Botanical Studies (2009) 50: 149-158.

Cloning and expression of aspartic proteinase cDNA from sweet potato storage roots

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(Received June 13, 2008; Accepted November 27, 2008)

ABSTRACT. Aspartic proteinases (EC, 3.4.23) cDNA clone (SPAP) of sweet potato (*Ipomoea batatas* (L.) Lam. 'Tainong 57') storage roots were isolated by differential display. The open reading frame in this cDNA encodes a pre-pro-protein of 508 amino acids with a predicted molecular mass of 55,006 Da (pI 4.91). The SPAP gene shares 81% and 78% homology on the level of nucleotides and amino acids, respectively, with an aspartic proteinase cDNA of sweet potato senescent leaves (SPAPSL). SPAP amino acid sequence was different from other AP sequences in signal and propeptide portions. The deduced amino acid sequence contains the conserved features of plant aspartic proteinases, including the plant specific insert (PSI) and two active site aspartic acid residues. Examination of the expression patterns in sweet potato by northern blot analyses revealed that the transcripts of SPAP were specifically induced in the storage roots. Recombinant SPAP overproduced in *E. coli* (M15) was purified by Ni^{2+}-chelated affinity chromatography. Active recombinant SPAP was able to digest the 22 kDa sweet potato trypsin inhibitor (TI) when the latter was reduced by dithiothreitol (DTT). SPAP could not degrade bands of reduced TI when NTS (NADP/thioredoxin system) was used to reduce TI. These results suggest that SWAP has an in vivo proteolytic function of processing storage SPTI after its being degraded initially by a specific cysteine proteinase.

Keywords: Aspartic proteinase; cDNA sequence; Recombinant protein; Sweet potato; Trypsin inhibitor.

INTRODUCTION

Aspartic proteinases (APs) (aspartic endopeptidases, EC, 3.4.23) are one of the four main classes of proteinases, the others being serine, cysteine, and metallo-proteinases (Barrett, 1998) and are a widely distributed class of proteinases present in animals, microbes, viruses, and plants (Davies, 1990; Rawling and Barret, 1995; Mutlu and Gal, 1999; Simões and Faro, 2004). Plant APs have characteristics common with aspartic proteinase A1 family, are active at acidic pH, are specifically inhibited by pepstatin and have two aspartic acid residues responsible for the catalytic activity (Dunn, 2002). APs have been found in seeds, tubers, flowers, and petals of many species. A number of aspartic proteinases cDNAs have been isolated from different plants including *Arabidopsis*, *Brassica*, rice, barley, and tomato (Runeberg-Roos et al., 1991; Asakura et al., 1995; Schaller et al., 1996; D'Hondt et al., 1997; Hiraiwa et al., 1997; Xia et al., 2004). The typical plant AP sequences contain preproportions which are similar to those of the other species. Plant AP genes have an extra region of approximately 100 amino acids called as "plant specific insert" (PSI). This segment, inserted into the C-terminal domain of the plant APs precursors, is usually removed during the proteolytic maturation of the proteinases. The PSI sequence shows no homology with mammalian or microbial APs, but is highly similar to that of saposin-like proteins (SAPLIPs) (Guruprasad et al., 1994). PSI has been reported to function as signals both for transport of AP molecules from the endoplasmic reticulum (ER) and for their targeting to the vacuole (Terauchi et al., 2006).

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APs were shown to perform many different and diverse biological functions, including specific protein processing (e.g. rennin and cathepsin D), protein degradation (e.g. chymosin and pepsin) or viral polyprotein processing (human immunodeficiency virus AP) (Rawlings et al., 1995; Hiraiwa et al., 1997; Mutlu and Gal, 1999). Plant AP functions are predicted in studies of the processing or degradation of putative protein substrates in vitro and/or specific expression in certain tissues or under specific conditions. Plant APs have been implicated in protein processing and/or degradation in different plant organs, as well as in plant senescence, stress responses, programmed cell death and reproduction (Simões and Faro, 2004).

In this paper we report the isolation and characterization of a sweet potato cDNA encoding AP. Active recombinant SPAP protein was able to digest the reduced 22 kDa trypsin inhibitor protein, one of the storage proteins of sweet potato tuberous roots.

MATERIALS AND METHODS

Plant materials

Fresh storage roots of sweet potato (*Ipomoea batatas* (L.) Lam. 'Tainong 57') were purchased from a local market. After cleaning with water, the roots were placed in a thermostated (28ºC) growth chamber and sprayed with water twice a day. Sprouted plants were cultivated in the greenhouse to collect roots, stems and full expanded green leaves for experiments.

PCR-based subtractive hybridization and RACE PCR

Total RNA was isolated separately from the storage roots and sprouts of roots of sweet potato according to the method of Sambrook et al. (Sambrook et al., 1989). Then, mRNA was purified with a purification kit (Promega) and used for the differentially-expressed first strand cDNA synthesis using a PCR-based subtractive hybridization kit (Clontech) following the protocol supplied by the manufacturer. The double-strand cDNAs of the storage roots were subtracted by the sprouts of roots, then ligated to the pGEM-T easy vector for *E. coli* DH5α competent cell transformation. Recombinant plasmids were isolated for DNA sequencing using the ABI PRIZM 337 DNA Sequencer. Nucleotide sequence data were analyzed using the Genetics Computer Group (GCG) programs. Full-length cDNA clone was obtained by performing 5′ and 3′ RACE (5′ and 3′ rapid amplification of cDNA ends) using the Marathon cDNA amplification kit (Clontech) according to the manufacturer's instructions. The gene-specific primers (5′-TCTCC AGATA GGTTT TGCTG AAGCT GCATG-3′) were used to amplify the double strand cDNAs.

Expression of aspartic proteinase in *E. coli*

Its prosequence was expressed in *E. coli.* The coding sequence was amplified from cDNA SPAP using an oligonucleotide (5′-GGA AA CCTTT GAGCA TGCCA TGGAA ATATC-3′), with a *Sph*I site (underlined) at the putative initial Met residue, and an oligonucleotide (5′-TCTCC AGATA GGTTT TGCGG TACC-3′), with a *Kpn*I site at the 3′ end. The PCR fragment was subcloned in pGEM T-easy vector. The plasmid was then digested with *Sph*I and *Kpn*I and the excised fragments were subcloned in pQE30 expression vector (QIAexpress expression system, Qiagen). The resulting plasmid, termed pQE-SPAP, was introduced into *E. coli* (M15). Cultures of the transformed *E. coli* (M15) overexpressed a protein of the expected molecular mass, which was purified by affinity chromatography in Ni-nitrilotriacetic acid (NTA) columns (Qiagen), according to Huang et al (Huang et al., 2007).

DNA isolation and Southern blot analysis

Young leaves of sweet potato were harvested and ground in liquid N_2. The powder was transferred to a centrifuge tube, mixed gently and thoroughly with CTAB (cetyltrimethylammonium bromide) buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 2% 2-mercaptoethanol, and 100 mM Tris-HCl pH 8.0), and kept at 60°C in a water bath for genomic DNA extraction according to the method of Huang et al. (Huang et al., 2004a). The total nucleic acid after precipitation with an equal volume of isopropanol was re-dissolved in sterile water, digested with various restriction enzymes and separated on 0.8% agarose gels. After electrophoresis, the DNA was transferred onto a Hybond-N^+ nylon membrane (Amersham) following the protocol of Molecular Cloning (Sambrook et al., 1989) for Southern blot hybridization.

RNA isolation and northern blot analysis

Total RNA was extracted from different tissues of sweet potato with TRIzol reagents kit (Invitrogen) according to the manufacturer's instructions. For northern blotting, 10 μg of total RNA isolated from storage roots, sprouts, sprouted roots, veins, fully expanded green leaves, and flowers were applied to a formaldehyde denaturing gel, then transferred to an Amersham Hybond-N^+ nylon membrane after electrophoresis, according to the methods of Huang et al. (Huang et al., 2005a). The filter was hybridized sequentially with α-^{32}P-labelled AP full-length cDNA. The procedures for hybridization and autoradiography were according to the methods of Molecular Cloning (Sambrook et al., 1989). Visualization of hybridization bands was carried out using X-ray film (Kodak).

Purification of sweet potato trypsin inhibitor

Sweet potato storage roots were washed and peeled, and then cut into strips that were extracted immediately and processed according to Huang et al. (Huang et al., 2004b; 2008a). The crude extracts were loaded directly onto a trypsin Sepharose-4B affinity column. The adsorbed TI was eluted by pH changes with 0.2 M KCl (pH 2.0).

Protein staining and thiol-label staining of trypsin inhibitors on 10% or 15% denaturing polyacrylamide gels

Samples were mixed with sample buffer, namely 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue with or without 2-mercaptoethanol. Coomassie brilliant blue G-250 was used for protein staining (Huang et al., 2008b). The method of thiol-label staining on an SDS-PAGE gel basically followed the report of Huang et al. (Huang et al., 2005b) using the mBBr (monobromobimane) reagent as a probe.

Production of polyclonal antibody and western blot hybridization

Expressed SPAP protein was cut from the 15% polyacylamide gel, and eluted with appropriate amount of pH 7.5 phosphate buffer saline (PBS) containing 0.1% SDS. The eluted proteins were precipitated with acetone containing 10% trichloroacetic acid (TCA) at -20°C for 2 h. After centrifugation at 13,000 *g* for 20 min, the pellet was washed with acetone twice, then, dried at room temperature. The acetone powder was re-dissolved in a small amount of PBS containing 0.1% SDS and used as antigens for subcutaneous injections of rabbit to prepare the first antigens (Taiwan Bio-Pharm Inc.). The second antigen (goat against rabbit Fc portion of Ig) was a product of Sigma (USA). Polyclonal antibodies obtained from rabbit antiserum were utilized for western blot hybridization to study the gene expression of SPAP in different tissues of sweet potato.

Protein extraction, electroblotting analysis of aspartic proteinase and TI

All steps were carried out at 4-8°C. Sweet potato leaves, sprouted storage roots, veins, and storage roots, were cleaned and homogenized with 4 volumes (v/w) of 50 mM Tris-HCl buffer (pH 7.5) in a Polytron homogenizer (Luzern, Swiss). The homogenate was filtered through two layers of cheesecloth and then centrifuged in a Sorvall RC-2B with an SS-34 rotor at 10,000 *g* for 20 min. The protein concentration of the supernatant was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA). The supernatant was saved for electroblotting. The crude extract was subjected to 15% SDS-PAGE according to Laemmli (Laemmli, 1970). After electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris-HCl, pH 8.3, 150 mM glycine and 10% (w/v) methanol). The separated proteins were transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA) in transfer buffer at pH 8.3 for 1 h at 100 V. Membranes were blocked for 2 h at room temperature in 5% nonfat dry milk powder and then incubated with polyclonal antibodies from rabbit as the primary antibodies against SPAP and TI. After incubation, membranes were washed in Tris-buffered saline with 0.05% Tween (TBST) three times, 10 min each, then incubated with anti-rabbit alkaline phosphatase-conjugated antibody, washed in TBST three times, 10 min each, and developed using NBT (nitro blue tetrazolium)/ BCIP (5-bromo-4- chloro-3-indolyl-phosphate) (Sigma, USA).

Phylogenic analysis of AP

Amino acid sequence alignment of AP after Genetics Computer Inc. (GCG)/Pileup comparison was used for phylogenic tree construction. The distances among entries were calculated with neighbor-joining method (Thompson et al., 1994). The internal support was evaluated by bootstrap analyses. In parsimony analysis, each of 1,000 bootstrap replicates was analyzed with the heuristic search option invoking one random addition replicate each, and not invoking the retention of multiple parsimonious trees. The phylogenic tree was drawn using NJ plot and redrawn by the graphic software of CLUSTALX 1.81.

Proteinase activity

The general proteolytic activity of the enzyme was monitored using denatured substrates such as casein, hemoglobin, azoalbumin, and azocasein by the method of Arnon (Arnon, 1970) with some modifications (Hou et al., 2002). In a total final volume of 1 mL, 2% azocasein (250 μL) and 0.5-3 μg of the enzyme were mixed and kept at 37°C in a water bath for 30 min. The control assay was performed without any enzyme in the reaction mixture. The reaction was terminated by adding 0.5 mL of 10% TCA and the absorbance at 340 nm of the supernatant was measured.

RESULTS

Isolation and nucleotide sequence of aspartic proteinase cDNA clone from sweet potato storage roots

AP cDNA clones of sweet potato storage roots were isolated by differential display. We have completed the sequencing of one of the clones, which was named SPAP (Gene Bank accession number: DQ903691). The open reading frame in this cDNA encodes a pre-pro-protein of 508 amino acids with a predicted molecular mass of 55,006 Da (pI 4.91). The SPAP gene shares 81% and 78% homology on the level of nucleotides and amino acids, respectively, with an aspartic proteinase cDNA of sweet potato senescent leaves (SPAPSL) (Chen et al., 2004; 2008). SPAP amino acid sequence was different from other AP sequences in both signal and propeptide portions. Figure 1 shows a multiple alignment of the sweet potato SPAP protein and other homologous plant precursor AP proteins available in the GenBank (Figure 1). The deduced amino acid sequence contains the conserved features of plant aspartic proteinases, including the plant specific insert (PSI).

A putative cleavage site on the hydrophobic N-terminal signal peptide for targeting to ER was predicted between Ser-24 and Glu-25 (Von Heijne, 1983; Nielsen et al.,

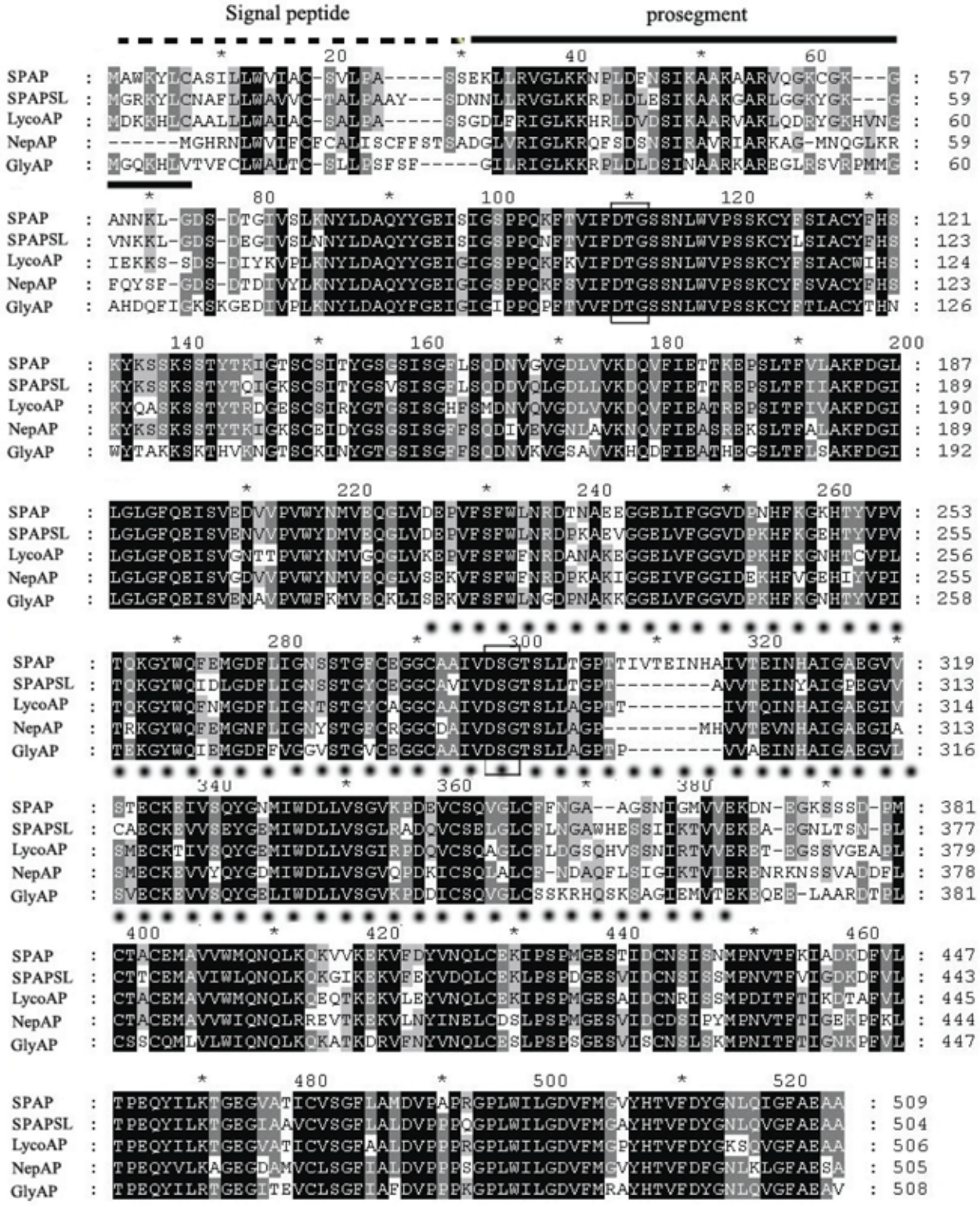

Figure 1. Multiple alignments of plant aspartic proteinase proteins. The sequences are aspartic proteinase SPAP (DQ903691) from sweet potato storage roots, aspartic proteinase SPAPSL (AF216783) of sweet potato senescent leaves; LycoAP (L46681) of *Lycopersicon esculentum*; NepAP (AB045894) of *Nepenthes alata*; and GlyAP (AB070857) of *Glycine max*. The proteins were aligned using the GCG program. The regions corresponding to the signal peptide (black dashed line), the prosegment (black solid line) and the PSI (black dotted line) are marked. The catalytic aspartic acid residues are boxed. Black shading indicates the same amino acid at that position among all sequences. Gray shading shows those amino acids with similar side-chain properties. The numbers above all sequences stand for the positions of the amino acids within individual proteins corresponding to the numbering system of *Glycine max* GlyAP, which is the longest among all sequences shown. The numbers at the end of the right hand side of each line stand for the cumulative total number of amino acids in each line of each preproprotein sequence.

1997). The two active site aspartic acid residues, one with the Asp-Thr-Gly motif and the other Asp-Ser-Gly, are consistent. Two putative *N*-glycosylation sites (270-273 NSST; 435-438 NVTF), one putative tyrosine kinase phosphorylation site (73-81; KNYLDAQYY) (Pcgene, Prosite program, Intelligenetics) (Richter, et al., 1998) and one prokaryotic membrane lipoprotein lipid attachment site (7-17; CASILLWVIAC) were predicted.

Phylogenic analysis of APs based on their amino acid sequences

The phylogenic tree of SPAP together with amino acid sequences of other 4 proteins were constructed (Figure 2). Sweet potato aspartic proteinase SPAPSL was found in the same subgroup, whereas SPAP was clustered in another subgroup that included LycoSP and NepAP.

Copy numbers of aspartic proteinase sequences in sweet potato

We performed Southern blot hybridization with *EcoRI* (E), *BamHI* (B) and *HindIII* (H) digests of sweet potato Tainong 57 DNA, using probe derived from 3′-noncoding sequence of the cDNAs to estimate the copy number of the gene. Tainong 57, an elite sweet potato cultivar derived from a cross between Tainong 27 and Nancy Hall, has a hexaploid number of chromosome (2n=6x=90). The results suggest that *SPAP* belongs to a small multigene family in sweet potato (Figure 3).

Expression of SPAP in *E. coli*

SDS-PAGE analysis of crude extracts from the transformed *E. coli* (M15) showed high amounts of a polypeptide with the molecular mass (ca. 55 kDa) of the SPAP prepropeptide (Figure 4A). This polypeptide was found as a soluble protein in the supernatant (Figure 4A, lane 2), and was absent in protein extracts obtained from *E. coli* transformed with pQE-30 vector (Figure 4A, lane 1). The expressed protein was purified from crude extracts as His-tagged SPAP (Figure 4A, lane 3). This polypeptide was then analyzed by proteinase activity assay according to Sundd et al (Sundd et al., 1998) (Figure 4B).

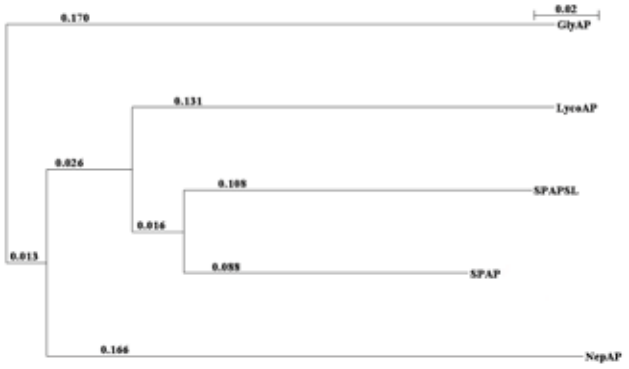

Figure 2. Phylogenetic analysis of aspartic proteinases based on their amino acid sequences. The scale bar represents 0.02 units.

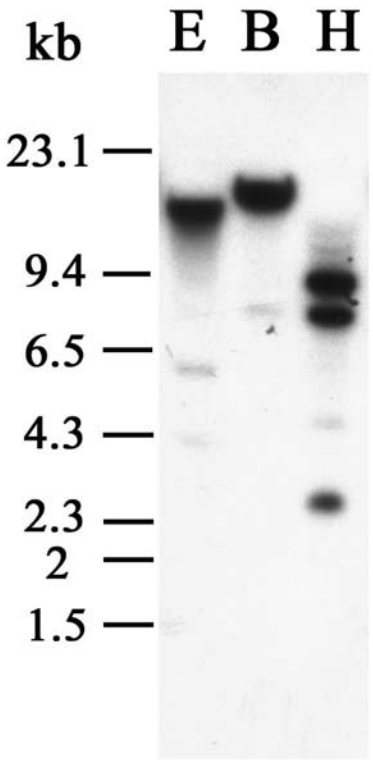

Figure 3. Southern blot detection of aspartic proteinase genomic sequences. Samples (10 μg) of genomic DNA from sweet potato Tainong 57 leaves were digested with *EcoRI* (E), *BamHI* (B) and *HindIII* (H). The DNA fragments were separated in 0.8% agarose gels, transferred to a Hybond-N^+ nylon membrane, and hybridized with PCR-labeled cDNA probes. Molecular size markers were λ DNA/*HindIII* fragments. The experiments were done twice and a representative one is shown.

The expressed protein was able to degrade the substrate azocasein indicating that SPAP has proteinase activity.

Aspartic proteinase mRNA levels were developmentally regulated

The presence and amounts of different sweet potato SPAP mRNAs were examined in various organs and tissues by northern blot analysis. *SPAP* was obtained from sweet potato tuberous roots. Figure 5A shows that SPAP probe hybridized to mRNA species of approximately 1.5 kb. SPAP mRNA levels were the highest in the storage roots, followed by that in sprouted roots and full expanded green leaves; while was the lowest in sprout and vein.

Western blot analysis of aspartic proteinase from sweet potato tissues

Western blot hybridization using SPAP polyclonal antibody from rabbit antiserum was used for the gene expression analysis of SPAP in crude extracts from different sweet potato tissues (Figure 5B). SPAP levels were the highest in the storage roots; followed by that in sprouted. There were no signals in sprout roots, veins and full expanded green leaves.

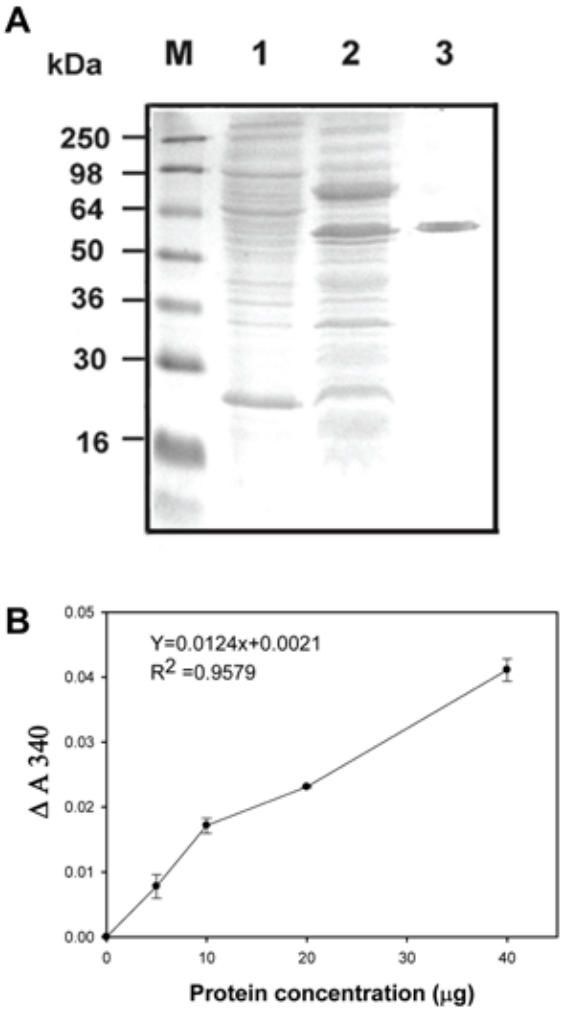

Figure 4. Purified recombinant sweet potato aspartic proteinase SPAP. A, 10% SDS-PAGE analysis. Crude extracts (5 μg protein) from *E. coli* (M15) transformed with pQE30 (lane 1) or with pQE30-SPAP (lane 2) were analyzed by 10% (w/v) SDS-PAGE, and then the gels were stained with Coomassie brilliant blue G-250. Molecular masses of standard proteins were indicated at the left of the figure. His-tagged SPAP was purified by Ni^{2+}-chelated affinity chromatography (lane 3); B, Proteinase activity analysis. The experiments were done twice and a representative one is shown. "M" indicated the see Blue™ pre-stained markers for SDS-PAGE.

SPAP expression protein can degrade the stored trypsin inhibitor proteins in vitro

It has been reported that inactivation of seed TI by reductants improved protein digestibility (Huang et al., 2005b). Formation of disulfide bonds of protein likely functions to increase its structural stability and decrease its water solubility, rendering it resistant against proteolysis. Therefore, it would be interesting to study whether reduction of stored TI by DTT or NTS can improve protein digestibility in vitro.

The purified TI contained two bands (38 and 22 kDa) on non-reducing SDS-PAGE under either protein-staining or thiol labeling condition (lanes 1, 2 of Figure 6A and 6B, serving as controls). The existence of two forms of the purified TI in controls could be explained by equilibrium between TI38 and TI22 via interchain disulfide bond formation, TI22 being a monomer and thermodynamically favored. The evidence came from the reproducible observations that when TI38 was cut from the gel and stored in sample buffer at 4°C overnight and then run non-reducing SDS-PAGE again, two bands (38 and 22 kDa) still could be observed; On the contrary, if TI22 was treated similarity only one band (TI22) was detected (our unpublished data).

SPAP protein could not degrade bands of native TI (lanes 3 of Figure 6A). However, DTT could transform TI38 into TI22 at room temperature. TI22 appeared concomitantly with the disappearance of TI38 (lane 4,

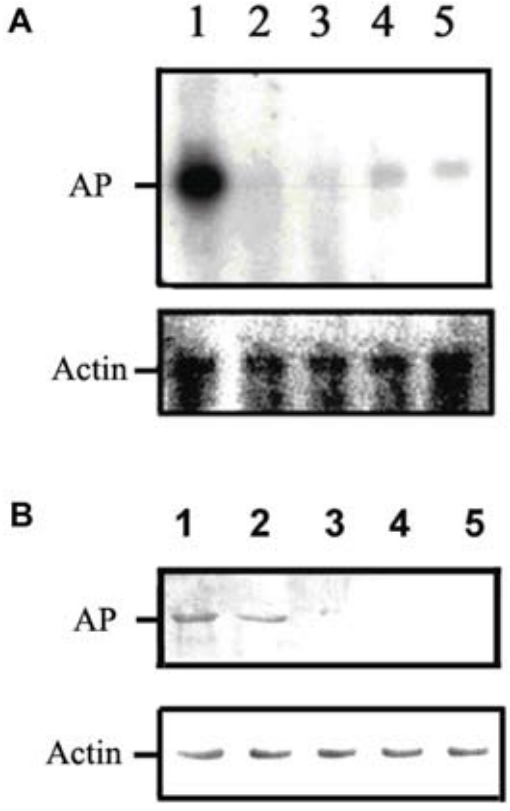

Figure 5. Northern and western blot detections of sweet potato aspartic proteinase SPAP. A, Northern blot analysis. Samples (10 μg each) of total RNA were isolated from different tissues of sweet potato and actin (AY905538) was utilized as an internal control of mRNA from sweet potato. Blots were hybridized to α-^{32}P-labeled 3′ specific cDNA probes; B, Western blots analysis. Ten μg of crude extracted proteins from sweet potato were analyzed by 15% (w/v) SDS/PAGE, and then the gels were transferred onto PVDF membranes that were probed with a 1:1000 (v/v) dilution of rabbit antibodies raised against SPAP using goat-antirabbit alkaline phosphatase as the second antibody. Lane 1: storage roots, lane 2: sprout, lane 3: veins, lane 4: sprouted roots, and lane 5: fully expanded green leaves. The experiments were done twice and a representative one is shown.

Figure 6A). Scanning the mBBr-TI bands with a laser densitometer revealed that TI22 increased after DTT treatment (lane 4, Figure 6B). In the presence of DTT and expressed SPAP protein, TI22 was degraded completely (lane 5, Figure 6A). At the same time, the mBBr-protein band of TI22 disappeared (lane 5, Figure 6B).

When NTS (NADP/thioredoxin system) was used to reduce TI, SPAP protein could not degrade bands of reduced TI (data not shown). The results indicated that SPAP protein could degrade TI reduced by DTT that cause drastic 3-D conformational changes of TI.

DISCUSSION

We have presented a nucleotide sequence of an aspartic proteinase cDNA clone from sweet potato. The protein encoded by the open reading frame contains 508 amino acids. Plant SPAP cDNAs have been detected and isolated from many different plant species. SPAP amino acid sequence was different from other AP sequences in both signal and propeptide portions. The deduced amino acid sequence contains the conserved features of plant aspartic proteinases, including the plant specific insert (PSI). According to the cDNA sequence analysis *SWAP*, SPAP protein like most plant APs has an internal domain (PSI) with a high homology to saposin-like proteins (SAPLIPs) type B. This protein family has been associated with antitumoral and antimicrobial activity due to their membrane leakage activity. The PSI presence in mature APs could explain bifunctional activity (proteolytic and antimicrobial) (Guevara et al., 2005).

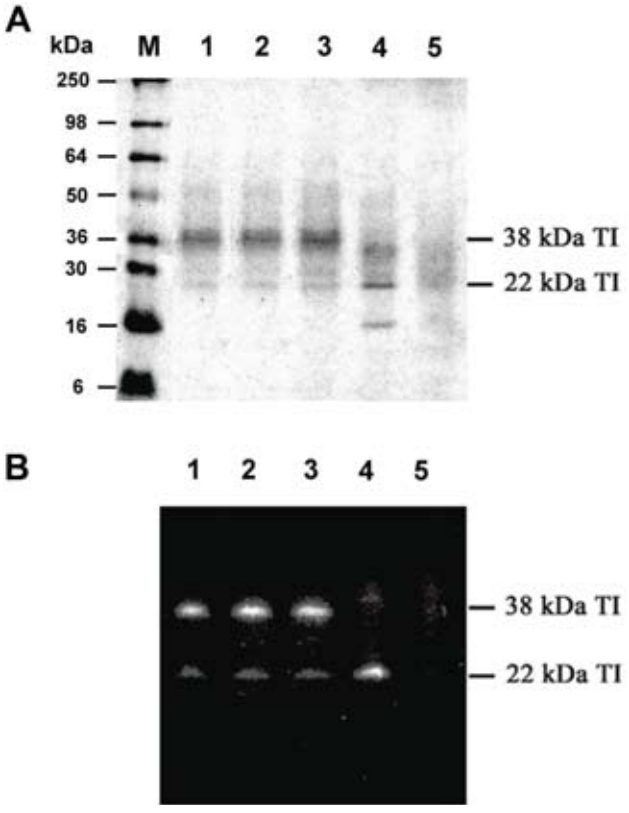

Figure 6. Reduction of trypsin inhibitor from sweet potato storage roots by DTT and digestion with the recombinant SPAP protein. (A) Protein stainings of TI with Coomassie brilliant blue G250 were performed on 15% SDS-PAGE gels; (B) The fluorescence of samples (thiol-labeling) after reduction was detected on 15% mBBr-containing SDS-PAGE gels. Lane 1, trypsin inhibitor at 0ºC for 24 h; lane 2, trypsin inhibitors incubated at 37ºC for 24 h; lane 3, trypsin inhibitors and 1 μg SPAP were incubated at 37ºC for 24 h; lane 4, trypsin inhibitor plus 2 mM DTT was incubated at 37ºC for 24 h; lane 5, trypsin inhibitor, 2 mM DTT and 1 μg SPAP were incubated at 37ºC for 24 h. The experiments were done twice and a representative one is shown. Each lane contained 10 μg purified SPTI. "M" indicated the see Blue™ pre-stained markers for SDS-PAGE.

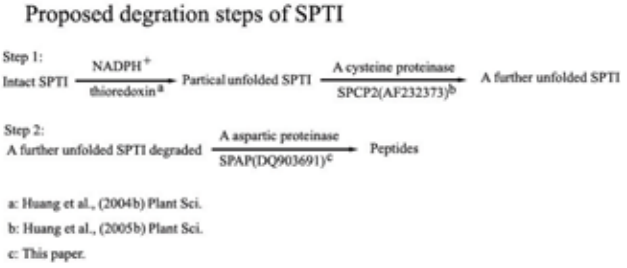

Figure 7. A proposed degradation steps of trypsin inhibitor from sweet potato storage roots.

The functions of the PSI are still unclear, however, an important role in vacuolar targeting of plant AP precursors has been proposed based on its possible direct interaction with lipid bilayers (Munford et al., 1995; Vaccaro et al., 1999). Thus, this saposin-like domain in plant APs may be responsible for bringing AP precursors into contact with membranes or membrane-bound receptor proteins mediating the sorting of enzyme precursors during Golgi-mediated intracellular transport to the vacuoles (Egas et al., 2000).

SPAP protein could degrade TI22 reduced by DTT. When NTS was used to reduce TI, SPAP protein could not degrade bands of reduced TI (data not shown). In our previous paper, the NTS could reduce TI proteins in vitro rendering them to be degraded by suitable proteinases such as SPCPRPP protein (Huang et al., 2005b). These results suggest that CPR is responsible for initiation of degradation and re-mobilization of stored 22 kDa TI during sprouting of SP storage roots after the reduction of 22 kDa TI by the NTS (step 1, Figure 7). And SPAP protein may be responsible for the later step of degradation (step 2, Figure 7).

In castor bean aspartic endopeptidase cannot directly convert pro2S albumin into the mature form, but it may play a role in trimming the C-terminal propeptides from the subunits that are produced by the action of the vacuolar processing enzyme (Hiraiwa et al., 1997). In citrus leaf extracts, an AP has been implicated in the proteolysis of the photosynthetic enzyme ribulose-1, 5-bisphosphate

carboxylase/oxygenase which plays a significant role as a nitrogen source during the growth of new organs (Garciamartinez et al., 1986). Participation of plant APs in storage protein degradation during the mobilization of reserve proteins in seed germination has been proposed for rice and wheat. In rice seeds AP could be involved in the hydrolysis of γ-globulin during the initial stage of germination because both proteins are similarly distributed in the seeds (Doi et al., 1980). In wheat seeds AP could hydrolyse the storage protein, gliadin, in vitro (Belozersky et al., 1989).

Therefore, our data provide evidence for the first time that stored TI could be degraded initially by a specific cysteine proteinase and then by AP more efficiently in vitro and possibly in vivo.

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甘藷塊根天門冬胺酸蛋白酶 cDNA 之選殖和表現

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利用差異表現法 (differential display) 分離甘藷 (*Ipomoea batatas* [L.] Lam. 'Tainong 57') 塊根之天門冬胺酸蛋白酶 (Aspartic proteinases; EC, 3.4.23; SPAP)。SPAP 之開放轉譯架構（open reading frame）轉譯成 508 個胺基酸，預測其分子量為 55,006 Da (pI 4.91)。SPAP 基因和甘藷老化葉中之天門冬胺酸蛋白酶基因 (SPAPSL) 在 DNA 和蛋白質序列比對時，具有 81% 和 78% 相似性。SPAP 胺基酸序列和其他之 AP 胺基酸序列差異在於訊息胜肽和前胜肽的部分。胺基酸序列含有天門冬胺酸蛋白酶基因保守性特徵，包括 plant specific insert (PSI) 和兩個活性區之天門冬胺酸基。藉由北方點墨法檢視 SPAP 在甘藷表現量的多寡發現 SPAP 之轉錄專一性的表現在塊根中。在大腸桿菌 (M15) 中大量表現重組蛋白質 SPAP，然後利用鎳離子螯合之親和性管柱純化。當 22 kDa 甘藷胰蛋白酶抑制因子 (trypsin inhibitor, TI) 被 dithiothreitol (DTT) 完全還原後，可被具活性之重組蛋白質 SPAP 分解。但是當 TI 被 NTS (NADP/thioredoxin system) 部分還原後，卻不能夠被具活性之重組蛋白質 SPAP 分解。這些結果顯示當 22 kDa SPTI 由 NTS (NADP/thioredoxin system) 部分還原後，先由專一性之半胱胺酸蛋白酶進行初步之降解 ； 接著再由本文所描述之 SPAP 進行後續的水解過程。

關鍵詞 ： 天門冬胺酸蛋白酶 ； 基因表現 ； 重組蛋白質 ； 甘藷 ； 胰蛋白酶抑制因子。