Aspartic proteinases are a well-characterized class of proteinases. In plants, all nascent aspartic proteinases possess a 100-amino-acid, plant-specific sequence (PSS) within their C-terminal lobe, presumed to possess a targeting role in vivo. In this study, the PSS domain from the Arabidopsis thaliana aspartic proteinase was inserted into porcine pepsinogen at the identical location found in nascent plant aspartic proteinases, to create a chimaeric mammalian–plant enzyme. Based on enzymic activity, this chimaeric enzyme demonstrated increased in pH stability above 6 and temperature stability above 60 °C compared with commercial pepsin. Differential scanning calorimetry of the chimaeric enzyme illustrated an approx. 2 °C increase in denaturation temperature (T_m), with increases in co-operativity and similar enthalpy values.

Kinetic analysis indicated an increase in K_m and decreased k_cat compared with pepsin, but with a catalytic efficiency similar to the monomeric plant aspartic proteinase from wheat. Using oxidized insulin B-chain, the chimaeric enzyme demonstrated more restricted substrate specificity in comparison with commercial pepsin. This study highlights the use of a chimaeric enzyme strategy in order to characterize unique protein domains within enzyme families, and, for the first time, a putative structure–function role for the PSS as it pertains to plant aspartic proteinases.

Key words: aspartic proteinase, chimaeric enzyme, pepsin, structure–function.
oxidized insulin B-chain and methyl-D-α-mannopyranoside were purchased from Sigma (St. Louis, MO, U.S.A.). Concanavalin A was purchased from Amersham Biosciences (Baie D’Urfé, Quebec). *Escherichia coli* biochemical use.

CA, U.S.A.). All other chemicals were of analytical grade for vector pHIL-S1, were purchased from Invitrogen (San Diego, CA, U.S.A.).

**Cloning and mutagenesis**

The following oligonucleotide primers were synthesized for the QuikChange mutagenesis procedure (Stratagene, La Jolla, CA, U.S.A.). Porcine-N-1, 5'-GAC ATC GGA GCC GCC GCC ACC TCA TAT GG-3'; Porcine-N-2, 5’-GCC ATA TGA GGT CGC GCC GCC CGC TCC GAT GTC G-3'; Porcine-C-1, 5’-GGA GCA GCT TAA GAC CAT CTC GCC-3'; Porcine-C-2, 5’-GGC GAG ATG GTC TTA AGC TGC TGC TCC-3'; PSS-N-1, 5’-CAT GCT ATT GGA GCC GCC GGA GTT GTT AGC-3'; PSS-N-2, 5’-GCT AAC AAC TCC GGC CGC TCC AAT AGC ATG-3'; PSS-C-1, 5’-GGA GAG TAT GCC TTA AGC TGC TGC TCC GAT GTC G-3'; PSS-C-2, 5’-CAG TTG TGC TGC TTA AGC TGC TCC GAT GTC G-3'; PSS-C-3, 5’-GGA TCT GCC TTA AGC TGC TGC TCC GAT GTC G-3'; PSS-C-4, 5’-CGC TCG AGT ATT CAT GCC TCC GAT GTC G-3'; PSS-C-5, 5’-CC ATA TGA GGT CGC GCC GCC GGC TCC GAT GTC G-3'; PSS-C-6, 5’-GGA GCA GGC GAG GCC GCC GCC ACC TCA TAT GG-3'; PSS-C-7, 5’-GGA GCA GGC GAG GCC GCC GCC ACC TCA TAT GG-3'.

The cDNA for the porcine pepsinogen was subcloned into the vector pHIL-S1 resulting in plasmid pPSP2000, which was used to transform *E. coli* TOP10F cells [16]. Selection of the transformants containing the chimaeric aspartic proteinase sequence was performed using the 5' AOX1 and 3' AOX1 primers (Invitrogen). The amplified fragment was purified from the agarose gel and confirmed to contain the complete chimaeric sequence by sequencing.

**PSS insertion into porcine pepsinogen**

Chimaeric construction was performed by ligating three independent DNA portions, corresponding to the N-terminal fragment of porcine pepsinogen, the PSS and the C-terminal fragment of porcine pepsinogen, in a stepwise fashion. The successful ligation was purified and amplified using the EcoRI-F and XhoI-R primers. The chimaeric DNA fragment was purified from the agarose gel and double digested with EcoRI and XhoI restriction endonucleases. The chimaeric sequence was ligated into the pHIL-S1 vector. The ligation reaction was transformed into *E. coli* TOP10F and selected using LB agar with ampicillin (150 μg/ml). Colonies were selected, screened by restriction digestion and sequenced. The plasmid containing the chimaeric aspartic proteinase sequence was renamed pKGP2.

**Expression using P. pastoris**

The pKGP2 plasmid was linearized using the restriction endonuclease *SalI*, electroporated into *P. pastoris* (KM71) cells according to the manufacturer’s instructions (Invitrogen) and expressed as described in [14]. The clones that produced supernatants that were positive upon activation for milk-clotting activity and via Western blot, using the anti-pepsinogen antibody, were frozen at −86°C in the presence of sterile glycerol (25% final concentration). One clone (KP1-2), which was positive using both screening methods, was grown on a large scale and used for further purification and characterization (see below). The genomic DNA of *P. pastoris* KP1-2 was isolated using a Qiagen DNA-extraction kit. Amplification of the integrated region containing the chimaeric aspartic proteinase sequence was performed using the 5' AOX1 and 3' AOX1 primers (Invitrogen). The amplified fragment was purified from the agarose gel and confirmed to contain the complete chimaeric sequence by sequencing.

**Chimaeric aspartic proteinase purification**

After 72 h of incubation in BMIMY medium (buffered methanol complex medium) at 30°C (supplemented with 0.75% methanol every 24 h), the *P. pastoris* cells were pelleted at 3000 g for 5 min at 4°C. The supernatant (containing the chimaeric enzyme) was removed and concentrated via ultrafiltration using a 30 kDa molecular-mass-cut-off membrane and washed with 1 litre of 20 mM Tris/HCl. The concentrated post-(NH4)2SO4 material (50 ml) was applied five times to a 20 ml concanavalin A-Sepharose column and the column washed with 20 mM Tris/HCl, pH 7.5, with 0.5 M NaCl. Elution was performed using 0.4 M methyl-D-α-mannopyranoside in 20 mM Tris/HCl, pH 7.5, with 0.5 M NaCl. The eluted material was concentrated using a Centricon YM-30 with 20 mM Tris/HCl, pH 7.5. Anion-exchange chromatography was performed on the post-concanavalin A–Sepharose sample using an HR 5/5 Source Q column (Amersham Biosciences) equilibrated with 20 mM Tris/HCl and 30 mM NaCl, pH 7.5, at a flow rate of 1 ml/min−1. Elution from the column was performed using an increasing NaCl gradient from 0.03 to 1 M over 80 min. Fractions were collected manually, activated and assessed for milk-clotting activity. Those fractions possessing activity were concentrated (Centricon YM-30) and tested via Western blot using an anti-pepsinogen antibody, and used for further enzyme characterization.

**SDS/PAGE and Western blotting**

All steps of the purification were assessed by SDS/PAGE [17] using 10% Bio-Rad Ready Gels and stained using either SYPRO Ruby Gel stain or SYPRO Orange G stain (Bio-Rad), or transferred to a PVDF membrane (Bio-Rad) for Western blotting [18]. For N-terminal analysis, the post-electroblotting membrane was stained with Coomassie Brilliant Blue R solution and prepared as described in [19].

**Enzyme assays and activation**

Activation of both the chimaeric aspartic proteinases and commercial porcine pepsinogen was initiated unimolecularly as described in [20] for 30 min periods, and the released
prosegment removed using a Centricon YM-10 filtration unit (10 kDa molecular-mass-cut-off). Protein concentrations were assessed in duplicate using the Bio-Rad DC assay and absorbance calculations at 280 nm [21]. Preparations of the chimaeric aspartic proteinase and commercial pepsinogen were activated and kinetic measurements were made using the synthetic substrate 1 [SS1; Lys-Pro-Ala-Glu-Phe-Phe(NO3)-Ala-Leu], synthesized at Queen’s University, Kingston, ON, Canada [20]. For kinetic studies, a minimum of 10 SS1 concentrations in the range 0.02–0.35 mM, pH 1.5, at 25 °C (determined to be the pH optimum of the chimaeric enzyme) were used. The analysis consisted of monitoring the change in absorbance at 300 nm using a DU 640 Spectrophotometer (Beckman Instruments, Fullerton, CA, U.S.A.), where progress curves were measured to give a change in absorbance/min. $K_m$ and $V_{\text{max}}$ values were estimated using non-linear regression [20]. The $k_{\text{cat}}$ values were estimated using the estimated $V_{\text{max}}$ values and the protein concentration from spectrophotometric measurements at 280 nm [21]. Three independent samples assessed in triplicate were used for kinetic constant determination with means and S.D. calculated.

Stability studies

The purified pepsin, or chimaeric enzyme, was activated as described in the previous section and tested for pH or temperature stability. Activity measurements using SS1 were obtained by independently incubating each enzyme at pH 6.5, 7.0 and 7.5 using 0.4 M phosphate buffer for 10 min. The pH was adjusted to 5.2, using 3 M sodium acetate. Temperature stability was determined by incubation at specified temperatures (35, 45, 55, 65 and 75 °C) for 10 min and subsequent adjustment of the temperature back to 25 °C. All measurements were performed in triplicate with three replicates and comparisons were made to the control conditions (pH 5.2 and 25 °C, respectively).

Hydrolytic study

Oxidized insulin B-chain (100 μg) was dissolved in 1 ml of 0.1 M sodium citrate, pH 1.5, and incubated with activated chimaeric enzyme or activated pepsin (100:1, w/w) at 37 °C [22]. Aliquots were removed at 1, 3, 7, 24 and 48 h intervals and frozen at –25 °C to stop the reaction. The peptides were analysed by reversed-phase HPLC (RP-HPLC) with a µBondapak C18 RP column (Waters Associates, Milford, MA, U.S.A.) using a linear gradient of acetonitrile from 3 to 80 % in 0.1 % trifluoroacetic acid over 75 min at 0.7 ml.min⁻¹. The eluant was monitored at 215 nm and the peaks detected were manually collected and dried using a vacuum concentrator and submitted for amino acid analysis. Control reactions were run using identical conditions without the enzyme and monitored from 1 to 24 h.

Differential scanning calorimetry (DSC)

Calorimetric measurements were carried out using a MicroCal MC-2 differential scanning calorimeter (MicroCal, Northampton, MA, U.S.A.). The activated chimaeric aspartic proteinase and pepsin samples (0.6–0.8 mg/ml final concentrations) were subjected to calorimetric measurements from 20 to 100 °C at a heating rate of 1.5 °C/min. Data analysis was performed using the Origin DSC ITC software (MicroCal). Runs were performed in duplicate.

RESULTS AND DISCUSSION

Construction, expression and purification of the chimaeric aspartic proteinase

The general scheme for the construction of the chimaeric enzyme is indicated in Figure 1 (upper panel), i.e. the catalytic aspartates from pepsin, the PSS from the A. thaliana aspartic proteinase and the C-terminal fragment from pepsin. With the exception of one amino acid change of Ile to Leu, both hydrophobic residues, to create one unique restriction endonuclease site, the entire chimaeric enzyme was designed from the available sequences of both aspartic proteinases (Figure 1, lower panel). Thus minimal loss or change to the naturally occurring amino acids for both polypeptides was achieved.

The PSS possesses a conserved N-glycosylation site modified in vivo with an oligomannose-type moiety containing proximal fucose and xylose residues [23]. In order to mimic conditions in vivo, the chimaeric enzyme was expressed in P. pastoris, as this lower eukaryote is capable of both N- and O-glycosylation using high-mannose residues [24]. Porcine pepsinogen, which does not possess the typical Asn-Xaa-Ser/Thr glycosylation site, was engineered into porcine pepsinogen at the identical location in which it is found in A. thaliana (approx. 100 amino acids from the C-terminus). The 100-amino-acid PSS region was boxed and labelled. Asterisks represent identical residues between the porcine pepsinogen and A. thaliana aspartic proteinase and were used to identify the insertion site of the PSS region into pepsinogen. The sole amino acid foreign to the sequence was Leu, in the N-terminal portion of porcine pepsinogen part B (light grey).
and a minor band at 58 kDa by SDS/PAGE and Western blot analyses (Figure 2, both panels, lanes 2). Based on the amino acid sequence, the predicted molecular mass of the zymogenic form of the chimaeric aspartic proteinase was predicted to be 51 100 Da. The cause of the heterogeneity (production of 68 and 58 kDa forms) of the chimaeric enzyme was determined to be 2-fold. First, the primary sequence was predicted to possess an N-glycosylation site within the PSS region and it was determined that the amount of glycosylation differed between the two forms. Both forms indicated, through the periodic acid–Schiff method, the presence of glycosylation (results not shown). Deglycosylation with PNGase F resulted in an SDS/PAGE and Western-blot profile of the chimaeric enzyme as a single band at the predicted 51 kDa (Figure 2, both panels, lanes 4). Secondly, the lower band (58 kDa) also possessed a truncated N-terminus (a loss of a 26 amino acids from the N-terminal region of the enzyme; Figure 3). The full (68 kDa) and truncated (58 kDa) forms of the chimaeric enzyme (from the Source Q anion-exchange purification step) were successfully separated by Superose 12 gel filtration; however, there was a substantial loss in yield (from approx. 20 % to 1 % of total protein). Since amino acid sequencing determined that the full and truncated forms of the chimaeric enzyme activated to the proper N-terminus of pepsin, and that the 68 and 58 kDa forms displayed similar properties to one another upon activation, e.g. stability and activity, to maximize yield of the chimaeric enzyme source, the heterogeneous preparation (68 and 58 kDa forms) was activated and used for stability and enzymic studies.

No evidence of contaminating yeast aspartic proteinases from the P. pastoris expression system was apparent. Aspartic proteinase activity was not detected in the medium (assayed using milk-clotting assay) until it was activated as described in

the Experimental section. Secondly, N-terminal analysis of the major and minor bands isolated from Source Q anion-exchange and Concanavalin A affinity chromatography revealed the full and truncated N-terminus of porcine pepsinogen. The absence of endogenous yeast aspartic proteinases in our chimaeric enzyme preparation is consistent with the findings of other groups that have used the P. pastoris system for over-expressing, purifying and characterizing aspartic proteinases [14,16].

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Figure 2  SDS/PAGE and Western-blot analysis of the purified chimaeric aspartic proteinase

Top panels: SDS/PAGE analysis performed under reduced conditions, stained with SYPRO Ruby Red and photographed using a Syngene gel documentation system. All protein amounts were approx. 1 μg. Molecular-mass markers are indicated on the figure. Lane 1, commercial pepsinogen; lane 2, zymogenic chimaeric enzyme; lane 3, activated chimaeric enzyme; lane 4, PNGase F-treated zymogenic chimaeric enzyme; lane 5, PNGase F-treated activated chimaeric enzyme. Bottom panels: Western-blot analysis using the anti-pepsinogen antibody. All protein amounts were approx. 0.1 μg. Lane designations were as described for the top panel. Molecular-mass markers are indicated.

Figure 3  N-terminal sequence data for the zymogenic and active forms of the chimaeric aspartic proteinase

(A) The N-terminal sequence of pepsinogen (zymogen) (accession number J04601). Underlining indicates the 44-amino-acid prosegment sequence, while bold letters indicate the N-terminal sequence of the active form of the enzyme, pepsin. 
(B) The N-terminal sequence of the 68 kDa chimaeric aspartic proteinase (zymogenic form). 
(C) The N-terminal sequence of the truncated 58 kDa chimaeric aspartic proteinase (zymogenic form). The sequence of the truncated 58 kDa form indicates that the sequence starts 26 amino acids from the anticipated N-terminus of the pepsinogen sequence. 
(D) and (E) indicate the N-terminal sequences derived from the activated forms of the 68 and 58 kDa chimaeric enzymes, respectively. [ ] Indicates a minor amount of amino acid detected during the cycle.
All eukaryotic aspartic proteinases are produced in a stable, zymogenic form [7]. The prosegment of the zymogenic form of the chimaeric enzyme was predicted to consist of a 44-amino-acid stretch that resides in the active site of the enzyme cleft and prevents premature functioning [25] until either a unimolecular or bimolecular reaction occurs resulting in an active protease [26]. Activity was not present until the chimaeric enzyme was activated unimolecularly (lowering the pH below 2), resulting in loss of the prosegment (confirmed via N-terminal analysis; Figure 3). The reasons for the production of a minor amount of the chimaeric enzyme with a truncated prosegment were not clear. It is possible that an endogenous proteinase may have cleaved the 26 amino acids of the prosegment, as postulated for the activation of the cyprosin aspartic proteinase produced in P. pastoris [14]. However, porcine pepsinogen produced in this system does not result in a prosegment cleavage event [16]. The loss of the 26 amino acids could indicate the first stage of intermolecular activation from the zymogenic form to an intermediate form of the enzyme, as found for the conversion of procathepsin D into pseudocathepsin D in vitro [27]. However, production of the mature active enzyme was not achieved after prolonged incubations at room temperature (>48 h). Alternatively, the P. pastoris overexpression system is documented to produce N-terminal heterogeneity [28] and the truncated form of the prosegment may simply be an inherent trait of the expression system.

Structural properties of the chimaeric protein

The inclusion of the PSS resulted in a chimaeric enzyme with increased pH stability above pH 6. After incubation at pH 6.5, the chimaeric enzyme possessed almost twice the relative activity compared with pepsin (Figure 4, upper panel) and after incubation at pH 7, pepsin was without activity, while the chimaeric enzyme retained approx. 20% of its original activity. The chimaeric enzyme was found to be marginally stable at pH 7.5, where approx. 5% residual activity remained. Temperature studies indicated that the chimaeric enzyme demonstrated an increase in stability in comparison with pepsin (Figure 4, bottom panel). The chimaeric enzyme showed almost 40% of residual activity after incubation at 65 °C, whereas porcine pepsin retained only 10%.

DSC was used to assess the thermal properties of the chimaeric enzyme and pepsin, where higher denaturation temperatures (T_m) would indicate a more thermostable structure. The chimaeric enzyme demonstrated an increased T_m (68.3 °C) compared with pepsin (66.5 °C; Figure 5). Analysis also indicated that the temperature range at which the chimaeric enzyme began unfolding and was completely unfolded (64.2–69.5 °C) was narrower than that of pepsin (60.3–69.4 °C). The narrower temperature range for unfolding represented a greater co-operativity during denaturation for the chimaeric enzyme compared with pepsin. Enthalpy calculations determined for the chimaeric enzyme and pepsin were 2.7 ± 0.1 compared with 2.64 ± 0.08 kcal/g (1 kcal = 4.184 kJ) respectively. The DSC data would tend to indicate that the thermostability of pepsin was lower than that of chimaeric enzyme. An initial loss of co-operativity in pepsin structure occurs at a lower temperature (60.3 °C) compared with the chimaera (64.2 °C) and can explain the higher relative activity of the chimaera at 65 °C (Figure 4, bottom panel), where pepsin had lost most of its activity due to a loss of co-operativity.

The described stability changes of pepsin, through the introduction of the PSS, could be attributed to the α-helical design of the PSS domain. DSC studies of α-helical domains indicate that a single α-helical structure is not highly stable in aqueous media.
Analyses were performed in duplicate using 0.2 mg/ml enzyme. The values of several plant aspartic proteinases from the literature were the average of the four scans corrected by subtracting the four scans of the buffer. Analyses were performed in duplicate using 0.2 mg/ml enzyme.

However, as the number of helices increases, lateral interactions can lead to increases in co-operativity [29]. The PSS is characterized by five amphipathic $\alpha$-helices folded into a single compact entity with three disulphide linkages [4], and CD spectra supported the increase in $\alpha$-helical content (via the presence of a minimum in the 207–222 nm region) for the chimaeric enzyme (Figure 6). DSC demonstrated that the inclusion of the PSS has made the pepsin enzyme a more thermodynamically compact structure, i.e. greater co-operativity during denaturation. Glycosylation of the PSS region in the chimaeric enzyme did not appear to contribute to the stability of the enzyme as deglycosylating the chimaeric enzyme did not demonstrate altered pH–activity profiles, nor thermostability changes when compared with the glycosylated chimaera (results not shown).

**Kinetic properties of the chimaeric protein**

The $K_m$ and $k_{cat}$ values of the chimaeric enzyme were approx. 0.117 mM and 8.3 s$^{-1}$, respectively, using SS1. This $K_m$ value was found to be approx. 2-fold higher than the reported literature values of pepsin and a reduced catalytic-centre activity (‘turnover number’) in comparison with pepsin (Table 1). The kinetic characterization was performed at the optimal pH for the chimaeric enzyme (pH 1.5) and compared with the kinetic values of several plant aspartic proteinases from the literature at various pH values (Table 1). Monomeric aspartic proteinases are poorly characterized and a search among the monomeric aspartic proteinases resulted in a single plant aspartic proteinase (isolated from the bran of soft wheat) being characterized using a chromophoric synthetic substrate [30]. This proteinase, at pH 3.3, possessed an elevated $K_m$ value compared with our enzyme (Table 1). However, comparing catalytic efficiencies, our monomeric chimaeric enzyme demonstrated a low catalytic efficiency ($k_{cat}/K_m$) of 70.5 mM$^{-1}$·s$^{-1}$ compared with pepsin, and this may be supported by the overall lower catalytic efficiency of the monomeric aspartic proteinase from wheat [30]. While it is speculative to conclude that this chimaeric enzyme has acquired the properties of a monomeric plant aspartic proteinase, there is evidence to suggest that chimaeric proteins can exhibit activities found in one or both parent proteins [31]. In the present study, an entire domain has been incorporated within pepsin that is naturally present in some of the enzymes within the same family. The plant-like changes described herein, with respect to kinetic parameters, may be due to the incorporation of this domain. However, we recognize that the changes determined may simply be due to the disruption of the catalytic residues. Additionally for the chimaeric enzyme, and unlike pepsin, the insertion of the PSS resulted in an enzyme with inconsistent pepstatin binding, i.e. the pepstatin-inhibition curve was non-linear. A PSS-deficient monomeric aspartic proteinase (from rice) has been produced in vitro, and while kinetic and specificity data were not presented [32], it would be of interest to determine if this modified plant enzyme possessed altered specificity and kinetic parameters from its parent enzyme.

Glycosylation of the PSS may interfere with the catalytic efficiency, yet crystallographic data of phytepsin indicated that the glycosylation site within the PSS is far removed from the active site [4]. Speculation is that the role of the glycosylation may be due to the incorporation of this domain. However, we recognize that the changes determined may simply be due to the incorporation of this domain. However, we recognize that the changes determined may simply be due to the disruption of the catalytic residues. Additionally for the chimaeric enzyme, and unlike pepsin, the insertion of the PSS resulted in an enzyme with inconsistent pepstatin binding, i.e. the pepstatin-inhibition curve was non-linear. A PSS-deficient monomeric aspartic proteinase (from rice) has been produced in vitro, and while kinetic and specificity data were not presented [32], it would be of interest to determine if this modified plant enzyme possessed altered specificity and kinetic parameters from its parent enzyme.

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with other aspartic proteinases using the same method [22,34]. Evidence exists for a monomeric plant aspartic proteinase with a limited cleavage ability [34]; however, in comparison with the better-characterized heterodimeric enzymes it is difficult to determine whether confined cleavage specificity is an inherent trait of monomeric aspartic proteinases, or a function of the PSS. To assess the subsite binding and specificity of this chimaeric aspartic proteinase in greater detail, studies using glucagon and melittin [22] are underway.

Role of the PSS in pepsin and plant aspartic proteinases

Pepsin is a gastric aspartic proteinase involved in the digestion process of vertebrates. It is a bilobar protein with N- and C-terminal lobes that are comprised mainly of β-sheets. While optimally active below pH 2 with broad specificity, pepsin is active up to pH 6. However, between pH 6 and 7, pepsin is documented to undergo irreversible denaturation [20,35]. Speculation is that denaturation in one particular domain of pepsin is responsible for the loss of function. However, the domain responsible for unfolding remains controversial [36–38]. Attempts in our lab to stabilize pepsin above pH 6 have demonstrated partial success through site-directed mutagenesis experiments [38]. However, the limitations of this technique are well documented, and the development of a chimaeric enzyme represents an attempt at a more robust engineering method. Through the inclusion of the PSS in the C-terminal lobe of pepsin, the chimaeric enzyme demonstrated modifications in specificity, thermostability and pH stability. However, this stability appears to come at the expense of reduced catalytic activity. The changes in stability and specificity of pepsin through the construction of the chimaeric enzyme could provide some insight into the role of the PSS as pertains to its presence in the plant proteinases.

The exact role of the PSS is unknown, but much speculated upon. Recent work demonstrated that the PSS might be responsible for membrane targeting, as the presence of the PSS resulted in a bifunctional molecule containing a proteolytic portion and a membrane-associating/-destabilizing domain [5,12]. However, the present study suggests that the PSS can contribute to changes in stability, activity and structure, in an enzyme that does not normally possess this domain. Thus we suggest adding a putative structure–function role to the list. The tertiary structure of the PSS appears to be conserved between the fully sequenced plant aspartic proteinases; however, the PSS shows limited identity to other plant aspartic proteinase PSS regions and may represent an evolutionary divergence greater than that of other parts of the enzyme [3,6,7]. Perhaps the divergence in the PSS provides the flexibility for different enzyme stabilities, or allows variation in specificity in response to substrate changes. Further characterization of other chimaeric enzymes using the PSS from a different plant source could prove fruitful in elucidating this possibility. It is our belief that the PSS is an operationally relevant domain [39], as the present study has demonstrated that it can be transferred into a related structure and used to create a functional protein that has altered substrate specificity and moderately enhanced stability. Typically, more successful enzyme engineering advances to construct enzymes with desired functionality have been achieved via error-prone PCR and DNA shuffling. Combining both methodologies, this chimaeric enzyme may be a point of introduction to directed evolution studies [40] that could provide insight into eukaryotic aspartic proteinase evolution and/or improved kinetic parameters for optimizing aspartic proteinases in industrial endeavours.

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