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Expression and characterization of the recombinant aspartic proteinase A1 from *Arabidopsis thaliana*

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ABSTRACT

The present study reports the recombinant expression, purification, and partial characterization of a typical aspartic proteinase from *Arabidopsis thaliana* (AtAP A1). The cDNA encoding the precursor of AtAP A1 was expressed as a functional protein using the yeast *Pichia pastoris*. The mature form of the rAtAP A1 was found to be a heterodimeric glycosylated protein with a molecular mass of 47 kDa consisting of heavy and light chain components, approx. 32 and 16 kDa, respectively, linked by disulfide bonds. Glycosylation occurred via the plant specific insert in the light chain. The catalytic properties of the rAtAP A1 were similar to other plant aspartic proteinases with activity in acid pH range, maximal activity at pH 4.0, K_m of 44 μM, and k_{cat} of 55 s^{-1} using a synthetic substrate. The enzyme was inhibited by pepstatin A.

1. Introduction

Aspartic proteinases (APs, E.C. **3.4.23**) constitute a heterologous class of proteases and are widely distributed in animals, microbes, viruses and plants (Davies, 1990; Dunn, 2002). APs share common characteristics and properties, e.g., high β-sheet content, aspartic acid active site residues, sequence homology, preferences for hydrophobic amino acids, activity at low pH and inhibition by pepstatin A (Dunn, 2002; Ramalho-Santos et al., 1998). APs have been implicated in a variety of functions including digestion (pepsin and chymosin), proprotein processing (cathepsin D, carboxypeptidase A) (Baldwin et al., 1993; Cooper, 2002), control of blood pressure (renin) (Cooper, 2002; Davies, 1990), the processing of the polyproteins during human immunodeficiency virus type1 activation (retroviral protease) (Davies, 1990; Ramalho-Santos et al., 1998) as well as food processing aids (e.g., chymosin in cheese making) (Andreeva et al., 1995; Jiang et al., 2005).

Unlike their animal and microbial counterparts, plant APs have received limited attention in the past and only a few have been characterized (Simoes and Faro, 2004). Plant APs have been detected and identified from many different plants sources including monocotyledons (e.g., barley, rice, maize and wheat) (Bi et al., 2005; Brodelius et al., 2005; Glathe et al., 1998; Radlowski et al., 1996; Tamura et al., 2007) and dicotyledons (e.g., cucumber, squash, tomato, cacao, *Arabidopsis*) (Guilloteau et al., 2005; Schaller and Ryan, 1996; Simoes and Faro, 2004). Plant APs are isolated principally from seeds, but have detected in flowers, leaves and roots (Kervinen et al., 1995; Mutlu and Gal, 1999). Biological functions include protein turnover, germination, senescence and defense against insect or microbial invasion (Mendieta et al., 2006; Mutlu and Gal, 1999; Rodrigo et al., 1989; Simoes and Faro, 2004). Unlike mammalian APs, a characteristic feature of most typical plant APs is the presence of an insert of approx. 100 amino acids (referred to as the plant specific insert, PSI) which separates the enzyme sequence into two regions (Simoes and Faro, 2004). It was thought that all plant APs contained the PSI in the nascent sequence; however, atypical APs lacking the PSI have been recently reported (e.g., nucellin from barley and rice (OSAsp1) and the CDR1 and PCS1 from *Arabidopsis* (Chen and Foolad, 1997; Xia et al., 2004; Bi et al., 2005; Ge et al., 2005). The PSI shows no homology with APs from non-plant sources, but has high similarity with the mammalian saposins, animal sphingolipid-activator proteins (Mutlu and Gal, 1999; Ramalho-Santos et al., 1998). Functions such as targeting to the vacuole, vesicle leakage, processing and a structure–function role in the mature enzyme have been proposed (Egas et al., 2000; Payie et al., 2003; Simoes and Faro, 2004); however, their role(s) is still speculative. The PSI domain assumes a compact globular structure formed by 5 alpha-helices linked to each other by three disulfide bridges (Park et al., 2001;

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Simoes and Faro, 2004). The PSIs have been called swaposins since they comprise the C-terminal portion of one saposin followed by N-terminal portion of another saposin linked by a sequence of about 20 amino acids (Brodelius et al., 2005) tentatively called saposin-linker (S-linker). Typical plant APs possess the PSI in the zymogen form; however, its partial or total removal can occur by a sequential processing mechanism (Mutlu and Gal, 1999) during activation yielding either monomeric or heterodimeric forms (Mutlu and Gal, 1999). Heterodimeric plant APs, consists of entities known as heavy chain (HC) and light chain (LC) which can be linked by disulfide bonds. Processing to form the active enzyme can be initiated in the S-linker region (Glathe et al., 1998) and can result in the partial or total removal of the PSI (Simoes and Faro, 2004). The reason for this differential mechanism of excision of the PSI remains unclear (Mutlu and Gal, 1999; Sarkkinen et al., 1992), but it appears to be a crucial step in regulating the activity of APs (Asakura et al., 2000; Castanheira et al., 2005).

Arabidopsis thaliana has facilitated the study of gene functions, providing valuable insights into plant development and environmental responses including disease, cold, and drought resistance thereby enhancing production in crop species (Meinke et al., 1998). Most of the knowledge regarding structure–function of plant APs has been generated from the study of phytepsin from barley seeds (Kervinen et al., 1999) and cardosin A from the flowers of cardoon (Frazao et al., 1999). However, the complete sequencing of the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000) and the prediction of more than 50 sequences that potentially encode APs (Beers et al., 2004; Faro and Gal, 2005) will allow for further studies to be conducted. Presently only three typical (possessing a PSI) APs (A1, A2 and A3) (Chen et al., 2002) and two atypical (lacking of a PSI) APs (CDR1 and PCS1) (Ge et al., 2005; Xia et al., 2004) have been identified in *Arabidopsis*; however, no AP from this plant has yet been fully characterized.

Typical APs from *A. thaliana* have been implicated in protein processing during seed development and degradation of storage proteins during seed germination (D'Hondt et al., 1993; Mutlu and Gal, 1999). The three typical APs present in *Arabidopsis* are differentially expressed in the plant suggesting that they may play multiple roles in addition to those proposed in seeds (Chen et al., 2002). The aspartic proteinase, AtAP A1, from *A. thaliana* was previously purified from the natural source (seeds), although, due to the limited amount obtained and its co-purification with AtAP A2, its complete characterization was not accomplished (Chen et al., 2002; Mutlu et al., 1998). The cDNA of AtAP A1 was isolated (D'Hondt et al., 1997) and its recombinant expression in *Escherichia coli* resulted in a non-functional protein product (Mutlu et al., 1999). Since AtAP requires posttranslational modification such as disulphide bond formation and glycosylation, the use of eukaryotic expression systems such as the yeast *Pichia pastoris* is necessary. Only a few reports have described the over-expression of plant APs, i.e., cardosin and cyprosin from cardoon (Castanheira et al., 2005; White et al., 1999), oryzasin from rice (Asakura et al., 2000), phytepsin from barley (Glathe et al., 1998) and recently an atypical AP from *A. thaliana* which is involved in disease resistance signaling (CDR1) (Simoes et al., 2007). This study reports the successful expression, purification and partial characterization of the recombinant aspartic proteinase AtAP A1 from *A. thaliana*.

2. Results and discussion

2.1. Selection of Pichia strain and construct

Previous attempts to express AtAP A1 in *E .coli* resulted in a nonfunctional enzyme (Mutlu et al., 1999). Our initial attempts using *P. pastoris* strain KM71 in combination with the expression vectors pHIL-S1 were also unsuccessful. Proteolysis of the recombinant proteins as well as the low expression level made KM71 an unsuitable strain to express rAtAP A1 (data not shown); therefore, a protease deficient strain of *Pichia* was tested. The use of protease-deficient strains has been found to enhance the yield and the quality of various heterologous proteins (Cereghino and Cregg, 2000; Macauley-Patrick et al., 2005; Sreekrishna et al., 1997).

P. pastoris SMDll68H is a strain that is protease deficient for proteinase A (*PEP4*) and has been shown to be effective in reducing degradation of some foreign proteins (Cereghino and Cregg, 2000; Daly and Hearn, 2005). This strain was employed in order to reduce the premature proteolysis of the recombinant proteins. Also the vector system was replaced with pPICZαA since this vector possesses important features such as the gene conferring resistance to the antibiotic zeocin, a useful marker during the selection of transformants, the signal sequence (α-mating factor) from *Saccharomyces cerevisiae* for secretion of the recombinant protein, and the c-*myc* epitope and $His_{(6)}$-tag within the C-terminus for both detection and purification.

2.2. Confirmation of rAtAP A1 expression

Expression of soluble rAtAP A1 was confirmed by anti-$His_{(6)}$ Western blot analysis where an expected band of approx. 56 kDa was observed (Fig. 1). The band of interest was subsequently in-gel trypsin digested and analyzed by MALDI-TOF-MS (Tables 1 and 2). Fourteen masses of peptides matched the sequence of the aspartic proteinase of *A. thaliana* (AtAP A1) which further confirmed that the rAtAP A1 was successfully expressed.

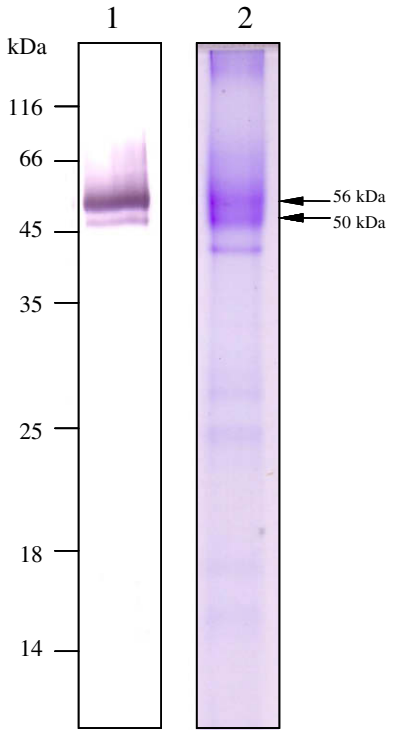

Fig. 1. Identification of rAtAP A1 purified by immobilized metal affinity chromatography. (A) Identification of the rAtAP A1 Western blot using anti-$His_{(6)}$ (lane 1) and SDS–PAGE (lane 2) analysis. Sample purified after 36 h of expression. Analysis was performed under non-reducing conditions (10 μg of protein). The arrows in the gel indicate the bands used for MS identification.

Table 1
Amino acid sequence of the recombinant pro-AtAP A1 His-tagged protein

1	AEAE FGRFAE	RNDGTFRVGL	KKLKLDSKNR	LAARVESKQE	KPLRAYRLGD
51	SGDADVVVLK	NYLDAQYYGE	IAIGTPPQKF	TVVFDTGSSN	LWVPSSKCYF
101	SLACLLHPKY	KSSRSSTYEK	NGKAAAIHYG	TGAIAGFFSN	DAVTVGDLVV
151	KDQEFIEATK	EPGITFVVAK	FDGILGLGFQ	EISVGKAAPV	WYNMLKQGLI
201	KEPVFSFWLN	RNADEEEGGE	LVFGGVDPNH	FKGKHTYVPV	TQKGYWQFDM
251	GDVLIGGAPT	GFCESGCSAI	ADSGTSLLAG	PTTIITMINH	AIGAAGVVSQ
301	QCKTVVDQYG	QTILDLLLSE	TQPKKICSQI	GLCTFDGTRG	VSMGIESVVD
351	KENAKLSNGV	GDAACSACEM	AVVWIQSQLR	QNMTQERILN	YVNELCERLP
401	SPMGESAVDC	AQLSTMPTVS	LTIGGKVFDL	APEEYVLKVG	EGPVAQCISG
451	FIALDVAPPR	GPLWILGDVF	MGKYHTVFDF	GNEQVGFAEA	ATTAAAASFL
501	EQKLISEEDL	NSAV*D*HHHHH	H		

Shaded residues represent tryptic peptides detected by MALDI-TOF mass spectrometry analysis (see Table 2).

2.3. Determination of conditions of expression and activation for rAtAP A1

Activation of rAtAP A1 resulted in loss of the C-terminal His_6-tag as evidenced by the loss of immunoreactivity against the antibody (anti-$His_{(6)}$) at low pH (Fig. 2a). Proteolytic processing of rAtAP was most efficient at pH 3–4 and samples activated at pH 3.5 showed a plateau of maximum activity after approx. 60–90 min of incubation at 37 °C (Fig. 2b). This activation process was inhibited by pepstatin A (data not shown). The autoactivation of plant APs at low pH values has also been observed in others plant APs *in vitro* (Castanheira et al., 2005; Domingos et al., 2000; Glathe et al., 1998) and has significance for autoprocessing *in vivo* (Otegui et al., 2006).

Partial purification of rAtAP A1 using immobilized metal affinity chromatography (IMAC) resulted in low protein recovery and the purity of the protein decreased with increasing induction time (data not shown). It is suggested that some activation and/or degradation of the rAtAP A1 occurred during expression and IMAC purification. Total proteolytic activity against the synthetic substrate SS1 showed an increase with induction time, reaching a maximum after 4 days (data not shown). The proportion of active enzyme in the expression medium was variable from batch to batch (approx. 10–50% of relative activity) and was dependent on induction time. The accumulation of the active form has been also reported for recombinant expression of cyprosin in *P. pastoris* (White et al., 1999) and the post-translational loss of the $His_{(6)}$-tag located at the C-terminal also has been reported during the recombinant expression of β-mannanase in *P. pastoris* and polyamine oxidase in *E. coli* (Tavladoraki et al., 2006; Xu et al., 2002). Proteases secreted by the host cells may be involved in the degradation and possible activation of the rAtAP A1 since it has been speculated that the activation of plant APs could occur at neutral pH by action of other proteases (Dostál et al., 2005; White et al., 1999). In the

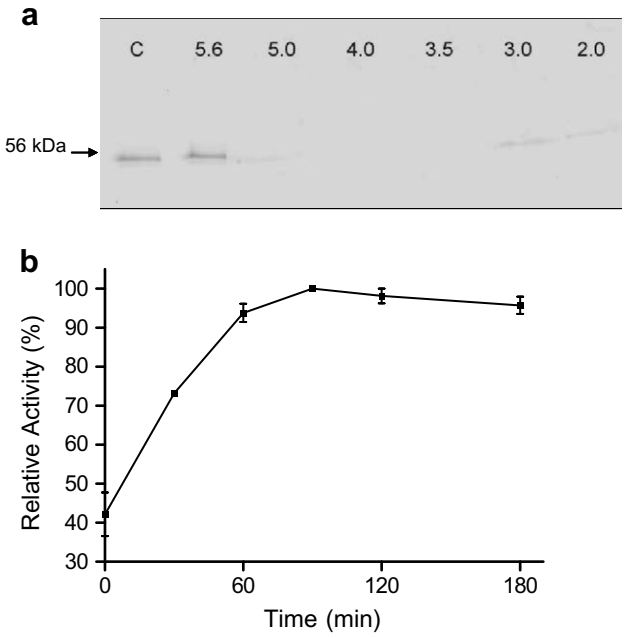

Fig. 2. pH-dependence of rAtAP A1 activation. (a) Western blot (anti-$His_{(6)}$-tag) of rAtAP A1 activated at acidic pHs for 2 h at 37 °C (4 μg of protein). Sample control (C) was at pH 7.0. (b) Increase of specific activity after incubation at pH 3.5, 37 °C. Samples were harvested after 48 h of expression. Values are the mean ± SD of four determinations from two independent experiments.

Table 2
Molecular masses of the expected and observed tryptic peptides matched to the sequence of the pro-AtAP A1 His-tagged protein

Expected	Observed	Residues	Missed cut	Amino acid sequence
2140.05	2140.52	61–79	0	NYLDAQYYGEIAIGTPPQK
1969.98	1970.39	80–97	0	FTVVFDTGSSNLWVPSSK
2763.42	2763.97	124–151	0	AAAIHYGTGAIAGFFSNDAVTVGDLVVK
2720.48	2722.85	161–186	1	EPGITFVVAKFDGILGLGFQEISVGK
1678.89	1679.17	171–186	0	FDGILGLGFQEISVGK
2259.01	2259.61	212–232	0	NADEEEGGELVFGGVDPNHFK
2360.25	2360.81	304–324	0	TVVDQYGQTILDLLLSETQPK
1754.84	1755.20	325–339	1	KICSQIGLCTFDGTR
1626.75	1627.04	326–339	0	ICSQIGLCTFDGTR
1678.82	1679.17	340–355	1	GVSMGIESVVDKENAK
2721.27	2722.85	356–380	0	LSNGVGDAACSACEMAVVWIQSQLR
1421.70	1421.92	388–398	0	ILNYVNELCER
1421.75	1421.92	427–438	0	VFDLAPEEYVLK
2252.16	2252.75	439–460	0	VGEGPVAQCISGFIALDVAPPR
2001.89	2002.35	474–491	0	YHTVFDFGNEQVGFAEAA

present study, *P. pastoris* SMD1168H, a proteinase A (proA) deficient strain, was used. ProA (*PEP4*) is a vacuolar aspartic proteinase necessary for the activation of vacuolar proteases such as carboxypeptidase Y (*CPY*) and proteinase B (*PRB1*) (Macauley-Patrick et al., 2005; Parr et al., 2007). Although certain yeast strains are deficient in proA (*pep4*) some proteinase B activity (approx. 50%) is present which could activate other vacuolar proteases (Romanos et al., 1992; Glesson and Howard, 1994; Macauley-Patrick et al., 2005).

AtAP A1 is found in plant tissues and is localized in the multivesicular body (MVB) forming part of the vacuolar processing enzymes (VPEs) where activation occurs as lumen gradually acidifies (Otegui et al., 2006). Activation of typical plant APs involves removal of the prosegment (located at the N-terminus) and, by a multi-step autoproteolytic processing, the partial or total removal of the PSI (located between the N- and C-terminal domains) (Mutlu and Gal; 1999; Simoes and Faro 2004). Also, although less common, removal of some amino acids at the C-terminal may also occur (Park et al., 2001). The loss of the C-terminal His-tag in rAtAP during activation did not affect the processing into the mature enzyme.

2.4. Purification of the mature rAtAP A1

The use of IMAC was discontinued in the purification protocol for the rAtAP A1 since activation was occurring during expression, e.g., the loss of the $His_{(6)}$-tag in the rAtAP A1 mature form, which limited the amount of enzyme obtained by this procedure. An alternative purification protocol was then established. The first step involved ion-exchange chromatography (IEC) using DEAE-Sepharose followed by IEC using a Mono Q column which was effective in increasing the specific activity as well as removing the yellow pigment associated with yeast expression medium (White et al., 1999). The sample obtained from IEC using a Mono Q column was then subjected to acid incubation at pH 3.5, 37 °C which resulted in the final mature form of the rAtAP A1 via autoactivation. This acid incubation step included centrifugation to remove acid-precipitable contaminant proteins. An increase in total activity was observed in the first 3 h of incubation. After 24 h of incubation, 10–20% of total activity was lost; however, acid conditions were helpful in eliminating contaminating proteins through hydrolysis.

The acid-activated sample consisted predominantly of two protein bands, 35 and 32 kDa, as seen via SDS–PAGE under reducing conditions. This sample was then further purified using a two step gel filtration procedure. The first gel filtration (Fig. 3) permitted the separation of the 35 and 32 kDa protein and a second gel filtration step resulted in improved purification by isolating mainly the 32 kDa fraction polypeptide which was associated with proteolytic activity (Fig. 3). The antibody anti-AtAP A1 (for the heavy chain, HC) recognized both the mature form of 48–50 kDa as well as the 32 kDa band (data not shown) which was identified as the HC (see Section 2.5). The 32 and 35 kDa fractions were shown to be different in composition (see Section 2.5 and Fig. 4a).

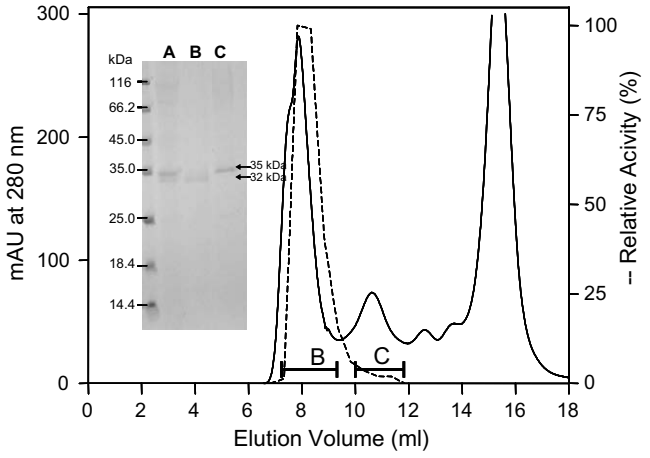

Fig. 3. Purification of rAtAP A1 by gel filtration chromatography. Fractions (0.25 ml) were analyzed for proteolytic activity (dashed line) using the substrate SS1. Bars indicate the pooled fractions (eight fractions) and analyzed by SDS–PAGE (insert in the figure) under reducing conditions. Lane A: sample before gel filtration; lane B: pool of fractions with high proteolytic activity (elution from 7.5 to 9.5 ml); lane C: fractions eluted from 10 to 12 ml. The gel was stained with Commassie Blue.

The expression and purification protocol established in this study yielded 300–400 μg of rAtAP A1 preparation per liter culture (25–30 nanokatals). Previously, only a few reports have described the overexpression of functional plant APs; however, their recombinant expression was associated with low yield. White et al. (1999) reported 1 mg l^{-1} for the expression of cyprosin B using *P. pastoris*, while Glathe et al. (1998) reported a yield of 500 μg l^{-1} for prophytepsin using bacuolovirus-infected insect cells. A lower yield of AtAP A1 in our study could be related to the co-expression of the non-glycosylated incorrect folded form described above which was largely removed during purification.

2.5. Characterization of the heavy and light chains in the rAtAP A1 mature form

rAtAP A1 preparation in the absence of β-ME was visualized as a broad band (approx. 48–50 kDa), indicating microheterogeneity (Fig. 5). The microheterogeneity observed in the heterodimeric mature rAtAP A1 is largely attributed to the consensus glycosylation site located in the PSI (Chen et al., 2002) which is part of the light chain (LC). The smear of protein bands (lane 1) appeared as a sharp band of approx. 47 kDa after enzymatic deglycosylation (lane 3) under non-reducing conditions. In addition, the HC (ca. 32 kDa) and the LC (ca. 16 kDa) (lane 4), in the presence of β-ME, also became visible. The above results suggested that rAtAP A1 is a heterodimeric glycosylated protein with its HC and LC disulfide linked.

N-terminal analysis for the HC (32 kDa) of rAtAP A1 mature form indicated an amino acid sequence of **RLGDSG** while the sequence for the LC of rAtAP A1 was **SNGVGD** which indicated that the long fragment of the PSI (44 AAs, starting at residue 61 of the PSI) was retained (Fig. 4a).

N-terminal sequencing of the 35 kDa polypeptide indicated a sequence of **LGLGF** which corresponded to the sequence of a degradation product of the mature AtAP A1 (Fig. 4a). Based on its estimated molecular mass and N-terminal sequence, this fraction may be a truncated form of AtAP A1 consisting of the latter part of the HC (starting at residue 129 of the HC) follow by the entire PSI containing the consensus glycosylation site and the LC (Fig. 4a and b) although no glycosylation was evident (Fig. 5). It has been previously shown that expression systems which generate high yields of recombinant material can result in misfolded proteins (Kukuruzinska et al., 1987) which may prevent post-translational glycoslylation. In the present study, it would appear that *Pichia* secreted both the correctly folded glycosylated and possibly the incorrect folded non-glycosylated form.

Previous attempts to obtain the N-terminal sequence of the native AtAP A1 have been unsuccessful most likely due to contamination of preparation by AtAP A2 (Mutlu et al., 1998; Chen et al., 2002). The presence of several polypeptides in AtAP preparations (e.g., 31, 28.5, 16 and 6 kDa) (Mutlu et al., 1998) supports the assumption that processing of APs in natural systems involves additional processing events such as trimming of remaining parts of PSI or alternative cleavage sites which can result in various shorter chains. Additionally, the presence of other fragments (98, 24 and 23 kDa fractions) detected with anti-AtAP A1 by Mutlu et al. (1999) suggested the presence of incompletely processed forms

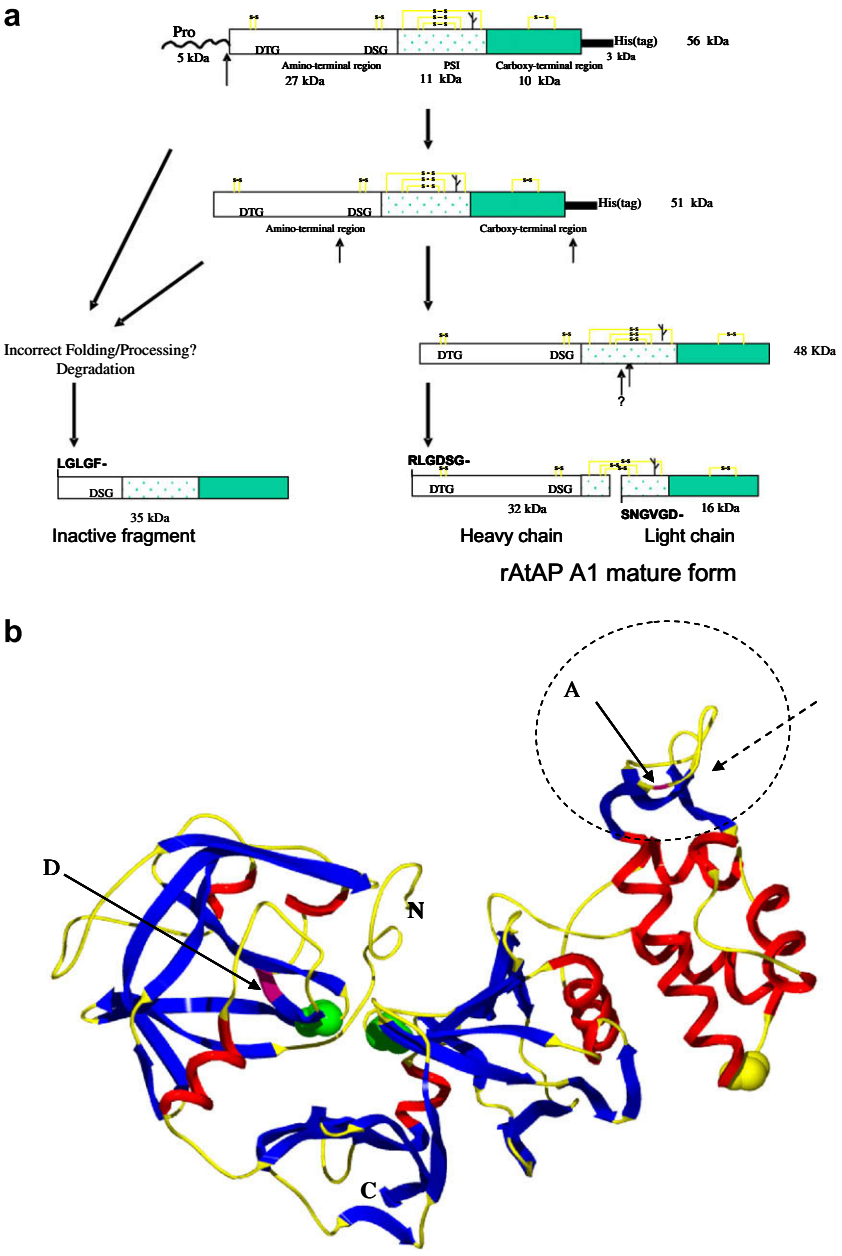

Fig. 4. Hypothetical processing for the rAtAP A1 during activation and degradation and ribbon diagram of rAtAP A1. (a) Schematic representation of processing events of rAtAP A1. N-terminal sequences of the identified polypeptides and molecular mass are indicated. The arrows indicate a possible cleavage sites; the proteolytic degradation of the putative incorrectly folded non-glycosylated form is also displayed. (b) Ribbon diagram representation for rAtAP A1. The area of the PSI where most of the plant APs starts its processing is highlighted by a dashed circle. Arrow with letter A shows the cleavage site identified in the light chain. Catalytic Asp residues and the putative *N*-glycosylation site are in green and yellow space-filling, respectively. The prosegment is not included in the model. Coordinates were obtained using first approach by Swiss-Model.

with no AP activity. In this study we also detected a faint band of approx. 20 kDa in the rAtAP preparation (Fig. 5) which was not recognized by the antibody anti-AtAP A1 (against the heavy chain).

The present study was successful in reporting the N-terminal sequence for the recombinant AtAP A1. However, since no conclusive data for N-terminal sequence of the natural source has been reported until now (Mutlu et al., 1998; Chen et al., 2002), a clear difference between *in vivo* and *in vitro* processing for AtAP A1 cannot be made.

2.6. The pH- and temperature-dependence of rAtAP A1 activity

pH- and temperature-dependence of rAtAP A1 activity are shown in Fig. 6a and b, respectively. Using SS1 as the substrate, rAtAP A1 exhibited activity in the acidic pH range from 2.0 to 6.0 with a maximum activity at pH 4.0 (Fig. 6a). A pH optimum of 3.5 was previously reported for the wild-type AtAP A1 using hemoglobin as a substrate (Mutlu et al., 1998) although it was reported that AtAP A2 was also co-purified from *Arabidopsis* seeds (Chen et al., 2002). The pH optimum of rAtAP A1 is similar to that previously reported for barley phytepsin, which is between pH 3.5 and 3.9 (Sarkkinen et al., 1992). In general, the pH optimum for plant aspartic proteinases is found in the acidic pH range. Maximum activity at pH 2.0–2.5 was reported for nephentensins from carnivorous plants and oryzacin from rice (Asakura et al., 2000; Athauda et al., 2004), with the highest pH optimum of 4.5–5.0 reported for cardosin A. The temperature-dependence of rAtAP A1 activity was also examined. rAtAP A1 displayed maximum activity from

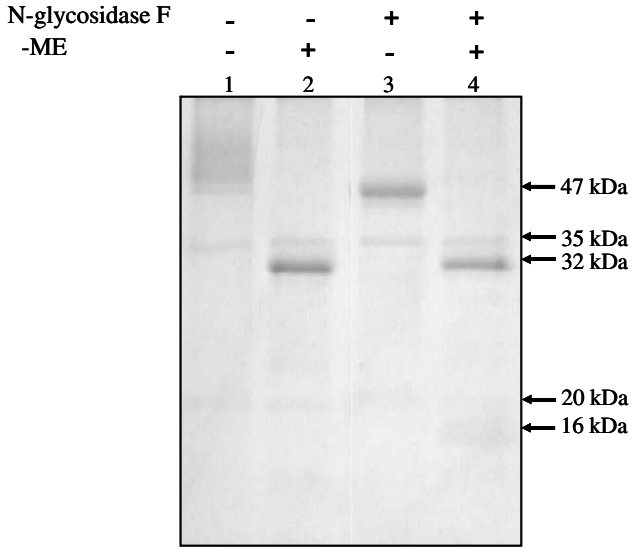

Fig. 5. Analysis by Tricine–SDS–PAGE of the purified rAtAP A1. Sample was analyzed before (−) and after (+) enzymatic *N*-deglycosylation in the presence (+) or absence (−) of β-ME. Note the visualization of the light chain, LC (approx. 16 kDa) after *N*-deglycosidase F treatment. Six micrograms of protein were loaded per well.

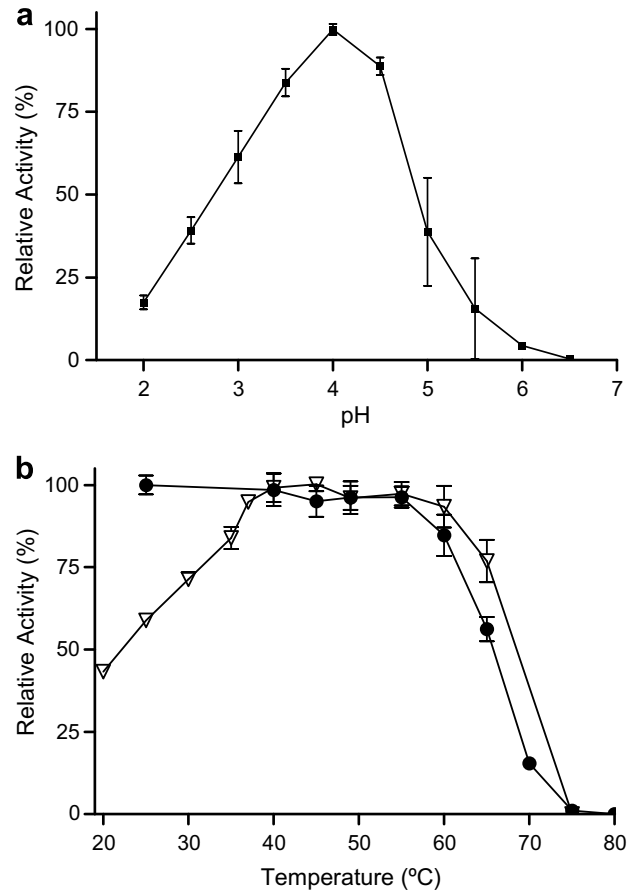

Fig. 6. Effect of reaction pH and temperature on the activity of rAtAP A1. (a) Activity was determined using 0.1 mM SS1 at 37 °C at the indicated pH values. The relative activity was expressed as a percentage of the highest activity over the pH range examined. (b) Effect of the temperature on the activity (▽) of rAtAP. Relative activity was expressed as a percentage of the highest activity at the temperature examined. For rAtAP stability (●) samples at pH 3.5 were incubated at each temperature for 10 min and incubated at 0 °C for 30 min before activity determination at pH 3.5 and 37 °C. The mean residual activity was compared with the mean activity of the sample kept at 25 °C (control). Each point represents the mean ± SD of six determinations from two independent experiments.

45 to 60 °C at pH 3.5 (Fig. 6b). Temperatures higher than 60 °C led to a loss in activity and most of the activity (85%) was lost after 10 min at 70 °C and pH 3.5.

Similar results were observed for other plant APs. Maximal activities were reported at 55 °C and pH 3.5 for the heterodimeric AP isolated from *Theobroma cacao* seeds (Guilloteau et al., 2005), at 50 °C and pH 4.7 for cardosin A from *Cynara cardunculus* (Castanheira et al., 2005) and at 55 °C at pH 3.0 for nephentensin I from *Nephentes distillatoria* (Athauda et al., 2004).

2.7. Kinetic properties of rAtAP A1, inhibition and action against hemoglobin

The K_m and k_{cat} obtained for rAtAP A1 were 44 ± 3.9 μM and 55.9 ± 5.2 s^{-1}, respectively, using the synthetic substrate SS1. Using the SS1 synthetic substrate, the K_m value for rAtAP A1 was comparable to both commercial and recombinant pepsin as well as recombinant cyprosin (≈50 μM); however, k_{cat} was 2 and 3 times lower than those reported for commercial and recombinant pepsin, respectively (White et al., 1999; Yoshimasu et al., 2002). The catalytic efficiency (k_{cat}/K_m) for rAtAP A1, 1240 ± 200 mM^{-1} s^{-1}, was similar to that reported for cardosin B (808 mM^{-1} s^{-1}) (Verissimo et al., 1996) and cyprosin (1100 mM^{-1} s^{-1}) (White et al., 1999), both from *C. cardunculus* using the same synthetic substrate.

Denatured hemoglobin, a common substrate for aspartic proteinases (Mutlu et al., 1998), was hydrolyzed by rAtAP A1 (Fig. 7). rAtAP A1-catalyzed hemoglobin degradation was strongly inhibited by the AP inhibitor pepstatin A but not by PMSF, leupeptin and EDTA. Similar effects were observed when the synthetic peptide SS1 was used as the substrate (Table 3) and a K_i for pepstatin A of ∼0.5 nM was determined. Interestingly, the compound ALLN, a cysteine protease inhibitor which is highly selective towards calpain and cathepsin L (Zhang et al., 1999), partially inhibited the activity of rAtAP A1 against hemoglobin (Fig. 7) and SS1

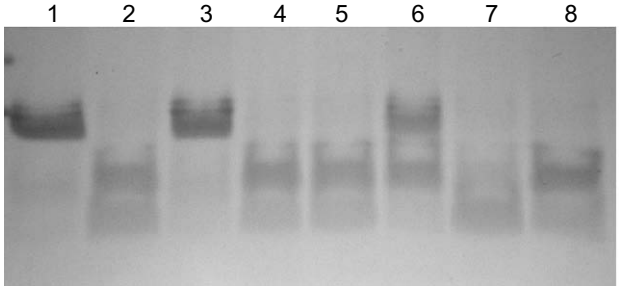

Fig. 7. Degradation of hemoglobin by rAtAP A1 and the effect of various protease inhibitors. SDS–PAGE (15%) analysis of denatured human hemoglobin degradation by rAtAP A1 after incubation in 100 mM sodium acetate buffer pH 3.5 at 37 °C for 2 h. Five micrograms of protein in a 20 μl volume were loaded per lane. Lane 1: hemoglobin; lane 2: hemoglobin with 10 nM rAtAP A1; lanes 3–7: hemoglobin with rAtAP A1 in the presence of pepstatin A (1 μM), PMSF (1 mM), leupeptin (100 μM), ALLN (15 μM) and EDTA (4.5 mM), respectively; lane 8: DMSO was the control.

Table 3
Residual activity of rAtAP A1 after incubation with protease inhibitors[a]

Inhibitor	Concentration	Residual activity (%)
None	–	100
Pepstatin A	1 μM	0
ALLN	10 μM	7
PMSF	1 mM	96
Leupeptin	100 μM	85
EDTA	4.5 mM	96

[a] The reactions were carried out with the synthetic substrate SS1 (100 mM) in 0.1 M sodium acetate buffer (pH 3.5) at 37 °C after 10 min of incubation of the enzyme with the inhibitor.

(Table 3). This inhibitor has also been shown to be active against plasmepsins II and IV, aspartic proteinases from *Plasmodium falciparum* (Kim et al., 2006).

3. Conclusions

A functional, soluble recombinant aspartic proteinase AtAP A1 from *A. thaliana* expressed in *P. pastoris* was reported for the first time and a purification procedure was established. The rAtAP A1 was capable of autoactivation at acid pH with an optimum of pH 3–4. The proteinase was heterodimeric (32 + 16 kDa) and displayed microheterogeneity, likely attributed to glycosylation. The N-terminal sequences of the HC and LC of rAtAP A1 are reported for the first time.

4. Experimental

4.1. General

The *P. pastoris* expression strains SMD1168H (*pep4*), *E. coli* TOP 10F′, expression vectors pPICZαA and the sequencing primers 5′AOX1 and 3′AOX1 were purchased from Invitrogen (San Diego CA). Other oligonucleotides primers and the synthetic octapeptide KPAEFF(NO_2)AL (SS1) were synthesized by Sigma Genosys (Oakville, ON, Canada). All other chemicals and reagents unless otherwise indicated were purchased from Fisher Scientific.

4.2. AtAP gene cloning and transformation

A cDNA clone for AtAP A1 previously isolated by D'Hondt et al. (1997) (At1g11910; EMBL Accession No. U51036), was subcloned in the pHIL-S1 vector in the *Eco*R1 site was amplified by PCR using primers 5′-AOX1: 5′-GACTGGTTCCAATTGACAAGC-3′ and RevA-tAP*Sac*II: 5′-AAATCACCGCGGTCGTGGCTGCCTCTGC-3′; the *Sac*II restriction site is underlined. The resulting fragment, along with vector pPICZαA, were digested with restriction enzymes *Eco*R1 and *Sac*II, and purified using the QIAquick PCR DNA and Gel purification kits (Qiagen Inc.). The insert (1472 bp) was subcloned into the expression vector and used to transform *E. coli* (Top10F′) cells. The selection of transformants containing the vector was made on low salt LB agar plate containing zeocin (25 μg ml^{-1}). The recombinant plasmid was purified and sequenced by DNA sequencing using the primers 5′AOX1 and 3′AOX1 to confirm the insertion into the correct translational reading frame. The purified plasmid AtAP-A1-pPICZαA*EcoR1Sac*II (AtAP A1 gene inserted into the vector pPICZαA in the restriction sites *Eco*R1 and *SacII)* was linearized by digestion with *Pme*I and electroporated into *P. pastoris* (SMD1168H strain) competent cells pretreated with lithium acetate and DTT to improve transformation efficiency (Wu and Letchworth, 2004). The transformed *Pichia* were selected in YPDS agar plates containing 500 μg ml^{-1} of zeocin.

The incorporation of the AtAP gene in the *Pichia* genome was confirmed by PCR (Linder et al., 1996) using 5′AOX1 and 3′AOX1 primers and in combination with AtAP A1 specific primers AtAPScrFor: 5′-GGATCCAGAGCCAGTTGAGGCAAAACATGACTC-3′ and AtAPScrRev: 5′-GAGTCATGTTTTGCCTCAACTGGCTCTGGATCC-3′.

The transformed *Pichia* clone was stable at −80 °C in glycerol and expressed after replating on YPD plate supplemented with zeocin (250 μg ml^{-1}) to ensure pure colonies (Macouzet et al., 2005).

4.3. Extracellular expression of the rAtAP A1 in P. pastoris

A recombinant clone of *P. pastoris* with AtAP A1 was cultured in 20 ml YPD medium containing zeocin (100 μg ml^{-1}) overnight at 30 °C with shaking at 250 rpm. Five milliliters of this culture were used to inoculate 1 l buffered (pH 6.0) glycerol complex media BMGY [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, 1.34% (w/v) yeast nitrogen base, 0.4 mg l^{-1} biotin, 0.5% (v/v) glycerol] containing ampicillin (50 μg ml^{-1}). The culture was grown for 24–36 h to an OD_{600} = 6–8 AU (absorbance units) and harvested by centrifugation 2000g for 5 min. The yeast pellet was resuspended in 1 l of induction medium BMMY (similar to BMGY but containing 2% (v/v) MeOH instead of glycerol) containing 0.5% (w/v) casaminoacids and 50 μg ml^{-1} ampicillin. A volume of 500 ml in a 2 l baffled flask was used during the induction phase with constant shaking (250 rpm) at 28 °C. The induction was maintained by the addition of 1% (w/v) of MeOH every 24 h. The expressed protein in soluble form was harvested by centrifugation (8000g for 20 min) after a total of 4 days of induction and subjected to purification.

4.4. Purification of the rAtAP A1 by immobilized metal affinity chromatography (IMAC)

The purification of the $His_{(6)}$-tagged recombinant AtAP A1 in the zymogen form using immobilized metal affinity chromatography (IMAC) was evaluated. The supernatant (1 l) from the culture with less than 36 h of induction was harvested and concentrated using a stirred ultrafiltration cell (Model 8400, Millipore, Bedford, MA) with a 30 kDa cut-off membrane (YM30) and washed with 500 ml of 20 mM Tris–HCl, pH 7.5. The sample (approx. 25 ml) obtained was diluted with the same volume of binding buffer (100 mM sodium phosphate, 600 mM NaCl, 5 mM of imidazole, pH 7.5) and applied to a nickel affinity column (6.4 ml His-Select cartridge, Sigma Co.). The column was washed with approx. 30 ml of washing buffer (50 mM sodium phosphate, 300 mM NaCl with 2.5 mM of imidazole, pH 7.5) and the bound protein eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM of imidazole, pH 7.5).

4.5. Purification of the rAtAP A1 in the active form

Samples harvested after 4 days of expression (1 l) were diluted two times with buffer A (20 mM Tris-HCl, pH 7.5) and applied to DEAE Sepharose CL6B (Amersham Biosciences) open column (2.6 × 10 cm) at a flow rate of 150 ml h^{-1} at 4 °C. After extensive washing with buffer A, the protein was eluted with 600 mM NaCl (300 ml) in buffer A. After desalting with buffer A by ultrafiltration (YM30), the sample (approx. 100 ml) was applied to a FPLC Mono Q column (HR 10/10 Amersham Biosciences). The column was washed with buffer A (80 ml) followed by washing with 100 mM NaCl (50 ml) at a flow rate of 2 ml min^{-1} and the protein eluted with 450 mM NaCl in buffer A. Active fractions were pooled (15–20 ml) and diluted with the same volume of sodium acetate buffer 200 mM, pH 3.5. After 3 h of incubation at 37 °C, the sample was centrifuged (5000g for 10 min) and the supernatant was incubated at 37 °C. After 24 h incubation, the sample was centrifuged (5000g for 10 min) and the supernatant concentrated and washed with 20 mM Tris–HCl, pH 7.0, by ultrafiltration (YM30). The concentrated sample (approx. 2 ml) was filtered (0.22 μm) and aliquots (400 μl) were applied to size exclusion chromatography (Superose 12 10/30 GL column) pre-equilibrated with buffer B (Tris–HCl 50 mM, pH 7.0 with 150 mM NaCl). Protein was eluted using buffer B at a flow rate of 0.5 ml min^{-1} in a FPLC AKTA system (Amersham Biosciences). Purity was checked by SDS–PAGE and a second gel filtration was performed. Active fractions were pooled and concentrated to 2.0 ml and frozen at −80 °C in glycerol (25% (v/v) final concentration) for further analysis.

4.6. Identification of the rAtAP A1 expressed in P. pastoris

The expression and purification were monitored by SDS–PAGE according to Laemmli (1970) using 15% acrylamide gels in a Mini-Protean II electrophoretic cell (BioRad, Hercules, CA) at 200 V for 1 h. Separated proteins were transferred to PVDF membrane (Roche). Electroblotting was conducted for 50 min at a constant voltage (100 V, 4 °C) on a mini TransBlot electrophoretic transfer cell (BioRad, Hercules CA). Anti-$His_{(6)}$ mouse monoclonal antibody (Roche), and goat anti-mouse IgG (H + L)-alkaline phosphatase conjugate (BioRad, Hercules, CA) were used for the detection of the recombinant $His_{(6)}$-tagged protein. Additionally, anti-AtAP rabbit antibody against the HC (Ab319) (kindly provided by Dr. Susannah Gal, State University of New York, Binghamton) (Mutlu et al., 1999) and goat anti-rabbit IgG (H + L)-alkaline phosphatase conjugate (BioRad) were used. The Alkaline phosphatase was detected using the conjugate substrate kit from BioRad. Protein concentration was determined using the BioRad DC assay using BSA as standard.

To confirm that AtAP was successfully expressed, the in-gel trypsin digestion of the partially purified protein was analyzed by mass spectrometry at the University of Guelph Biological Mass Spectrometry Facility (Katayama et al., 2001). Protein bands were excised, destained, reduced with DTT and cysteine residues alkylated with iodoacetamide, after which trypsin (Sigma–Aldrich, St. Louis, MO, USA) was added overnight to digest protein. Peptides were extracted using CH_3CN/HCO_2H, concentrated and desalted using ZipTip μ-C18 (Millipore). Peptides were eluted on the target with 4 μl of α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich) and analyzed using a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker, Germany) in positive-ion, linear detection mode with external calibration. Monoisotopic masses generated were searched using Mass Profound (http://www.prowl.rockefeller. edu).

4.7. pH-dependence of activation

Determination of the optimum pH for autoactivation of the rAtAP A1 was performed by mixing 20 μl (0.8 mg ml^{-1}) of the partially purified protein with the same volume of buffer, i.e., 100 mM NaOAc for pH values 2.0, 2.5, 3.0, 4.0 and 5.0 and 100 mM sodium phosphate for pH values 6.0 and 7.0. After incubation for 2 h at 37 °C, the reaction was terminated by heating at 100 °C in SDS–PAGE sample buffer without β-ME and loaded onto 15% polyacrylamide gel. Gels were run and transferred to PVDF membrane for Western blot analysis using anti-$His_{(6)}$ antibody.

4.8. Deglycosylation and N-terminal sequencing of the rAtAP A1

Deglycosylation of the rAtAP A1 preparation was performed by incubating 50 μl of the purified sample (0.4 mg ml^{-1}) with 1 unit of N-glycosidase F (Roche Biochemicals) in 100 mM sodium phosphate buffer pH 7.2. After incubation for 15 h at 30 °C, the reaction was terminated by heating the sample for 5 min at 100 °C in SDS–PAGE sample buffer in the presence of β-ME (0.75%, v/v) and analyzed by Tricine–SDS–PAGE (16.5% separation gel). The separated peptides were transferred to PVDF membrane and stained with Gelcode blue following the manufacture's protocol (Pierce Chemical Co.). Peptides bands were excised from the membrane and their N-terminal amino acid sequence determined by the Edman degradation method at the Hospital for Sick Children (Toronto, ON).

4.9. Activity determination and kinetic measurements

Activity was determined at 37 °C using 0.1 mM of the synthetic chromogenic substrate SS1 diluted in 100 mM sodium acetate buffer at pH 3.5. Five microliters of enzyme solution were used in a final reaction volume of 100 μl. The rate of substrate hydrolysis was monitored at 300 nm in a Biochrom Ultrospec 3100 pro UV–Vis spectrophotometer using a temperature controlled cell holder set at 37 °C and a molar absorptivity coefficient of 1350 M^{-1} cm^{-1} (Tanaka and Yada, 1996). For the kinetics studies, 6 nM of rAtAP A1 and different substrate concentrations of SS1 in the range of 0.01–0.25 mM at pH 3.5 and 37 °C were used. Non-linear least-square fit of the data was used to calculate K_m and V_{max} using the software GraphPad Prism v4.0 (San Diego, CA). Three determinations were done in triplicate. In calculating of k_{cat}, the enzyme concentration was determined by pepstatin titration assuming an equimolar ratio 1:1 enzyme:inhibitor (Knight, 1995; Knight and Barret, 1976).

4.10. pH optimum for activity

The pH optimum for rAtAP A1 activity was analyzed using 0.1 mM of the above synthetic substrate SS1 at 37 °C. The reaction was carried in the following buffers: 100 mM sodium citrate, pH 2.0, 2.5 and 3.0; 100 mM NaOAc, pH 3.5, 4.0, 4.5, 5.0 and 5.5; 100 mM sodium phosphate, pH 6.0 and 7.0 (Athauda et al., 2004; Castanheira et al., 2005). The activity was determined in triplicate and two determinations were performed.

4.11. Effect of temperature on activity and stability of rAtAP A1

Activity was determined at different temperatures using 0.1 mM SS1 in 100 mM sodium acetate, pH 3.5. Relative activity was expressed as a percentage of the highest activity at the temperature examined. For stability samples at pH 3.5 (100 mM sodium acetate) were incubated at 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C for 10 min. Samples were then placed in an ice bath and activity was determined at pH 3.5/37 °C using the SS1 as described above. Relative activity was determined relative to a control (samples were incubated at 25 °C).

4.12. Activity of rAtAP A1 against hemoglobin and its inhibition

Hemoglobin degradation by rAtAP A1 was performed by incubating 5 μg of denatured hemoglobin (Sigma–Aldrich, Oakville, ON, Canada) with 0.1 μg of rAtAP A1 in 100 mM sodium acetate, pH 3.5 at 37 °C for 2 h. The reaction was terminated by heating the sample to 100 °C for 5 min in SDS–PAGE sample buffer. In order to test for inhibition by various inhibitors the following inhibitors were used: pepstatin A (10 μM) phenylmethylsulfonyl fluoride (PMSF, 1 mM), leupeptin (100 μM), *N*-acetyl-leucyl-leucyl-norleucinal (ALLN, 15 μM), EDTA (5 mM) and finally dimethyl sulfoxide (DMSO, 3%) as a control (Kim et al., 2006). The degradation of hemoglobin was detected by Gelcode blue stained SDS–PAGE (Xiao et al., 2006). The percent of inhibition of the rAtAP A1 by the above inhibitors was determined using the synthetic substrate SS1 at standard conditions (pH 3.5/37 °C).

4.13. Molecular modeling of the rAtAP A1

The three-dimensional structure modeling of the rAtAP A1 using its cDNA deduced amino acid sequence (At1g11910; EMBL Accession No. U51036) was conducted using the coordinates obtained using first approach mode by Swiss-Model and displayed with DeepView software v3.7 (SwissPdb Viewer).

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