Recombinant expression and characterization of *Solanum tuberosum* aspartic protease

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Unique to aspartic proteinases from plants are the presence of approximately 100 amino acid regions, which are usually excised during activation of the zymogen. These sequences are termed 'Plant-Specific Inserts' and are implicated in membrane interactions of their parent enzymes, including vacuolar targeting and host defense. The need to further characterize the structure-function role(s) of plant-specific inserts stimulated the current study of the characterization of *Solanum tuberosum* AP (StAP). Recombinant expression of wild-type StAP resulted in the 54 kDa protein being visualized by SDS-PAGE analysis with a protein yield of 0.03%. A protein purification factor could not be established since activation of the protein at pH 2.2, 3.0, 3.7 and 5.5 was not achieved as evidenced by a lack of change in band patterns on SDS-PAGE as well as acidification and hemoglobin degradation assays. To potentially improve enzyme folding and activation ability, two mutants, (1) lacking the pre-signal sequence and (2) lacking both the signal sequence and the prosegment, were designed, sub-cloned, and expressed. Both products proved to be insoluble and inactive. New constructs were designed for the expression of StAP inclusion bodies for insoluble expression and subsequent re-folding of the protein. Additionally, Cys→Ala mutations for each PSI Cys residue were made to investigate the role(s) of plant-specific insert disulfide bonds in plant aspartic proteinase enzyme folding and structure. All PSI cysteine mutations (eight mutants) were successfully created using QuickChange Mutagenesis™.

Aspartic proteases (APs) are a ubiquitous, heterologous class of proteinases that have been extensively studied.¹ APs are characterized by having a prosegment that is cleaved when the zymogen is activated. Although APs display differences in activities and specificities, they share several common characteristics, including the fact that they contain two catalytic aspartate residues within two amino acid stretches that share a high degree of homology.^{1,2} The catalytic motifs are Asp-Thr-Gly and Asp-Ser-Gly in all plant APs belonging to the A1 family of proteases.³ APs also have catalytic activity at low pH, a preference for hydrophobic amino acids, and are most often inhibited by pepstatin A.^{1,2} In addition, these proteases are characterized by a hydrophobic N-terminal signal sequence responsible for translocation into the ER, followed by a 40 amino acid prosegment that is cleaved upon zymogen activation.⁴ Currently, plant APs have not been studied as extensively as their mammalian counterparts. Known biological functions of plant APs are limited, and there is inadequate structure availability.^{1,2,4,5} Unique to plant APs, is the presence of a 100 amino acid sequence known as the plant-specific insert (PSI).² The PSI is an independent subunit of five amphipathic α -helices linked by three disulfide bridges inserted into the C-terminal domain.² The PSI has been found to have high homology with mammalian saposins which are lysosomal, sphingolipid-activating proteins involved in membrane

degradation.⁵ The PSI has been found to exhibit antimicrobial activity by inducing membrane leakage.⁵ A membrane-destabilizing domain of the PSI containing a high hydrophobic region possibly causes lipid disruption in vesicle membranes and resulting in pore formation and eventually cell death.^{5,6} It has also been suggested that the PSI is involved in vacuolar targeting of APs and is excised during activation.^{1,3}

Only two crystal structures have been established for plant APs, including cardosin A and prophytpsin.³ Cardosin A, from *Cynara cardunculus* L. (Cardoon), is a milk-clotting AP used in cheese-making. The Cardosin precursor, Procardosin A, undergoes proteolytic processing as the cardoon flower matures. During this maturation, likely to occur in vacuoles, the PSI is removed before the removal of the prosegment.⁶

The present study seeks to further characterize the PSI in terms of its potential roles in membrane interactions and protein structure and function using *Solanum tuberosum* AP (StAP) as a model plant AP system. StAP has been shown to retain its PSI upon activation,⁵ thereby offering an opportunity to study PSI roles in an active AP conformation. The gene for StAP has previously been found to contain a coding region of 1494bp long including 497 amino acids out of a predicted 54kDa molecular mass.⁵ Neither StAP crystal structure nor the characterization of the StAP activation

process have been studied to date –that is, both areas are poorly understood among plant APs. Guevara et al. determined that StAP possesses antimicrobial activity against *P.infestans* possibly by inducing membrane destabilization.⁴ Additionally, the disulfide bonds present in the PSI are proposed to function as a stabilizing factor for the AP.⁸ The goal of the present study was to express, purify, and partially characterize wild-type StAP proteolytic activity and activation in anticipation of a final crystal structure. Also, cysteine mutations were created in order to better understand the role of disulfide linkages within the PSI structure in StAP folding / structure, activation and activity.

MATERIALS AND METHODS

Materials

QuickChange Site-Directed Mutagenesis™ kit purchased from Stratagene (La Jolla, CA). Plasmid isolation kit purchased from Sigma (Oakville, ON). All primers were ordered from Sigma Genosys (Oakville, ON). *Pfu* Polymerase and PageRuler™ Pre-Stained Protein Ladder purchased from Fermentas Life Sciences (Burlington, ON). DC Protein Assay kit purchased from Bio-Rad Laboratories (Mississauga, ON). StAP sequence optimized for *E.coli* expression was purchased from Mr. Gene (Regensburg, Germany). pET vector system purchased from END Biosciences (San Diego, CA). All other reagents and supplies were purchased from Fisher Scientific (Ottawa, ON).

PSI Mutations

The vector used to clone the optimized StAP sequence was a modified version of pET32b+, containing a deletion of the S-Tag and enterokinase coding sequences. Primers for Cys→Ala mutagenesis were produced for each Cys residue of the PSI region of StAP. Cys→Ala mutations were located at C271A, C306A, C331A, C337A, C368A, C371A, C399A, and C413A corresponding to full-length (including signal sequence) StAP numbering. Primers were designed using PrimerX for QuickChange™ site-directed mutagenesis (Table 2).

Sub-cloning of truncated StAP forms

StAP was produced without its pre-signal sequence (-P) in addition to StAP with pre-signal and prosegment deletion (-PP) in an attempt to increase solubility of the protein. Primers (Table 2) were ordered to clone -P StAP and -PP StAP into pET32(z)+ and a PCR reaction was completed using; 5uL of 10x *Pfu* MgSO₄ reaction buffer, 0.5uL (250ng) of oligonucleotide fwd/rev primer, 1uL dNTP mix, 1uL *Pfu* DNA polymerase (2.5U/uL), 1uL dsDNA template (50ng/uL), and 41uL of ddH₂O. Cycle parameters consisted of denaturing at 95°C for 30s followed by 18 cycles of 94°C (30s), 58°C (1 min) and 72°C (15 min).

Construct verification

Mutagenesis / ligation reactions were transformed into *E.Coli* TOP10F' competent cells using the Hanahan method. DNA sequencing was done by ACC Genomic Facility (Guelph, ON) to verify mutations. If correct, transformations were transformed into expression strain *Rosetta Gami B DE3 pLysS* (RG) using 1uL of stock plasmid DNA. Stock cultures were stored in 20% glycerol at -80°C.

Expression of StAP

One litre LB (Ap,Kn,Cm,Tet) was inoculated with 25mL overnight culture and grown at 37°C. Cultures were induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD₆₀₀ reached between 0.5-0.8. Cultures were further incubated at 30°C for 3h. Cells were collected by centrifugation at 4,800 x g at 4°C for 6min. Cell pellets were stored at -20°C. Additionally, -P StAP and -PP StAP strains were expressed at RT and 30°C to determine if expression of soluble fusion protein increased at lower expression temperature.

Purification of StAP

Frozen expression pellets were resuspended in 20mL 20mM Tris-HCl pH 7.5 per L original expression culture. Samples were rotated for 60min at RT to allow cell lysis (via pLysS). To reduce viscosity, 1uL Benzonase-Nuclease (EMD Biosciences, San Diego, CA) was added for every 40mL of solution. The reaction mixture was continuously rotated at room temperature for 15min, after which the solution was then centrifuged at 4,800 x g at 4°C for 10min, after which the supernatant was re-centrifuged at 21,000 x g at 4°C for 30min.

Supernatant was loaded onto an Umac cobalt column (EMD Biosciences, San Diego, CA) using a Bio-Rad Econo Pump. Sample was washed with Buffer A (20mM Tris/300mM NaCl pH 8.0) until the absorbance stabilized. Buffer B (20mM Tris/300mM NaCl/250mM Imidazole pH 8.0) was added at 4%, 10% and 100% step intervals to purify desired His-tagged fusion protein (StAP). Fractions containing StAP were dialyzed overnight in 20mM Tris-HCl pH 7.5 (total dilution factor 40,000-fold) in Fisher Brand dialysis tubing (12,000-14,000 MWCO) and protein concentration was assessed using DC protein assay (Bio-Rad, Mississauga, ON). Thrombin (0.02mg/mL stock) was added to the dialysate at a protein mass ratio of 3000:1. Samples were left overnight on a rotator and aliquots were taken at 2 and 24 hours to view cleavage progress. Cobalt affinity HPLC was used to separate released StAP (no His-tag) from fusion partner thioredoxin and uncleaved fusion protein (His-tag) by collecting flow-through. Samples were re-dialyzed as described above. Samples were loaded onto a 1mL MonoQ column (GE Healthcare), washed with 20mM Tris-HCl pH 7.5, and eluted using a gradient 20mM Tris-HCl, 2M NaCl, pH 7.5.

Figure 1: Bottom: Western blot using HisProbe™ to detect His-tagged protein; Top: same blot stained with Coomassie G-250. Lane 1: Crude protein; Lane 2: Dilute crude protein; Lane 3-5: WP StAP post Co-HPLC; Lanes 6-8: a second isolate WP StAP Co-HPLC elution

Activation full length, -P and -PP StAP

Post-thrombin StAP was acidified by addition of the following buffers at the indicated concentrations to test for autoactivation similar to the method of Glathe et al.⁷ 50mM Glycine-HCl pH2.2, 50mM Na-citrate pH 3.0, 50mM Na-acetate pH 3.7, and 50mM Na-acetate pH 5.5. Samples at various stages (time and pH) of experimentation were analyzed using 12% and 18% SDS-PAGE to identify band shifting resulting from acidification. Aliquots were taken at 1 h, 5 h, and 24 h. Acidified StAP samples at the same pH values were also prepared in the presence of 1 mg/mL hemoglobin substrate to test for enzymatic activity

Refolding of -P StAP and -PP StAP

After 24h dialysis in 20mM Tris-HCl pH 7.5, -P StAP fusion protein solution samples appeared cloudy. 50uL of sample was centrifuged for 10 min 14,000 x g to visualize insoluble protein on SDS-PAGE. The supernatant was collected and the pellet washed twice with 20mM Tris-HCl pH 7.5. Insoluble -P StAP pellets were refolded using 8M urea containing 100mM β -mercaptoethanol as done with Cardosin A by Castanheira et al.⁸

RESULTS AND DISCUSSION

First attempts at mutagenesis were successful for C271A, C306A, C331A, C368A, and C413A StAP, however, C337A, C371A and C399A StAP yielded wild-type. A second attempt at mutagenesis yielded successful C337A and C371A mutants. After reviewing primers for C399A primers, secondary structures that could interfere with mutagenesis reaction were identified and thus a new primer was designed and used yielding C399A StAP. Wild-type and mutants were transformed using *E. coli* strain RG. Wild-type, and truncated, StAP were expressed as described in the Materials and Methods section.

When purifying StAP using cobalt affinity HPLC, it was found that washing using 4%, 10% buffer B, followed by eluting at 100% buffer B, yielded improved purity relative to washing using only Buffer A (data not shown). Only 100% B eluate contained target fusion protein recovery was determined to be 0.03% of the total soluble crude protein (Table 1). Fusion protein samples were dialyzed prior to thrombin cleavage to remove imidazole in order to allow for a second Cobalt affinity HPLC step, allowing for the retention of His-tagged

Figure 2: 12% SDS-PAGE. WT StAP isolation from fusion tag; Lanes 1-3: Co HPCL eluate fractions post-thrombin; Lanes 4-6: Flow-through Co HPCL fractions post-thrombin; Lanes 7-9: Co HPCL eluate fractions post-thrombin. The solid arrow indicates uncut Trx-StAP fusion protein MW; the white arrow indicates StAP MW.

Figure 3: 18% SDS_PAGE; Lanes 1 and 3: -P StAP post-Co HPLC; Lanes 2 and 4: Post thrombin cleavage -P; Lanes 5 and 8: -PP StAP post -Co HPLC; Lanes 6 and 7: Post-thrombin cleavage -PP; Lanes 9 and 11: Soluble fraction -P StAP; Lanes 10 and 12: insoluble fraction -P StAP

Figure 4: 18% SDS-PAGE; Hemoglobin degradation assays after 17h incubation at 37°C; lanes 1-4: Controls for hemoglobin acidification tests at pH 2.2, 3.0, 3.7 and 5.5 respectively; Lanes 5-8: -P StAP at the same pH values, respectively; Lanes 9-12: -PPStAP at above pH values.

Figure 5: 18% SDS-PAGE. Lane1: -P StAP post-thrombin soluble fraction; Lane 2: Post-thrombin -P StAP soluble fraction; Lane 3: -‘refolding’ -P StAP; Lanes 4-7 and 8-11 represent 2h and 18h acidification of ‘refolded’ -P StAP, respectively.

thioredoxin, while target StAP was allowed to flow through (Figure 2). Following dialysis and further HPLC of the cleaved wildtype StAP, a new band on SDS-PAGE appeared corresponding to the molecular weight of wild-type StAP (54.3 kDa). An additional band appeared just below 70kDa corresponding to the uncleaved fusion protein (68.2kDa). Therefore, thrombin cleavage was incomplete. Thioredoxin tag containing the His-tag and thrombin cleavage site was visualized just below the 17 kDa standard. After attempting activation of the wild-type StAP at pH 2.2, 3.0, 3.7 and 5.5 similar to the method of Glathe et al.⁷ no band shifting was observed, i.e., a band shift indicates cleavage of the zymogen to produce mature enzyme (data not shown). Furthermore, no hemoglobin proteolysis was apparent under the above pH condition upon incubation for up to 48 h at 37°C.

In an attempt to improve StAP folding and activation ability, two truncated forms were created, -P and -PP, eliminating the pre- signal sequence (Gly25 N-terminus) and both the pre- signal sequence and the prosegment (Asp68 N-terminus), respectively. The signal sequence in StAP consists of residues 1-24.⁵ AP signal sequences are removed

prior to activation,⁹ thus it was hypothesized that -P StAP expression may result in an enzyme capable of activation *in vitro*. -PP StAP was designed to test if StAP requires prosegment for correct folding as active form StAP has been shown to be stable at non-acidic pH values.¹⁰ Expression levels of both StAP truncated forms at room temperature versus 30°C were compared to determine if lowered induction temperature improved target protein expression. -P StAP RT and 30°C contained 1.8 mg and 2.9 mg total protein, respectively. -PP StAP RT and 30°C samples contained 0.50 mg and 3.1 mg total protein, respectively (Table 1). Thus, RT induction yielded lower total protein content, however, SDS-PAGE revealed that RT induction yielded increased purity as fewer contaminant bands were visualized (data not shown).

Fusion protein masses for -P-Trx and -PP-Trx were 66 kDa and 61 kDa, respectively. Thrombin treatment of the truncated StAP forms (-P and -PP) produced the expected new bands at approximately 52kDa for -P StAP and 47kDa for -PP StAP (Figure 3). Upon dialysis of -P StAP with 20mM Tris-HCl pH 7.5 and thrombin cleavage, the sample

Table 1: Protein purification table for WT Trx-StAP partial purification. Protein purification factor could not be calculated in the absence of specific activity measurements. –P and –PP samples are also shown for comparison to WT StAP

	Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Recovery (%)
Crude Trx-StAP	150	3.0	450	100
Co Elution WT	1.5	0.83	1.2	0.027%
Co Elution -P StAP RT	3.0	0.98	2.9	-
Co Elution -P StAP 30°	3.0	0.61	1.8	-
Co Elution -PP StAP RT	2.5	0.20	0.50	-
Co Elution -PP StAP 30°	3.5	0.89	3.1	-

Table 2: Designed primers using PrimerX for QuickChange™ site-directed mutagenesis. Primers were ordered to clone –P StAP and –PP StAP into pET32(z)+

C271A mut primer 37bp	
Fwd	GATATTGTGCTGGCGGTGCCGCTGCTATTGTGGATTC
Rev	GAATCCACAATAGCAGCGGCACCGCCAGCACAATATC
C306A mut primer 43 bp	
Fwd	GATTGTAAGCATGGAGGCCAAAACCTATTGTTAGCCAGTATGG
Rev	CCATACTGGCTAACAAATAGTTTGGCCTCCATGCTTACAATCC
C331A mut primer 27 bp	
Fwd	CCTGACCAAGTGGCTTCACAGGCTGGG
Rev	CCCAGCCTGTGAAGCCACTTGGTCAGG
C337A mut primer 32 bp	
Fwd	CACAGGCTGGGCTGGCTTTTGTGATGGTGCC
Rev	GGCACCATCAACAAAAGCCAGCCCAGCCTGTG
C368A mut primer 33 bp	
Fwd	GGAGAAGCACCTCTGGCCACAGCATGTGAGATG
Rev	CATCTCACATGCTGTGGCCAGAGGTGCTTCTCC
C371A mut primer 29 bp	
Fwd	CTCTGTGTACAGCAGCTGAGATGGCCGTG
Rev	CACGGCCATCTCAGCTGCTGTACACAGAG
C399A mut primer 37 bp (1)	
Fwd	GAGTATGTGAATCAACTGGCTGAAAAAATCCCGAGTC
Rev	GACTCGGGATTTTTTCAGCCAGTTGATTACATACTC
C399A mut primer bp (2)	
Fwd	CTGGAGTATGTGAATCAACTGGCGGAAAAAATCCC
Rev	GGGATTTTTTCCGCCAGTTGATTACATACTCCAG
C413A mut prime 38 bp	
Fwd	GTGAGTCCGCTATTGACGCCAATAACATCAGCTCAATG
Rev	CATTGAGCTGATGTTATTGGCGTCAATAGCGGACTCAC
P StAP Pimers 26 bp	
Fwd	GACCATGGGAGATCTGCTGCGTATTG
Rev	ATCTCGAGTTAAAGGCTATTATGCACTTCAGCAA
PP StAP Pimers 26 bp	
Fwd	GACCATGGACTCCGACATCGATATTG
Rev	ATCTCGAGTTAAAGGCTATTATGCACTTCAGCAA

appeared 'cloudy' suggesting loss of solubility of StAP upon release from the highly soluble fusion partner thioredoxin (Trx). Centrifugation yielded an insoluble protein pellet. The pellet was solubilized in SDS-PAGE sample buffer and compared to the sample's soluble fraction on SDS-PAGE. Results indicated that the majority of the desired -P StAP became insoluble upon thrombin cleavage and dialysis (**Figure 3**). Apparently -P StAP solubility was dependent on the Trx fusion tag.

Activation of -P StAP and -PP StAP was attempted at pH 2.2, 3.0, 3.7 and 5.5 as used for full-length StAP. No clear band shift was visible suggesting that autoactivation of -P StAP protein had not occurred (data not shown). -PP StAP would not be expected to change masses as no prosegment was present by design. Additionally, proteolytic activity testing by hemoglobin degradation assays for -P and -PP StAP resulted in no detectable change in band patterns on SDS-PAGE as compared to the controls indicating no activity (**Figure 4**).

After dialysis, -P StAP was insoluble as noted above. Pellets collected from -P StAP centrifugation, as well as post-thrombin (soluble) -PP StAP sample were subjected to the denaturing conditions of 8M urea and 100mM β -mercaptoethanol, followed by washing 3 times using 60mL 20mM Tris-HCl pH 7.5 via dia-filtration (27,000-fold total dilution factor). Activation was again attempted at four different pH values and subsequent hemoglobin degradation assays were completed. No bands were visible on a SDS-PAGE gel (Fiomg. 5) indicating that the protein reprecipitated upon washing with 20mM Tris-HCl pH 7.5 during refolding.

FUTURE EXPERIMENTATION

Future studies will attempt to obtain pure, folded StAP via expression in *E.coli* BL21 from refolded protein inclusion bodies, as refolding of protein inclusion bodies has been successfully used for Cardosin A.⁸ Expression of soluble StAP could be attempted using a Baculovirus-infected insect cell expression system. Such expression systems sometimes produce high levels of soluble target protein while allowing for post-translational modifications. Such modifications may help with the folding of StAP.

REFERENCES

1. Payie, K., Tanaka, T., Gal, S., and Yada, R. (2003) Construction, expression and characterization of a chimaeric mammalian-plat aspartic proteinase. *Biochem. J.* **372**, 671-678.
2. Mazorra-Manzano, M. and Yada, R. (2008) Expression and characterization of the recombinant aspartic proteinase A1 from *Arabidopsis thaliana*. *Phytochemistry*. **69**, 2439-2448.
3. Simoes, I. and Faro, C. (2004) Structure and function of plant aspartic proteinases. *Eur. J. Biochem.* **271**, 2067-2075.
4. Mendieta, J.R., Pagano, M.R., Munoz, F.F., Daleo, G.R. and Guevara, M.G. (2006) Antimicrobial activity of potato aspartic proteases (StAPs) involves membrane permeabilization. *Microbiology*. **152**, 2039-2047.
5. Guevara, M.G., Almeida, C., Mendieta, J.R., Faro, C.J., Verissimo, P., Pires, E.V. and Daleo, G.R. (2005) Molecular cloning of a potato leaf cDNA encoding an aspartic protease (StAsp) and its expression after *P.infestans* infection. *Plant Physiology and Biochemistry*. **43**, 882-889.
6. Pereira, C., da Costa, D.S., Pereira, S., de Moura Nogueira, F., Albuquerque, P.M., Teixeira, J., Faro, C. and Pissarra, J. (2008) Cardosins in postembryonic development of cardoon: towards an elucidation of the biological function of plant aspartic proteinases. *Protoplasma*. **232**, 203-213.
7. Glathe, S. Kervinen, J., Nimtz, M., Li, G.H., Tobin, G.J., Copeland, T.D., Ashford, D.A., Wlodawer, A. and Costa, J. (1998) Transport and activation of the vacuolar aspartic proteinase phytepsin in barley (*Hordeum vulgare* L.) *J. Biol. Chem.* **273**, 31230-31236.
8. Castanheira, P., Samyn, B., Sergeant, K., Clemente, J.C., Dunn, B.M., Pires, E., Van Beeumen, J. and Faro, C. (2005) Activation, proteolytic processing, and peptide specificity of recombinant cardosin A. *J. Biol. Chem.* **280**, 13047-13054.
9. Runeberg-Roos, P., Kervinen, J., Kovaleva, V., Raikhel, N.V. and Gal, S. (1994) The aspartic proteinase of barley is a vacuolar enzyme that processes probarley lectin in vitro. *Plant Physiol.* **105**, 321-329.
10. Guevara, M.G., Daleo, G.R. and Oliva, C.R. (2001) Purification and characterization of an aspartic protease from potato leaves. *Physiologia plantarum* **112**, 321-326.