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Functional chimera of porcine pepsin prosegment and *Plasmodium falciparum* plasmepsin II

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Proplasmepsin II (zPMII) represents a unique member of the aspartic proteinase family, with a prosegment–enzyme interaction that is thus far unique among the pepsin-like proteases. The role of the prosegment in aspartic proteinase structure and function was investigated by generating two chimeric proteins, one with the pepsinogen prosegment fused to the mature region of PMII (pepproPMII) and a second with the prosegment of PMII fused to pepsin (PMIIpropep). Both chimeras were expressed using *Escherichia coli*; however, PMIIpropep was extremely unstable suggesting protein misfolding. Alternatively, pepproPMII was capable of both autoactivation and hydrolysis of a synthetic substrate. Similarly, when the PMII enzyme was expressed without a prosegment, it too exhibited activity against the synthetic enzyme. CD measurements indicated that pepproPMII had reduced thermal stability when compared with zPMII. This reduction of temperature stability may have resulted from the inability of the pepsinogen prosegment to stabilize the C-terminal domain of the PMII enzyme. The ability of PMII to fold in the presence of a completely non-homologous prosegment and in its absence suggests that prosegment is not critical to obtaining a functional enzyme in all pepsin-like enzymes but likely plays a role in protein stabilization.

Keywords: aspartic proteinase/chimeras/plasmepsin II/prosegment

Introduction

Eukaryotic aspartic proteinases are expressed as inactive zymogens. The inactive precursor generally consists of an approximately 40 amino acid prosegment that physically blocks the active site preventing access of the substrate. The malaria aspartic proteinases, termed plasmepsins (PMs), however, are expressed as zymogens (zPMs) with much larger prosegments, approximately 120 amino acids in length. The excess amino acids include a stretch of approximately 19 hydrophobic amino acids that have been implicated in type II membrane anchoring (Francis *et al*., 1994). In addition, it has been reported that the malaria aspartic proteinase PMII has a novel zymogen structure (Bernstein *et al*., 1999). In pepsin-like aspartic proteinases, the prosegment sterically blocks the active site region, preventing substrate binding. Activity is further hindered by the interaction of the catalytic aspartic acid residues with Lys 36p (pepsin numbering, p denotes prosegment), and two tyrosine residues (Tyr 37p and 9) (Richter *et al*., 1998). Notably, the active site of pepsin-like aspartic proteinases is completely preformed in the zymogen form of the enzyme and undergoes only minor changes upon activation. In contrast, the prosegment of zPMII does not block substrate access to the active site, but rather associates with the C-terminal domain of enzyme (Bernstein *et al*., 1999). Through its interaction with the C-terminal domain, the prosegment acts as a 'harness' pulling the C-terminal domain away from the N-terminal domain. In addition, several amino acids in the mature region of PMII interact with the psi loops (named after the resemblance of the Greek symbol psi) which form the active site. Such an interaction may serve to force apart and then stabilize the catalytic dyad in a distorted conformation (Bernstein *et al*., 1999). Despite differences in the prosegment, the structure of the mature PMII is typical of aspartic proteinases (Asojo *et al.*, 2003).

It has been generally accepted that the prosegment of pepsin-like aspartic proteinases is essential for obtaining a correctly folded mature region (Foltmann, 1988). It is interesting that despite having two vastly different zymogen conformations, both PMII and the classic aspartic proteinase pepsinogen have typical active aspartic proteinase mature structures (Cooper, 2002). In this study, two zymogen chimeras were produced to determine the role of the prosegment on aspartic proteinase structure and function.

Materials and methods

Materials

The pET32b(+) plasmid and *Escherichia coli* Rosetta-gami B (DE3)pLysS cells were purchased from Novagen (Mississaga, ON, Canada). GenElute™ Plasmid Miniprep Kit was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). *Pfu* DNA polymerase was obtained from Fermentas Life Sciences (Burlington, ON, Canada) and primers were synthesized by Sigma Genosys (Oakville, ON, Canada). QIAquick® PCR Purification Kit was purchased from Qiagen Sciences (Germantown, MD, USA). HIS®-Select 6.4 ml cartridges were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Centrifugal filter units were supplied by Millipore Corp. (Bedford, MA, USA). All chemicals and media were obtained from Fisher Scientific (Nepean, ON, Canada) or Sigma-Aldrich Co. (St Louis, MO, USA).

Generation of chimeric proteins

Overlap PCR was used to generate the chimeric constructs. Truncated PMII in the expression vector pET32b(+) (Novagen, Mississauga, ON, Canada) obtained from our laboratory was used for amplification of the PMII prosegment and the mature region of PMII. The PMII prosegment was amplified with a 5′ overhang coding for an NcoI restriction enzyme site and a 3′ overhang coding for a portion of the 5′-end of mature pepsin. The following primers (Sigma Genosys, Oakville, ON, Canada) were used to amplify the

PMII prosegment: 5′-GGCCATGGAACATTTAACTATT GG-3′ and 5′-GGGCTCATCGCCTATACCTAAATAATTT GTTTAGTAAGTCC-3′. The mature region of PMII was amplified with a 5′ overhang coding for a portion of the 3′-end of the pepsin prosegment and a 3′ overhang encoding for a HindIII restriction enzyme cut site. The following primers were used to amplify the mature region of PMII: 5′-CCCCGAGGCCGCTGCCCTGAGTTCAAATGATAATA-TCG-3′ and 5′-CCAAGCTT TTATAAATTCTTTTTAGC-3′. The pepsin prosegment was amplified with a 5′ overhang coding for an NcoI restriction enzyme site and a 3′ overhang encoding for a portion of the 5′-end of mature PMII. The following primers were used to amplify the prosegment of pepsin: 5′-GGCCATGGGCTCGTCAAGGTCCCGCTGG-3′ and 5′-CGATATTATCATTTGAACTCAGGGCAGCGGCC TCGGGG-3′. The mature region of pepsin was amplified with a 5′ overhang coding for a portion of the 3′ of PMII prosegment and a 3′ overhang encoding for HindIII. The following primers were used to amplify the mature region of pepsin: 5′-CTAAAACAAATTATTTAGGTATAGGCGAT GAGCCC-3′ and 5′-GGAGCTTTCAGAATTATCTACATGG AAGCG-3′. The amplified prosegment and mature regions were fused and amplified using overlap PCR methodology. Chimeric DNA was cloned into pET32b(+) using the HindIII and NcoI cut sites. Constructs were sequenced (Laboratory Services, University of Guelph, Guelph, ON, Canada) using an ABI prism DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Generation of PMII without prosegment (noproPMII)

Truncated PMII in the expression vector pET32b(+) (Novagen, Mississauga, ON, Canada) obtained from our laboratory was used for amplification of the mature region of PMII. The mature region of PMII was amplified with a 5′ overhang coding for NcoI restriction enzyme cut site and the 3′ overhang encoding for HindIII restriction enzyme cut site. The following primers were used for the amplification: 5′-GGCCATGGGGAGTTCCAAATGATAATATCGAATTA-G-3′ and 5′-CCAAGTCTTTTATAAATTCTTTTTAGC-3′. The amplified PCR product was cloned into a modified pET32b(+) expression vector using the HindIII and NcoI cut sites. The modified vector has its native thrombin cut site removed and replaced just upstream of the NcoI cut site in the multiple cloning site. Constructs were sequenced (Laboratory Services, University of Guelph, Guelph, ON, Canada) using an ABI prism DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Protein expression

All the constructs were transformed into *E.coli* Rosetta-gami™ B (DE3)pLysS cells (Novagen, Mississauga, ON, Canada) for expression. Cells were cultured in 1.0 l Luria-Bertani media containing 15 μg/ml kanamycin, 34 μg/ml chloramphenicol, 12.5 μg/ml tetracycline and 50 μg/ml ampicillin to an OD_{600} of 1.0, and then induced with isopropyl-β-D-thiogalacto-pyranoside (1 mM). After expression cells were collected by centrifugation (2500 g for 10 min).

Purification of zymogen

Cell pellets were resuspended in 50 mL 1X BugBuster (Novagen, Mississauga, ON, Canada), diluted in 50 mM $NaPO_4$ pH 7.5 and incubated at room temperature for 1 h with gentle shaking. The sample was then centrifuged at 16 000 g for 20 min at 4°C to remove cell debris. The supernatant was applied to a HIS®-Select Cartridge (Sigma-Aldrich, Oakville ON, Canada) on a AKTA™ FPLC system (GE Healthcare, Chalfont St Giles, UK). The column was washed with 50 mM $NaPO_4$/0.3 M NaCl/10 mM imidazole pH 7.5 wash buffer and a gradient of 0–10% 50 mM $NaPO_4$/0.3 M NaCl/250 mM imidazole pH 7.5 buffer was applied to the column over 8 column volumes. Recombinant thioredoxin fusion protein was eluted with 50 mM $NaPO_4$/0.3 M NaCl/250 mM imidazole pH 7.5 buffer. The sample was concentrated and washed with 20 mM Tris–HCl, pH 7.5 using an Amicon Ultra-15 centrifugal filter unit with an Ultracel-50 membrane (Millipore Corp., Bedford, MA, USA). The protein was then applied to a Mono Q 10/100 GL (GE healthcare, Chalfont, St Giles, UK) anion exchange column. The column was washed with 20 mM Tris–HCl pH 7.5, and a gradient of 0–100% 20 mM Tris–HCl, 1 M NaCl, pH 7.5 was applied to column over 16 column volumes. Fractions containing the zymogen were concentrated and washed with 50 mM $NaPO_4$, pH 7.5. Protein was then digested with thrombin (Sigma-Aldrich, Oakville, ON, Canada) (50 000:1,w:w) for 6 h at 37°C to cleave the thioredoxin tag from the protein. After thrombin digestion, the sample was applied to a Superose™ 12 10/300 GL column (GE Healthcare, Chalfont St Giles, UK) in 50 mM $NaPO_4$, pH 7.5 to recover pure zymogen protein.

Purification of mature protein

Cell pellets were lyzed and initially purified using a HIS®-Select Cartridge (Sigma-Aldrich, Oakville ON, Canada) similar to the protocol used for the zymogen. After nickel affinity chromatography, the sample was concentrated in 50 mM $NaPO_4$, pH 7.5. The concentrated sample was then applied to a Superose™ 12 10/300 GL column (GE Healthcare, Chalfont St Giles, UK). After separation, protein was concentrated in 50 mM $NaPO_4$, pH 7.5. Activation was accomplished by incubating the samples for 2 h in 50 mM CH_3COONa pH 4.7. After activation, the sample was applied to a Superose™ 12 10/300 GL column (GE Healthcare, Chalfont St Giles, UK) to recover pure protein. Protein sample was finally washed in 50 mM $NaPO_4$, pH 7.5 and stored at 4°C. The product resulting from activation was N-terminally sequenced by Edman Sequencing (The Hospital for Sick Children, Toronto, ON, Canada) to confirm its identity.

Purification of noproPMII

Cell pellets were lyzed and initially purified using a HIS®-Select Cartridge (Sigma-Aldrich, Oakville ON, Canada) similar to the protocol used for zymogen protein. After nickel affinity chromatography, the sample was concentrated in 50 mM $NaPO_4$, pH 7.5. The concentrated sample was then applied to a Superose™ 12 10/300 GL column (GE Healthcare, Chalfont St Giles, UK). After separation, protein was concentrated in 50 mM $NaPO_4$, pH 7.5, and digested with thrombin (50 000:1, PMII:thrombin, w:w) at 37°C from 4 h. After digestion, the protein sample was applied to a Superose™ 12 10/300 GL column for final purification.

pH of activation

Protein samples were incubated in pH 2–7 for 30 min at 37°C. Samples were run on SDS–PAGE and stained with Commassie Brilliant Blue stain to visualize the bandshift associated with the zymogen activating to the smaller active enzyme.

Trypsin digestion of pepsinogen and PMIIpropep

Zymogen protein was purified using nickel affinity and gel filtration chromatography. Protein was washed and concentrated with 0.1 M $NaPO_4$, pH 7.5 and total protein concentration was determined. Trypsin (Sigma-Aldrich, Oakville, ON, Canada), dissolved in 0.1 M $NaPO_4$, pH 7.5 was added (50:1, PMII:trypsin, w:w). In addition, a sample of protein was incubated without the addition of trypsin to ensure no protein degradation occurred in the absence of trypsin. Samples were incubated at 37°C and 25 μL aliquots were removed at predetermined time points. The reaction was stopped by heating at 90°C and the addition of SDS–PAGE sample buffer. All aliquots were separated and visualized using 15% SDS–PAGE with Coomassie blue staining.

Activation kinetics

The activation kinetics were determined using densitometry (Sorsa *et al*., 1997). Activation was initiated by lowering the pH by adding 0.1 M CH_3COONa, pH 4.7 and at predetermined time intervals aliquots were removed. The reaction was stopped by the addition of SDS–PAGE sample buffer and heating at 90°C. Activation was analyzed over the linear range of the curve and rate was expressed as relative amount of mature enzyme over time. Activation was carried out in triplicate with the experiment repeated twice ($n = 6$).

N-terminal sequencing

The product resulting from activation was N-terminally sequenced by Edman Sequencing (The Hospital for Sick Children, Toronto, ON, Canada) to confirm cleavage sites and identities.

pH optimum

The proteolytic activity of the enzymes was determined at the following pH values: 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0. Activity was determined using the following reaction conditions: 100 mM CH_3COONa pH 3.5–6.0, 1 μM fluorescent peptide substrate EDANS-CO-CH_2-CH_2-CO-Ala-Leu-Glu-Arg-Met-Phe-Leu-Ser-Phe-Pro-Dap-(DABCYL)-OH (2837b) (AnaSpec Inc., San Jose, CA, USA) (Istvan and Goldberg, 2005) and 2 nM enzyme. The assay was performed at 37°C using a Victor 2 1420 multilabel counter (Perkin Elmer, Woodbridge ON, Canada) with excitation at 335 nm and emission at 535 nm. The reaction rates were determined by calculating the slope of the linear portion of the curve (fluorescence/min) (Xiao *et al*., 2006).

Kinetic parameters

Kinetic parameters were determined using synthetic substrate 2837b (Istvan and Goldberg, 2005). The assay was carried out in 100 mM CH_3COONa, pH 5 using 0.03125–8 μM substrate and 2 nM enzyme. The concentration of enzyme was determined using pepstatin A titration (Tanaka and Yada, 1996). The measured fluorescence was converted to moles per second using a conversion factor derived from a standard curve for the complete digestion of the substrate by *Saccharomyces cerevisiae* proteinase A (Sigma-Aldrich, Oakville, ON, Canada) (Xiao *et al*., 2006). Nonlinear regression with the Michaelis–Menten model was used to determine both K_m and k_{cat}. Each sample was done in triplicate, with the experiment repeated twice ($n = 6$) (Xiao *et al*., 2006).

Far-UV circular dichroism

Far-UV circular dichroism (CD) spectra were determined from 250 to 185 nm at room temperature using a 100 μl quartz cuvette with a 0.1 cm pathlength. The CD spectra were determined for both zymogen and mature enzyme forms. A Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) was used to determine the spectra. The CD scans were done in triplicate with the average buffer spectra being subtracted from the sample spectra. The scans were all completed in 0.1 M $NaPO_4$ pH 7.5. All samples were filtered prior to measurement. Ellipticity values (mdeg) were recorded as a function of wavelength. Spectra were converted from mdeg units to mean residue ellipticity (degrees cm^2 $dmol^{-1}$) using the following equation:

$$[\theta]_{mrw\lambda} = (MRW)(\theta_\lambda)/(10)(d)(c)$$

where MRW is mean residue weight, θ_λ the measured ellipticity at a particular wavelength (mdeg), d the pathlength (cm) and c the concentration of enzyme (g/ml).

CD results were analyzed using Dichroweb (http://dichroweb.cryst.bbk.ac.uk), an online CD analysis tool (Lobley *et al*., 2002; Whitmore and Wallace, 2004). Three analysis programs (Selcon3, Contin and CDSSTR) and two data sets (4 and 7) were used to determine percentages of secondary structure. The average of two determinations was used for secondary structure determination (Richter *et al*., 1999).

Thermal denaturation

Thermal denaturation of the enzymes was monitored using CD signals between 260 and 195 nm. Samples (0.1 mg/ml protein) were scanned from 25 to 90°C with CD scans being conducted every 2°C with a scan rate of 500 nm/min and response time of 0.25 s. Thermal denaturation was monitored at 200 nm and the melting temperature determined using GraphPad Prism software 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

PepproPMII and PMIIpropep chimeric enzymes were generated to investigate the role of the prosegment in protein folding and function. PMII and pepsin were chosen for this study since their mature forms represent a typical aspartic proteinase yet both have drastically different zymogen forms. Pepsinogen has a very typical aspartic proteinase zymogen structure with the prosegment sterically blocking and interacting directly with the active site (Richter *et al*., 1999), while PMII has a unique zymogen structure with the prosegment acting as a harness that forces apart the active site rendering it inactive (Bernstein *et al*., 1999).

Autoactivation of chimeric enzymes

Both pepproPMII and PMIIpropep were generated using overlap PCR as indicated in the Material and methods section. Both chimeric proteins were successfully expressed from *E.coli* Rosetta gami cells. After expression, the zymogen enzyme was purified using nickel affinity and size exclusion chromatography and tested for autoactivation. To investigate the pH dependence of activation, both chimeric and wild-type (Wt) zymogen proteins were incubated at pH 2–7 (Fig. 1A–D). The zpepproPMII chimeric protein activated at pH 3–5 as indicated by bandshift (Fig. 1B). Activation at these pH values was consistent with the pH-dependent autoactivation observed for zPMII (Fig. 1A). To confirm the band shift observed upon acidification was in fact due to autoactivation the protein sample was acidified in the presence of pepstatin A, a tight-binding inhibitor of aspartic proteinases known to inhibit autoactivation of this class of enzymes (Dunn, 2002). In the presence of pepstatin A, no band shift was observed indicating the enzymes autoactivate under acidic conditions (data not shown). N-terminal sequencing was conducted on the autoactivation products and revealed that zpepproPMII activates between two alanine residues (N-terminal sequence AALSS), 3 amino acids upstream of the N-terminus of the mature region of PMII. Alternatively, WtPMII had an N-terminal sequence of SSNDN. Densitometric measurements indicated that zPMII exhibited an initial rate of activation (Table I) of $0.273 \pm 0.094\ \text{min}^{-1}$. Alternatively, zpepproPMII exhibited an activation rate of $0.1075 \pm 7.0 \times 10^{-4}\ \text{min}^{-1}$, approximately 40% slower than zPMII.

Unlike zpepproPMII, zPMIIpropep did not exhibit autoactivation at any pH (Fig. 1D). To investigate the possibility that misfolding was preventing proper functioning of this chimeric enzyme, PMIIpropep and pepsinogen were subjected to trypsin digestion (Fig. 2A and B). Pepsinogen exhibited limited digestion with a band shift resulting in two bands. This is consistent with the known trypsin cleavage site in the pepsinogen-thioredoxin fusion protein (Tanaka and Yada, 1996). Prolonged incubation after the initial cleavage did not further degrade pepsinogen suggesting the enzyme is resistant to further trypsin digestion, indicative of a folded enzyme. Alternatively, zPMIIpropep was rapidly degraded by trypsin, suggesting it was misfolded. Trypsin digestion has been widely used to evaluate the folding of mutant proteins (Liu *et al.*, 1996; Tamarappoo *et al.*, 1999; Pagant *et al.*, 2007). Since PMIIpropep was misfolded, it was not further characterized.

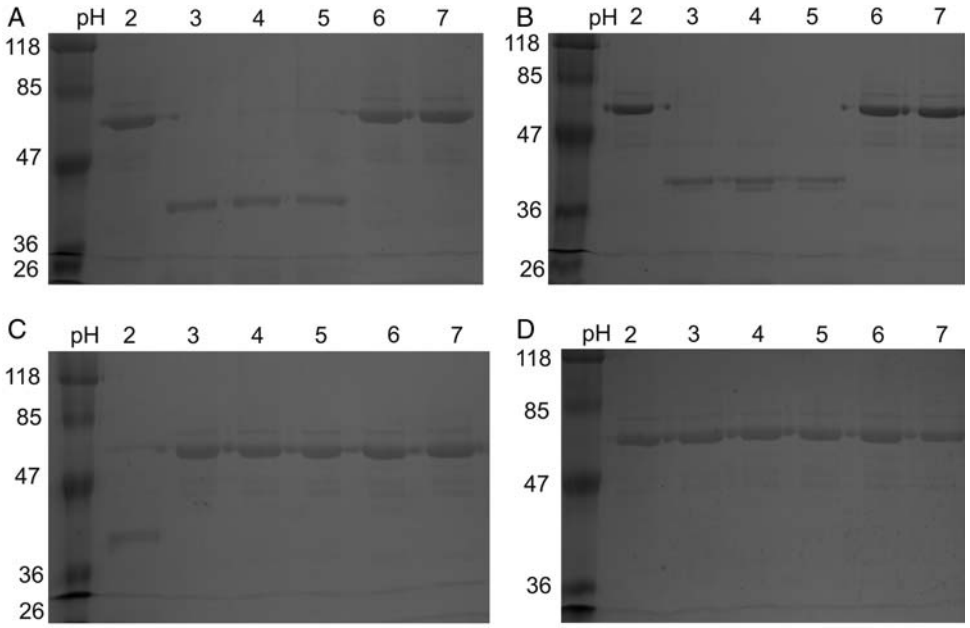

Fig. 1. Enzyme autoactivation. Coomassie Brilliant Blue stained 15% SDS–PAGE gel demonstrating processing of WtzPMII (**A**), pepproPMII (**B**) and pepsinogen (**C**) after incubation at 37°C for 30 min in pH 2–7. PMIIpropep (**D**) did not exhibit any processing. f, fusion protein; m, mature protein. Sizes of molecular weight markers are indicated on the *y*-axis in kDa, and pH values are indicated on the *x*-axis.

Table I. Summary of measured kinetic parameters for WtPMII, pepproPMII and noproPMII[1]

Enzyme	Rate of autoactivation (min^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1}\mu\text{M}^{-1}$)
WtPMII	$0.273 \pm 9.4 \times 10^{-2}$ [a]	1.2 ± 0.3[c]	2.10 ± 0.4 [f]	1.50 ± 0.9 [i]
Chimeric PMII	$0.0175 \pm 7.0 \times 10^{-4}$ [b]	$0.70 \pm 3 \times 10^{-2}$ [d]	1.11 ± 0.3 [g]	1.66 ± 0.3 [i]
noproPMII	—	$0.17 \pm 3 \times 10^{-2}$ [e]	$0.103 \pm 9\ \text{x}10^{-3}$ [h]	0.620 ± 0.2[j]

[1]Obtained using least-squares for best fit to the Michaelis–Menten model. All values are the means ± standard deviations of two replicates with 3 determinations per replicate ($n = 6$). Means sharing the same letter are not significantly different ($P > 0.05$).

Secondary structure determination

Since zpepproPMII did exhibit autoactivation, it is likely that this chimeric enzyme was folded (Richard *et al*., 2005). Since PMII did not require its native prosegment to obtain a functional enzyme, a PMII enzyme without its prosegment, noproPMII, was generated. To determine whether PMII requires its prosegment to obtain its native secondary structure, far-UV circular dichroism (CD) spectra for the various forms (zPMII, mature (m)PMII, noproPMII, zpepproPMII and mpepproPMII) of the PMII enzyme were determined. The spectra were used to predict secondary structure of the enzymes in both their mature and zymogen forms. The predicted secondary structures (Table II) were not significantly different ($P > 0.05$) with the zymogen form of the enzymes being ~10% alpha helix, ~40% beta sheet and ~19% turn, and the mature forms and noproPMII being ~9% alpha helix, ~41% beta sheet and ~19% turn. The similarity of predicted secondary structures indicates that the alteration of prosegment in pepproPMII did not alter the secondary structure of this enzyme.

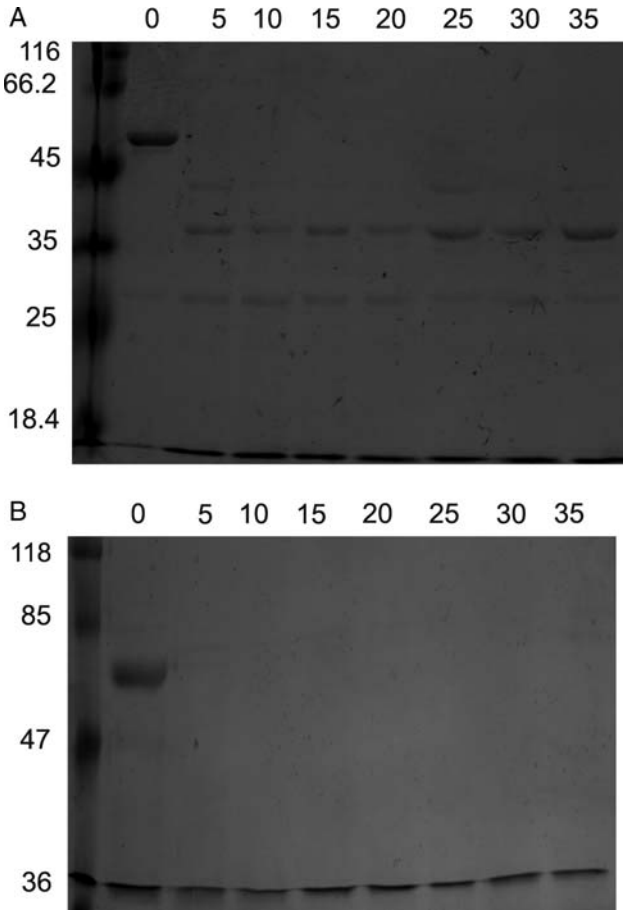

Fig. 2. Trypsin digestion. Coomassie Brilliant Blue stained 15% SDS–PAGE gel demonstrating digestion of pepsinogen (**A**) and PMIIpropep (**B**) by trypsin after incubation at pH 7.0 over 35 m. f, fusion protein. Sizes of molecular weight markers are indicated on the *y*-axis in kDa, and time (mins) are indicated on the *x*-axis.

Determination of kinetic parameters and pH optimum

Kinetic parameters (Table I) and pH optimum (Fig. 3) for mPMII, mpepproPMII and noproPMII were determined using the quenched fluorescent substrate 2837b previously used for the kinetic characterization of PMII (Istvan and Goldberg, 2005). All enzymes exhibited a pH optimum of approximately 5 (Fig. 3), consistent with that previously reported for PMII. Kinetic parameters were determined using the Michaelis–Menten model. mPMII exhibited a K_m of 1.2 ± 0.26 μM, and a k_{cat} of $2.05 \pm 0.37\ s^{-1}$. These values are consistent with those previously reported for mPMII (Istvan and Goldberg, 2005). mpepproPMII, alternatively, exhibited a K_m of 0.70 ± 0.27 μM, and a k_{cat} of $1.11 \pm 0.28\ s^{-1}$. NoproPMII also exhibited measurable activity against the synthetic substrate although significantly reduced ($P \leq 0.05$), i.e. K_m of 0.16 ± 0.16 μM and k_{cat} of $0.103 \pm 9.9 \times 10^{-3}\ s^{-1}$.

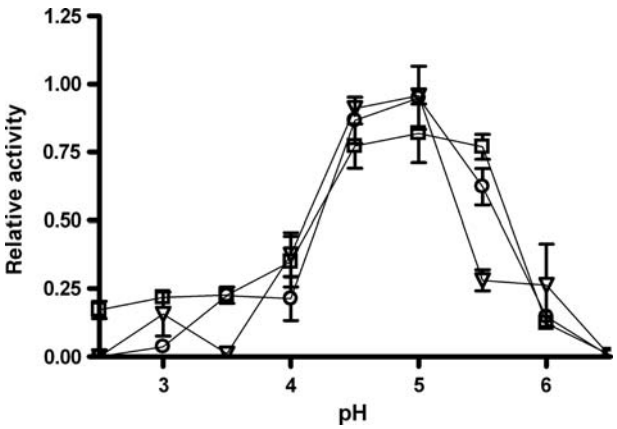

Fig. 3. Determination of pH optimum. Effect of pH on WtPMII (□), pepproPMII (○) and noproPMII (▼) activity in 100 mM CH_3COONa pH 3.5–6.0. Assays were conducted with 2 nM enzyme and 1 μM peptide substrate 2835b. Each data point represents the mean of 3 determinations and standard deviation.

Table II. Predicted secondary structure composition of zPMII, zpepproPMII, mWTPMII and mpepproPMII[1]

Enzyme	α-Helix	β-Sheet	Turn
zPMII	$0.110 \pm 7.4 \times 10^{-2}$ a	$0.415 \pm 8.1 \times 10^{-2}$ b	$0.184 \pm 4.5 \times 10^{-2}$ c
zpepproPMII	$0.107 \pm 7.1 \times 10^{-2}$ a	$0.390 \pm 8.0 \times 10^{-2}$ b	$0.193 \pm 4.6 \times 10^{-2}$ c
mWtPMII	$0.085 \pm 8.0 \times 10^{-2}$ a	$0.420 \pm 8.4 \times 10^{-2}$ b	$0.187 \pm 4.9 \times 10^{-2}$ c
mpepproPMII	$0.094 \pm 2.5 \times 10^{-2}$ a	$0.409 \pm 8.1 \times 10^{-2}$ b	$0.200 \pm 3.9 \times 10^{-2}$ c
noproPMII	$0.104 \pm 5.7 \times 10^{-2}$ a	$0.374 \pm 1.2 \times 10^{-2}$ b	$0.201 \pm 2.0 \times 10^{-2}$ c

[1]All values are the means ± standard deviations of two replicates with 4 scans per replicate. Means sharing the same letter are not significantly different ($P > 0.05$).

Table III. Melting temperature of zymogen and mature forms of PMII and pepproPMII and of noproPMII[1]

Enzyme	T_m (°C)
zPMII	52.27 ± 0.67^a
zpepproPMII	49.19 ± 0.74^b
mWtPMII	48.56 ± 0.51^b
mpepproPMII	49.01 ± 0.83^b
noproPMII	58.30 ± 2.4^c

[1]Protein was scanned from 25 to 90°C with CD scans being conducted every 2°C with a scan rate of 500 nm/min. Proteins were scanned at a concentration of 0.1 mg/ml. All values are the means ± standard deviations of two replicates with 4 scans per replicate. Means sharing the same letter are not significantly different ($P > 0.05$).

Melting temperature determination

CD was used to assess the melting temperature (T_m) of all forms of the PMII enzyme (Table III). The exchange of the PMII prosegment for pepsinogen prosegment resulted in a chimeric protein with a T_m (49.19°C ± 0.75) 3.08°C lower than that observed for zPMII (52.27 ± 0.67). mpepproPMII, and mPMII, exhibited a T_m of ~49°C, while noproPMII exhibited a T_m of 58.30°C.

Discussion

In this study, two chimeric proenzymes, pepproPMII and PMIIpropep and a protein without a prosegment, noproPMII were produced to investigate the role of the prosegment in aspartic proteinase folding and function. Many reports have suggested that the prosegment is required to obtain the active mature form of gastric-like aspartic proteinases (Foltmann, 1988; van den Hazel *et al*., 1993; van den Hazel *et al*., 1995; Richter *et al*., 1999). Notable is the ability for PMII to assume a classic aspartic proteinase mature structure despite having an atypical zymogen structure (Bernstein *et al*., 1999) thereby raising the question of the role of the prosegment aspartic proteinase folding.

Results of the present study suggested that zPMIIpropep was misfolded as indicated by the inability of zPMIIpropep to autoactivate. To confirm misfolding, zPMIIpropep was digested with trypsin. Limited cleavage of pepsinogen by trypsin resulted in two products, consistent with the known cleavage site in the fusion protein (Tanaka and Yada, 1996). Both bands were stable and were not subject to further cleavage during extended incubation. Alternatively, upon incubation with trypsin zPMIIpropep was rapidly degraded suggesting that zPMIIpropep was misfolded (Liu *et al*., 1996; Tamarappoo *et al*., 1999; Pagant *et al*., 2007). It has been shown that residues in the prosegment of pepsinogen are critical to the ultimate conformation of pepsin (Richter *et al*., 1999). In particular, Lys36p (p denotes prosegment) was identified as a critical residue for both enzyme stability and folding (Richter *et al*., 1999) and its mutation led to enzymes that were extremely unstable and degraded rapidly. It is thought that Lys36p is involved in correctly aligning the active aspartic acid residues (Richter *et al*., 1999). In addition, work with another aspartic proteinase, *S.cerevisiae* proteinase A, found that no particular residue of the prosegment was required for obtaining a functioning enzyme with the exception of Lys36p (van den Hazel *et al*, 1995) which for both vertebrate and invertebrate aspartic proteinases is highly conserved (Davies, 1990; Dunn, 2002). Considering the significance of this particular prosegment residue, it is not surprising that zPMIIpropep enzyme was inactive and misfolded. Unlike most known aspartic proteinases PMII prosegment does not contain a conserved Lys residue and the absence of this residue in zPMIIpropep may have prevented proper folding, activation and ultimately leading to enzyme degradation by trypsin. The likely cause of unfolding is charge repulsion by the two active site aspartic acid residues. At near neutral pH, these residues are both negatively charged and require an interaction with Lys36 to neutralize their respective charges, allowing them to occupy their active conformations without repelling one another (James, 1983). The absence of a Lys36p complement in the PMII prosegment means the negative charges of the active site aspartic acid residues are not neutralized, allowing for repulsion and ultimately protein unfolding to occur. Given the inability to activate the enzyme, the PMIIpropep protein was not further characterized.

Alternatively, the zpepproPMII enzyme was capable of autoactivation. Both zPMII and zpepproPMII were incubated in the pH range of 2–7 for 30 min at 37°C. Both exhibited activation at pH 3–5, consistent to that previously reported for PMII (Hill *et al*., 1994). The prosegment of pepproPMII was cleaved three amino acids upstream of the N-terminus of the PMII mature sequence indicating that such a cleavage may have been more sterically driven as opposed to sequence driven (i.e. sequence is not as important as its location near the active site residues). The ability of zpepproPMII to activate only three amino acids upstream of its native cleavage may indicate that the zymogen protein undergoes similar conformational changes in the initial phase of cleavage, placing the prosegment in a reasonable position for activation. Reports have suggested that during activation the mature region, which is identical in both zpepproPMII and zPMII, undergoes large conformation changes (Bernstein *et al*., 1999; Friedman and Caflisch, 2008). It is possible then that the changes in the mature region of the PMII enzyme are unaffected by the alteration to the prosegment, and fortuitously place the prosegment in a position of cleavage. Densitometric analysis did, however, indicate that zpepproPMII activated at half the rate of PMII. The reduction in activation rate of PMII may be the result of cleavage occurring at a non-native activation sequence.

The ability of pepproPMII to autoactivate indicates a folded, functional enzyme suggesting that the sequence of the prosegment is not critical to PMII protein folding since the sequence conservation between pepsinogen prosegment and PMII prosegment is limited, sharing only 18.6% identity. To further investigate the role of the prosegment in PMII folding, a PMII enzyme without its prosegment, noproPMII, was generated. Along with mPMII and mpepproPMII, the kinetic parameters of noproPMII were determined. Analysis of kinetic parameters also revealed a 2-fold reduction in both k_{cat} and K_m measured for mpepproPMII against synthetic substrate 2837b. Alternatively, noproPMII exhibited a 20-fold reduction in k_{cat} and a 7-fold reduction in K_m. The reduction of the measured kinetic parameters suggests that although the PMII prosegment is not required to achieve a functional enzyme, it may prevent slight misfolding and

therefore play a role in contributing to conformation in the active site required for optimal catalysis. Recent molecular dynamics simulations work by Friedman and Caflisch (2008) suggested that Ser2 in the mature region of PMII may be an analog of the typically conserved Tyr9 residue in other pepsin-like aspartic proteinases. Given the difference in position of the N-terminus of the enzyme in its zymogen form to its active form, the N-terminus must make considerable movement during activation. Friedman and Caflisch suggest that during the transition from zymogen to mature enzyme Ser 2 interacts with one of the active site aspartic acid residues to donate a hydrogen atom, and stabilize the unprotonated state of the catalytic aspartates, ultimately enabling them to achieve their active conformation. In the two altered forms of PMII investigated here (pepproPMII and noproPMII), Ser 2 is obviously conserved and thus is conceivably available to interact with the active site residues. However, in pepproPMII, the N-terminal amino acid sequence is altered and contains an additional amino acid which may interfere with the interaction of Ser 2 with the active site aspartic acid residues. If these interactions are impeded, it is possible that the conformation of the active site aspartic acid residues is also altered which may be reflected in the change in both K_m and k_{cat}. However, in the case of noproPMII, there is no activation process, given the lack of prosegment, which is in contrast to the work by Friedman and Caflisch (2008) who demonstrated that the activation process was germane to the conformation of the active enzyme. In the absence of the movements associated with the typical activation process, it is not surprising that noproPMII had a drastically reduced k_{cat} and K_m. This result indicates that the presence of a prosegment does appear to have at least a minor affect on tertiary structure and ultimate functionality of the enzyme.

Although the prosegment of PMII was not critical in producing an active, functional enzyme, it may play a role in stabilizing the zymogen. CD wavelength and temperature scans were used to assess the temperature stability of the zymogen forms of the enzymes. It was determined that the zPMII had a significantly ($P \leq 0.05$) higher T_m when compared with zpepproPMII (52.27 versus 49.19°C, respectively). This difference suggests that disruption of the native interactions which exist between the prosegment and the mature region of PMII had a detrimental affect on the enzyme stability. In the zymogen form of PMII, there are several interactions between the prosegment and the mature region of the enzyme including one between Asp 4—Tyr 122p that fastens the prosegment to the C-terminal domain of the enzyme. It has been proposed that this interaction is important in 'prying' or forcing apart the two lobes of the enzyme (Bernstein *et al*., 1999), but it is also possible that this interaction stabilizes the charge repulsion of the active site aspartic acid residues by preventing the C-terminal lobe from distorting due to charge repulsion. This important interaction does not exist in the zpepproPMII chimeric enzyme since the sequence conservation between pepsinogen and PMII prosegments is limited. In the absence of the native interactions between the prosegment and mature enzyme, it is possible that that prosegment is no longer able to contribute to the stability of the protein and allows an even greater distortion of the zymogen structure due to charge repulsion of the negatively charged active site aspartic acids. The stability of zpepproPMII was not significantly ($P > 0.05$) different from mpepproPMII, suggesting that the pepsinogen prosegment did not enhance the overall stability of the zymogen enzyme as a prosegment typically does. Such an increased distortion may have resulted in the observed 2-fold decrease in activation rate of the zymogen protein. If the active site aspartic acid residues are allowed to separate to an even larger degree, than a greater conformation alteration would be necessary to achieve autoactivation. It is unclear why noproPMII had a significantly higher T_m than all other forms of the PMII proteins. This result may be indicative of an alteration in the structure of the protein which enhances the thermal stability of the enzyme. Further structural work would be necessary to elucidate the true cause of this unexpected result.

The inability to generate correctly folded pepsin when a different prosegment was attached was expected, since the significance of Lys36p to pepsin folding has been highlighted in a number of studies. In contrast, PMII was able to fold and function with its native prosegment replaced with a non-homologous sequence or in its absence. The alteration in kinetic parameters, particularly in the case of noproPMII, suggests that the prosegment is not absolutely essential to protein folding although its presence does enhance protein folding. However, the ability to obtain a functioning enzyme even without the prosegment in this study indicates that the region encoding for mature region of the enzyme likely plays a large role in the structure and function of the protein. While it is apparent that the prosegment has little role in dictating protein structure, we suggest that the prosegment in PMII has a definite role in enhancing the enzyme stability.

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