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# Primary Structures of Two Low Molecular Weight Proteinase Inhibitors from Potatoes†

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ABSTRACT: The amino acid sequences of two low molecular weight proteinase inhibitors from Russet Burbank potatoes have been determined. One of these, a chymotrypsin inhibitor, is a peptide of 52 amino acid residues, while the second inhibitor, which is specific for trypsin, contains 51 amino acid residues. These peptides are highly homologous, differing at only nine positions. At position 38, the chymotrypsin inhibitor possesses leucine and the trypsin inhibitor an arginine. This difference probably represents the $P_1$ sites, which are consistent with the respective specificities of the two inhibitors. The inhibitors are also homologous with potato inhibitor II and with an inhibitor previously isolated from eggplants.

Russet Burbank potato tubers have been shown to contain a variety of heat-stable proteinase inhibitors. In addition to inhibitor I ($M_r$ 40 000; Melville & Ryan, 1972) and inhibitor II ($M_r$ 19 500; Bryant et al., 1976; Iwasaki et al., 1972), several low molecular weight inhibitors have been detected. The latter group contains a carboxypeptidase inhibitor, which has been extensively characterized (Rancour & Ryan, 1968; Hass & Ryan, 1981), and at least three inhibitors of serine proteinases (Pearce et al., 1981). This report describes the determination of the amino acid sequences of two of the polypeptide serine proteinase inhibitors, a chymotrypsin inhibitor I (PCI-I)[1] and a trypsin inhibitor (PTI). PCI-I is a potent inhibitor of chymotrypsin with little effect on trypsin (Hass et al., 1976), and, conversely, PTI is specific only for trypsin (Pearce et al., 1981). The three polypeptide serine proteinase inhibitors are highly homologous, since they all exhibit strong immunological cross-reactivity against rabbit anti-PCI-I serum (Pearce et al., 1981).

## Experimental Procedures

*Materials.* The polypeptide chymotrypsin inhibitor (PCI-I) and the polypeptide trypsin inhibitor (PTI) were prepared as described by Hass et al. (1976) and by Pearce et al. (1981), respectively. The *S*-Cm derivatives were prepared by reduction of the inhibitors with 0.1 M 2-mercaptoethanol in 6 M guanidine hydrochloride and 0.1 M Tris-HCl (pH 8.0) followed by treatment with a 1.5-fold M excess of bromo[1-$^{14}$C]acetate (Crestfield et al., 1963).

Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone, chymotrypsin, and carboxypeptidase B were purchased from Sigma Chemical Co. Pyridine was redistilled from ninhydrin prior to use in chromotographic systems (Hill & Delaney, 1967), and all other chemicals were reagent grade or better and were used without further purification.

*Analytical Procedures.* Amino acid analyses were performed according to Spackman et al. (1958) on samples which had been hydrolyzed for 18 h in 6 N HCl at 110 °C (Moore & Stein, 1963). Peptide purity was assessed by high-voltage paper electrophoresis at pH 3.6 or 6.5 (Bennett, 1967). Peptides were routinely detected on dried chromatograms by using fluorescamine (Mendez & Lai, 1975).

*Automatic Edman Degradation.* The amino acid sequences were determined with a Beckman 890C sequencer according to the procedures of Hermodson et al. (1972) with the exceptions that Polybrene (3 mg) was added to the sequencer cup with the peptides (100–200 nmol) and in the case of the whole protein 0.1 M Quadrol buffer (Pierce Chemical Co.) was used. The program used was identical with that used by Hermodson et al. (1977) except that a 10-min extraction with ethyl acetate was performed immediately following the benzene-extraction step. Pth-amino acids were identified by high-pressure liquid chromatography employing a slight modification of the procedures of Zimmerman et al. (1977). No identifications were made unless the peak to background ratio was at least 3.

*Cleavage at the Asn–Gly Bond.* The *S*-Cm derivative of each protein (800 nmol) was cleaved at the $Asn_{19}$–$Gly_{20}$ bond (Bornstein & Balian, 1970). Hydroxylamine hydrochloride (2.8 g) and guanidine hydrochloride (6 g) were dissolved in enough water to make 15 mL of solution. The solution was titrated to pH 7 with 12 N NaOH while stirring in an ice bath and then adjusted to 20 mL with water. Three milliliters of this solution was added to the protein, the pH was adjusted

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[1] Abbreviations: *S*-Cm, *S*-carboxymethyl; PCI-I, polypeptide chymotrypsin inhibitor I; PTI, polypeptide trypsin inhibitor; Pth, phenylthiohydantoin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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                          5                  10                  15                  20                  25
PCI-I     Pro Ile Cys Thr Asn Cys Cys Ala Gly Tyr Lys Gly Cys Asn Tyr Tyr Ser Ala Asn Gly Ala Phe Ile Cys Glu
                          5                  10                  15                  20                  25
PTI       Arg Ile Cys Thr Asn Cys Cys Ala Gly Tyr Lys Gly Cys Asn Tyr Tyr Ser Ala Asn Gly Ala Phe Ile Cys Glu
                          5                  10                  15                  20                  25
a>        PCA Ile/Leu Cys Thr Asn Cys/Asn Cys Ala Gly Arg Lys Gly Cys Ser Tyr Phe Ser Glu Asp Gly Thr Phe Ile Cys Lys

                         30                  35                  40                  45                  50
PCI-I     Gly Gln Ser  Asp Pro Lys Lys Pro Lys Ala Cys Pro Leu ↓ Asn Cys Asp  Pro His Ile Ala Tyr Ser Lys Cys Pro Arg Ser
                         30                  35                  40                  45                  50
PTI       Gly Glu Ser  Asp Pro Lys Asn Pro Asn Val Cys Pro Arg ↓ Asn Cys Asp  Thr Asn Ile Ala Tyr Ser Lys Cys Leu Arg
                         30                  35                  40                  45                  50
a>        Gly Glu Ser  Asn Pro Glu Asn Pro Lys Ala Cys Pro Arg ↓ Asn Cys Asp  Gly Arg Ile Ala Tyr Gly Ile Cys Pro Leu Ser
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FIGURE 1: Amino acid sequences of the polypeptide chymotrypsin inhibitor I (PCI-I) and the polypeptide trypsin inhibitor (PTI). Sequences were determined primarily by automatic Edman degradation as described in the text. The sequence of a trypsin inhibitor from eggplant (a) is included for comparison (Richardson, 1979). The arrows (↓) indicate the presumed reactive site.

to 9.0 with 0.5 N NaOH, and the temperature was maintained at 45 °C for 4 h with occasional adjustment of the pH to 9.0. The solutions were then chromatographed on a column (2.5 × 90 cm) of Sephadex G-50 (Pharmacia Fine Chemicals), equilibrated, and eluted with 10% (v/v) propanol containing 1 mL/L concentrated $NH_4OH$. Three peaks were obtained; the first at 315 mL was the uncleaved protein, the second at 350 mL was the COOH-terminal fragment, and the third at 420 mL was the $NH_2$-terminal 19 residues. The yield of cleavage products was about 70% as judged by the 280-nm absorbance of the peaks. Each fraction was then lyophilized and chromatographed on a 1.5 × 80 cm column of Sephadex G-75 SF in 5% formic acid prior to amino acid analysis.

*Digestion of S-Cm PCI-I with Chymotrypsin.* The *S*-Cm inhibitor (2 μmol) was dissolved in 3 mL of 0.2 M ammonium bicarbonate and treated for 4 h at 37 °C with 5% (mol/mol) α-chymotrypsin. The digest was lyophilized, dissolved in 3 mL of 0.1 M pyridine–acetic acid (pH 3.1), and applied to a 0.9 × 15 cm column of Dowex 50-X2 at 55 °C. The initial fractionation of the chymotryptic peptides was achieved by using the following: (1) 0.1 M pyridine–acetic acid (pH 3.1); (2) gradient of 150 mL each 0.1 M pyridine–acetic acid (pH 3.1) and 0.5 M pyridine–acetic acid (pH 3.75); (3) gradient of 150 mL each 0.5 M pyridine–acetic acid (pH 3.75) and 2.0 M pyridine–acetic acid (pH 5.0) (Bradshaw et al., 1969). Peptide elution was monitored by reaction of 50-μL aliquots from alternate fractions (4 mL) with fluorescamine (Stein et al., 1973). Five pools were taken and pool C3 was rechromatographed on a 0.9 × 15 cm column of Dowex 1-X2 at 37 °C by using a linear gradient of 500 mL total from 3% pyridine to 1.0 M pyridine–acetic acid (pH 5.5) (Bradshaw et al., 1969). Fractions of 4.0 mL were collected and monitored for peptide content by reaction of aliquots (50 μL) with fluorescamine (Stein et al., 1973).

*Digestion of S-Cm PCI-I with Trypsin.* The *S*-Cm inhibitor (3 μmol) was dissolved in 3 mL of 0.2 M ammonium bicarbonate and incubated with 0.2 mg of trypsin for 3 h. An additional 0.2 mg of trypsin was added, and digestion was terminated by lyophilization after 1.5 h. The tryptic peptides were dissolved in 3 mL of 3% pyridine and chromatographed at 37 °C on a 0.9 × 15 cm column of Dowex 1-X2. A linear gradient of 250 mL each of 3% pyridine and 2 M pyridine–acetic acid (pH 5.0) was applied, and fractions of 3 mL were collected (Bradshaw et al., 1969). Peptide elution was monitored by reaction of 50-μL aliquots with fluorescamine (Stein et al., 1973).

*Cleavage of S-Cm PCI-I at Aspartyl–Proline Bonds.* The *S*-Cm inhibitor (0.5 μmol) was incubated at 40 °C for 48 h in 10% acetic acid which had been adjusted to pH 2.5 with pyridine (Fraser et al., 1972). The lyophilized cleavage fragments were dissolved in 3 mL of 0.02 M ammonium bicarbonate and chromatographed on a 1.5 × 80 cm column of Bio-Gel P2 (Bio-Rad Laboratories). Fractions of 1.5 mL were collected, and the peptide elution was monitored by absorbance at 224 nm.

*Digestion of S-Cm PTI with Carboxypeptidase B.* *S*-Cm PTI (0.1 μmol) was dissolved in 0.5 mL of 0.1 M NaCl and 20 mM Hepes (pH 7.5). Carboxypeptidase B (40 μg) was added, and digestion was effected at 25 °C. Aliquots of 75 μL were removed at various times, mixed with 75 μL of 0.2 M sodium citrate (pH 2.2), and subjected to amino acid analysis. Controls containing only enzyme or only inhibitor were also analyzed at each time interval.

## Results

*Amino Acid Sequence of PCI-I.* The polypeptide chymotrypsin inhibitor was shown by amino acid sequence analysis (Figure 1) to contain 52 amino acid residues. Positions 1–39 were identified by automatic Edman degradation of the intact molecule and of the C-terminal peptide fragment (residues 20–52) generated by hydroxyalamine cleavage of the $Asn_{19}$–$Gly_{20}$ bond. The remainder of the sequence (residues 40–52) was assigned by subtractive Edman degradation (Koningsberg & Hill, 1962) of chymotryptic and tryptic peptides. The inhibitor contained two additional resiudes of half-cystine than were expected on the basis of an earlier report (Hass et al., 1976). However, when extreme care was taken to minimize oxidation of *S*-Cm-Cys, a value of eight residues of *S*-Cm-Cys per molecule was observed upon hydrolysis.

*Edman Degradation of the Intact Molecule. Identification of Residues 1–22.* Degradation of the *S*-Cm derivative of the inhibitor yielded the sequence of residues 1–22 (Figure 1; Table I). The stepwise yield decreased dramatically at the $Asn_{19}$–$Gly_{20}$ peptide bond, preventing the assignment of a longer segment.

*Cleavage of the $Asn_{19}$–$Gly_{20}$ Bond. Identification of Residues 20–39.* The *S*-Cm derivative of the inhibitor was treated with hydroxylamine to effect cleavage of the $Asn_{19}$–$Gly_{20}$ peptide bond. Chromatography of the resulting fragments on Sephadex G-50 produced partial separation of intact inhibitor ($NH_2OH$-1) and the Asn-Gly fragment, $NH_2OH$-2 (residues 20–52). Fragment $NH_2OH$-3 (residues 1–19) eluted later and

Table I: Automated Edman Degradations[a]

| cycle no. | PCI-I intact | PCI-I $NH_2OH$-2 | PTI intact | PTI $NH_2OH$-2 |
|---|---|---|---|---|
| 1 | Pro (436) | Gly (400) | Arg (~80) | Gly (101) |
| 2 | Ile (564) | Ala (348) | Ile (135) | Ala (87) |
| 3 | Cys (180) | Phe (294) | Cys (63) | Phe (122) |
| 4 | Thr (120) | Ile (274) | Thr (33) | Ile (114) |
| 5 | Asn (236) | Cys (*) | Asn (83) | Cys (50) |
| 6 | Cys (160) | Glu (164) | Cys (57) | Glu (90) |
| 7 | Cys (198) | Gly (234) | Cys (65) | Gly (72) |
| 8 | Ala (458) | Gln (136) | Ala (70) | Glu (72) |
| 9 | Gly (316) | (Ser) (–) | Gly (56) | Ser (20) |
| 10 | Tyr (278) | Asp (92) | Tyr (60) | Asp (32) |
| 11 | Lys (234) | Pro (60) | Lys (82) | Pro (35) |
| 12 | Gly (354) | Lys (92) | Gly (48) | Lys (57) |
| 13 | Cys (86) | Lys (128) | Cys (48) | Asn (32) |
| 14 | Asn (100) | Pro (50) | Asn (36) | Pro (32) |
| 15 | Tyr (276) | Lys (98) | Tyr (57) | Asn (36) |
| 16 | Tyr (356) | Ala (60) | Tyr (65) | Val (47) |
| 17 | Ser (40) | Cys (*) | Ser (12) | Cys (17) |
| 18 | Ala (298) | Pro (30) | Ala (56) | Pro (22) |
| 19 | Asn (60) | Leu (40) | Asn (27) | Arg (~10) |
| 20 | Gly (104) | Asn (15) | Gly (24) | Asn (16) |
| 21 | Ala (60) | | Ala (46) | Cys (9) |
| 22 | Phe (58) | | Phe (31) | Asp (18) |
| 23 | Ile (84) | | Ile (38) | Thr (4) |
| 24 | | | Cys (21) | Asn (11) |
| 25 | | | Glu (17) | Ile (18) |
| 26 | | | Gly (12) | Ala (19) |
| 27 | | | Glu (17) | Tyr (13) |
| 28 | | | Ser (6) | Ser (6) |
| 29 | | | Asp (3) | Lys (10) |
| 30 | | | (Pro) | Cys (6) |
| 31 | | | (Lys) | Leu (11) |
| 32 | | | | Arg (~5) |

[a] The *S*-Cm derivatives of PCI-I and its hydroxylamine fragment $NH_2OH$-2 (~500 nmol each) and of PTI and its hydroxylamine fragment $NH_2OH$-2 (~150 nmol each) were degraded in the presence of Polybrene. Identifications were made by HPLC, and the quantities of each residue (in nanomoles) were estimated from peak areas. Some Cys residues were detected by radioactivity (*). $Ser_9$ in $NH_2OH$-2 of PCI-I was detected but did not meet the peak to background criteria specified.

was completely resolved from contaminating peptides by this procedure. After rechromatography on Sephadex G-75, fragments $NH_2OH$-2 and $NH_2OH$-3 yielded amino acid

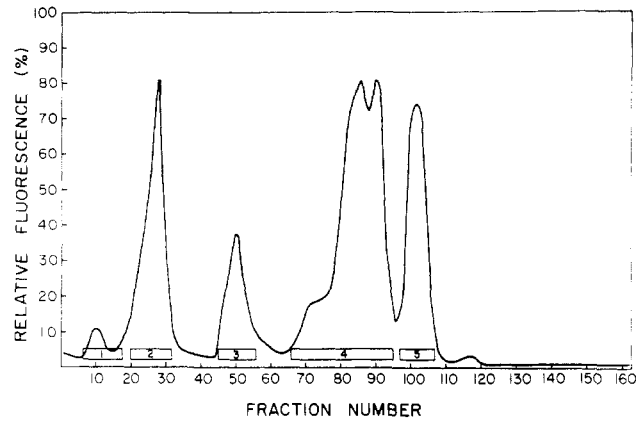


FIGURE 2: Separation of the chymotryptic peptides of the *S*-Cm derivative of PCI-I. Ion-exchange chromatography was performed on a 0.9 × 15 cm column of Dowex 50-X2 at 55 °C. Fractionation was achieved by using a double-linear gradient of pyridine–acetic acid buffers as described in the text. At fraction 130, 2.0 M pyridine–acetic acid (pH 5.0) was applied. Peptide elution was monitored by reaction of 50-μL aliquots of alternate fractions with fluorescamine.

compositions (Table II) which were consistent with the proposed sequence.

Automatic Edman degradation of fragment $NH_2OH$-2 (residues 20–52) provided positive identification of residues 20–27 and 29–39, with a tentative assignment of Ser at position 28 (Table I). Ser-28 was confirmed by the amino acid analysis of fragment $NH_2OH$-2 (Table I) as well as by the amino acid compositions of a chymotryptic peptide (residues 23–38) and of a tryptic peptide (residues 12–31) (data not shown).

*Digestion with Chymotrypsin and Edman Degradation of Peptides C-3-2 (Residues 39–46) and C-5 (Residues 47–52).* The preliminary separation of the chymotryptic peptides derived from *S*-Cm inhibitor was achieved by chromatography on Dowex 50-X2 (Figure 2). After rechromatography on Dowex 1-X2, peptide C-3-2, which eluted in fractions 24–29, was pure, as judged by amino acid analysis (Table III) and by paper electrophoresis. The amino acid sequence of C-3-2 was determined by subtractive Edman degradation (Table III). The carboxyl terminus of C-3-2 was Tyr, consistent with the specificity of chymotrypsin. Position 41 in the inhibitor was assigned as Asp rather than Asn on the basis of the cleavage of the bond between residues 41 and 42 under conditions

Table II: Amino Acid Compositions of the Inhibitors and Their Hydroxylamine Fragments

| amino acid | PCI-I integral | PCI-I sequence | PCI-I $NH_2OH$-2 | PCI-I $NH_2OH$-3 | PTI integral[b] | PTI sequence | PTI $NH_2OH$-2 | PTI $NH_2OH$-3 |
|---|---|---|---|---|---|---|---|---|
| Cm-Cys | 6 (8)[c] | 8 | 1.0 (4)[d] | 0.8 (4) | 8 | 8 | 2.6 (4) | 2.7 (4) |
| Asp | 6 | 6 | 3.3 (3) | 2.6 (3) | 7 | 9 | 5.3 (6) | 3.1 (3) |
| Thr | 1 | 1 | 0.2 (0) | 1.2 (1) | 2 | 2 | 0.9 (1) | 1.0 (1) |
| Ser | 3 | 4 | 2.1 (2–3) | 1.1 (1) | 3 | 3 | 2.0 (2) | 1.1 (1) |
| Glu | 2 | 2 | 2.0 (2) | 0.2 (0) | 2 | 2 | 2.0 (2) | 0.2 (0) |
| Pro | 6 | 6 | 5.4 (5) | 1.7 (1) | 3 | 3 | 2.4 (3) | 0.1 (0) |
| Gly | 4 | 4 | 2.6 (2) | 2.0 (2) | 4 | 4 | 2.2 (2) | 2.2 (2) |
| Ala | 5 | 5 | 3.0 (3) | 1.9 (2) | 4 | 4 | 2.1 (2) | 2.1 (2) |
| Val | 0 | 0 | 0 | 0 | 1 | 1 | 1.0 (1) | 0.0 (0) |
| Ile | 3 | 3 | 1.9 (2) | 1.2 (1) | 3 | 3 | 1.5 (2) | 0.9 (1) |
| Leu | 1 | 1 | 1.0 (1) | 0.0 (0) | 1 | 1 | 1.0 (1) | 0.2 (0) |
| Tyr | 4 | 4 | 1.6 (1) | 2.2 (2) | 3 | 4 | 1.1 (1) | 2.6 (3) |
| Phe | 1 | 1 | 1.2 (1) | 0.1 (0) | 1 | 1 | 0.6 (1) | 0.0 (0) |
| His | 1 | 1 | 1.0 (1) | 0.1 (0) | 0 | 0 | 0.0 (0) | 0.0 (0) |
| Lys | 5 | 5 | 4.2 (4) | 1.3 (1) | 3 | 3 | 2.3 (2) | 1.2 (1) |
| Arg | 1 | 1 | 0.9 (1) | 0.2 (0) | 3 | 3 | 2.0 (2) | 1.1 (1) |
| residues | | | 20–52 | 1–19 | | | 20–51 | 1–19 |
| total | 49 (51)[c] | 52 | | | 48 | 51 | | |

[a] Taken from the data of Hass et al. (1976). [b] Taken from the dat of Pearce et al. (1981). [c] Determined on hydrolysates which included thioglycolic acid. [d] Values in parentheses are those expected from the proposed amino acid sequences (Figure 1).

Table III: Subtractive Edman Degradation of C-3-2 (Residues 39–46) from *S*-Cm Chymotrypsin Inhibitor[a]

| amino acid | composition | step 1 | step 2 | step 3 | step 4 | step 5 | step 6 | step 7 |
|---|---|---|---|---|---|---|---|---|
| Cm-cysteine | 0.8 | 0.9 | *0.2* | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 |
| aspartic acid | 1.8 | *1.1* | 1.0 | *0.3* | 0.2 | 0.3 | 0.3 | 0.3 |
| proline | 1.0 | 1.1 | 1.1 | 1.0 | *0.1* | 0 | 0 | 0 |
| alanine | 1.0 | 1.0 | 1.0 | 1.0 | 1.1 | 0.9 | 1.3 | *0.3* |
| isoleucine | 1.0 | 1.0 | 0.9 | 0.9 | 1.0 | 1.1 | *0.5* | 0.2 |
| tyrosine | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| histidine | 1.0 | 0.8 | 0.9 | 0.9 | 1.0 | *0.2* | 0.1 | 0.2 |

[a] Italic entries indicate the amino acid removed at each step.

Table IV: Subtractive Edman Degradation of C-5 (Residues 47–52) from *S*-Cm Chymotrypsin Inhibitor[a]

| amino acid | composition | step 1 | step 2 | step 3 | step 4 |
|---|---|---|---|---|---|
| Cm-cysteine | 0.8 | 0.8 | 0.8 | *0.3* | 0.3 |
| serine | 1.2 | *0.7* | 0.6 | 0.5 | 0.4 |
| proline | 1.1 | 1.0 | 1.0 | 1.0 | *0.5* |
| lysine | 1.1 | 1.0 | *0.2* | 0.3 | 0.1 |
| arginine | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

[a] Italic entries indicate the amino acid removed at each step.

specific for the hydrolysis of Asp–Pro linkages (Fraser et al., 1972) (see below).

The amino acid composition of C-5 (Figure 2) was Cm-$Cys_{0.8}$, $Ser_{1.2}$, $Pro_{1.1}$, $Lys_{1.0}$, and $Arg_{1.0}$, with only trace amounts of other amino acids. Since this peptide did not contain an amino acid for which chymotrypsin exhibits specificity, it was assumed to be the carboxyl-terminal chymotryptic peptide. Subtractive Edman degradation of C-5 (Table IV) yielded the partial sequence of Ser-Lys-Cys-Pro-(Arg, Ser).

*Digestion with Trypsin.* The separation of the tryptic peptides of *S*-Cm inhibitor was accomplished by chromatography on Dowex 1-X2 (Figure 3). Peptides T2, T3, and T4, all of which required no further purification, were derived from the carboxyl-terminal region of the inhibitor. Peptide T4 (residues 35–48) provided by composition (Cm-$Cys_{1.9}$, $Asx_{2.0}$, $Ser_{0.9}$, $Pro_{2.1}$, $Ala_{2.0}$, $Ile_{1.0}$, $Leu_{1.1}$, $Tyr_{1.0}$, $His_{0.9}$, and $Lys_{0.9}$) the overlap between the sequences obtained by degradation of peptides $NH_2OH$-2 and C-3-2 (see above). T3 exhibited the composition Cm-$Cys_{0.8}$, $Pro_{1.0}$, and $Arg_{1.0}$ and, thus, corresponded to residues 49–51. T2 contained free serine, which was thus identified as the carboxyl-terminal residue of the inhibitor. These data together with the partial sequence of C-5 (see above) established the sequence of residues 47–52 as Ser-Lys-Cys-Pro-Arg-Ser.

*Cleavage of the Asp–Pro Bonds.* Separation of the peptides generated by incubation of *S*-Cm inhibitor under conditions specific for cleavage of aspartyl–proline bonds was achieved by gel filtration on Bio-Gel P2 (Figure 4). Pool 1 contained a mixture of uncleaved inhibitor and partially hydrolyzed material. Pool 2 contained some contaminants but exhibited an amino acid composition expected of residues 30–41. Pool 3 appeared to be nearly pure and gave the following composition: Cm-$Cys_{1.6}$, $Ser_{1.8}$, $Pro_{1.7}$, $Gly_{1.3}$, $Ala_{1.7}$, $Ile_{1.1}$, $Tyr_{0.9}$, $His_{0.9}$, $Lys_{1.0}$, and $Arg_{1.0}$. This peptide provided a convincing overlap for peptides C-3-2 and C-5 and established the identity of residue 41 as aspartic acid (see above).

*Amino Acid Sequence of PTI.* The amino acid sequence of PTI was determined by automatic Edman degradation of the whole protein and of the COOH-terminal hydroxylamine fragment. In addition to direct identification of the *S*-Cm cysteine derivatives by HPLC, $^{14}C$ labeling at 35 cpm/nmol was also employed, and the results were consistent with those in Table II.

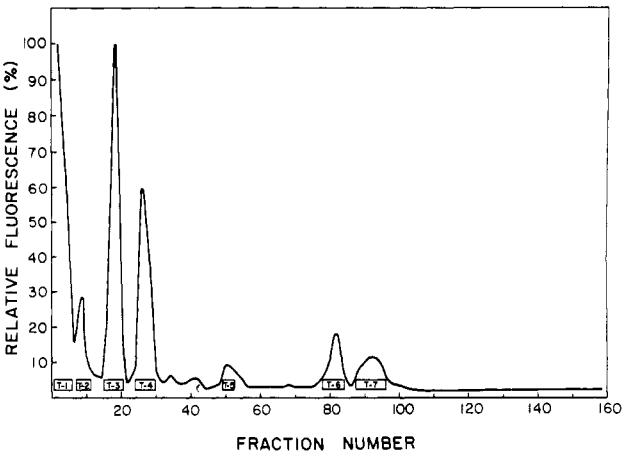


FIGURE 3: Separation of the tryptic peptides of *S*-Cm PCI-I. Ion-exchange chromatography was performed on an 0.9 × 15 cm column of Dowex 1-X2 at 37 °C. Fractions of 3 mL were collected. The peptide content was assessed by reaction of 50-μL aliquots with fluorescamine.

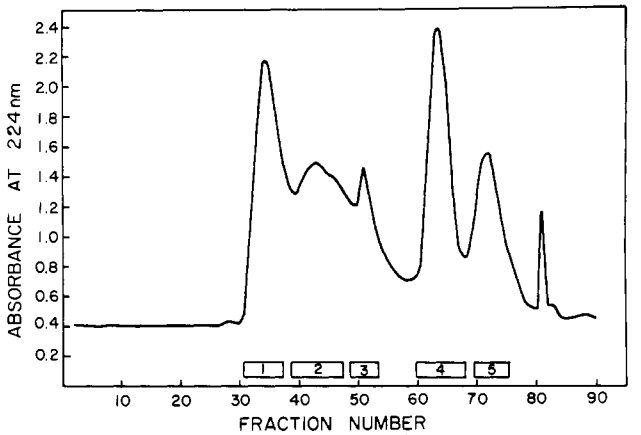


FIGURE 4: Separation of the peptides generated by cleavage *S*-Cm PCI-I at its aspartyl–proline bonds. Gel filtration was performed on a 1.5 × 80 cm column of Bio-Gel P2 in 20 mM ammonium bicarbonate. Fractions of 1.5 mL were collected, and the peptide elution was monitored by absorbance at 224 nm.

Identification of residues 1–29 was achieved by degradation of the intact *S*-Cm inhibitor. Cleavage of the inhibitor at the $Asn_{19}$–$Gly_{20}$ bond by treatment with hydroxylamine as in the case of PCI-I and separation of the resulting fragments by chromatography on Sephadex G-50 were effected as in the case of PCI-I (see above). Amino acid compositions of the hydroxylamine fragments (Table II) were consistent with the proposed sequence of PTI (Figure 1) except for routinely low values for *S*-Cm-Cys, a residue particularly labile to oxidation during acid hydrolysis.

Automatic Edman degradation of the COOH-terminal hydroxylamine fragment ($NH_2OH$-2) provided the identification of residues 20–51 (Table I). That Arg-51 was in fact the COOH terminus of PTI was confirmed by digestion of *S*-Cm PTI with carboxypeptidase B. Under the conditions described in the text, 1.0–1.1 residues of arginine per molecule were released within 15 min of digestion. Leucine was released much more slowly, yielding 0.7 residue per molecule after 3 h of incubation.

## Discussion

The amino acid sequences of PCI-I and PTI are presented in Figure 1. The amino acid sequence of PCI-I is in good agreement with its amino acid composition (Table II; Hass

et al., 1976). However, earlier sequence data on this inhibitor clearly indicated that position 6 was lysine rather than half-cystine as reported herein (Hass et al., 1976). It is highly likely that earlier analyses were performed on inhibitor derived from a different variety of potato. With respect to residue 6, it is interesting to note that the corresponding position in the highly homologous trypsin inhibitor from eggplant (Figure 1; Richardson, 1979) exhibits microheterogeneity, with half-cystine and asparagine occurring in almost equal amounts.

The amino acid sequence of PTI (Figure 1) is in agreement with its amino acid composition, with the exception of values for tyrosine and aspartic acid (Table II; Pearce et al., 1981). Tyrosine values are often slightly low in acid hydrolysates due to partial oxidation of this amino acid; however, the discrepancy between our earlier estimation of aspartic acid (7 residues per molecule) and that observed in the sequence (9 residues per molecule) remains to be resolved.

A comparison of the sequences of PCI-I and PTI reveals considerable homology, as expected from immunological comparisons (Pearce et al., 1981). The N-terminal regions of these inhibitors are particularly similar with only one amino acid replacement (Pro-1 → Arg) occurring in residues 1–26. Residues 27–52 contain the remaining eight replacements including the most dramatic change in residue character (Leu-38 → Arg). In contrast, the trypsin inhibitor from eggplant exhibits a minimum of 18 amino acid replacements with respect to PTI. Thus, it is likely that the gene duplication event which gave rise to PTI and PCI-I occurred much later than the duplication which led to PTI and the eggplant inhibitor.

Although reactive site cleavage (Laskowski & Sealock, 1971) has not been effected for either PCI-I (Eddy et al., 1980) or PTI, the Arg-38–Asn-39 peptide bond has been identified as the reactive site of the trypsin inhibitor from eggplant (Richardson, 1979). This assignment is consistent with our identification of Leu-38 as the corresponding $P_1$ position in PCI-I, a chymotrypsin inhibitor, and of Arg-38 as the $P_1$ position in PTI, a trypsin inhibitor. In addition to the fact that the $P_1$ position appears to dictate specificity, other structures near the reactive sites of these inhibitors are consistent with the generalizations proposed by Laskowski & Kato (1980). For example, half-Cys, which occupies positions $P_3$ and $P_{2'}$ in these inhibitors, is a common feature near inhibitor reactive sites. Also, $P_2$ (Pro-37) is typically a neutral amino acid, and Pro is often found at $P_3$, $P_2$, $P_{3'}$, or $P_{4'}$ to produce the correct reactive site orientation.

It is interesting to note that amino acid replacements other than at $P_1$ are not observed in the immediate vicinity of the reactive sites of the three inhibitors (Figure 1), with the nearest changes occurring at the $P_4$ (residues 35) and $P_{4'}$ (residue 42) positions. This is in contrast to the family of the avian ovomucoid third domains, in which the $P_2$ and $P_{3'}$ as well as $P_{1'}$ exhibit considerable variability (Kowalski & Laskowski, 1976).

Based in part upon this study, all of the polypeptide inhibitors isolated from Russet Burbank potato tubers appear to be homologous. The three serine proteinase inhibitors, PCI-I, PTI, and PCI-II (whose amino acid sequence is yet to be determined), all cross-reacted strongly with rabbit anti-PCI-I serum (Pearce et al., 1981). The other polypeptide proteinase inhibitor that has been isolated is a powerful inhibitor of metallocarboxypeptidases (Ryan et al., 1974; Hass & Ryan, 1981). This inhibitor exhibits striking homology at the N terminus with PCI-I (Hass et al., 1976). Five of the 18 amino terminal positions are identical, including three half-cystine residues and the tripeptide Pro-Ile-Cys (Hass et al., 1976). The amino acid sequences of PCI-I and PTI are also highly homologous with the known partial sequence of potato inhibitor II (Iwasaki et al., 1972). Thus the polypeptides all belong to the inhibitor II class of proteinase inhibitors (Laskowski & Kato, 1980). Whether the polypeptide inhibitors are products of posttranslational modifications of inhibitor II isoinhibitor protomers or are products of small inhibitor genes is not yet known.

The polypeptide inhibitors are present in significant quantitites in potato tubers where they are thought to be part of a natural defense system against pests (Pearce et al., 1981). The entire spectrum of the inhibitor II class of inhibitors can potentially render all five major pancreatic digestive enzymes inert. Therefore, these peptides must be considered to be important antinutrient compounds in raw potato tubers.

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