
Proteins of circularly permuted sequence present within the same organism: The major serine proteinase inhibitor from *Capsicum annuum* seeds

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Abstract

The major serine proteinase inhibitor from bell pepper (*Capsicum annuum*, paprika) seeds was isolated, characterized, and sequenced, and its disulfide bond topology was determined. PSI-1.2 is a 52-amino-acid-long, cysteine-rich polypeptide that inhibits both trypsin ($K_i = 4.6 \times 10^{-9}$ M) and chymotrypsin ($K_i = 1.1 \times 10^{-8}$ M) and is a circularly permuted member of the potato type II inhibitor family. Mature proteins of this family are produced from precursor proteins containing two to eight repeat units that are proteolytically cleaved within, rather than between, the repeats. In contrast, PSI-1.2 corresponds to a complete repeat that was predicted as the putative ancestral protein of the potato type II family. To our knowledge, this is the first case in which two proteins related to each other by circular permutation are shown to exist in the same organism and are expressed within the same organ. PSI-1.2 is not derived from any of the known precursors, and it contains a unique amphiphilic segment in one of its loops. A systematic comparison of the related precursor repeat-sequences reveals common evolutionary patterns that are in agreement with the ancestral gene-duplication hypothesis.

Keywords: Protein chemistry; protein structure; proteinase inhibitors; evolution

Supplemental material: See www.proteinscience.org.

Most plants produce proteinase inhibitors as a defense against insects and pathogenic organisms (Ryan 1980, 1989). One of the well-studied groups is that of the serine proteinase inhibitors belonging to the so-called potato type II (PT-II) family (Richardson 1977; Casaretto and Corcuera 1995; Bowles 1998). The members of this group are ~50-residue-long, cysteine-rich polypeptides that inhibit both trypsin and α -chymotrypsin. Their three-dimensional structure is characterized by four disulfide bonds, a triple-stranded β -sheet, and a long loop that interacts with the

active site of the proteinase (Greenblatt et al. 1989; Bode and Huber 1992; Nielsen et al. 1994, 1995). These proteins are expressed in various tissues of the members of the *Solanaceae* family, sometimes at very high levels (Graham et al. 1985a; Keil et al. 1986; Johnson and Ryan 1990; Balandin et al. 1995; Miller et al. 2000). Several genes encoding PT-II proteins have been identified (for a recent overview, see Miller et al. 2000). These genes usually encode a larger precursor protein, which is subsequently cleaved into several slightly different PT-II inhibitor molecules (Table 1). The precursors contain a variable number of cysteine-rich repeats that we had termed inhibitor precursor (IP)-repeats after the Swiss-Prot name of the parent proteins (Murvai et al. 1999). Proteolytic cleavage of the precursor occurs within, rather than between, repeats (Atkinson et al. 1993). It has been hypothesized that the gene encoding the

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Table 1. Members of the potato proteinase inhibitor type II family used in the phylogenetic analysis.

Entry name	Accession No.	Protein name (gene name)	Species, tissue	No. of repeats	Reference
S72492 (PIR)	S72492	probable proteinase inhibitor precursor–tomato, AT2 protein (PIN2)	<i>L. esculentum</i> (tomato), shoot	2	Brandstadter et al. 1996
O82735	O82735	potato (<i>S. tuberosum</i>) mRNA 2 for proteinase inhibitor II [fragment]	<i>S. tuberosum</i> (potato).	2	Sanchez-Serrano et al. 1986
IP25_SOLTU	Q41488	proteinase inhibitor type II P303.51 [precursor]	<i>S. tuberosum</i> (potato), tuber	2	Jongsma et al. 1995
IP27_SOLTU	Q43652	proteinase inhibitor type II CM7 [precursor] (PIN2–CM7)	<i>S. tuberosum</i> (potato), leaf	2	Murray and Christeller 1994
IP2Y_SOLTU	Q41489	proteinase inhibitor type II [precursor]	<i>S. tuberosum</i> (potato)	2	ds
IP2K_SOLTU	P01080	proteinase inhibitor type II K [precursor], IIK, (PIN2K)	<i>S. tuberosum</i> (potato)	2	Keil et al. 1986
IP2X_SOLTU	Q00782	proteinase inhibitor type II [precursor]	<i>S. tuberosum</i> (potato)	2 ⁺	Choi et al. 1990
IP2T_SOLTU	Q41435	proteinase inhibitor type II T [precursor], (PIN2T)	<i>S. tuberosum</i> (potato)	2	Park and Thornburg et al. 1996
IP21_LYCES	P05119	wound-induced proteinase inhibitor type II [precursor]	<i>L. esculentum</i> (tomato), leaf		Graham et al. 1985b
IP21_TOBAC	Q40561	proteinase inhibitor type II [precursor]	<i>N. tabacum</i> (common tobacco), leaf	3	Balandin et al. 1995
IP22_LYCES	Q43710	proteinase inhibitor type II TR8 [precursor] (ARPI)	<i>L. esculentum</i> (tomato), seedling, root	3	Taylor et al. 1993
IP23_LYCES	Q43502	proteinase inhibitor type II CEVI57 [precursor] (CEVI57)	<i>L. esculentum</i> (tomato), leaf	3	Gadea et al. 1996
X99095	X99095	proteinase inhibitor type II (pin2) gene	<i>S. tuberosum</i> (potato), leaf	3	Dammann et al. 1997
Q9SDL4, IP21_CAPAN	Q9SDL4	Hypothetical 22.5 KDA protein (CAPIN II)	<i>C. annuum</i> (paprika, bell pepper), seed	3'	ds
IP22_CAPAN	Q49146	wound-induced proteinase inhibitor II [precursor]	<i>C. annuum</i> (paprika, bell pepper), pericarp	3'	ds
Q9SQ77	Q9SQ77	proteinase inhibitor [precursor]	<i>N. alata</i> (winged tobacco) (Persian tobacco), stigma	4	Miller et al. 2000
Q40378	Q40378	proteinase inhibitor [precursor]	<i>N. alata</i> (winged tobacco) (Persian tobacco), stigma, styles	6	Atkinson et al. 1993
Q9SDW7, ICE3 (PDB)	Q9SDW7	proteinase inhibitor type II precursor NGPI-2 [fragment]	<i>N. glutinosa</i> (tobacco)	6	Choi et al. 1993
Q9SDW8	Q9SDW8	proteinase inhibitor type II precursor NGPI-1 [fragment]	<i>N. glutinosa</i> (tobacco)	8	Choi et al. 1993
PSI-1.2	n.a.	Paprika seed proteinase inhibitor	<i>C. annuum</i> (paprika, bell pepper) seed	?	this work.

2⁺, two IP-repeats plus a C-terminal partial repeat (see Fig. 5); 3', two IP-repeats flanked by two partial repeats (see Fig. 6); ds, database submission.

precursor protein arose from the duplication of a gene originally encoding a single IP-repeat, which was then followed by a frame shift in the proteolytic processing (Scanlon et al. 1999). To support this hypothesis, Scanlon and associates designed and expressed in *Escherichia coli* an hypothetical ancestral protein corresponding to a single repeat of the *Nicotiana alata* precursor protein and determined its three-dimensional structure by nuclear magnetic resonance (NMR). This product actually inhibits trypsin and chymotrypsin, and its fold is very similar to that of the naturally occurring inhibitors (Scanlon et al. 1999). In other terms, the *N. alata* repeat has the ability to fold both as a structural repeat (similar to mature PT-II inhibitors) and as a sequence repeat (similar to aPII; Scanlon et al. 1999).

Capsicum annuum (bell pepper, paprika) seeds express several regular PT-II inhibitors, including PSI-1.1 (Antcheva

et al. 1996). Here we report the isolation, amino acid sequencing, disulfide bond topology, and characterization of PSI-1.2, a proteinase inhibitor that corresponds to a single IP sequence repeat and thus has a fold similar to the putative ancestral inhibitor protein aPII. To our knowledge, this is the first case in which two proteins related to each other by circular permutation are shown to exist in the same organism. The structure of the PSI-1.2 protein is discussed with the help of a structural model as well as in the light of a systematic comparison of IP sequence repeats.

Results

Isolation and characterization

Isolation of PSI-1.2 was based on a procedure previously developed for bell pepper seed inhibitors (Antcheva et al. 1996). This process is based on affinity chromatography on

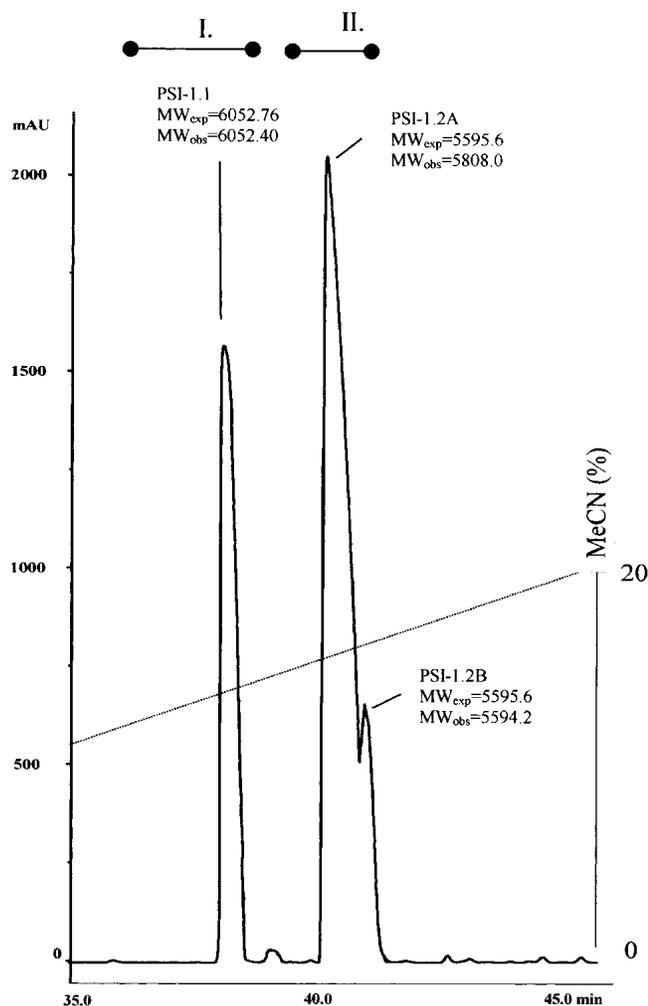


Fig. 1. Separation of various proteinase inhibitors from *Capsicum annuum* seeds by reverse phase–high-performance liquid chromatography. The observed molecular weights (MW_{obs}) were determined by mass spectrometry. The expected molecular weights (MW_{exp}) are based on the sequences shown in Figs. 2, 3. The bars over the elution profile correspond to fractions I and II.

α -chymotrypsin-Sepharose, and yields two main fractions shown in Figure 1. Mass spectrometry analysis indicated that fraction I contains PSI-1.1, a member of the PT-II family of inhibitors that had been identified previously (Antcheva et al. 1996). Fraction II contained two products designated as PSI-1.2A and PSI-1.2B. These components were repurified by narrow-bore reverse phase–high-performance liquid chromatography (RP-HPLC) before sequencing (data not shown).

Protein sequencing

The major inhibitor fraction (II in Fig. 1) consisted of two overlapping peaks. Initial sequencing attempts of the larger peak (A) failed to detect a sequenceable N terminus. A sample was thus reduced, pyridylethylated, and digested separately with either CNBr in 70% HCOOH or trypsin.

The resulting peptides (Fig. 2, PSI-1.2A-F1 and PSI-1.2A-F2, respectively) were isolated by narrow-bore RP-HPLC and sequenced. The smaller peak (B), on the other hand, gave a full sequence of 52 amino acids, identical with that of peak A. A comparison of the sequence (Fig. 2) and the observed molecular mass (Fig. 1) indicates that the difference between peak A and peak B results from an unidentified N-terminal modification of peak A. The PSI-1.2 sequence (Fig. 2) has eight cysteines, same as in the previously isolated PSI-1.1 (Antcheva et al. 1996).

The sequence of PSI-1.2 does not correspond to any published sequence found in the databases. On the other hand, the sequence search revealed that the previously determined PSI-1.1 is identical with one of the predicted proteolytic fragments of the recently published PT-II family precursor Q9SDL4 (Fig. 3A). The Q9SDL4 precursor can, in principle, yield three mature PT-II proteins. Interestingly, PSI-1.1 is identical with the first putative cleavage product.

Disulfide bridges of PSI-1.2

As PSI-1.2 is not identical with any natural mature proteinase inhibitor, its disulfide topology was experimentally determined from a set of enzymatic digests combined with mass spectrometry and N-terminal sequencing (Table 2). This analysis in itself did not yield the complete disulfide topology of the protein. In particular, the connectivity of two adjacent cysteines, Cys31 and Cys32, is not unambiguous (see inset in Fig. 4). Additional data were collected by Edman degradation combined with phenylhydantoin (PTH) analysis at 313 nm, which makes it possible to identify PTH-dehydroalanine (PTH-DHA), the β -elimination product of PTH-cystine. PTH-DHA forms when the process of N-terminal sequencing reaches a Cys residue that is disulfide bonded to a sequentially upstream Cys residue (Li and Liang 1999). This analysis showed PTH-DHA at positions 28, 32, 38, and 49; an example is shown in Figure 4. These results, combined with the data of Table 2, confirmed that PSI-1.2 has the disulfide topology Cys₃-Cys₃₂, Cys₇-Cys₂₈, Cys₁₆-Cys₃₈, Cys₃₁-Cys₄₉. The disulfide bridges of PSI-1.2 thus correspond to those of the aPII hypothetical ancestral protein (Scanlon et al. 1999).

Enzyme inhibitor assays

PSI-1.2B, the product with a fully known sequence was isolated for enzymatic analysis. It is a strong inhibitor of trypsin ($K_i = 4.6 \times 10^{-9}$ M) and a somewhat weaker inhibitor of α -chymotrypsin ($K_i = 1.1 \times 10^{-8}$ M), whereas elastase and subtilisin DY are not inhibited (Table 3). The enzymes thrombin and factor Xa, related to the blood clotting system, are only weakly inhibited by PSI-1.2 ($K_i = 1.1 \times 10^{-6}$ M and $K_i = 2.6 \times 10^{-5}$ M, respectively). As a whole, PSI-1.1 appears to be a stronger inhibitor of thrombin (100 \times), trypsin (10 \times), and factor Xa (10 \times) than the

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PSI-1.2A-F1          VCPSSGERIIRKVCNTNCCAAQKGCKLFRSNGSIKCTGT
PSI-1.2A-F2          NCDTDIAYMVCPSSEGER
PSI-1.2A            NCDTDIAYMVCPSSEGERIIRKVCNTNCCAAQKGCKLFRSNGSIKCTGT
PSI-1.2B            KACPRNCDTDIAYMVCPSSEGERIIRKVCNTNCCAAQKGCKLFRSNGSIKCTGT

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Fig. 2. The sequence of PSI-1.2 as determined by automated Edman sequencing after reduction and pyridylethylation. PSI-1.2B gave a complete sequence corresponding to its observed molecular weight determined by mass spectrometry. PSI-1.2A failed to produce an N-terminal signal, so its fragments obtained by CNBr cleavage (PSI-1.2A-F1) and trypsin (PSI-1.2A-F2) were subjected to sequencing.

presently isolated PSI-1.2. Pepsin was found not to hydrolyze PSI-1.2 over a period of 30 min at pH 2.0. Heat treatment (100°C) at pH 4.0 for 10 min had no effect on the anti-trypsin activity of PSI-1.2B.

Sequence similarity searches

The sequence of PSI-1.2 is not identical with any known protein found in the protein and DNA databases. However, it shows a sequence similarity to various protein precursors of the PT-II family proteinase inhibitors. Comparing the sequence with the domain database SBASE, it becomes apparent that the sequence of PSI-1.2 corresponds to a complete IP-repeat (Fig. 3B), the repeat unit of the PT-II family precursors (Murvai et al. 1999). A comparison with mature PT-II inhibitors reveals, on the other hand, that the sequence of PSI-1.2 is circularly permuted compared with that of the mature proteins, as if a domain swapping event had taken place (Bennett et al. 1995; Heringa and Taylor 1997). For example, of the 45 residues of the potato tuber inhibitor PCI-1 (PDB code 4SGB_I) that can be aligned with PSI-1.2, 25 are identical (56%). If we divide PSI-1.2 into three fragments, A-P-B, PCI-1 should be represented as B-A, with P denoting the putative processing site, which is missing in the mature PCI-1 protein (Fig. 3B).

A database search for IP-repeats similar to PSI-1.2 in databases resulted in the identification of several proteinase precursor proteins containing from two to eight IP-repeats (Table 1). In the precursor proteins from *Nicotiana* (tobacco), *Solanum* (potato), and *Lycopersicon* (tomato), the number of IP-repeats can vary from two to eight. In general, the precursor consists of an integer number of IP-repeats, the only exception being IP2X_SOLTU, for which a short

C-terminal partial repeat containing two cysteines is present. The *C. annuum* precursors IP22_CAPAN and Q9SDL4 are unique in the sense that they contain two IP-repeats flanked by N- and C-terminal partial repeats. Their N-terminal partial repeat contains six cysteines, whereas the C-terminal partial repeat contains three cysteines only. In several cases, different IP-repeats share the same amino acid sequence. For example, five of the Q9SDW7 repeats (R2 to R6) are 100% identical to R4 through R8 from Q9SDW8. The previously isolated PSI-1.1 (IP21_CAPAN) protein (Antcheva et al. 1996) is identical to residues 36–90 of Q9SDL4, indicating that it derives from the proteolytic processing of the Q9SDL4 precursor.

The exon/intron structure was reported for a number of genes. In the known cases, all the IP-repeats (i.e., the entire precursor apart from the signal peptide) are coded by one single exon.

We performed a systematic sequence comparison on the individual IP-repeats found in the databases. For better identification, the repeats are numbered as R1 through R8, starting from the N terminus of the precursors, and the precursors are denoted by the number of IP-repeats indicated in Table 1. For example, a precursor with two IP-repeats is denoted IP-2. The multiple alignment (deposited) revealed that only the cysteine pattern is completely conserved. The results of the comparison are displayed as a phylogenetic tree (Fig. 5) as well as in a graphical form (Fig. 6). One major cluster includes R1 and R2 repeats from precursors with two and three IP-repeats (IP-2 and IP-3), including IP2X_SOLTU, which has an additional short truncated repeat at the C terminus (IP-2⁺). This group includes all the known potato precursors as well as the one expressed in tobacco leaves. The second group contains all the IP-repeats

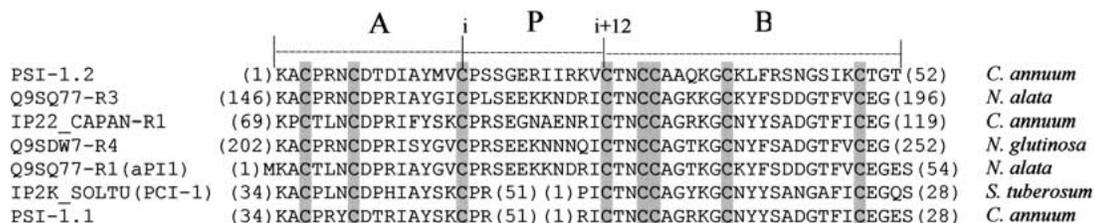


Fig. 3. Multiple alignment of PSI-1.2 with selected PT-II sequences. (A multiple alignment comprising all inhibitor precursor IP-repeat sequences was deposited as supplemental material.) The dashed line indicates the region in which proteolytic cleavage of the IP-repeats takes place (PSI-1.2 is not cleaved in this region). The segments A, P, and B are explained in the text and also shown in Fig. 7. The conserved cysteines are highlighted in grey. Q9SQ77-R1 corresponds to the aP11 (PDB code 1CE3), IP2K_SOLTU to PCI-1 (PDB code 4SGB), and PSI-1.1 to IP21_CAPAN.

Table 2. The disulfide structure of PSI-1.2 as determined by enzymatic digestion and electron spray ionization mass spectrometry

Enzyme	Native peptide fragment (predicted structure)	Molecular mass [Da] (S–S form)		Reduced fragment (predicted structure)	Molecular mass [Da] after reduction		S–S bridge
		Observed	Predicted		Observed	Predicted	
T + C		1860.6	1862.16	² ACPR ₅ ²⁷ VCTNCCAAQK ₃₆ ⁴⁹ CTGT ₅₂	445.5 1040.3 380.4	445.54 1040.24 380.42	3–32 31–49
T + C		1270.2	1269.47	¹⁴ MVCPSSEGER ₂₂ ³⁷ GCK ₃₉	965.2 306.4	965.12 306.38	16–38
P		3603.3	3604.14	³ CPRNCDTDIA ₁₂ ²² RIIRKVCTNCCA ₃₃ ⁴² RSNGSIKCTGT ₅₂	1107.6 1379.8 1123.6	1107.22 1379.72 1123.25	3–32 7–28 31–49
P		1354.6	1354.62	¹⁴ MVCPSSEGE ₂₁ ³⁶ KGCKL ₄₀	808.3 547.3	808.92 547.71	16–38

T + C indicates trypsin/chymotrypsin; P, pepsin; at pH 3.0.

of the *N. alata* and *N. glutinosa* precursors that consist of four to eight IP-repeats (IP-4, IP-6, and IP-8). These precursors are expressed in floral organs and young tissues of the plants.

Finally, the precursors that contain three repeats form a further group that can be subdivided into two subgroups. One group, IP-3', is represented by two bell pepper inhibitor precursors that have an unusual architecture, as they contain one repeat consisting of two partial repeats attached to the N and C terminus of two intact repeats. The other subgroup of three-repeat precursors, IP-3, consists of three regular IP-repeats. In these sequence comparisons, PSI-1.2 was found to be most similar to the R3 (the third IP-repeat) of the IP-3 precursors that form a separate cluster (Fig. 5).

Model building

To identify the putative region responsible for proteinase inhibition, we built a three-dimensional model of PSI-1.2 based on the NMR structure of the proteinase inhibitor aPI1 (Scanlon et al. 1999). PSI-1.2 and aPI1 have a 53% sequence identity and the same disulfide bond pattern, so they are likely to adopt the same fold. Figure 7 compares the aPI1-based model of PSI-1.2 with the crystallographic structure of the potato chymotrypsin inhibitor-1 (PCI-1) complexed with the proteinase B from *Streptomyces griseus* (PDB code 4SGB). Of the available proteinase/inhibitor complexes, 4SGB_I shows the highest sequence similarity to PSI-1.2. Figure 7 clearly shows that despite the different positions of the N and C termini, the overall fold of the two proteins and the orientation of the disulfide bonds are very similar, with a conserved core composed of a triple-stranded anti-parallel β -sheet and a network of four disulfide bridges. The PCI-1 residues involved in contacts with the active site of the *S. griseus* proteinase are located in the long loop

connecting strands 2 and 3. These residues (33-PKAC-PLNCDP-42) correspond to the N-terminal region in PSI-1.2. The central eight residues of PSI-1.2 (34-KACPRNCD-41) are well conserved, with the only exception of the L→R substitution. Although constrained by two disulfide bridges

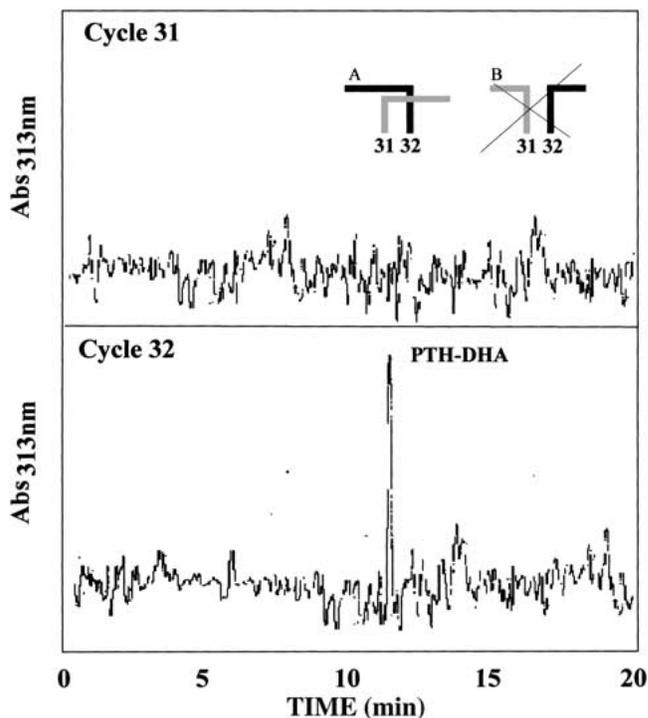


Fig. 4. Identification of dehydroalanine (DHA) during Edman sequencing by monitoring the high-performance liquid chromatography–effluent of the phenylhydantoin (PTH) degradation products at 313 nm. Appearance of PTH-DHA in position 32 indicates that this residue is disulfide bonded to a sequentially upstream cysteine, which by exclusion is Cys₃. This arrangement is compatible with scheme A and not with scheme B.

Table 3. Inhibition constants of PSI-1.2

Inhibitor	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	Trypsin K_i (M)	α -chymotrypsin K_i (M)	Thrombin K_i (M)	Factor Xa K_i (M)
PSI-1.2	A	C	P	R	N	C	D	4.6×10^{-9}	1.1×10^{-8}	1.1×10^{-6}	2.6×10^{-5}
PSI-1.1	A	C	P	R	Y	C	D	4.8×10^{-10}	4.7×10^{-8}	1.7×10^{-8}	1.1×10^{-6}

Inhibition effects were compared with PSI-1.1 isolated from *C. annuum* seeds (Antcheva et al. 1996). The numbering of residues in contact with the protease is taken from Bode and Huber (1992)

at positions 3 and 7, this region shows a high root mean square derived value in the NMR structure of aPII and is thus likely to be conformationally flexible.

The segment 19–28 of PSI-1.2 is of particular interest. This segment is not part of any other mature protein isolated so far; moreover, the corresponding segment of aPII is not well defined. A helical wheel plot reveals, on the other hand, that the distribution of hydrophobic and hydrophilic amino acids in this segment of PSI-1.2 is characteristic of amphiphilic helices (Fig. 7, inset). In the hypothetical helix, residues S, E, R, R, and K are facing the solvent, whereas residues G, I, I, V, and C face the interior of the protein. This asymmetric distribution of hydrophilic and hydrophobic residues is unique to PSI-1.2 among the known IP-repeats.

Discussion

Of the bell pepper seed inhibitors described in this work, PSI-1.1 is apparently a proteolytic cleavage product of the Q9SDL4 precursor, whereas PSI-1.2 is different from any known protein. PSI-1.2 is the first naturally occurring inhibitor that shows the circularly permuted topology present in the hypothetical ancestral PT-II protein aPII (Scanlon et al. 1999). Circular permutation of sequences had been reported in other cases (for a review, see Heringa and Taylor 1997; Lindqvist and Schneider 1997). Until now, however, circular rearrangements were observed only between species, such as the β -lectin of *Vicia faba* and the lectin concanavalin A from *Canavalia ensiformis* (Edelman et al. 1972), or the plant aspartyl proteinases and human lung surfactant proteins (Ponting and Russell 1995). PSI-1.2 is apparently the first example in which circularly permuted members of a protein family are expressed within the same organism, moreover within the same organ. As both proteinase inhibitors and lectins are proteins that play roles in the defense mechanisms of plants, it is tempting to speculate that the underlying sequence rearrangements are part of a general scenario by which plants produce functional diversity against pathogenic attack. In fact, the specificity of PSI-1.2 is slightly different from that of the previously isolated PSI-1.1 (Table 3).

Homology modeling confirmed that the sequence can be built into the structure of aPII without steric clashes. In the region that corresponds to the *in vivo* proteolytic processing

site in the aPII sequence, PSI-1.2 has the theoretical capability to form an amphiphilic helix that would have the hydrophobic face toward the interior of the protein and the hydrophilic face toward the solvent. As no other IP-repeat sequence shows similar distribution of hydrophobic and hydrophilic amino acids in the corresponding region, we speculate that this feature may be of significance and worth of structural studies. For example, the amphiphilic helix might contribute to the stability of PSI-1.2 in solution; moreover, it might provide protection against proteolytic cleavage as it might not be recognized by the proteases in the plant secretory pathway (Heath et al. 1995).

The similarities that exist between the individual IP-repeats of the various PT-II precursors (indicated by gray lines in Fig. 5) reveal a consistent pattern that suggests that insertion of double repeats may have taken place several times during the evolution of this protein family. For example, an insertion of two repeats between R3 and R4 of the *N. alata* precursor Q9SQ77 may have given rise to Q40378. In a similar way, the structure of the eight-repeat precursor Q9SDW8 can be deduced as a result of an analogous insertion N-terminal to R2 in Q9SDW7. This supposition has an interesting implication regarding the potential origins of PSI-1.2. The sequence of PSI-1.2 is most similar to R3 of the three-membered precursors IP-3. The similarity leads us to speculate that IP-3 proteins may in fact have evolved by insertion of a double repeat into a putative ancestral protein IP-1 (Fig. 8A), and PSI-1.2 might in fact be the product of the ancestral IP-1 precursor as predicted by Scanlon and associates (1999). This hypothetical precursor might then give rise to PSI-1.2 by cleavage of the signal peptides without further proteolytic processing. In other terms, all members of the PT-II family might have emerged via a combination of duplications, insertions, and subsequent or concomitant modification events (e.g., emergence of proteolytic cleavage sites, sequence losses in the case of IP-3' precursors) from the same ancestral sequence, as schematically shown in Figure 8A. This explanation is speculative and awaits experimental confirmation. It is equally possible that PSI-1.2 is derived via an alternative proteolytic processing scheme from an unknown precursor consisting of several IP-repeats (Fig. 8B). Irrespective of the precise evolutionary route, it appears that the general mechanisms producing the permuted precursor genes and protein prod-

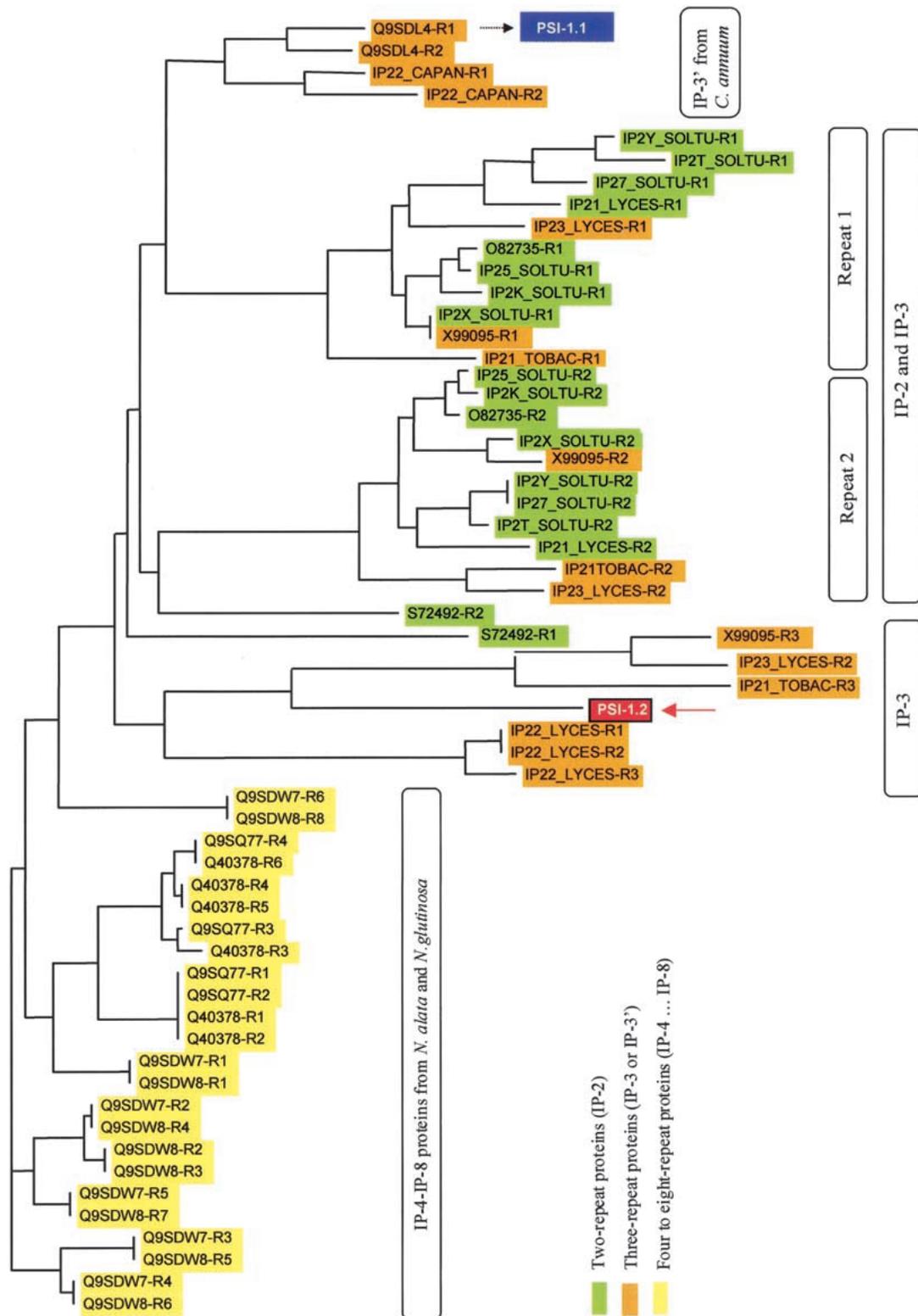


Fig. 5. Phylogenetic tree of the proteinase inhibitor precursor IP-repeats. The repeat number is abbreviated as R1, R2, R3, etc., starting from the N terminus. The amino acid sequence of PSI-1.1 (Antcheva et al. 1996) exactly corresponds to the first putative mature product of the hypothetical 22.5-kD protein of *Capsicum annuum* (Q9SDL4).

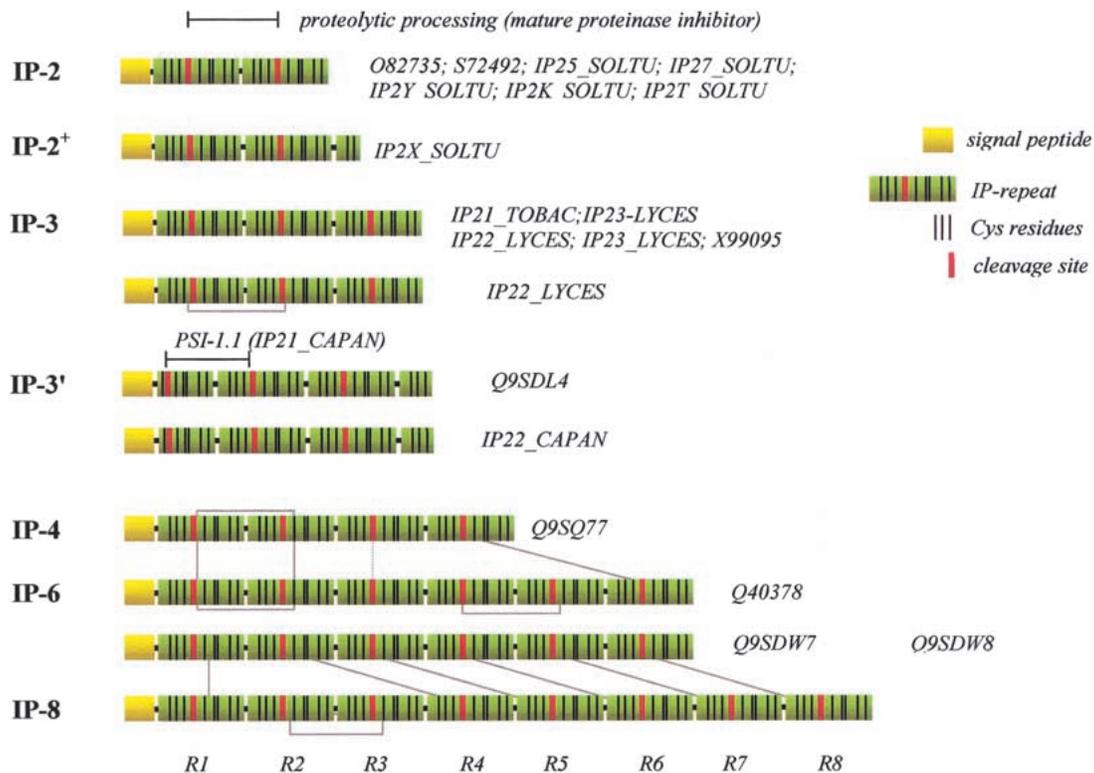


Fig. 6. The domain structure of the potato II inhibitor family precursors that show sequence similarity to PSI-1.2. IP-2 through IP-8 designate the number of inhibitor precursor IP-repeats in the group. Gray lines indicate sequence identity (>98%); dashed line, similarity. It is noted that the C-terminal truncated repeats in IP-2⁺ and IP-3' are not of the same length.

ucts in this family of *Solanaceae* genes are presumably similar to those invoked for the explanation of circular rearrangements in other species (Heringa and Taylor 1997; Lindqvist and Schneider 1997; Jeltsch 1999).

The emergence of the processing sites (red in Figs. 5, 7) may have had an interesting implication specific for this family of proteins. The ancestral repeat unit must have formed intra-repeat disulfides only, such as those in aPII and in PSI-1.2. In other terms, the ancestral repeat must have been an independent folding unit—as indirectly proved by Scanlon et al. (1999). In contrast, those repeats that are cleaved in their central sections form inter-repeat disulfides (e.g., PCI-1), so that the folding units are now formed from segments of two adjacent sequence repeats. The two disulfide topologies are different, as shown in Figure 7, even though the positions and the spatial orientation of the disulfide bridges are in good agreement. The emergence of an alternative folding ability, followed by the formation of a new disulfide topology, might have been a prerequisite for the new proteolytic processing to appear.

We can conclude that PSI-1.2 represents a novel type of plant serine proteinase inhibitor, which is related to the mature PT-II inhibitors by a circular permutation in the amino acid sequence. It corresponds to a complete IP-repeat

unit of the precursor proteins and is derived from a hitherto unidentified member of PT-II precursors. In the members of this protein family present in *Solanaceae*, rearrangements have apparently taken place both at the DNA level (duplications, insertions, loss of flanking sequences) as well as on the protein level (proteolytical processing variants, single-chain versus two-chain analogs). Nevertheless, the resulting protein products all seem to fold into the same overall three-dimensional structure, which may be required for binding to proteinases. The case of PSI-1.2 is apparently the first example in which circularly permuted members of a protein family are expressed within the same organism.

Materials and methods

Materials

Bell pepper (*C. annuum*) seeds were from the Canning Factory, Pazardjik, Bulgaria. Bovine trypsin (L-1-p-tosylamino-2-phenylethyl chloromethyl ketone-treated), α -chymotrypsin, elastase, and human plasma thrombin were purchased from Sigma; human plasma coagulation factor Xa, from Calbiochem; and pepsin, from Merck. Subtilisin DY was isolated according to Nedkov and Bobatinov (1976). H-D-Phe-Pec-Arg-pNA·AcOH was purchased from Bachem; *N*-methoxycarbonyl-Nle-Gly-Arg-pNA·AcOH (Chromozym X), from Boehringer Mannheim; Suc-Ala-Ala-Pro-

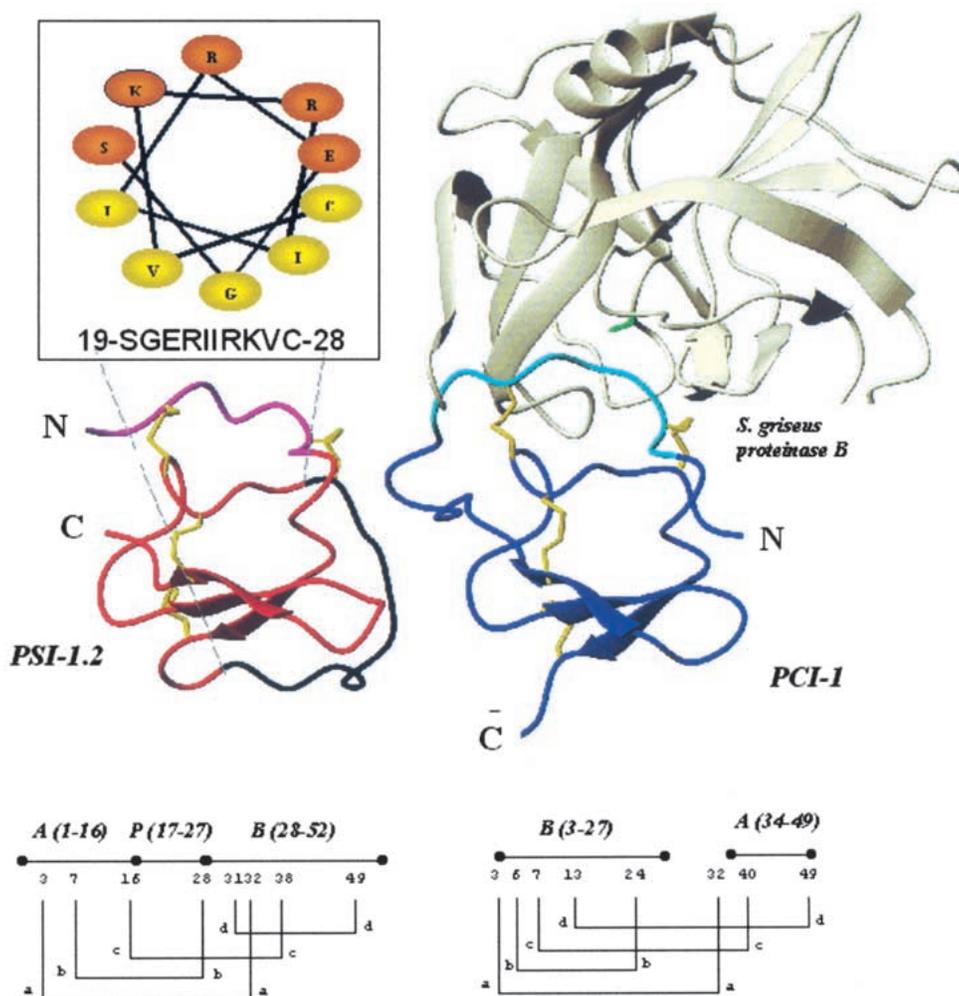


Fig. 7. (Left) The model of PSI-1.2 built by homology modeling. (Right) The structure of the complex between the polypeptide chymotrypsin inhibitor-1 from potato tubers (PCI-1, in blue) and the proteinase B from *Streptomyces griseus* (PDB code 4SGB; Greenblatt et al. 1989). The PCI-1 residues involved in contacts with the enzyme are shown in light blue; the corresponding residues of PSI-1.2, in magenta. The catalytically active residue (S195) of the enzyme is shown in green. Although the orientation of the disulfides (yellow) is identical in the two molecules, the disulfide topologies (*below*) are different: *abcdacd* in PSI-1.2 and *abcdcbad* in PCI-1. (*Inset*) The helical wheel diagram of the central region of PSI-1.2. In the model, the same segment is shown in black.

Phe-pNA (Suc-X-pNA) and N-benzoyl-L-arginine-pNA hydrochloride (L-BApNA), from Fluka; Casein Hammarsten Servabacter, from Serva; and p-nitrophenyl-*p'*-guanidino-benzoate hydrochloride (p-NPGB) and *N*-trans-cinnamoyl-imidazole (NTCI), from Sigma.

Inhibitor isolation

The isolation of the inhibitors was performed essentially as described previously (Antcheva et al. 1996). The final purification was performed by RP-HPLC on a semi-preparative Delta Pak 300 15RP18 column (250 × 10 mm) using a Millipore Waters HPLC system. The two solvents used were 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). A linear gradient was used for elution (flow rate, 6.0 mL/min) in which the solvent composition changed from 0% to 20% solvent B in 45 min. The purity of the isolated proteins was

checked on an analytical HPLC column (LiChrosphere 5RP18, 4 × 250 mm, Merck); chromatography was performed at a flow rate of 1 mL/min and gradient from 0% to 45% solvent B in 40 min, using the same buffer system as for the preparative runs. The molecular weight of inhibitors were determined by electron spray mass spectrometry on a Perkin Elmer Sciex API 150EX.

Enzyme inhibition assays

Inhibition of serine proteinases (except subtilisin) were followed by monitoring the rate of hydrolysis of p-NA substrates spectrophotometrically at 405 nm using a Ultraspec 3000 UV/Visible spectrophotometer (Pharmacia Biotech) using L-BApNA (trypsin), Suc-Ala-Ala-Pro-Phe-pNA (α -chymotrypsin), Suc-(Ala)₃-pNA (elastase), H-D-Phe-Pec-Arg-pNA·AcOH (thrombin), and *N*-methoxycarbonyl-Nle-Gly-Arg-pNA·AcOH (factor Xa). Precise concentrations of the active enzymes were determined by standard

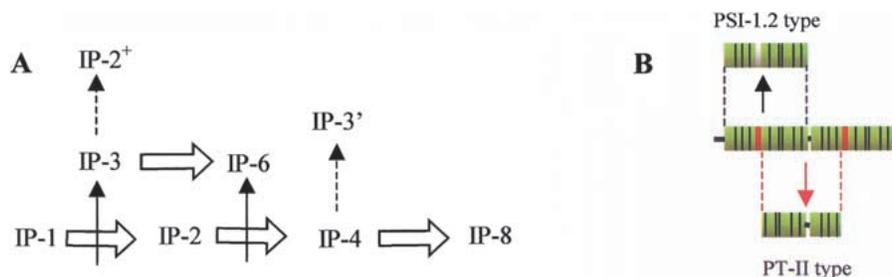


Fig. 8. (A) Comparison of PSI-1.2 with the inhibitor precursor IP-repeats and hypothetical evolutionary pathways capable of producing the current proteinase inhibitor precursor genes of the PT-II family. Fat arrow indicates duplication events; thin arrow, insertion event; and dashed arrow, modification events (loss of N- and/or C-terminal sequence). (B) The possible products arising from alternative proteolytic processing pathways are also shown.

active site titration procedures (Chase and Shaw 1970). In the inhibition assays, stoichiometric concentrations in the 10^{-7} M range of the enzymes (trypsin, α -chymotrypsin) and the inhibitors (PSI-1.1 and PSI-1.2) were used. The residual enzyme activity was measured after 5-min incubation of the enzymes with the inhibitors at 22°C in 50 mM Tris-HCl buffer at pH 7.8, containing 0.02 M CaCl_2 . The substrates (10^{-4} M) were added, and the reaction was followed at 405 nm (Geiger and Fritz 1984). In the inhibition assays of human plasma thrombin and blood coagulation factor Xa, concentrations in the 10^{-9} M range of the enzymes, 10^{-7} M of the inhibitors, and 10^{-4} M of the substrates were used. The measurements were performed in 50 mM Tris/HCl, 100 mM NaCl, 0.5 mg/mL BSA, 20 mM EDTA at pH 7.8 and 22°C (van Dam-Mieras et al. 1984). PSI-1.2 was assayed for possible inhibitory activity against subtilisin DY using 1.2% casein as a substrate. The dissociation constants (K_i) of the enzyme-inhibitor complexes were determined by the method of Cha (1975).

Protein sequence determination

Reduced and pyridylethylated samples (Hampton et al. 1992) were digested separately either with CNBr in 70% HCOOH overnight at room temperature or with trypsin for 3 h at 37°C (E:S, 1:20). The resulting peptides were isolated by narrow-bore RP-HPLC on an analytical Aquapore OD300 column (220×2.1 mm, 7-mm particle size, Applied Biosystems) using gradients of acetonitrile in 0.1% (v/v) aqueous TFA. The pyridylethylated protein and the peptides were sequenced by automated Edman degradation using an Applied Biosystems protein sequencer (model 471 A) equipped with a 120A PTH analyzer (Hunkapiller et al. 1983).

Determination of disulfide bridges

The disulfide topology of PSI-1.2B was deduced from mass spectrometric and N-terminal sequencing analysis of enzymatic digests of the protein using a combination of methods described previously (Chagolla-Lopez et al. 1994; Lu et al. 1999). Peptide samples corresponding to 75 μg PSI-1.2B were dissolved in 150 μL of 0.2M Tris-HCl buffer at pH 7.3 and digested with a mixture of trypsin (3 μg) and chymotrypsin (3 μg) for 15 h at 37°C. The mixture was separated by RP-HPLC on a Vydac C_{18} column (2.1×250 mm) and subjected to mass spectrometry analysis, and the molecular weights were compared with those predicted for the expected peptides. Selected peaks were collected, lyophilized, and subjected to automated Edman degradation. The released amino acid phenylthiohydantoin were detected at 269 and 313 nm (Li and Liang 1999).

Sequence similarity searches

The sequence homology search in the SWISS-PROT, trEMBL (Bairoch and Apweiler 2000), SBASE (Murvai et al. 2000), and PIR (Barker et al. 2000) protein databases for proteinase inhibitor precursor proteins was performed using BLAST (Altschul et al. 1997), FASTA (Pearson and Lipman 1988), and BLITZ (Collins and Coulson 1990). Full-length precursors only were retained. The precursors were dissected into repeats homologous to the PSI-1.2 sequence and clustered using CLUSTALW (Thompson et al. 1994). The phylogenetic tree was built using the PHYLIP package and drawn using DRAWGRAM according to Felsenstein (1981) using a neighbor-joining algorithm (Saitou and Nei 1987).

Modeling

The three-dimensional model of PSI-1.2 was built from the NMR structure of the *N. alata* *aP11* putative inhibitor. PSI-1.2 and *aP11* were aligned manually, and the first five structures of the NMR ensemble (PDB code 1CE3) were used as templates. The model was built by satisfaction of spatial restraints and energy minimization using MODELLER (Sali and Blundell 1993). The molecules were displayed using MOLMOL (Koradi et al. 1996).

Electronic supplemental material

The multiple sequence alignment of the IP-repeats used in this work is available.

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