCCQQLRNIS} PP\textbf{RCQ}</td>
<td>(Gifoni et al., 2012)</td>
</tr>
<tr>
<td>Sesame 2S albumin (Sesamum indicum)</td>
<td>MAKKLALAVLLVAMVLA SATTYTT TVTITTAIDENQQS\textbf{QQCRQ} QLQRGR QFRSCQRYLSQGRSPYGGEDEVLEMS TGNQQSEQSLRDC\textbf{QQLRN} VDERCRC EAIRQA\textbf{VRQQQEGQ} QGQS\textbf{QQVY QR}A\textbf{DLPRCNMRPQQC} FRVIFV</td>
<td>(Tái et al., 2001)</td>
</tr>
<tr>
<td>* β-lactoglobulin fragment 1–8</td>
<td>\textbf{LIVTQTMK}</td>
<td>(Pouliot et al., 2009)</td>
</tr>
</tbody>
</table>

* This example is shown because it contains the skeleton of the motif in question; hydrophobic patch, Gln, single positive charge.
Table 3.2: Proteins in the RCSB PDB databank that contain the [N/Q]-[N/Q]-[N/Q]-[A/L/I/V]-[R/K]-[N/Q] motif.

<table>
<thead>
<tr>
<th>PDB ID</th>
<th>PDB Macromolecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>3RF1</td>
<td>Saposin-like domain of plant aspartic protease from Solanum tuberosum</td>
</tr>
<tr>
<td>3HJ1</td>
<td>Phosphatidylycerine synthase Haemophilus influenzae Rd KW20</td>
</tr>
<tr>
<td>3KN1</td>
<td>Golgi phosphoprotein 3 N-term truncation variant</td>
</tr>
<tr>
<td>3U0C</td>
<td>N-terminal region of type III secretion first translocator Invasin IpaB</td>
</tr>
<tr>
<td>1XS5</td>
<td>Membrane lipoprotein TpN32 from Treponema pallidum</td>
</tr>
<tr>
<td>5AL6</td>
<td>Central coiled-coil domain of Drosophila melanogaster anala spindle 2</td>
</tr>
<tr>
<td>1BWM</td>
<td>Single-chain T-cell receptor D10 from major histocompatibility complex class II-restricted</td>
</tr>
<tr>
<td>4U49</td>
<td>Pectate Lyase Pel3 from Pectobacterium carotovorum</td>
</tr>
<tr>
<td>4ULV</td>
<td>Cytochrome C prime from Shewanella frigida marina</td>
</tr>
<tr>
<td>4QGO</td>
<td>DNA-entry nuclease (NucA) from Streptococcus agalactiae</td>
</tr>
<tr>
<td>4Q98</td>
<td>Fimbriulin - Major fimbrial subunit protein from Porphyromonas gingivalis</td>
</tr>
<tr>
<td>4L1K</td>
<td>Catalytic subunit of human DNA primase</td>
</tr>
<tr>
<td>4HOW</td>
<td>Isomaltulose synthase from Erwinia rhapontici NX5</td>
</tr>
<tr>
<td>4GL</td>
<td>RHS-repeat containing B,C component of the secreted ABC toxin complex (Versinia entomophaga)</td>
</tr>
<tr>
<td>4I5S</td>
<td>Sensor histidine kinase - Putative histidine kinase CovS; VicK-like protein</td>
</tr>
<tr>
<td>2LY1</td>
<td>Repetitive domain (RP) of aciniform spindrin 1 from Nephila antipodiana</td>
</tr>
<tr>
<td>3W14</td>
<td>Insulin receptor domains - L1,CR,L2,FNII-1 alpha肽</td>
</tr>
<tr>
<td>4DR0</td>
<td>Bacillus subtilis dimanganese(II) Ribonucleoside-diphosphate reductase subunit beta</td>
</tr>
<tr>
<td>3MVP</td>
<td>TetR/AcrR transcriptional regulator from Streptococcus mutans</td>
</tr>
<tr>
<td>3A09</td>
<td>Sphingomonas sp. A1 alginate-binding protein AlgQ1</td>
</tr>
<tr>
<td>2WDQ</td>
<td>E. coli succinate dehydrogenase hydrophobic membrane anchor subunit</td>
</tr>
<tr>
<td>3GBE</td>
<td>Isomaltulose synthase SmuA from Protaminobacter rubrum</td>
</tr>
<tr>
<td>3F3S</td>
<td>Human lambda-crystallin CRYL1</td>
</tr>
<tr>
<td>2EJ9</td>
<td>Riboflavin biosynthesis protein - Biotin protein ligase from Methanococcus jannaschii</td>
</tr>
<tr>
<td>2G6V</td>
<td>RibD from E. coli</td>
</tr>
<tr>
<td>2HR7</td>
<td>Insulin receptor domains 1-3</td>
</tr>
<tr>
<td>1YCK</td>
<td>Human peptidoglycan recognition protein PGRP-S</td>
</tr>
<tr>
<td>4ZXB</td>
<td>Human insulin receptor ectodomain - heavy chain, light chain</td>
</tr>
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</table>
Figure 3.15: Protein domain structure alignments. A structure alignment search of the RCSB PDB database using the Protein Structure Comparison Tool v.4.2.0 (Prlić et al., 2010) running the FATCAT algorithm (Ye and Godzik, 2003) based on protein domains (as opposed to whole protein chains) yielded matches to PSI (3RFI) for diverse surface active/membrane interacting proteins. The alignments presented here were selections based upon a combination of high alignment score, low P-value and/or high sequence similarity: (A) Endosomal sorting complex, (B) sodium channel protein type 5 subunit α, (C) vacuolar transport chaperone 4, (D) calmodulin, (E) jellyfish calcium-regulated photoprotein cp-aequorin, (F) sweet protein mabinlin-2.
In conclusion, inspection of the structural elements that make up StAP PSI has offered insights into its membrane interaction requirements, and has offered more questions for future consideration. The present study showed that, nested within its primary structure, PSI contains a peptide (H3) that is itself bilayer-active. Oddly, this peptide takes up a β-stand configuration when alone as opposed to its normal helix character within the PSI tertiary structure. H3 deserves further investigation to assess its biological activity and mutability as it may present a novel sequence for membrane active protein applications (e.g., nano-particle targeting), irrespective of its apparent central role in PSI-bilayer structure-function. The H1 region, part of a very hydrophobic region of PSI that tightly binds within the dimer (Bryksa et al, 2011), was shown to be the sole portion of PSI that is intrinsically pH-sensitive albeit adopting a strand configuration at acidic pH and is otherwise unstructured, as is the case at all pH values for H2, “X” and H4. Thus, the overall tertiary and quaternary structures of PSI are essential to the folding of every component native helix, including both the dimer and monomer PSI states. Ironically, elimination of PSI’s disulfide bonds, which ubiquitously hold the N- and C-terminal portions of SAPLIPs together, does not result in appreciable loss of native secondary structure. This leaves one evident source of PSI-induced helix: the extensive hydrophobic contacts that reside within both the extended dimer as well as the canonical monomeric saposin fold. At present, investigations into the PSI transition to the bilayer environment as well as the roles of charged residues seek to better illuminate the structural basis for PSI bilayer interactions. More broadly, the apparent structural cohesion among surface/lipid active proteins across protein families is a vast topic that calls for detailed comparative structural investigation in the context of plant immunity as well as pathogen invasion factors.
3.6 References


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aspartic protease precursor from *Cirsium vulgare* (Asteraceae). *Phytochemistry*, 81, 7-18.


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3.7 Appendices

3.7.A Considerations for using DLS to characterize vesicle fusion - As fusogenic activity by PSI on PL vesicles proceeds, samples become increasingly polydispersed, both in terms of multiple sizes appearing and disappearing within the vesicle population (not only multiple sub-populations but also a relatively dramatic increase to the particle diameter size range), and in terms of newly formed aggregates, thereby further increasing the complexity of light scatter by the overall population (Pence & Hallett, 2003). Deviation from the initial vesicle population uniformity of size and particle shape (spherical) eventually becomes too complex for correlation of light intensity and position across the detector with the dynamics of a population of particles (Pence & Hallett, 2003). PSI-induced vesicle size change can be detectable within minutes, and therefore, the use of lengthy measurement times and multiple incident light angles (necessary for accurate size determination in polydispersed populations) are precluded. Therefore, when considering DLS data for use in vesicle fusion assays, the data must be treated as suitable for qualitative characterization of relative changes within a controlled experiment, but should not be compared across platforms or experimental designs.

3.7.B Stability of large unilamellar vesicles - In terms of vesicle stability for stock LUV preparations, Z-average diameter was monitored over long periods for test batches. Figure 3.16 outlines data from monitoring vesicle stability far past storage times used for experiments, unexpectedly revealing relatively small fluctuations in average diameter at 38 days (1:1 POPE:POPS LUVs) and 50 days (1:1:1 POPC:POPE:POPS LUVs) post-extrusion stored in the dark at ambient temperature. Negative control LUV samples post-experiment were also randomly verified for size by DLS prior to being discarded (data not
Figure 3.16: Stability testing on LUV stocks after storage at ambient temperature
shown) to ensure that assay conditions did not cause size distortions (e.g., shear stress from shaking polyethylene microplate-based leakage assays, pipetting in and out of cuvettes and stock tubes, repeated UV light exposure during time trial assays). These tests were intended as assurance of vesicle stability; that fusion assays and cryo-TEM experiments were in the context of substrate that is not prone to destabilizing without PSI or other intervention. Also anecdotally, but potentially important to many researchers, it has been noted repeatedly that LUV preparations used in the present study are more stable when refrigeration temperatures are avoided and the authors recommend re-evaluating protocols that call for refrigeration during LUV storage.

3.7.C Considerations for comparing leakage rates at low pH values - Although the results in Figure 3.4 are shown normalized across groups (to the highest rate measured), we caution that comparison of calcein fluorescence rates of change between different pH conditions is troublesome due to low fluorescence efficiencies at low pH. The signal ranges from negative control (intact LUVs) to positive control (full calcein release) are essentially equal and highest at pH 7.4 and pH 6.2 whereas range is approximately 4-fold lower at pH 4.5 and 12-fold lower at pH 3.0. These values were variable, but of typical LUV preparations and assays over many experiments. Noise introduced greater relative error to pH 3.0 rates compared to those at pH 4.5. Furthermore, fluorescence emission as a function of concentration was not consistent between pH values below 6.2 presumably reflecting the effects of varying quantum yield on fluorescence emission itself as well as self-quenching efficiency / inner filter effect. At constant pH, the latter can be ignored for measuring initial leakage rates due to the leaked calcein concentration in the reaction volume being low (Kendall & MacDonald, 1982), however, comparison of leakage rates
for reactions with self-quenching fluorophore reporter at different quantum yields (i.e., pH 3.0 vs, pH 4.5) is likely unreliable quantitatively, particularly at pH 3.0. The important finding in the context of the present study is that PSI appears to have strong PL bilayer perturbation activity at both pH 3.0 and pH 4.5.
Chapter 4: Comparative Structure-Function Characterization of the Saposin-Like Domains from Potato, Barley, Cardoon Thistle and Arabidopsis Aspartic Proteases

Note: The findings and content of following chapter were submitted (January 2017) as a revised manuscript to Biochimica et Biophysica Acta - Biomembranes for review.

4.1 Abstract

The present study characterized the aspartic protease saposin-like domains of four plant species in terms of bilayer fusion, bilayer perturbation, and structure pH-dependence. Recombinant saposin-like domains from Solanum tuberosum (potato), Hordeum vulgare L. (barley), Cynara cardunculus (Cardoon thistle) and Arabidopsis thaliana were compared revealing that all had vesicle leakage activities against simple phospholipid bilayer vesicles with the relative rates as Arabidopsis > barley > Cardoon > potato. When compared against a bilayer composed of a vacuole-like phospholipid mixture, leakage was more than five times higher for potato saposin compared to the others. In terms of fusogenic activity, all showed faster and larger particle size increases relative to potato saposin, particularly for barley and Arabidopsis saposins. Bilayer fusion assays in reducing conditions caused clear alterations in the fusion product profiles except for the case of Arabidopsis saposin which was virtually unchanged. Secondary structure contents were similar across all four proteins under different pH conditions, although Cardoon saposin appeared to have higher overall helix structure. In terms of Trp emission for saposin interactions with bilayer, rates for reaching equilibrium indicated that more than half of the structural rearrangement for saposin-like domain bilayer interactions occurred in less than two minutes upon encountering vesicles under experimental conditions. Overall, the
present findings serve as a foundation for future studies seeking to attribute functionality differences to bilayer interacting structural motifs or other variants among the plant saposin domains as well as between the domains and other saposin-like proteins.

4.2 Introduction

For several decades, the relationship in food plants post-harvest (Yomo & Srinivasan, 1973) as well as during seed maturation (Jacobsen & Varner, 1967) and germination (Garg & Virupaksha, 1970) with increased proteolytic activity has been noted. Also of importance is the association of acid protease activity within the lysosome (Yatsu & Jacks, 1968), one of two functions of a type of plant acid protease-associated protein that is the focus of the present study. Protein degradation and processing being an important function of this organelle, more than two dozen proteases are in fact found in the plant vacuole (Arabidopsis thaliana) (Carter et al, 2004). In addition to cysteine proteases (McGrath, 1999) and serine proteases (Breddam, 1986), aspartic proteases (AP) are part of the ensemble of vacuolar enzymes, analogous to its lysosomal equivalent in animal cells (Marty, 1999).

The two earliest structures to be reported for plant APs were phytpepsin (Kervinen et al, 1999) from barley (Hordeum vulgare L.) and cardosin A (Frazão et al, 1999) from the flowers of Cardoon (Cynara cardunculus L.), the latter being a source of vegetable rennet specifically due to cardosin milk-clotting activity (Veríssimo et al, 1995). Phytpepsin, crystalized in its zymogen form, displayed the usual bilobal AP tertiary structure, and also revealed the first structural detail of a domain uniquely found in plant APs termed the plant-specific insert (PSI) or plant-specific sequence (PSS) (Runeberg-Roos et al, 1991;
Sarkkinen et al, 1992), most closely related to saposins in terms of primary, and predicted secondary and tertiary structures (Guruprasad et al, 1994). This predicted structural similarity was first verified for phytepsin (Kervinen et al, 1999) and more recently for the PSI of *Solanum tuberosum* AP (Bryksa et al, 2011) for a recombinantly expressed product sans its usual AP structural partner. The particular interest in the latter stems from its well-demonstrated anti-pathogen activities both *in vivo* (Guevara et al, 2002; Mendieta et al, 2006; Pagano et al, 2007) and *in vitro* (Muñoz et al, 2010).

The structure-function relationships of the saposin-like domains of plant aspartic proteases have only recently begun to be unraveled despite the large body of research on other saposin-like proteins, as well as their importance to agricultural success and stability, and food security by way of their roles in plant resistance to pathogens. Compared to many saposin-like proteins (SAPLIP) (Bruhn, 2005; Vaccaro et al, 1999), particularly NK-lysin and surfactant protein B and others having long standing recognition for their critical roles in health and potential directed usage in disease treatments (Gaspar et al, 2013), the saposin-like domains of plant aspartic proteases remain largely untouched with respect to their biochemical properties. Throughout the saposin literature, a wide array of different PLs, vesicle sizes and temperatures can be readily found. It was reported that PL composition has an important impact on bilayer disruption (Muñoz et al, 2011), rendering it difficult to compare across platforms. Lastly, temperature is potentially important to PL physical state so variability between projects could be problematic even if studying the same PL vesicles.

Generally, delineating structure-function relationships within a given group of proteins is aided by the existence of highly similar proteins having small sequence differences,
providing an experimental framework in which to operate, and such is the situation presented by plant AP PSIs. The present study compared the PSIs of four species, namely *Solanum tuberosum*, *Hordeum vulgare* L., *Cynara cardunculus* L. and *Arabidopsis thaliana*, in terms of their bilayer interactions and protein structural features. The investigation appears to be the first comparative study of multiple plant saposins wherein all received the same treatments, environmental conditions and LUVs. The results highlight some basic functional differences among the four PSIs despite their highly similar structures.

4.3 Experimental Procedures

4.3.1 Materials - Synthetic genes for each PSI were optimized for expression in *E. coli* and purchased from Mr. Gene GmbH (Regensburg, Germany). Plasmid pET32b(+), *E. coli* Rosetta-gami B (DE3)pLysS, and u-MAC™ columns were obtained from EMD Biosciences (San Diego, CA, USA). *E. coli* TOP10F™ was from Invitrogen (San Diego, CA, USA). GenElute™ Plasmid Miniprep Kit was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The QIAquick® PCR Purification Kit and QIAquick® Gel Extraction Kit were from Qiagen (Germantown, MD, USA). Restriction enzymes, T4 DNA ligase and *Pfu* DNA polymerase were obtained from Fermentas Life Sciences (Burlington, ON, Canada). Primers were synthesized by Sigma Genosys (Oakville, ON, Canada) and thrombin was purchased from Fisher Scientific Co. (Ottawa, ON, Canada). The RPC column was from GE Healthcare (Piscataway, NJ, USA). Phospholipids (PL) were from Avanti Polar Lipids (Alabaster, AL, USA).
4.3.2 PSI Expression and Purification - Sub-cloning the Solanum tuberosum AP gene into pET23b was described previously as was the method used for recombinant expression and purification for all PSIs in the present study (Bryksa et al, 2011). Primers used for sub-cloning the three other PSI synthetic genes for the present study were:

Phytepsin PSI: 5’-atccatggcgggtggttagtcagggttaaacg, and 5’-atctcaggttagccagcacggttaacagtt;
Cardosin A PSI: 5’-atccatggcgggtgatgaaccagcagtgtaaacc, and 5’–atctcaggttagcaggttaacacagt
AtAP PSI: 5’-atccatggcgggtggtttcagcagtgtaaacc, and 5’-atctcaggttagccagcacggttaacagtt

4.3.3 Preparation of Large Unilamellar Vesicles (LUV) - LUV stocks were prepared as per (Bryksa et al, 2011) with the exception that 80 mM calcein / 140 mM NaCl / 25 mM Na-acetate pH 4.5 was used in order to suspend dried PL mixtures at 42º C after removal of storage solvent under nitrogen flush. Phospholipids used were 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphothanol-amine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) for 1:1 POPE:POPS or 1:1:1 POPC:POPE:POPS. For vacuole-like PL mixture vesicles, the relative amounts of each PL are detailed in Table 4.1.

4.3.4 Circular Dichroism Spectropolarimetry (CD) - CD analysis of PSI secondary structure was carried out using a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD, USA). PSIs (10 µM) were scanned over 196-260 nm at 100 nm/min, 0.5 s response, standard sensitivity, and at ambient temperature using a 1 mm pathlength quartz cell.

4.3.5 LUV Disruption Assays - PSI-caused perturbation of LUVs was measured by calcein-loaded vesicles and leakage was detected using a Victor2 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA) or a Shimadzu RF-540 spectrofluorophotometer.
Table 4.1: Phospholipid composition used for preparing LUVmix. The composition mimics those of barley root endoplasmic reticulum, tonoplast and Golgi membrane bilayers (Brown & DuPont, 1989). The commercially available PLs used in the present study were selected for their stabilities at ambient temperature and shelf-life / cost, in addition to their similarities to the lipid mixtures of barley root PLs.

<table>
<thead>
<tr>
<th>PL</th>
<th>Charge</th>
<th>Barley root (%)</th>
<th>Commercial PL Used in the present study</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>0</td>
<td>53.6</td>
<td>Soy Lyso PC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lysophosphatidylcholine (Soy)</td>
</tr>
<tr>
<td>PE</td>
<td>0</td>
<td>23.0</td>
<td>Soy PC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-α-phosphatidylcholine (Soy)</td>
</tr>
<tr>
<td>PS + PI</td>
<td>-1</td>
<td>11.6</td>
<td>18:1 PS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1,2-dioleoyl-sn-glycero-3-phospho-L-serine</td>
</tr>
<tr>
<td>PA</td>
<td>-2</td>
<td>1.9</td>
<td>16:0-18:1 PA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate</td>
</tr>
<tr>
<td>PG</td>
<td>0</td>
<td>6.0</td>
<td>18:1 Cardiolipin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1',3'-bis(1,2-dioleoyl-sn-glycero-3-phospho)-sn-glycerol</td>
</tr>
<tr>
<td>DPG</td>
<td>-2</td>
<td>0.6</td>
<td>16:0-18:1 PG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)</td>
</tr>
<tr>
<td>LPC</td>
<td>0</td>
<td>3.2</td>
<td>16:0-18:1 PE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
</tbody>
</table>
(Shimadzu Corporation, Kyoto, Japan) at 25° C. 200 µL reactions were set up in 96-well microplates or a quartz ultra-microcuvette with varying concentrations of LUVs, 500 nM PSI and 140 mM NaCl / 25 mM Na-acetate pH 4.5. Leakage was detected using excitation at 385 nm and emission at 435 nm. Endpoints were measured by incubating LUVs in 0.5% triton for each condition. Non-linear regression analyses were done using GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA).

4.3.6 Bilayer Fusion Assays - Ten µM PSI or peptide was incubated with 100 nm LUVs (100 µM total PL; 1:1 molar ratio of POPE:POPS) at 25° C in either 25 mM Na-acetate / 140 mM NaCl pH 4.5 or 5 mM Na-phosphate / 140 mM NaCl pH 7.4 (control). Mixtures were then monitored for changes in average LUV size by dynamic light scattering in a Malvern Zetasizer Nano-S (Malvern Instruments, Malvern, Worcestershire, UK) using a disposable polystyrene 1.5 mL semi-microcuvette. Three consecutive measurements of five 30 s runs each were averaged using the refractive index for polystyrene.

4.3.7 Tryptophan Intrinsic Fluorescence Spectrometry - Fluorescence spectra were recorded using a Shimadzu RF-540 spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan) with a 1-cm quartz three-sided ultra-micro cuvette in a water-circulating temperature-controlled cell holder at 25° C. The settings used were λ_{excitation} 295 nm with 3 nm slit width and λ_{emission} scan 300-400 nm with 3 nm or 5 nm slit width. PSI monomer-dimer characterization was done at 10 µM, and PSI/peptide-bilayer scans used 8.5 µM protein and 100 nm LUVs (100 µM total PL; 1:1:1 molar ratio of POPC:POPE:POPS).
4.4 Results and Discussion

4.4.1 Primary Structure Comparison - Primary structure identity as well as charge inversions and regional differences in hydrophobicity were assessed by multiple sequence alignments of StAP, phytepsin, cardosin A and AtAP (Figure 4.1.A) using PRALINE (Simossis & Heringa, 2005). Among the four sequences, identity was 0.61 with a clear majority of the sequences indicating highly conserved regions in terms of both residue type and hydrophobicity. An 11-residue near perfectly conserved sequence between the four PSI species (indicated with a red box in Figure 4.1.B) is located centrally within the C-terminal half of the PSIs, surrounding the singular requisite Trp77 (StAP PSI numbering). Among the four PSIs, some noteworthy differences indicated in Figure 4.1.B are the N-terminal domain Trp unique to StAP (green diamond) as well as various unmatched or inverted charges (red or orange diamonds, respectively) for acidic and basic residues.

The sequences of the canonical SAPLIPs saposin B and saposin C were aligned with the swapped N- and C-terminal halves of StAP PSI (Figure 4.1.C). Relative to other SAPLIPs, helices 1 and 2 of the N-terminal half of saposin are structurally equivalent to PSI helices 3 and 4 of its C-terminal half while H3 and H4 of saposin are equivalent to PSI H1 and H2 (Figure 4.1.C), hence the term “swaposin” (Heinemann & Hahn, 1995). The alignment produced high scoring sequence conservation (49% sequence identity), and secondary structure alignment. Since the vast majority of research to date on SAPLIPs has been done on non-plant sources, most available information must be applied to PSIs through saposin-swaposin residue equivalencies indicated in Figure 4.1.C.
Figure 4.1: PSI primary structure. (A) PRALINE multiple sequence alignments indicating amino acid conservation (top) and type/hydrophobicity (bottom) for AtAP (1), phytepsin (2), cardosin A (3) and StAP (4) PSIs. (B) Multiple sequence alignment generated using CLUSTRAL W for AtAP (1), phytepsin (2), cardosin A (3) and StAP (4) PSIs, identifying charge differences (red diamonds), charge inversions (orange diamonds) and the additional Trp exclusive to StAP PSI (green diamond). (C) PRALINE multiple sequence alignment for human saposin B (1), human saposin C (2) and StAP PSI (3) coloured based on sequence conservation. StAP PSI is presented with its N- and C-terminal halves swapped to align with the saposins.
4.4.2 Bilayer Disruption and Fusion - StAP, phytepsin, cardosin A and AtAP PSIs were initially compared based upon calcein leakage rates for POPE:POPS LUVs at three PL concentrations in buffered saline pH 4.5 (Figure 4.2.A). No statistically significant differences in maximum leakage rates were measured between PSI species at 100 µM and 20 µM PL, respectively (P>0.05), whereas all pairs were significantly different (P≤0.05), respectively, at 500 µM PL. Although all were within an order of magnitude, StAP PSI had the lowest membrane perturbation activity, up to 3-fold lower relative to the other 3 species.

The present study principally used vesicles composed of simple PL mixtures (1:1 POPE:POPS and 1:1:1 POPC:POPE:POPS) as previously (Bryksa et al, 2011; Bryksa & Yada, 2017), however, being a comparison between multiple PSIs, a new PL blend that mimics the PL composition of barley vacuolar plasma membrane (Brown & DuPont, 1989), as outlined above in Table 4.1, was incorporated for making LUVs. As a set of parallel measurements to the aforementioned simpler PL blends, the vacuole-like bilayer was meant to provide a balance between a more realistic substrate for PSIs and simplicity (i.e., no membrane proteins, plant cholesterol, carbohydrates) for optimal signal-to-noise and repeatability.

Although slower than the rates for POPE:POPS LUVs, PSIs were each active against the vacuole-like LUV mix bilayer vesicles, with StAP having significantly higher leakage rate (P≤0.05, respectively) than the other three PSI species (Figure 4.2.B). Thus, the differences in leakage activity among the PSIs were somewhat unclear. As noted above for POPE:POPS vesicles, the PSIs had similar activity levels at 20 µM and 100 µM PL, but significantly higher (P≤0.05) leakage for cardosin A, phytepsin and AtAP PSIs, in that order, at 500 µM PL. Contrasting this were the leakage results for LUV mix where StAP PSI
Figure 4.2: PSI-induced PL bilayer vesicle leakage in isoionic saline buffers at 25° C. Rates were calculated and expressed as relative to the highest data point measured across all data sets. (A) StAP, phytepsin, cardosin A and AtAP PSIs compared at three POPE:POPS concentrations. (B) The same compared using the vacuole-like PL composition LUV_{mix} at 100 µM PL. Error bars indicate +/- one standard deviation.
had much higher activity (Figure 4.2) with the other three PSIs having less than 15% of the relative rate of leakage.

PSI-induced bilayer fusion was characterized by monitoring POPE:POPS LUV size at 25°C in buffered saline pH 4.5 with and without 10 mM DTT (Figures 4.3 and 4.4, respectively). Assays were ended when vesicle size populations became overly polydisperse for DLS measurements. Under native conditions, phytepsin, cardosin and AtAP PSIs all caused markedly faster onset of larger and more complete transitions of the vesicle size populations (Figure 4.3). Within 30 min, both phytepsin and AtAP PSIs caused 100% of the original size population to convert to larger apparent sizes. By contrast, vesicle size increased more gradually for 95-98% of the overall population upon incubation with StAP PSI (i.e., the initial peak broadened incrementally), and 2-5% of the overall population formed relatively large structures (Figure 4.3). Although overall cardosin A PSI-induced size shifting was slower compared to phytepsin and AtAP, it nonetheless also caused enlargement of the entire original population by 20 h. Notably, relatively narrow vesicle size populations were maintained throughout the apparent fusion process (Figure 4.4.C). Also, the action of both cardosin A and phytepsin PSIs on POPE:POPS LUVs resulted in a lack of fused structures much greater in size than 1000 nm. In reducing conditions, fusogenic activity appeared to be virtually unaffected for phytepsin and cardosin A PSIs (Figure 4.3.B-C compared to Figure 4.4.B-C) whereas AtAP activity resulted in a wider range of vesicle sizes relative to non-reducing conditions. StAP PSI action was most affected by the absence of its disulfide bonds compared to the other PSIs. Although StAP PSI produced the narrowest overall vesicle size range in native conditions (Figure 4.3), StAP, phytepsin and AtAP PSIs all produced similar fusion profiles upon
Figure 4.3: Bilayer fusion of 100 μM POPE:POPS as 100 nm LUVs. Size was monitored by DLS 25° C upon incubation with 10 μM of either StAP, phytpepin, cardosin A or AtAP PSI. Three consecutive measurements of five 30 s runs each were averaged using the refractive index for polystyrene.
Figure 4.4: Bilayer fusion of 100 µM POPE:POPS as 100 nm LUVs in reducing conditions. Size was monitored by DLS 25°C upon incubation with 10 µM of either StAP, phytpepsin, cardosin A or AtAP PS. Three consecutive measurements of five 30 s runs each were averaged using the refractive index for polystyrene.
elimination of their disulfide bonds (Figure 4.4). This may implicate a common tertiary structural feature dependent on the PSI disulfide bonds that, when lost, results in a loss of differentiation of function/specificity.

Fusion assays were additionally carried out for vacuole-like LUV\textsubscript{mix} vesicles (Figure 4.5), where all four PSIs were indicated to have fusogenic activity, and phytpepsin PSI being the fastest. Due to the slower fusion rates relative to POPE:POPS LUVs, Figure 5 utilizes a linear diameter scale to better visualize changes, and time points at 5, 15 and 30 min are not shown to improve clarity in these figures. The overall lower bilayer effects compared to the simpler PL mixes was in general agreement with (Muñoz et al, 2011). The lower particle sizes obtained were not surprising in that these vesicles contain approximately half of the total negative charge compared to 1:1 PE:PS bilayers, and therefore, lowered contact incidence would be expected. Phytepsin displayed the highest fusogenic activity, possibly reflective of the fact that the LUV\textsubscript{mix} PL content is based upon barley root vacuolar plasma membranes (Brown & DuPont, 1989). After 9 h incubation, fusion mixtures were reduced with DTT and left for 24 h to verify that PSI-induced vesicle size increases for LUV\textsubscript{mix} vesicles remained stable (i.e., no unexpected profile changes) despite reduction of PSI disulfide bonds. This verification was done in the context of the findings that reduction of PSI disulfide bonds did not prevent vesicle fusion (Fig. 4.4 and 4.5). Similarly, vesicle size increase did not appear to have deviated, qualitatively, from the trend observed through 9 h in non-reducing conditions for the four PSIs.

Since the vacuole-like LUV\textsubscript{mix} vesicles had not been studied previously, the stability of vesicle stocks were assessed upon a 3-month storage period at ambient temperature in
Figure 4.5: Bilayer fusion of 100 µM LUVmix as 100 nm LUVs. Size was monitored by DLS 25° C upon incubation with 10 µM of either StAP, phytepsin, cardosin A or AtAP PSI. Three consecutive measurements of five 30 s runs each were averaged using the refractive index for polystyrene.
buffered saline pH 4.5. No detectable size changes were noted for two separately prepared batches (i.e., 126 vs. 127 nm), while the vesicles leaked only ~16%, indicating that LUV_mix vesicles are highly stable. Although more susceptible to action by PSIs, 1:1 POPE:POPS and POPC:POPE:POPS vesicles were also found to be stable, to a lesser extent, over five weeks (i.e., size increases of ~3% and ~7%, respectively), reinforcing that effects on bilayers observed within experimental timeframes (minutes or hours) were strictly due to PSI.

4.4.3 Comparison of pH-dependence of secondary structure - StAP, phytepsin, cardosin A and AtAP PSIs were compared qualitatively in terms of secondary structure in buffered saline pH 3.0, 4.5, 6.2, and 7.4 (iso-ionic across the four buffers). The CD spectral changes for the respective PSIs as well as inter-species comparisons are presented in Appendices A-B. For all four PSIs, helix content increased with decreasing pH, evidenced by the stronger ellipticity measurements at 222 and 208 nm. In terms of secondary structure differences between the PSIs across pH conditions, cardosin A PSI appeared to have consistently higher helix content while phytepsin appeared to have consistently lower overall helix content across all pH values, based on the relative magnitudes of the negative peaks at 222 and 208 nm. With the possible exception of phytepsin, the distinct secondary structure states between neutral and acidic pH, previously reported for StAP PSI (Bryksa et al, 2011), were consistent with cardosin A and AtAP PSIs (see Appendices A-B) of the present study. Despite the presence of an extra Trp in the N-terminal portion of StAP PSI, which is suspected to influence secondary structure (Bryksa et al, 2011; Lai & Tamm, 2007), CD scans and patterns of CD spectral changes over the pH range studied did not produce clear differences. Although Trp is important to the fusogenic helical boomerang
configuration (Lai & Tamm, 2007), the overall helical content would not necessarily be appreciably altered, possibly explaining the apparent lack of CD spectral characteristics between StAP and the other PSIs.

4.4.4 Intrinsic Trp fluorescence in solution and PSI-bilayer interactions - Intrinsic Trp fluorescence was used to detect evidence for monomer-dimer equilibrium as reported for StAP PSI (Chapter 3). StAP PSI was to thus serve as a known benchmark since it had been measured by analytical centrifugation (Chapter 3). The emission scans are shown in Figure 6 where the distinct two-state pH-dependent curve magnitudes for StAP PSI coincide with its inactive/active pH profile as well as confirmation by analytical centrifugation (Bryksa et al, 2011). The optimal wavelength for emission among the spectra were also plotted for detecting red- or blue-shift as an indicator of changes in Trp environment, showing that \( \lambda_{\text{max}} \) was unchanged across all datasets (P>0.05; data not shown). Although the emission spectra in Figure 4.6 were all measured under the same conditions and for the same PSI concentration (8.5 \( \mu \)M), the absolute fluorescence emission for StAP at its lowest point (pH 7.4) was still almost double the highest signal strength emitted at any pH for the other three PSIs due to the presence of a second Trp. The emission differential was in spite of using an emission slit width of 5 nm for phytepsin, cardosin and AtAP PSIs instead of 3 nm used for StAP (Figure 4.6) to improve signal-to-noise as emission peaked approximately 2.5-fold lower at 3 nm slit width compared to that at 5 nm.

The relatively low emission signals for phytepsin, cardosin A and AtAP PSIs were consistent with saposin C (Wang et al, 2003), but problematic for trying to characterize an increase in signal that was small (~10% increase for the dimer relative to the monomer) for StAP PSI (Figure 4.6). Overall, among the respective emission spectra, no clear evidence
Figure 4.6: Intrinsic Trp fluorescence emission spectra for StAP, phytepsin, cardosin A and AtAP PSIs in buffered saline at varying pH. The respective $\lambda_{\text{max}}$ measurements at the indicated pH values for each PSI are inset, respectively. Error bars indicate +/- standard error.
was presented for an StAP-like monomer-dimer arrangement among the other three PSIs that followed an interpretable pattern, however, such an arrangement is not precluded either. There were differences within the data sets shown in Figure 4.6 (i.e., higher emission at pH 7.4 for cardosin A PSI; lower emission at pH 4.5 for phytepsin PSI; and higher emission for pH 3.0 AtAP PSI). Among these results, the emission increase at lower pH for AtAP was the only one that fit the monomer-dimer model given by StAP PSI, however, this could not be interpreted in the context of phytepsin and cardosin A PSIs having seemingly erroneous emission spectra at pH 4.5 and pH 7.4, respectively (i.e., phytepsin monomer at pH 4.5 and cardosin A dimer at pH 7.4). If these emission changes are reflections of Trp environment then they could not be explained. A possible confounding factor may be differences in local structure proximate to Trp77 (the Trp common to all 4 PSIs) such that solvent exposure differences not directly related to monomer-dimer status cause altered fluorescence emission. Further study of the monomer-dimer status of the phytepsin, cardosin A and AtAP PSIs in solution will require a different experimental design. At present, structural studies are ongoing which will aid in understanding local effects of Trp77.

Spectral changes in Trp fluorescence emission upon encountering 1:1:1 POPC:POPE:POPS LUV bilayer were also compared (Figure 4.7). No $\lambda_{\text{max}}$ shifts were observed for the four PSIs. Kinetic analyses of Trp fluorescence increase were done as a measure of the rate at which PSI equilibrated such that spectra were collected over time periods that approached the endpoints for increasing emission (preliminary spectra were collected well past detectable changes in emission to define these amounts of time; 300–650 s). Data were then normalized to their respective endpoints so that signal strength
Figure 4.7: Trp fluorescence emission of PSIs upon incubation with anionic bilayer vesicles. (A) Intrinsic Trp fluorescence emission spectra for 10 µM PSI with 100 µM 1:1:1 POPC:POPE:POPS LUVs. (B) Kinetics of emission increase indicating equilibration of PSI with bilayer (one-phase non-linear association).
differences between samples were comparable. Individual plots are shown in Figure 4.7.A, and the kinetics for increasing maximum emission measurements for the four PSIs are summarized in Figure 4.7.B. After several scans of consistently increasing emission over the first 200–500 s, spectra began to overlap and eventually superposed, indicating that a new equilibrium state had been reached. The equilibration processes for the four PSIs were calculated for the increase in maximum emission over time yielding the rates listed in the table inset within Figure 4.7.B, suggesting that equilibration was 50% complete within the first two minutes for all PSIs. StAP PSI half-time was 52+/−3 s, and approximately 90% of the change occurred within the first 3 min. In a previous study (Chapter 3) which focused on StAP PSI, timed CD scans in the presence of vesicles was also complete around the 4.5 min mark. The possible link between these two data (i.e., secondary structure change on a similar timeline as tertiary/quaternary structure environment change) will require further investigation.

The lack of \( \lambda_{\text{max}} \) shifting observed for the four PSIs, a result that was previously found for StAP PSI alone (Chapter 3), was unexpected considering the presumed insertion of Trp into the bilayer (Popovic et al, 2012; Qi & Grabowski, 2001; Wang et al, 2003; Willis et al, 2011). Furthermore, all measured \( \lambda_{\text{max}} \) values were notably low (blue-shifted; elevated Trp environment hydrophobicity) compared to previous results for saposins (Popovic et al, 2012; Qi & Grabowski, 2001; Wang et al, 2003). The present findings provide new context to the emission spectral anomalies previously reported (Chapter 3) where it was suggested that a suspected *Internal Stark Effect* (Vivian & Callis, 2001) was manifesting within the H3 region of StAP PSI based upon: (i) the lack of blue shifting in emission spectra despite the presence of a membrane penetrating motif in a proven bilayer-active protein despite
exposure to a favorable bilayer target; and (ii) the presence of Glu19 neighboring Trp18. Since StAP PSI contains two Trp, it was postulated that the Glu-Trp-induced red shift and a concomitant bilayer-Trp-induced blue shift, thereby offsetting expected change in $\lambda_{\text{max}}$. Although the above may be correct for StAP PSI, the present findings from a comparative study that included three single-Trp PSIs may further clarify the phenomena in that phytpepsin, cardosin A and AtAP PSIs all contain Trp77 as their only source of Trp fluorescence emission (via excitation at 295 nm) and none have a neighbouring charged residue. Therefore, all of the elements that made the Internal Stark Effect a possible explanation for the lack of blue shift in emission spectra of StAP PSI are absent for phytpepsin, cardosin A and AtAP PSIs, yet each showed the same lack of blue shift. Speculatively, an alternative explanation for this apparent unchanging high-hydrophobicity status may be that Trp77, upon encountering bilayer, essentially transitions from a dimer hydrophobic pocket (Bryksa et al, 2011) to bilayer interior, effectively trading one highly hydrophobic environment for another. The PSI Trp77-bilayer relationship will be characterized by ongoing protein-bilayer investigations at the atomic scale. Although the study of the saposin-like domains of plant APs is in its infancy, the subtle primary structure distinctions and accompanying subtle differences in bilayer functionalities between the PSIs of the present study are suggestive of the potential for exploiting swaposins to gain detailed insight into membrane-active protein sequences, essential for emerging SAPLIP-related targeted delivery, cell penetrating and surface active biotechnologies (Fonseca et al, 2009; Frauenfeld et al, 2016; Walther et al, 2016).
4.5 References


4.6 Appendices

4.6-Appendix A Comparison of the secondary structures of StAP, phytepsin, cardosin A and AtAP PSIs at different pH in iso-ionic buffered saline.

Figure 4.8: Far-UV CD spectra comparing PSIs at different pH in iso-ionic buffered saline. Spectra are arranged for comparison between pH values for the respective PSIs.
4.6-Appendix B Comparison of the secondary structures of StAP, phytase, cardosin A and AtAP PSIs at different pH in iso-ionic buffered saline

Figure 4.9: Far-UV CD spectra comparing PSIs at different pH in iso-ionic buffered saline. Spectra are arranged for comparison between PSIs at given pH values.
Chapter 5: Concluding Discussion

The experiments of the previous three chapters comprised a series of related protein biophysical and biochemical studies that sought to gain insight into the structural basis of saposin bilayer activity. From the beginning, the overriding focus was to identify structural elements that govern PSI-bilayer interactions. The findings represent a significant improvement to the current state of knowledge concerning the mode of action of the poorly understood and structurally unique plant-derived saposin-like AP domains.

5.1 Further Considerations for PSI-Induced Bilayer Fusion

The use of DLS in conjunction with electron microscopy has long been recognized as reliable for detecting vesicle fusion (Day et al, 1977). Additionally, the use of DLS for detecting surface active protein association with vesicles has been used up to and including a recent study on an amoeboid saposin-like protein (Michalek & Leippe, 2015) as well as for SNARE-induced membrane fusion (Brüning et al, 2013; Castorph et al, 2011; Trivedi et al, 2000; Versluis et al, 2014; Yang et al, 2015). Although the technique has long proven to be useful for assessing vesicle size changes in solution in a practical manner, it is not capable of giving morphological information for LUVs under the required assay conditions. In fact, measuring liposome size by DLS for the assays explicitly assumes that size changes result in approximately spherical particles. Although DLS is capable of assessing changes in particle shape for other experimental setups, tracking liposome size increases requires that the speed of measurements is a priority over precision.

The expectation to visualize PSI-induced changes on bilayers by cryo-TEM initially resulted in empty fields of view or inconsistent and distorted images. This perhaps was
fortuitous because it induced added scepticism for images that would follow, and further consideration of potential experimental pitfalls. After experimental adjustments, various shapes were visible for PSI-treated vesicles (Chapter 3, Figure 3.8), many of which were drastic deviations from control vesicles. It had been anticipated that ~100 nm vesicles would be observed, mixed with an array of approximately spherical liposomes, some several times their original size. This expectation was based on apparent size as measured by DLS, the only indication, to that point, of fusion product changes by PSIs on bilayer vesicles (see Chapter 2, Table 2.3). Initially, high quality images were selected for their contrast and sharpness as well as exemplifying the drastic shape changes between control and test conditions. In re-assessing all images that had been collected for the cryo-TEM experiments, it was subsequently determined that some of the images having superior contrast were in fact showing vesicles that were frozen on the grid support itself as opposed to vesicles in solution frozen within the holes of the grid matrix as desired. This situation causes image distortions due to mobility of material on grid surfaces (as evidenced by streaking and/or odd elongated forms), resulting in unreliable sizes and shapes of mobile vesicles. Additionally, interaction with the grid surface results in artefacts arising from clumping/aggregation of vesicles. This phenomenon is not indicative of true vesicle characteristics regardless of test or control conditions. Phenomena observed for vesicles contacting grid surfaces cannot be considered as reliable. Illustrating this point, a high quality cryo-TEM image selected by the manufacturer of cryo-TEM imaging hardware and software shows clumping and distortion of vesicles on grid surfaces including contact with the edges of the pore/intra-grid space, while those properly situated within the pore showed uniformity of size and spherical shape (Goodwin & Khant).
Limiting consideration to vesicles not in contact with grid surfaces, the results of the cryo-TEM experiments were outlined and discussed previously in Chapter 3, Figures 3.8 and 3.9. Although it remains premature to assert a PSI-induced bilayer disruption scheme with confidence, the cryo-TEM results of Chapter 3 produced vesicle morphologies that were repeated using different samples on different days and in different concentrations/ratios of components. In the light of this reproducibility as well as the context provided by published reports for other bilayer/surface-active proteins/peptides (see the Discussion section of Chapter 3), it seems reasonable to postulate that the observed vesicle morphologies may constitute a coherent sequence of occurrence. It should be noted that the following arrangement is intended strictly as an exploratory supposition which can serve as a reference point for future imaging interpretation and comparison. In Figure 5.1, various distinct liposome shapes from the original cryo-TEM images (Chapter 3, Figure 3.8) have been arranged in a fashion that appears to reveal a progression of morphologies from intact spherical vesicles (upper left) to narrow rod-like objects having a clear lipid bilayer surrounding a dense core (lower left). When considering the scheme in Figure 5.1, consider that quantitation of the various morphologies and their sizes were presented in Chapter 3, including increased size range as well as overall size among the spherical vesicle population, suggesting fusion of spherical LUVs. Thus, the purported progression in Figure 5.1 would either represent phenomena separate from spherical vesicle growth, or possibly the latter constitutes a part of the progression in morphological change. At present, it remains for future investigations to systematically determine morphological changes to PSI-treated bilayers under an array of conditions and concentrations, and significant access to cryo-TEM facilities and technical expertise.
Figure 5.1: Morphologies of PSI-treated LUVs observed by cryo-TEM. The selected shapes are arranged to illustrate an apparent progression of morphological changes.
5.2 Bilayer Activity Via an Isolated Swaposin Structural Region

As discussed in Section 3.5 of Chapter 3, the singular portion of the PSI sub-structure that displayed activity of its own was the penultimate helix, H3, located in the C-terminal half of the overall primary structure. Recall the features of H3:

\[
\text{EAPLCTACEMAVWMQNQLKQ}
\]

That a sub-structure such as H3 was found to be “active” was not surprising in and of itself; in fact, it was hypothesized that the adjacent helix region would display membrane perturbation activity. A similar case for saposin was previously reported (Wang et al, 2003), albeit not in the same mode as the present case with respect to sequence. Furthermore, the fact that H3 is active, and Lys83 is essential for H3 bilayer activity, has now offered a simple and fortuitous case study to consider in future experiments. This is particularly true in consideration that the [Asn/Gln]-[Asn/Gln]-[Ala/Leu/Ile/Val]-[Arg/Lys]-[Asn/Gln] motif, found in H3, is present in many flocculating/coagulating proteins. Flocculating proteins such as Flo must have a positively charged, glutamine-rich portion of the peptide in order to cause flocculation (Suarez et al, 2005). There are variations on the general structural arrangement of having high Gln content, a hydrophobic moiety and at least one positive charge (Lys or Arg) within a relatively short sequence. To the best of our knowledge, the shortest such example that functions as a flocculent protein is β-lactoglobulin fragment 1–8 (Pouliot et al, 2009). Other examples of similar antimicrobial peptides/proteins are listed in Table 3.1.

As discussed in Chapter 3, StAP PSI contains an additional Gln (QNQLKQ) compared to the 2S/saposin 5-residue motif above. The fact that there appears to be just 28 non-redundant protein structures known, listed in the table in Table 3.2 for reference, attests
to its rarity. Furthermore, the apparent commonality of function among all of the known 28 structures surely points to a critical role for this rare motif, and a functionality that likewise presumably is manifested in H3. The seeming differences in modes of action between full PSI and H3, discussed in Chapter 3, may indicate that the functionality of the H3 sequence is available exclusively upon its structural availability for interaction, i.e., steric and/or other conditions that hinder or prevent bilayer- and/or protein-protein interactions. If this is true then presumably the presence of the rare motif, as opposed to a general abundance of amphiphilic character and Asn/Gln residues typical for antimicrobial sequences (Patrzykat & Douglas, 2005), is a chance event since there are no reports of PSI sequence fragments present \textit{in vivo}. Also, unlike StAP PSI, the motif is not present in the three other PSIs from Chapter 4 despite strict sequence conservation among the four species at multiple sequence portions (see Figure 4.1).

Irrespective of \textit{in vivo} roles, the presence of the unique motif in a subdivision of the PSI structure that has now been shown to target bilayers is a situation that deserves further investigation directly on H3 variants by residue and/or motif swapping, based on bioinformatics analyses of an array of species. Gaining insight into the variability among different PSI species will provide context to phenomena observed in the present work including the significance and occurrence of the QNQLKQ sequence among PSI species. As of this writing, knowledge and understanding of biochemical variability among SAPLIPS continues to exist mainly from the perspective of non-plant examples with the exception of StAP PSI, a situation that restrains predicting connections between measured functional differences and observed structural features. The focus of studies in the
immediate future should therefore include a comparative approach for a range of plant SAPLIPs to illuminate the structure-function landscape in which StAP PSI exists.

5.3 Considerations for in vitro Structure-Function Studies on Bioactive Proteins

By necessity, protein structure-function experiments rely on techniques that employ indirect means of detecting and measuring phenomena that exist on a sub-microscopic scale. Critical for techniques that provide indirect sensing of sample states/characteristics is a consistent consideration of the assumptions that underpin interpretations of observed phenomena. Minimally complex, highly predictable assay systems are the ideal experimental environment in which to conduct structure-function work, particularly for research targets that have not seen significant attention. For this reason, the decision was made to rely largely on a simple bilayer system consisting of 100 nm LUVs composed of few phospholipid types. Importantly, the system provided reproducibility, familiarity in terms of existing published findings, and reliability and confidence in observed test signals (i.e., the vesicles did not spontaneously leak or aggregate/fuse, and quality of batch-to-batch vesicle preparations was verifiable).

On the subject of PSI concentrations used throughout the various experiments, a range of PSI/peptide concentrations was used depending on detection limits of the technique and/or strength of leakage activity, mindful of remaining in a biologically-relevant range. All concentrations fell within 0.5-10 µM with the exception of vesicle leakage screening at higher concentrations in order to confirm the absence of activity for peptide/PSI in given conditions (e.g., PSIs at neutral pH, or inactive peptides). The only other exception was the use of 16 µM PSI in conjunction with elevated an phospholipid concentration, 1000 µM,
The above range of PSI/peptide concentrations is well-aligned with published data for StAP PSI antimicrobial activity (Muñoz et al, 2010) (see Table 5.1) as well as a varied selection of other plant antibacterial/antifungal proteins. IC$_{50}$ values of plant antimicrobial peptides (thionins, plant defensins, LTP, and snakins (Broekaert et al, 1997; Segura et al, 1999) against bacterial and fungal pathogens generally range from 0.10 to 5.0 µM. Examples of plant antifungal peptides’ IC$_{50}$ ranges against various fungal plant pathogens relevant to the respective biological situations are summarized in Table 5.2. Thus, the concentration range used in the present study is in line with the biologically-relevant concentrations for a wide variety of plant defense peptides/proteins.

5.4 Final Thoughts on Future Research Directions

Knowledge gained from the investigations detailed herein regarding PSI structural features (Chapter 2), apparent modular design for fusogenic and disruption activities (Chapter 3), and comparison of bilayer functionality among four plant species (Chapter 4) will hopefully contribute to initiating further swaposin structure-function investigations. The identification of H3 as a high interest sequence presents an opportunity to scan not only potato PSI sequence, but putative PSI sequences not yet confirmed. Although the focus of Chapter 3 leaned to the N-terminal portion of PSI due to the activities of H3, the pH-sensitive H1 and H1H2 regions appear to be involved in StAP PSI pH sensitivity which in turn may contribute to the overall pH–dependence of dimerization, critical to PSI activity. This same N-terminal region was identified as being structurally similar to hemagglutinin fusion peptide (Chapter 2), and is expected to be involved in membrane insertion based on its clear structural design as a fusion peptide. To shed light on the
Table 5.1: Biologically active concentrations for StAP PSI. Antimicrobial activities were reported by (Muñoz et al, 2010).

<table>
<thead>
<tr>
<th>Antimicrobial Activity</th>
<th>IC$_{50}$</th>
<th>IC$_{90}$</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of <em>Fusarium solani</em> spore germination</td>
<td>1.3 µM</td>
<td>10 µM</td>
<td>-</td>
</tr>
<tr>
<td>Inhibition of <em>Phytophthora infestans</em> spores</td>
<td>0.25 µM</td>
<td>0.70 µM</td>
<td>-</td>
</tr>
<tr>
<td>Inhibition of <em>Staphylococcus aureus</em></td>
<td>-</td>
<td>-</td>
<td>21.0 µM</td>
</tr>
<tr>
<td>Inhibition of <em>Bacillus cereus</em></td>
<td>-</td>
<td>-</td>
<td>3.5 µM</td>
</tr>
<tr>
<td>Inhibition of <em>Escherichia coli</em></td>
<td>-</td>
<td>-</td>
<td>3.8 µM</td>
</tr>
</tbody>
</table>

IC: Inhibitory concentration, MBC: Minimal Bactericidal Concentration

Table 5.2: Concentration ranges of antimicrobial peptides used *in vitro*.

<table>
<thead>
<tr>
<th>Antifungal peptide</th>
<th>Concentration Range</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histatin</td>
<td>1.4 µM</td>
<td>Kavanagh and Dowd (2004)</td>
</tr>
<tr>
<td>Melittin</td>
<td>6.0 – 25.0 µM</td>
<td>López-García <em>et al.</em> (2000)</td>
</tr>
<tr>
<td>Synthetic hexapeptide 66-10</td>
<td>10.0 – 45.0 µM</td>
<td>López-García <em>et al.</em> (2000)</td>
</tr>
<tr>
<td>Cecropin-derived peptide 2</td>
<td>6.5 – 30.0 µM</td>
<td>Cavallarin <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>French Bean Defensin-Like Antifungal Peptide</td>
<td>3.0 – 4.5 µM</td>
<td>Leung <em>et al.</em> (2008)</td>
</tr>
</tbody>
</table>
energetics and dynamics of the PSI Trp77 transition from protein-protein to protein-lipid contacts, in silico experiments should be designed and carried out. Understanding the factors that govern sequestering of hydrophobic residues within monomeric PSI at neutral (inactive) pH as well as within the dimer hydrophobic core in acidic conditions, and the possible subsequent transition to the bilayer hydrophobic interior is likely key for unravelling the detailed mechanism for saposin bilayer interaction and perturbation.

In the near future, it seems reasonable to expect an expansion of scientific enquiry regarding plant saposins from a biotechnological standpoint, partly because of their possible uses in recombinant technologies as antifungal agents and/or intelligent crop breed selection as a desirable trait for plant pathogen resistance. Two key studies regarding in vitro antimicrobial activities of potato PSI, including human pathogens, made it clear that plant saposins are potentially useful biotechnologically (Mendieta et al, 2006; Muñoz et al, 2010). At present, saposins are already fruitful in terms of wide-reaching and potentially impactful applications (Fonseca et al, 2009; Frauenfeld et al, 2016; Kaimal et al, 2011; Qi, 2010; 2012; Qi et al, 2009; Walther et al, 2016). The plant kingdom is not only potentially a near limitless source of biochemical variability, it can also be a source of evolutionarily-ancient template sequences and structures. One would expect expansion into the plant saposin realm by researchers with envisioned saposin-based biotechnological designs, seeking a broader base of natural templates and exotic or possibly unique motifs and/or activities. Exploiting the variability between highly similar plant saposin family members offers the potential to gain a fundamental understanding of how plants employ these critical defense proteins for different purposes/situations. In turn, such knowledge will enable predictive structure redesign / engineering based upon the hundreds, if not thousands, of
putative PSI gene sequences already stored in the world’s plant genome projects, ready to serve as templates for understanding the roles of plant SAPLIP in issues related to food security, agricultural disease control and sustainability, and possibly human disease treatment.
5.5 References


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