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Isolation and characterization of isoinhibitors of the potato protease inhibitor I family from the latex of the rubber trees, *Hevea brasiliensis*

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Dedicated to Prof. Rod Croteau to celebrate his 60th birthday.

Abstract

Three isoinhibitors have been isolated to homogeneity from the C-serum of the latex of the rubber tree, *Hevea brasiliensis* clone RRIM 600, and named HPI-1, HPI-2a and HPI-2b. The three inhibitors share the same amino acid sequence (69 residues) but the masses of the three forms were determined to be $14{,}893 \pm 10$, 7757 ± 5, and 7565 ± 5, respectively, indicating that post-translational modifications of the protein have occurred during latex collection. One adduct could be removed by reducing agents, and was determined to be glutathione, while the other adduct could not be removed by reducing agents and has not been identified. The N-termini of the inhibitor proteins were blocked by an acetylated Ala, but the complete amino acid sequence analysis of the deblocked inhibitors by Edman degradation of fragments from endopeptidase C digestion and mass spectrometry confirmed that the three isoinhibitors were derived from a single protein. The amino acid sequence of the protein differed at two positions from the sequence deduced from a cDNA reported in GenBank. The gene coding for the inhibitor is wound-inducible and is a member of the potato inhibitor I family of protease inhibitors. The inhibitor strongly inhibited subtilisin A, weakly inhibited trypsin, and did not inhibit chymotrypsin. The amino acid residues at the reactive site P_1 and P_1' were determined to be Gln45 and Asp46, respectively, residues rarely reported at the reactive site in potato inhibitor I family members. Comparison of amino acid sequences revealed that the HPI isoinhibitors shared from 33% to 55% identity (50–74% similarity) to inhibitors of the potato inhibitor I family. The properties of the isoinhibitors suggest that they may play a defensive role in the latex against pathogens and/or herbivores.

Keywords: *Hevea brasiliensis*; Euphorbiaceae; Proteinase isoinhibitors; Wounding; Plant defense

1. Introduction

Para rubber trees (*Hevea brasiliensis* from the Euphorbiaceae family) grow in hot humid intertropical regions and are cultivated for their latex, the main source of natural rubber used for many industrial purposes in the world. The physiological roles of latex remain unclear and considering the high-energy cost, it is unknown why plants produce latex. Latex secretion has been hypothesized to be part of the defense system targeted against pest and/or pathogen attacks (Farrell et al., 1991). In latex collection, rubber trees are regularly wounded by a systematic ''tapping'' procedure, in which the bark is shaved to a thin layer and pretreated with Ethrel® or Ethephon® (ethylene generators)

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to stimulate latex production. *Hevea* possesses articulated laticifers at the inner soft zone of the bark that produce the latex (de Faÿ and Jacob, 1989; Premakumari and Panikkar, 1992). Wounding in the manner described can cause a 10- to 50-fold increase in the expression of stress related genes (Kush et al., 1990). These genes produce the second most abundant transcripts in *Hevea* latex, with transcripts for rubber biosynthesis being the highest (Han et al., 2000; Ko et al., 2003). Defense-related proteins reported to be present in latex include chitinase, β-1,3-glucanase, hevamines, hevein, glucosidase, β-galactosidase, β-*N*-acetyl-glucosaminidase, polyphenol oxidase, and a protease inhibitor (D'Auzac and Jacob, 1989; Kim et al., 2003). Details on the biochemistry of natural rubber and of latex structure and compositions, including rubber biosynthesis and defense-related proteins have been extensively reviewed (Wititsuwannakul and Wititsuwannakul, 2001). Our recent interest is focused on the protease inhibitor activity that we found to be induced in the latex as a result of tapping. We report here the purification and characterization of three subtilisin A isoinhibitors (HPI) from the latex of *Hevea* that had been repeatedly tapped. These studies revealed that the three isoinhibitors contained an identical amino acid sequence, and that post-translational modifications of the single half-cystine residue with two small organic adducts resulted in the three isoinhibitor types. The isoinhibitors are members of the potato inhibitor I family and may play a defensive role in the latex against pests and/or pathogens.

2. Results

The presence of trypsin inhibitors in the non-rubber fractions of *Hevea* latex was investigated. The non-rubber fractions were separated from the rubber fractions by centrifugation of the latex, and included a clear serum called C-serum and a fluid derived from the lutoid or bottom fraction called B-serum (see Graphical Abstract). The latex of repeatedly tapped rubber trees contains about 3 times higher trypsin inhibitory activity than latex of rubber trees tapped for the first time (Fig. 1), indicating that the latex trypsin inhibitory activity was induced by wounding. Trypsin inhibitory activity was not detected in the B-serum fraction from repeatedly tapped trees.

Trypsin inhibitor activity found in 70–95% acetone fractions of the C-serum comprised over 80% of the total inhibitory activity of the original serum, while 60% of the total trypsin inhibitory activity was in an 80–95% fraction. SDS–PAGE analysis of the proteins present in the 80–95% acetone fraction, revealed the presence of only one major protein band with an estimated M_r of about 7 kDa (Fig. 2). The protein was called *Hevea* protease inhibitor, HPI.

The inhibitory activity in the 80–95% acetone fraction was assayed against several proteases in addition to trypsin, including chymotrypsin, pepsin, papain, subtilisin A, pronase E, protease type XIII, and thermolysin. Only subtilisin A and trypsin were inhibited, with HPI activity against subtilisin A being 40-fold higher. Therefore, subtilisin A was chosen to assay HPI activity during further investigations.

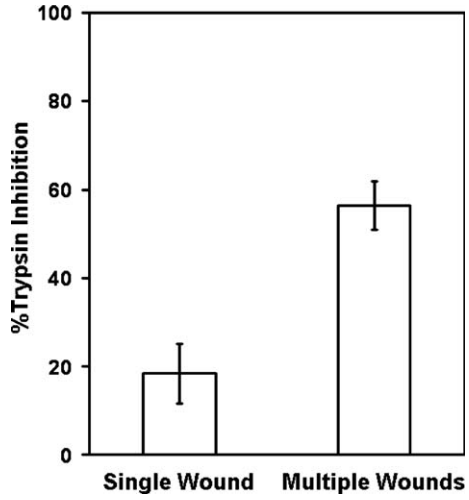

Fig. 1. Inhibition of trypsin activity by the C-serum of fresh latex collected from rubber trees during the first tapping (single wound) and from trees after several days of alternate-day tapping (multiple wounds). Equal amounts of C-serum protein (250 μg protein) were pre-incubated with 7.5 μg trypsin for 10 min at 37 °C and the activities assayed as described in Section 4. Data were performed in triplicate. The single wound data are the average from three singly wounded trees.

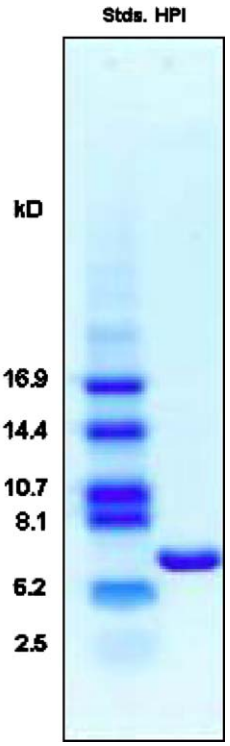

Fig. 2. SDS–PAGE separation of proteins *Hevea* latex C-serum that precipitated at 80–95% acetone. Electrophoresis of 3 μg HPI in Tricine-SDS–PAGE gels (16.5%).

Sephadex G-75 size-exclusion chromatography of the 80–95% acetone fraction revealed the presence of three components, of which two exhibited inhibitory activity against subtilisin A (Fig. 3). The inhibitory proteins were designated as HPI-1 and HPI-2, respectively.

Proteins in HPI-1 eluted from a C18-HPLC column separation as one major peak along with two minor peaks, and each exhibited inhibitory activity (Fig. 4A). HPI-2 eluted as two peaks, each having inhibitory activity, and were designated HPI-2a and HPI-2b (Fig. 4B). The major component of HPI-1 corresponds to M_r 14,893 ± 10 (m/z = 14,894, 7449, 4967, and 3725, +1, +2, +3, and +4 ions, respectively). The mass spectrum of HPI-2 exhibited major peaks corresponding to M_r values of 7757 ± 5 for HPI-2a (m/z = 7758 and 3879, +1 and +2 ions, respectively) and 7565 ± 5 for HPI-2b (m/z = 7566 and 3785, +1 and +2 ions, respectively). The same m/z values were observed in the spectrum of HPI-1, but as minor components, confirming that HPI-1 contains small amounts of the two proteins HPI-2a and HPI-2b.

Size-exclusion chromatography of HPI-1 on Sephadex G-75 indicated a mass of about 14 kDa, which is twice the M_r estimated by electrophoretic analysis using Tricine-SDS–PAGE under reducing conditions. This indicated that HPI-1 had a monomer molecular mass of approximately 7 kDa. HPI-I therefore, under oxidizing conditions, was engaged as a disulfide-linked dimer.

The isoelectric points of HPI-2a and HPI-2b, analyzed by isoelectric focusing, were 4.24 and 4.17, respectively.

All three isoinhibitors were fully active after incubation at 90 °C for 2 h. They were also stable between pH 3 and pH 11 when incubated at 37 °C for 24 h.

The inhibitory activities of HPI-1, HPI-2a, and HPI-2b against subtilisin A are shown in Fig. 5. The K_is for the three isoforms toward subtilisin A were estimated to be 0.21, 0.08, and 0.10 nM, respectively.

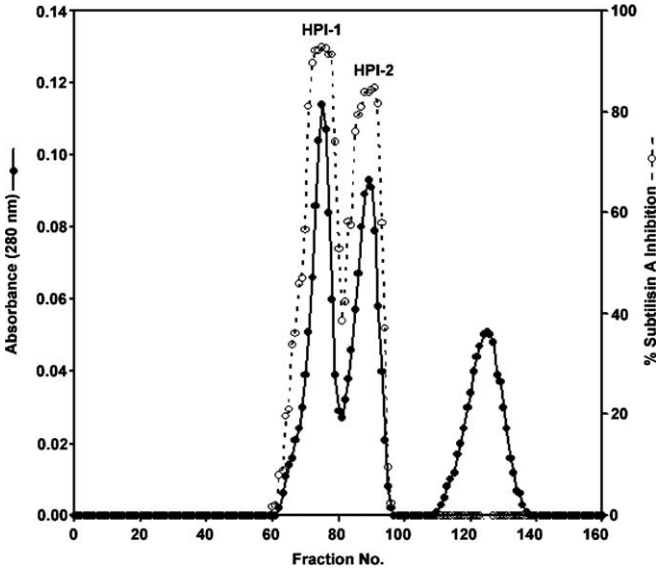

Fig. 3. Chromatography of HPI on Sephadex G-75 in 50 mM ammonium bicarbonate, pH 7.8. The absorbance of eluting proteins was recorded at 280 nm and subtilisin A inhibition was assayed using azocasein as substrate.

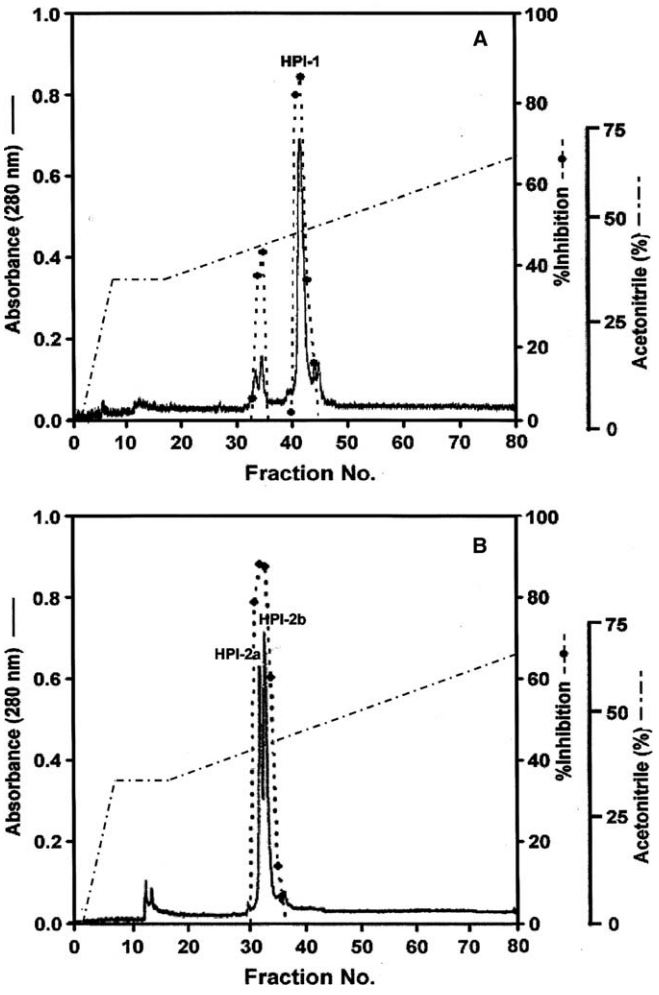

Fig. 4. Separation of (A) HPI-1 and (B) HPI-2 by C18-HPLC. Proteins were eluted with an acetonitrile gradient in 0.1% TFA. The absorbance was recorded at 280 nm and subtilisin A inhibition was assayed as in Fig. 3.

The N-terminal amino acids of all three isoinhibitors were blocked, precluding sequence analysis on the native proteins. The proteins were reduced and alkylated, and then digested with the endopeptidase Glu-C to obtain fragments for MALDI-MS analysis. The resulting peptides were separated by C18-HPLC and sequenced. Three of the peptide fragments derived from each digestion were determined to have the sequences NANVKAIVVKE, GLPITQDLNFNRVRVFVD, and NRVVTQVPAIG, respectively.

To obtain the complete amino acid sequences, the proteins were reduced and subjected to the deblocking enzyme, Ac-DAP, an enzyme that specifically cleaves N-terminal acyl-type blocking groups (such as formyl, acetyl, and myristyl) sequentially releasing amino acids from the N-terminus until it encounters an X-Pro bond. The deblocked N-terminal peptides were sequenced. The amino acid sequences of peptide fragments produced by endopeptidase

Glu-C and Ac-DAP cleavages from each protein produced the complete sequence that is shown in Fig. 6. This supported the mass spectroscopy data that all three isoinhibitors were derived from the same protein.

A search of the HPI sequence using the National Center for Biotechnical Information (NCBI) databases (NIH, USA), using the protein–protein BLAST (blastp) program, revealed high sequence homology with a conserved domain of the potato inhibitor I family, pfam00280 (score = 72.2 bits). The amino acid sequence of HPI showed the highest sequence identity with a family member called HbPI1 (GenBank accession no. AAP46156) that had been identified by ESTs and cDNA-AFLP as a protein from Para rubber tree (*Hevea brasiliensis*) (Han et al., 2000; Ko et al., 2003).

The amino acid sequence of HPI was identical to HbPI1 except at two residues. At residue 9 an N in HPI replaced D in HbPI1, and at residue 10, S replaced A, consistent with the MS/MS data. HPI isoinhibitors differ from the HbPI1 in several other respects. All HPI isoinhibitors have acetyl-Ala at their N-terminus, while HbPI1 has an N-terminal Met. Isoinhibitor HPI-2a is modified with a small molecule through a disulfide bond and released by reduction. Non-reduced HPI-2a, analyzed by LC/MS, produced a +2 ion with m/z 846.9 ± 0.5 Da, yielding a spectrum consistent with a species having a glutathione molecule linked to the cysteine residue by a disulfide linkage, including fragment ions that specifically relate to the glutathionyl moiety. In contrast to HPI-2a, reduction and alkylation of HPI-2b did not result in a change in its mass spectrum, indicating that the adduct with the cysteine residue was not through a disulfide linkage. LC/MS/MS analyses of HPI-2b yielded an MS/MS spectrum having a cysteine modified or substituted to produce a residue with mass 116 ± 1 Da greater than cysteine itself. This is consistent with the cysteine being alkylated with an adduct with the molecular formula $C_6H_{12}O_2$, although no additional structural information could be gleaned from the MS/MS spectrum.

The reactive site of HPI, analyzed by the amino acid sequences of cleavage products when incubated with subtilisin A at low pH, indicated that the P_1–P_1' residues of the reactive site of the HPI for subtilisin A were Gln45-Asp46.

3. Discussion

The tapping system in latex collection involves multiple wounds over a period of weeks that create important entry sites for pests and pathogens. Plants respond to wounding by activating genes that play roles in wound healing and defense (Ryan, 1990; Bowles, 1990; Ryan, 2000; Lawrence and Koundal, 2002; Valueva and Mosolov, 2004). Protease inhibitor proteins are among the defenses induced by wounding, and their defensive role in vegetative tissues and storage organs have been studied in detail in species of the Solanaceae, Leguminosae, Cucurbitaceae, and Graminaceae families (Ryan, 1990; Koiwa et al., 1997; Mosolov et al., 2001; Mosolov et al., 2004). The consistently higher trypsin inhibitory activity in the C-serum from the latex of rubber trees (*Hevea brasiliensis*) (Fig. 1) indicated that a wound inducible protease inhibitor was present in the latex. The presence of 60% of the inhibitory activity as a single electrophoretic band in the 80–95% acetone fraction of the C-serum (Fig. 2) facilitated the purification of the inhibitory activity. Although the inhibitory activity present in the latex was originally identified using trypsin inhibitor assays, the inhibitory activity of the C-serum was 40 times higher against subtilisin A than against trypsin.

Three isoinhibitors of subtilisin A were purified from the protein in the 80–95% acetone fraction of C-serum, (Figs. 3–5) and were named HPI1-1 (Mr 14, 893), HPI-2a (M_r 7757), and HPI-2b (M_r 7565). The isoinhibitors were heat stable, and acidic in charge, and the N-terminal residue of each isoinhibitor was blocked with an acetylated alanine. All three were powerful inhibitors of subtilisin A, having K_i's in the high pM range.

The complete amino acid sequences of the three isoinhibitors were achieved by removing the acetyl groups from the isoinhibitors with the enzyme Ac-DAP, and producing internal fragments using endopeptidase Glu-C. The amino acid sequences of the three isoinhibitors were revealed as being identical (Fig. 6), and the differences in the molecular masses determined by mass spectrometry were therefore due to post-translational modifications of a single translation product of 69 amino acids in length that is a member of the potato inhibitor I family. Family members are found widely distributed throughout the plant kingdom, as well as in the leech and common earthworm, indicating that the family has an ancient origin. HPI is the only isolated family member that has a single cysteine.

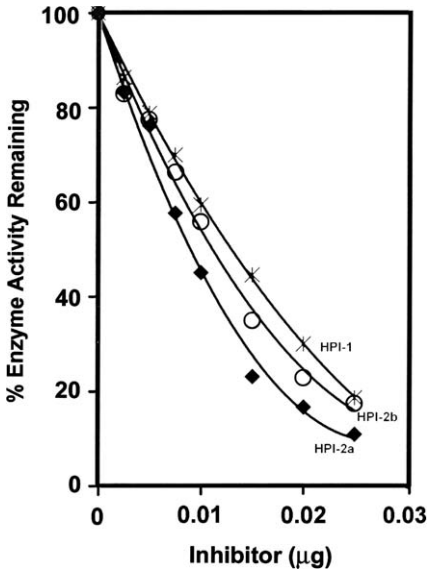

Fig. 5. Inhibition of the three purified isoinhibitor forms of HPI (HPI-1, HPI-2a, and HPI-2b) by subtilisin A. Each point was performed in triplicate.

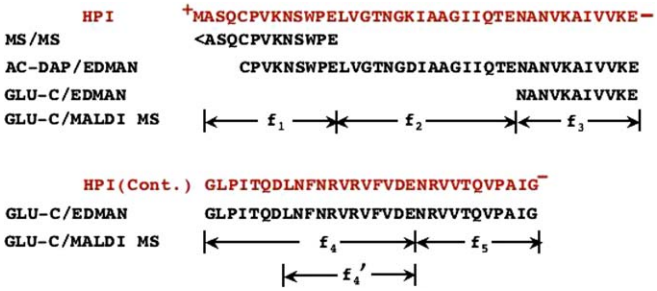

Fig. 6. The amino acid sequences of HPI. The peptides sequences of HPI fragments after enzyme digestion with endopeptidase Glu-C (Glu-C) and Pfu *N*-acetyl deblocking aminopeptidase (Ac-DAP) are shown, analyzed by either Edman degration or Maldi MS. (<) indicates the acetylated N-terminal. Fragments produced by Glu-C digestion and identified by MALDI MS are designated by f_1, f_2, f_3, f_4, f'_4, and f_5.

Structural analyses indicated that the single cysteine residue present in the inhibitor was engaged in three interactions. The first being the formation of an intermolecular disulfide bond through the cystine residue to cause dimerization, which explains the identification of a 14 kDa species (HPI-1). The other two modifications also involved the cystine residue in a disulfide bond with glutathione (HPI-2a) or by a small unknown adduct of mass 116 ± 1, in a covalent bond with cysteine that could not be dissociated under reducing conditions (HPI-2b). The post-translational modifications of the nascent polypeptide may have resulted from cross-linking of HPI-1 reduced monomers with itself, with glutathione, and an unknown adduct, in the oxidizing environment of the latex, either before or during its collection from trees.

The inhibitors may be components of the wound-inducible defenses of rubber trees, directed against secreted proteases of pathogens and/or digestive proteases of herbivores. The natural targets of HPI, and the wound signals that regulate its synthesis in *Hevea* latex, remain to be investigated.

4. Experimental

4.1. Materials

Trypsin (bovine pancreas, EC 3.4.21.4), chymotrypsin (bovine pancreas, EC 3.4.21.1), subtilisin A (Carlsberg Type VIII protease from *Bacillus lichenifermis*, EC 3.4.21.62), pronase E (*Streptomyces griseus*, EC 3.4.24.31), pepsin (porcin stomach mucosa, EC 3.4.23.1), papain (papaya latex, EC 3.4.22.2), protease type XIII (*Aspergillus saitoi*, EC 3.4.23.18), thermolysin (*Bacillus thermoproteolyticus* rokko, EC 3.4.24.27), casein, azocasein, hemoglobin, benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide (SAAPF *p*NA), and iodoacetic acid (IAA) were from Sigma (St, Louis, MO). *N*-benzoyl-L-tyrosine ethyl ester (BTEE) was from Nutritional Biochemicals Corporation (Cleveland, OH). Dithiothreitol (DTT) was from Pierce (Rockford, IL). Endoproteinase Glu-C (*Staphylococcus aureus*, EC 3.4.21.19) excision grade, was from Calbiochem (La Jolla, CA). *N*-acetyl deblocking aminopeptidase (*Pyrococcus furiosus*) was from Takara Mirus Bio Corporation (Madison, WI).

4.2. Methods

Rubber trees (*Hevea brasiliensis*, RRIM 600 clone), age about 20 years, were grown at Songkhla Rubber Research Center, Hat-Yai, Songkhla, Thailand. The trees were tapped for latex on alternate days. Fresh latex was collected in ice-chilled plastic cups, pooled and separated on the same day as collected. The pooled latex was sieved through cheesecloth to remove small particles and centrifuged (Beckman Model JA 2-21, Beckman Coulter, Fullerton, CA) at 5000*g* for 15 min at 4 °C. The latex separated into 3 layers; the top white zone containing rubber, a middle milky aqueous center layer called C-serum, and the sediment bottom layer containing lutoid particles. The C-serum was further subjected to ultracentrifugation (Beckman Model L8-70 M ultracentrifuge, Beckman Coulter, Fullerton, CA) at 59,000*g* for 45 min at 4 °C, and the supernatant containing the C-serum was collected and used for protease inhibitor purification. C-serum proteins were fractionated step-wise by the addition of acetone in increments of 0–50%, 50–70%, 70–80% and 80–95%. Each fraction was centrifuged at 59,000*g*, resuspended in distilled water, and assayed for trypsin inhibitory activity.

Amino acid sequencing from the N-terminus was performed on an Applied Biosystems (Foster City, CA) Procise Model 492 protein sequencer using Edman chemistry.

HPI-2a and HPI-2b were assayed for temperature stability in 50 mM citrate buffer, pH 6.

The reactive-site of the HPI with subtilisin A was identified by the methods of Finkenstadt and Laskowski, 1965. HPI (2 nmol) was incubated with 1% mol of subtilisin A in the solution of 0.5 M KCl and 0.05 M $CaCl_2$, pH 3.75 for 24 h at room temperature. After incubation, the reaction mixture was subjected to amino acid sequence analysis.

SDS–PAGE was performed according to the modified method of Laemmli (1970), using a 7–15% linear gradient of acrylamide, a 4% stacking gel and protein standards from Amersham Biosciences (Piscataway, NJ). After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 (Sigma, St, Louis, MO). Tricine-SDS–PAGE (polyacrylamide gel, 16.5%) was prepared using the method as described by Schägger and von Jagow (1987). Isoelectric focusing was performed with a BIO-RAD minigel IEF apparatus (Model 111 Mini IEF Cell, Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Protease inhibitory activity against trypsin and subtilisin A were assayed using azocasein as substrate. Aliquots from fractions were pre-incubated for 10 min at 37 °C with subtilisin A in a total volume of 300 μL containing 50 mM Tris–HCl pH 8.8. The assays were initiated by the addition of

200 μL substrate solution (1% (w/v) azocasein), further incubated for 20 min at 37 °C, and 235 μL of trichloroacetic acid (TCA, 20% w/v) was added to terminate the reaction. After 20 min at room temperature, the mixture was centrifuged at 10,000*g* for 10 min and the absorbance of the supernatant was measured at 335 nm. One unit of subtilisin A inhibitory activity was defined as the amount of inhibitor which decreases the absorbance at 335 nm by 0.1 OD when compared to a control solution lacking inhibitory activity. Activity assays against trypsin, chymotrypsin, pronase E and subtilisin A were measured as follows; aliquots of inhibitor-containing solutions were pre-incubated for 2 min with the appropriate enzyme in a total volume of 50 μL of appropriate buffer (see below) and the reaction was initiated by adding 500 μL substrate and adjusted to a final volume of 1 mL with buffer. The change in absorbance was recorded every minute for 5 min with a Hitachi Model U-2000 spectrophotometer. Trypsin and pronase E were assayed at pH 8.1 in 46 mM Tris–HCl, 11.5 mM $CaCl_2$ with the substrate BAPNA by a modified procedure based on the methods of Erlanger et al. (1961); Hummel (1959). Chymotrypsin was assayed at pH 7.8 in 80 mM Tris–HCl, 100 mM $CaCl_2$ with the substrate BTEE as described by Hummel (1959). Subtilisin A was assayed at pH 8.8 in 50 mM Tris–HCl, 5 mM $CaCl_2$ with *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAAPF *p*NA) as substrate by the method of Pekkarinen et al. (2002). Thermolysin and papain were assayed using casein as substrate (Eguchi and Yamashita, 1987; Shivaraj and Pattabiraman, 1981). Papain assays was carried out using 100 mM phosphate buffer, pH 6, with 0.38 mM EDTA and 1.9 mM cysteine-HCl. Pepsin and *Aspergillus* protease were assayed according to the Anson method (1938), using hemoglobin as substrate.

Purified inhibitors were reduced and alkylated using the method described by Pearce et al. (2001). The reduced and alkylated inhibitors were separated from the unreacted reagents prior to cleavage with enzymes by using $ZipTip_{C18}$ pipette tips containing C18 reversed-phase media (Millipore, Bedford, MA, USA), concentrated using a Speed Vac concentrator and then subjected to MALDI mass spectrometry analysis.

MALDI spectra were obtained using a PerSeptive Biosystems Voyager DE/RP time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm) (Framington, MA). Dried-droplet samples were prepared using α-cyano-4-hydroxycinnamic acid as the matrix, and spectra were acquired as averages of 256 laser shots with the laser fluency set approximately 20% above threshold. For LC/MS/MS analyses of peptide mixtures, samples separated with an LC Packings Ultimate Capillary LC system (Dionex Corporation, Sunnyvale, CA) were analyzed in data-dependant mode with a Bruker Daltonics (Billerica, MA) HCT ion trap with nanospray electrospray ionization (ESI). Aliquots of 5 μL were injected onto a 150 μm i.d. × 5 mm C18 packed capillary column (Dionex Corporation, Sunnyvale, CA) and eluted with a 60 min gradient of 0–100% methanol/0.1% trifluoroacetic acid.

Reduced and alkylated of each isoinhibitor (25 μg) were incubated with endopeptidase Glu-C (2.5 μg) in 50 mM ammonium bicarbonate buffer, pH 7.8, at 37 °C overnight to fragment the protein. The reaction was terminated by adding 1 μL TFA and dried in vacuo to remove ammonium bicarbonate and TFA. The dried fractions were dissolved in water, concentrated to small volumes and then subjected to mass spectroscopic analyses.

The internally calibrated positive ion MALDI-MS spectrum of the endopeptidase Glu-C digests exhibited peaks with *m*/*z* values within ±0.1 Da of the predicted singly charged ions of fragments f_3, f_4, and f_5 of the EST (1184.7, 2232.2, and 1153.7 Da, respectively), while the negative ion spectrum exhibited a peak within ±0.1 Da of the *m*/*z* expected for f_2 (1569.8 Da). The positive ion spectrum also showed a strong peak at *m*/*z* 1507.8 ± 0.1 Da, matching the mass expected for the fragment f'_4 = LNFNRVRVFVDE, which was obtained by endopeptidase Glu-C cleavage at an aspartate residue. Numerous other peaks of moderate intensity were observed in the positive ion spectrum of the endopeptidase Glu-C digest, but even though the f_1 fragment of the EST contains a basic residue and is therefore expected to appear in the MALDI spectrum, none of the observed *m*/*z* values matched the *m*/*z* predicted for f_1.

The endopeptidase Glu-C digest of purified HPI-2a included a prominent +2-charged peak of *m*/*z* 723.8 ± 0.5 Da at retention time 27.3 min. MS/MS spectra of this 723.8 Da +2 parent showed strong and interpretable series of b and y ions, containing the N and C termini, respectively, of a peptide tentatively interpreted to be Ac-ASQC*PVKNSWPE, where Ac-indicates N-terminal acetylation, and C* is the alkylation product of the SH group by iodoacetic acid.

N-acetyl deblocking aminopeptidase (Ac-DAP) was used to remove N-terminal acetyl groups from the inhibitor protein. Cleavage by Ac-DAP was performed according to the manufacturer's instruction (Takara Bio Inc, Shiga, Japan). The enzyme (25 μg) was added to the reduced and alkylated inhibitors (25 μg) in 50 mM *N*-ethylmorpholine-AcOH, 0.1 mM $CaCl_2$, pH 8.0 and incubated at 50 °C for 24 h. The reaction was terminated by adding 50% formic acid to a final concentration of 5% and the protein were subjected to amino acid sequence analyses.

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