Proteinase Inhibitors from Pea Seeds: Purification and Characterization

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Six protease inhibitors (denoted PSTI I, PSTI II, PSTI III, PSTI IVa, PSTI IVb, and PSTI V) have been purified from winter pea seeds (cv. Frilene) by ammonium sulfate precipitation, gel filtration, and anion and cation exchange chromatography. Their molecular masses were determined by electrospray mass spectrometry to be 6916, 6807, 7676, 7944, 7848, and 7844 Da, respectively. The sequences of the first 20 N-terminal amino acid residues of these six inhibitors were found to be identical and similar to those of *Vicia faba* and *Vicia angustifolia* inhibitors, which belong to the Bowman−Birk class of trypsin inhibitors.

Keywords: *Pisum sativum; Leguminosae; pea; purification; trypsin inhibitors; Bowman−Birk family*

INTRODUCTION

The seeds of many plants belonging to the legume family are rich sources of serine proteinase inhibitors. Inhibiting the pancreas proteinases of higher animals, these inhibitors impair the nutritional quality of seeds by reducing protein digestibility and adsorption, by inducing pancreatic hypertrophy, and by depressing growth (Liener and Kakade, 1980). Several functions for these proteins have been proposed, including the regulation of endogeneous proteinases during germination (Ryan, 1981), the storage of sulfur amino acids during dormancy, and the protection of the plant from insects and micro-organisms (Ryan, 1973). Two types of proteinase inhibitors are widely distributed in legume seeds (Belitz and Weder, 1990; Richardson, 1991): the Kunitz type inhibitors, characterized by a molecular mass of 21 kDa and four cysteines, and the Bowman−Birk type, which have a relatively low molecular mass (7−9 kDa), 14 cysteines linked into seven disulfide bridges, and two reactive sites. These so-called double-headed inhibitors interact simultaneously with two molecules of proteinases, not necessarily identical. Generally, they inhibit trypsin-like proteases on one site and chymotrypsin on the second one. The presence of various Bowman−Birk isoinhibitors in most of the species studied (Odany and Ikenaka, 1977; Norioka and Ikenaka, 1983; Ishikawa et al., 1985) is attributed either to the expression by distinct genes and/or to the post-translational proteolytic cleavage of few amino acids at the N- or C-terminal end of the inhibitors. In the case of peas, the existence of many isoforms has also been observed (Weder and Hory, 1972; Valdebouze et al., 1980; Tomé et al., 1981; Gaborit et al., 1989; Domoney et al., 1993; Ferrasson et al., 1995); some of them have been characterized. It was shown by cDNA sequencing (Domoney et al., 1993) as well as protein sequencing (Ferrasson et al., 1995) that some of these inhibitors belonged to the Bowman−Birk family. Complementary information is needed to know if the other isoforms were also members of this family. It was of interest to know if some of them were similar to the Kunitz type as in soybean.

The present paper reports the purification and characterization of six protease inhibitors from pea seeds. The amino acid composition, molecular masses, and N-terminal sequences of these proteins were determined and compared with those of Bowman−Birk type inhibitors of other legume seeds.

During purification procedures, partial proteolysis of the reactive site and modification by proteolytic cleavage of further peptide linkages may occur if affinity chromatography is performed on trypsin-bound gels (Belew, 1977; Kortt, 1979; Gatehouse et al., 1980; Mahoney et al., 1984). Even if an inactivated protease such as anhydrotrypsin is grafted to the affinity column, the pH shock generally used for eluting the bound inhibitors may modify their structure; especially, deamidation may be induced by extreme pH, leading to incorrect molecular mass values and sequences. For these reasons, the isolation of trypsin inhibitors from peas by conventional chromatographic procedures has been applied in the present study to avoid the production of modified forms that may give artifactual results.

MATERIALS AND METHODS

Materials. Pea seeds (*Pisum sativum* L.) cv. Frilene were purchased commercially. This cultivar was chosen because of its high trypsin inhibitory activity [12 TUI/mg of dry matter (dm)] (Valdebouze et al., 1980).

Isolation of the Crude Inhibitor Preparation. The pea flour was obtained by grinding dehulled seeds with an industrial roller mill. The crude protein extract was prepared by stirring a slurry of the pea flour (300 g) in 50 mM sodium acetate, pH 4.9, for 3 h at 4 °C. The flour/buffer ratio was 1 g/10 mL. The suspension was then centrifuged at 9000*g* for 30 min. The supernatant was brought to 4.25 M (80% saturation) with solid ammonium sulfate and stirred for 2 h at 4 °C. After centrifugation at 9000*g* for 15 min, the precipitate was suspended in 50 mM Tris-HCl, pH 8.8, and dialyzed for 24 h at 4 °C against this buffer. The resulting suspension was centrifuged at 15000*g* for 30 min. The supernatant was kept as the crude inhibitor preparation.

Gel Chromatography Purification. The crude inhibitor preparation was loaded on a preparative Sephadex G-75 column (95 × 10 cm) equilibrated with 50 mM Tris-HCl, pH

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Table 1. Purification of Proteinase Inhibitors from Pea Seeds[a]

	protein (mg)	TUI × 1000	recovery yield (%)	trypsin inhib sp act. (TUI/mg)
ground seeds	55500	2745	100.0	49
crude protein extract	12000	2480	90.3	206
crude inhibitor prepararion	7300	2350	85.6	321
G75 inhibitor fraction	1950	2250	81.9	1153
ion exchange fractions[b]				
DE-PSTI I	66	135	4.9	2045
PSTI I				3350
DE-PSTI II	225	550	20.0	2444
PSTI II				3280
DE-PSTI III	87	216	7.8	2482
PSTI III				2980
DE-PSTI IV	254	651	23.7	2563
PSTI IVa				3150
PSTI IVb				3020
DE-PSTI V	48	83	3.0	1729
PSTI V				3050

[a] From 300 g of seeds. [b] DE-PSTI refer to DEAE-Sepharose fractions, whereas PSTI are corresponding fractions purified to homogeneity by MonoS FPLC.

8.8. The column was eluted, and trypsin inhibitor activity (TI) in protein extracts and column fractions was assayed after adequate dilution using the conventional method (Kakade et al., 1974) modified for peas and faba beans (Valdebouze et al., 1980) and adapted in microtiter plates (Gaborit et al., 1993).

Ion Exchange Chromatography. Active protein fractions eluted from the Sephadex G-75 column were pooled and loaded on a 10 × 5 cm anion exchange column (DEAE-Sepharose FF from Pharmacia). Adsorbed proteins were eluted with a linear gradient of 0−500 mM NaCl in 50 mM Tris-HCl, pH 8.8. Active fractions were pooled, dialyzed against deionized water, freeze-dried, and subsequently fractionated by FPLC cation exchange chromatography using a Mono S HR 5/5 column (Pharmacia). Up to 10 mg of protein solubilized in 1 mL of 50 mM sodium acetate, pH 4.9, was loaded per run. Bound proteins were eluted using a NaCl gradient (0−300 mM) in the acetate buffer.

Isoelectric Focusing (CA-IEF). Carrier ampholyte isoelectric focusing (CA-IEF) was performed in a Multiphor chamber (Pharmacia) on precast polyacrylamide gels in the pH range 3.5−9.5 according to the manufacturer's instructions (Pharmacia).

Reduction and Alkylation. Before the protein preparations were subjected to amino acid analysis and sequence determinations, they were reduced with dithiothreitol and alkylated with 4-vinylpyridine. Proteins (2 mg) were dissolved in 0.2 mL of 0.1 M Tris, pH 8.5, containing 6 M guanidium hydrochloride. Dithiothreitol (11 mg) was added and the mixture kept under nitrogen overnight at 40 °C. Then, alkylation of SH groups was performed in the dark for 90 min by adding 15 mL of 4-vinylpyridine to the protein solution. The resulting pyridylethylated protein was isolated by reversed-phase HPLC on a 25 × 0.75 cm column packed with LiChrospher (Merck) (10 μm, 100 Å).

Amino Acid Analysis. Protein samples (1−2 nmol) were hydrolyzed in the gas phase of 6 M HCl at 110 °C for 24 h in tubes placed in closed containers in vacuum or with 4 M methanesulfonic acid. The amino acids were separated and quantified as phenylthiocarbamyl derivatives (Bidlingmeyer et al., 1984). The amino acid composition was expressed as the number of residues in the sequence.

Determination of Amino Acid Sequences. Sequencing by automated Edman degradation was performed on an Applied Biosystems gas-phase (Model 477A) sequencer with an on-line phenylthiohydantoin amino acid analyzer (Model 120 A).

Electrospray Ionization Mass Spectrometry (ESMS). All experiments were performed with a quadrupole mass spectrometer (the API I, Sciex, Toronto, Canada). Sample solutions were sprayed through a stainless steel capillary held at high potential, generating multiple charged ions. Positive ionization was used; the voltage on the sprayer was 5.2 kV. The nebulizer pressure was adjusted in the range of 50−60 psi. The sample was delivered to the sprayer by a syringe infusion pump at a flow rate of 5 μL/min. The interface between the sprayer and the mass analyzer was a conical orifice of 100 μm diameter. To enhance ion signals, the potential on the orifice was adjusted between 60 and 110 V. The instrument mass-to-charge ratio scale was calibrated with the ammonium adduct ions of polypropylene glycols. Mass spectra represent the average of 10 scans. Scan accumulation was performed with Tune 2.3 and mass calculation with Macspec 3.2.

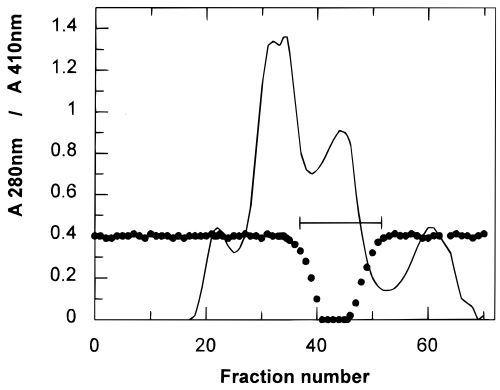

Figure 1. Separation of ammonium sulfate precipitated protein by size exclusion chromatography on a Sephadex G-75 column. The solid line shows absorbance at 280 nm. The dots indicate the absorbance at 410 nm of TI assays performed on every fraction after 1/50 dilution. Control trypsin reactions (without pea protein fractions) gave an A_{410nm} of 0.4.

RESULTS

Purification of the Pea Trypsin Isoinhibitors. Eighty percent ammonium sulfate saturation was needed to recover about 85% of the total inhibitor activity present in the flour (Table 1). By loading this crude-enriched fraction on Sephadex G-75 the whole trypsin inhibitor activity was eluted as a single peak (Figure 1). The total activity recovered in this peak represented 96.5% of the loaded activity and 82% of the original activity present in the seeds. By this step the specific activity was increased about 25-fold. Chromatography on DEAE-Sepharose of this TI-enriched fraction led to five active peaks (Figure 2). The pea seed trypsin inhibitors in these five peaks are referred to as DE-PSTI I−V. The cumulative activity of these five fractions represented about 60% of the initial activity. This rather low recovery yield is due to the fact that only the midsections of the peaks were kept in order to get highly enriched fractions as seen from the increased values of specific activity (Table 1). Purification to homogeneity of each active fraction was then performed

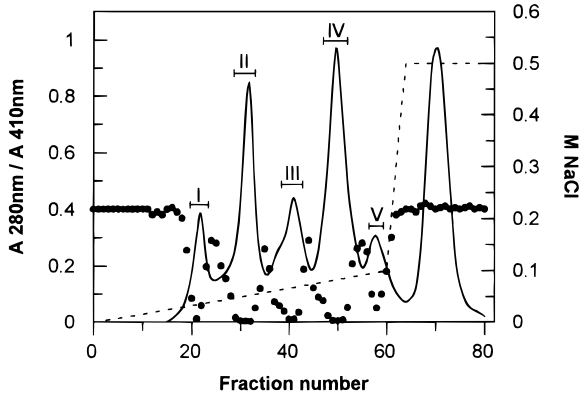

Figure 2. Separation of TI proteins by anion exchange chromatography on a DEAE-Sepharose column. The solid line shows absorbance at 280 nm. The dots indicate the absorbance at 410 nm of TI assays performed on every fraction after 1/80 dilution. Control trypsin reactions (without pea protein fractions) gave an A_{410nm} of 0.4. Dotted line indicates NaCl gradient.

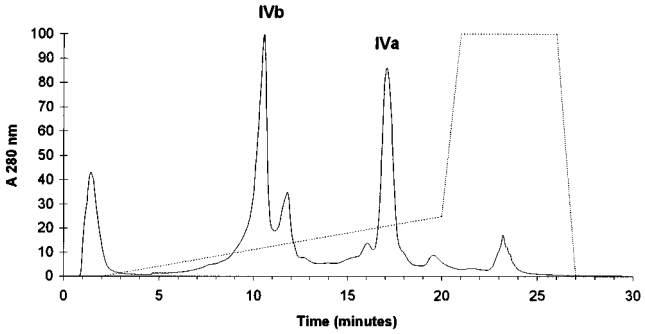

Figure 3. Separation of trypsin isoinhibitors from PSTI IV fraction eluted from the DEAE-Sepharose column on a Mono S HR 5/5 column. Up to 10 mg of protein solubilized in 1 mL of 50 mM sodium acetate, pH 4.,9 was loaded. Bound proteins were eluted using a gradient of NaCl (0−100 mM) in the same buffer. Dotted line indicates NaCl gradient.

by FPLC cation exchange chromatography on a Mono S column. By this technique DE-PSTI IV was separated into two peaks with trypsin inhibitory activity: PSTI IVa and PSTI IVb (Figure 3). The specific inhibitory activities toward trypsin of PSTI I−V have been determined (Table 1). The six isoinhibitors have rather close inhibitory specific activities, which were about a third higher than that found by Tomé et al. (1981) in a fraction isolated by affinity chromatography. The purity of the six inhibitor fractions was checked by HPLC reversed phase; a single peak was obtained in each case.

This purification procedure yielded, respectively, a total of 55, 40, and 60 mg of pure PSTI II, PSTI IVa, and PSTI IVb and 10 mg of pure PSTI I, PSTI III, PSTI V from 300 g of pea flour.

Biochemical Characterization. Isoelectric points (p*I*) were determined by CA-IEF for PSTI I−V and were, respectively 8.6, 7.9, 6.9, 5.7, 4.8, and 4.9. Thus, the order of elution from the DEAE-Sepharose column of the various trypsin inhibitor fractions is in agreement with these values.

The isotopic molecular masses, determined by ESMS, were between 6807 and 7944 Da (Table 2). These values were used to express the amino acid compositions as the number of each residue in the protein.

The amino acid compositions of PSTI I−V exhibited great similarities (Table 3). All of them contained 14 half-cystines and were rich in aspartate and asparagine and lacking in methionine and tryptophan. The difference of masses between intact and pyridylethylated proteins (Table 2) confirms from the weight of the 14 ethylpyridines fixed to the protein that all 14 cysteinyl residues are linked in disulfide bridges in the six isoforms. They differed, however, in the glutamate and glutamine and basic residues contents, which may explain their different isoelectric points.

The total number of residues in the sequence was 72 for PSTI IVa, PSTI IVb, and PSTI V, 70 for PSTI III, and only 63 for PSTI I and PSTI II.

Over the 20 residues determined in their N-terminal sequence (Figure 4), PSTI I−V were identical to each other and also to *Pisum abyssinicum* TI1 and TI2 inhibitors (Domoney et al., 1993). Moreover, they were extremely similar to trypsin inhibitors from *Vicia faba* (Asao et al., 1991) and *Vicia angustifolia* (Shimokawa et al., 1984). From the sequence comparison, the reactive sites of PSTI I−V correspond to Lys16−Ser17 and are trypsin-inhibiting reactive sites.

DISCUSSION

The purification procedure involving conventional chromatographic techniques appeared to be very satisfying for both quantitative and qualitative objectives. As previously explained, no risk of artifactual modification of the protein structure exists, in comparison to some affinity techniques previously used (Tomé et al., 1981). From a quantitative point of view, the recovery yields and the amount of purified proteins are rather high. The various isoforms could be prepared in large enough quantities to allow further biological experiments, such as studies on the toxicity of these proteins toward plant pathogens or insects. Moreover, for such objectives, the second step of ion exchange chromatography should not be necessary, according to the high specific activity reached in DE-PSTI fractions.

We may deduce, on the basis of the specific activity value reported and the fact that all of the isoforms have the same specific activity, that the trypsin inhibitors represent up to 1.6% (w/w) of the total protein in the cv. Frilene pea seeds.

Previous studies reported that the molecular masses of the pea trypsin inhibitors, estimated by SDS−PAGE, were in the range of 12 000−15 000 Da (Weder et al., 1972; Tomé et al., 1981). However, these values did not agree with those generally calculated from the sequences for Bowman−Birk type inhibitors, which are in the range of 7000−9000 Da, or with those reported for inhibitors of Kunitz type, around 21 000 Da. This discrepancy may result from abnormal migration, which has been previously observed for TIs (Wu and Whitaker, 1990). This artifact was explained as due to oxidation of the cysteine residues during electrophoresis, which leads to an overestimated M_r value, but this cannot be avoided even by a preliminary reduction of the disulfide bridges of the protein. The isotopically averaged masses measured by electrospray mass spectrometry show that they were in fact comprised of all the isoforms between 7000 and 8000 Da (Table 2).

Table 2. ESMS Measured Masses (MM) of Intact and Pyridylethylated PSTI I−V

MM (Da)	PSTI I	PSTI II	PSTI III	PSTI IVa	PSTI Vb	PSTI V
intact	6916	6807	7676	7944	7848	7844
pyridylethylated	8405	8293	9164	9432	9335	9335
no. of cysteines	14.02	14.01	14.01	14.00	14.02	14.01

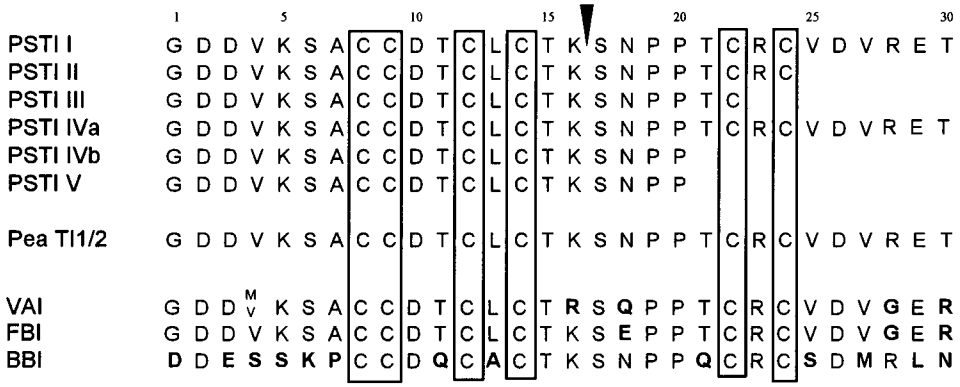

Figure 4. Comparison of the N-terminal sequences of PSTI I−V with those of legume Bowman−Birk inhibitors from *Pisum abyssinicum* (TI1/2 inhibitors) (Domoney et al., 1993), *Vicia faba* (FBI) (Asao et al., 1991), and *Vicia angustifolia* (VAI) (Shimokawa, 1984). Arrow indicates the trypsin reactive site. The conserved cysteines are enclosed in boxes.

Table 3. Amino Acid Compositions of PSTI I−V[a]

amino acid	PSTI I	PSTI II	PSTI III	PSTI IVa	PSTI IVb	PSTI V
Asx	9.4 (9)	8.3 (8)	8.3 (8)	10.4 (10)	9.2 (9)	9.3 (9)
Glx	1.9 (2)	2.9 (3)	4.8 (5)	4.8 (5)	5.8 (6)	4.8 (5)
Ser	4.9 (5)	4.9 (5)	5.8 (6)	5.7 (6)	5.8 (6)	5.7 (6)
Gly	1.0 (1)	1.9 (2)	1.0 (1)	1.0 (1)	2.0 (2)	1.9 (2)
His	2.8 (3)	2.0 (2)	2.8 (3)	2.8 (3)	1.9 (2)	2.8 (3)
Arg	2.0 (2)	1.0 (1)	1.0 (1)	2.0 (2)	1.0 (1)	1.0 (1)
Thr	4.7 (5)	4.7 (5)	4.8 (5)	4.9 (5)	4.6 (5)	4.7 (5)
Ala	4.1 (4)	4.3 (4)	4.0 (4)	4.2 (4)	4.0 (4)	4.2 (4)
Pro	4.3 (4)	4.2 (4)	4.3 (4)	4.3 (4)	4.7 (5)	4.2 (4)
Tyr	1.8 (2)	1.7 (2)	2.0 (2)	1.9 (2)	1.8 (2)	1.8 (2)
Val	2.9 (3)	2.9 (3)	4.7 (5)	4.8 (5)	4.7 (5)	4.9 (5)
PeC[b]	14.4 (14)	14.3 (14)	14.4 (14)	14.1 (14)	13.8 (14)	14.4 (14)
Ile	1.0 (1)	1.0 (1)	3.0 (3)	2.0 (2)	1.9 (2)	2.8 (3)
Leu	0.9 (1)	1.8 (2)	0.9 (1)	0.9 (1)	2.8 (3)	1.1 (1)
Phe	2.1 (2)	2.0 (2)	2.0 (2)	2.1 (2)	2.1 (2)	2.1 (2)
Lys	4.8 (5)	4.8 (5)	5.8 (6)	5.8 (6)	5.8 (6)	5.9 (6)
Met	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Trp	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
no. of residues	63	63	70	72	72	72

[a] Numbers in parentheses are integer residue values deduced on the basis of molecular masses. [b] PeC, pyridylethylcysteine.

According to their amino acid composition as well as their molecular masses, these six pea trypsin inhibitors may belong to the Bowman−Birk family. It was further confirmed by the similarity of their N-terminal sequences with those of both *V. faba* and *V. angustifolia* inhibitors as well as with that of PSTI IVa, which was recently sequenced by ourselves (Ferrasson et al., 1995). In particular, the six inhibitors possess the Pro−Pro sequence at positions 19−20 and the Lys16−Ser17 reactive peptide bond. The conservation of the cysteinyl residues, known for their role in the stabilization of the reactive sites, was also observed.

These similarities raise the question of the possible interconversion of these inhibitors. The complete sequences of these inhibitors compared to PSTI IVa should only allow us to explain the origin of these various inhibitors. According to our chromatographic procedure, it seems, however, that all of the inhibitors precipitated in the crude extract belong to the Bowman−Birk family. We cannot, however, exclude, in the nonextracted fraction (about 15% of the total activity), the presence of minor inhibitors belonging to other families.

The presence of multiple related low molecular mass protease inhibitors in pea resembles the situation in other species of legumes. It is interesting that there are so many inhibitors with close specific activity in pea seeds but which are present in very different quantities. PSTI II, PSTI IVa, and PSTI IVb represent major isoforms compared to PSTI I, PSTI III, and PSTI V. Further studies should define the relationships among these inhibitors since physiological functions of trypsin inhibitors are still largely unknown.

To explain the potential role of each isoform, further studies are under development to establish the sequence of the second active site and their inhibitory activity toward serine proteases of various origins.

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