Relative Abundance and Inhibitory Distribution of Protease Inhibitors in Potato Juice from cv. Elkana

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Protease inhibitors from potato juice of cv. Elkana were purified and quantified. The protease inhibitors represent ca. 50% of the total soluble proteins in potato juice. The protease inhibitors were classified into seven different families: potato inhibitor I (PI-1), potato inhibitor II (PI-2), potato cysteine protease inhibitor (PCPI), potato aspartate protease inhibitor (PAPI), potato Kunitz-type protease inhibitor (PKPI), potato carboxypeptidase inhibitor (PCI), and "other serine protease inhibitors". The most abundant families were the PI-2 and PCPI families, representing 22 and 12% of all proteins in potato juice, respectively. Potato protease inhibitors show a broad spectrum of enzyme inhibition. All the families (except PCI) inhibited trypsin and/or chymotrypsin. PI-2 isoforms exhibit 82 and 50% of the total trypsin and chymotrypsin inhibiting activity, respectively. A strong variation within the latter activities was shown within one family and between protease inhibitor families.

Keywords: *Potato; Solanum tuberosum; protease inhibitor; inhibiting activity; trypsin; chymotrypsin*

INTRODUCTION

Although protease inhibitors have been long considered only as antinutritional factors, they have regained interest in recent years because of their possible anticarcinogenic (*1*) and positive dietary effects (*2*). Potato tuber protease inhibitors have been reported to act as anticarcinogenic agents by interfering in tumor-cell proliferation (*3*), H_2O_2 formation (*4*), and processes resulting from solar-UV irradiation (*5*). In addition, by intervening with cholecystokinin (*2*), one of the protease inhibitors can act as a satiety agent.

Potato tubers (*Solanum tuberosum*) contain approximately 1.5% protein on a fresh weight basis (*6*). It has been reported that protease inhibitors represent about 30% of the total tuber protein (*7*). In contrast to patatin, the major potato tuber protein (*8*), the protease inhibitors are a more heterogeneous group of proteins. They differ with respect to molecular mass, amino acid sequence, and inhibitory activity. The most studied protease inhibitors from potato tuber are protease inhibitor I (PI-1), protease inhibitor II (PI-2), and potato carboxypeptidase inhibitor (PCI). PI-1 is a pentameric serine protease inhibitor composed of five 7−8-kDa isoinhibitor protomers and inhibits chymotrypsin (and with lower affinity also trypsin) (*9, 10*). PI-2 is a dimeric serine protease inhibitor composed of two 10.2-kDa subunits (*11*). A disulfide bridge links the subunits, and the protein behaves like a single domain protein (*12*). The 4.3-kDa PCI is the smallest inhibitor present in potato tuber, is a single subunit peptide (*13*) and is remarkably thermo-stable (*14, 15*). In addition to PI-1, PI-2, and PCI, other protease inhibitors have been identified in potato tubers as well. The reported protease inhibitors include Kunitz-family inhibitors (*16*), cysteine protease inhibitors (*17*), and cathepsin D inhibitors (*18*).

In industrial processes, potato proteins are recovered as a byproduct of potato starch production (*19*). This is done by an acidic heat-treatment of the so-called potato juice and results in irreversibly precipitated proteins which have lost all functionality (*19*). To use specific protease inhibitor fractions of the potato juice in industrial, food, or pharmaceutical applications, the proteins in this group should be identified and quantified. To our knowledge no reports have appeared which describe the relative abundance and activity of the different protease inhibitors in potato tuber. Therefore, in this report we describe a general fractionation method to obtain the most important protease inhibitors from potato tubers, in particular of cv. Elkana. The choice of Elkana cultivar was determined by the economical importance of this cultivar in the potato starch industry in The Netherlands. Subsequently, the inhibitors are identified on the basis of their subunit molecular mass, isoelectric pH, and their activity against various proteases. The amount of protein relative to the total protein content has been determined for each protease inhibitor fraction and an overview of the most abundant protease inhibitor families will be presented.

MATERIALS AND METHODS

Materials. Porcine pancreas trypsin (T-0134, lot 100H0658), bovine chymotrypsin (C-4129, lot 58H7001), papaya latex papain (P-9886, lot 66H7130), bovine pancreas carboxypepti-

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10.1021/jf010126v CCC: $20.00
Published on Web 05/25/2001

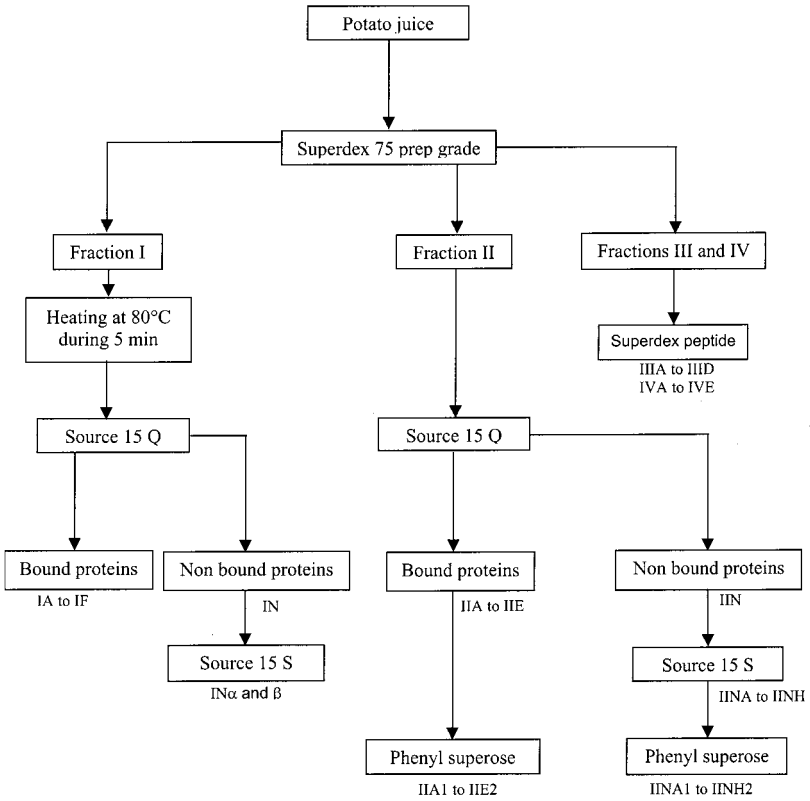

Figure 1. Schematic representation of the fractionation procedure.

dase A (C-0261, lot 116H8020), bovine spleen cathepsin D (C-3138, lot 103H8005), human leukocyte elastase (E-8140, lot 88H928), *p*-nitrophenyl-*p*′-guanidine benzoate, and N-trans-cinnamoylimidazole were obtained from Sigma Chemical Co.

Potato PI-2 (lot B14718), purified by affinity chromatography on immobilized chymotrypsin, and succinyl-Ala-Ala-Pro-Phe-*p*Na were purchased from Cal Biochem. N-furanacryloyl-L-Phe-L-Phe and succinyl-Ala-Ala-Val-*p*-nitroanilide were purchased from Bachem. Benzoyl-DL-Arg-*p*-nitroanilide (DL-BAPA) and benzoyl-L-Arg-*p*-nitroanilide (L-BAPA) were from Merck.

Potatoes of cultivar Elkana (AVEBE b.a., Veendam, The Netherlands) were stored at 4 °C in the dark at a relative humidity of 95 to 100% for a period of 6 months and used within this period.

Preparation of Potato Juice (PJ). Potatoes were chopped into large pieces (max. 8 × 2.5 cm) and subsequently mixed in the presence of sodium bisulfite or ascorbic acid, at a concentration of 0.5 g/kg or 4 g/kg of potatoes, respectively, to prevent polyphenol oxidation (5−6 potatoes representing 1 kg). The potato pieces were ground in a domestic type extractor (AEG). Starch was sedimented for 30 min at 4 °C and the supernatant was centrifuged (10000*g*, 30 min, 4 °C). After filtration through an 0.22-μm filter (Schleicher and Schüll, 301310), a clear extract, potato juice (PJ), was obtained (17.8 mg/mL of proteins according to Bradford analysis and 14.9 mg/mL according to Dumas analysis, after correction for non-protein nitrogen).

Protein Purification. Akta Purifier and Explorer protein chromatography systems and the columns used for the protein purification were from Pharmacia Biotech. The eluates were monitored at 280 and 320 nm.

A schematic representation of the fractionation procedure is shown in Figure 1. In the first step, 150 mL of PJ was applied to a Superdex 75 prep-grade gel filtration column (65 × 10 cm), equilibrated with 25 mM Tris−HCl buffer pH 7.0. The protein fractions were eluted with the same buffer.

The second general purification step was anion-exchange chromatography using a Source 15 Q column (15 × 2.6 cm). The column was equilibrated with 10 mM Tris−HCl buffer pH 8.0. After adjusting the pH of the sample to 8.0 with NaOH, the proteins were loaded and eluted with a linear gradient of 0−0.6 M NaCl for fraction I and 0−0.2 M NaCl for fraction II, derived from the Superdex 75 fractions.

The third general step was cation-exchange chromatography using a Source 15 S column (15 × 2.6 cm). For fraction IN, the column was equilibrated with 10 mM sodium acetate buffer pH 4.5 and the proteins were eluted with a salt gradient of 0−0.2 M NaCl in the same buffer. For fraction IIN, the same column was equilibrated with 10 mM sodium phosphate buffer pH 6.5 and the proteins were eluted with a salt gradient of 0−0.2 M NaCl in the same buffer.

The final general purification step, if required, was hydrophobic interaction chromatography using an HR 5/5 Phenyl Superose column equilibrated with 10 mM Tris−HCl buffer pH 7.0 containing 1.5 M $(NH_4)_2SO_4$; the proteins were eluted with a gradient of 1.5 to 0 M ammonium sulfate in the same buffer.

A Superdex peptide column (30 × 0.75 cm) was used specifically for the carboxypeptidase inhibitor purification from the Superdex 75 fractions III and IV. The column was equilibrated with 30 mM Tris−HCl buffer pH 7.0, containing 0.5 g/L NaCl, and the proteins were eluted with the same buffer.

Protein Purity. SDS−PAGE, with and without β-mercaptoethanol, and IEF electrophoresis were performed with a Pharmacia PhastSystem according to the instructions of the manufacturer using Gradient 8−25% and IEF 3−9 Phastgels, respectively. For a high sensitivity, the gels were stained according to the silver staining procedure provided by the manufacturer.

Protein Quantification. The protein concentration for each of the intermediate and purified fractions was determined using the Bradford assay (*20*), using bovine serum albumin (Sigma) as a standard. For PJ, patatin, and for at least one of the isoforms within each protease inhibitor family, the nitrogen content was also determined using the combustion (Dumas) method on the NA 2100 nitrogen and protein analyzer (Intersciences) according to the instructions of the manufacturer. For PJ, a distinction was made between protein nitrogen and

nonprotein nitrogen using the <1 kDa filtrate. A factor of 6.25 was used for the conversion of (protein) nitrogen to total potato protein content. The factors for patatin and the different inhibitor families were calculated from the known amino acid sequences. For patatin and the other proteins, the factor derived from the amino acid sequence was corrected when possible for the attached sugar moieties. Thus, the factors used were 6.30 for patatin, 5.85 for PI-1, 6.26 for PI-2, 6.12 for PCPI, 6.08 for PAPI, 5.57 for PCI, and 5.97 for PKPI and OSPI. Next, on the basis of the protein content as determined by the Dumas method, calibration curves were made for patatin and representative inhibitors of each of the protease inhibitor families in the Bradford assay. Subsequently, the values obtained using bovine serum albumin as standard were corrected using patatin and a representative inhibitor of each protease inhibitor family as standards. No major differences in Bradford response within each protease inhibitor family were expected and/or detected. All the assays were performed at least in duplicate.

Mass Spectrometry. MALDI−TOF MS analysis in the linear mode was performed using a Voyager DE RP instrument (Perseptive Biosystems). Protease inhibitor fractions were dialyzed against Nanopure purified water before analysis. Protein samples were prepared according to the drying droplet method using 10 mg/mL sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, Sigma), dissolved previously in 0.3% (v/v) aqueous TFA containing 50% (v/v) acetonitrile, as matrix. Spectra were externally calibrated using thioredoxin, insulin, and apo-myoglobin (Perseptive Biosystems) as standards.

Enzyme Titration. The concentrations of active enzyme were determined by titrating their active sites: trypsin with *p*-nitrophenyl-*p*′-guanidine benzoate (*21*) and α-chymotrypsin with *N*-*trans*-cinnamoylimidazole (*22*). The titration showed 75 and 25% activity for trypsin and α-chymotrypsin, respectively.

Inhibitor Assays. Proteinase activity in the presence or absence of inhibitor was measured by the hydrolysis of synthetic substrates with a microtiterplate reader (μQuant, Bio-Tek instruments Inc.), and a Beckman DU-62 or an Hitachi PC 3000 spectrophotometer. To estimate the inhibition, proteases were preincubated during 15 min with inhibitor, before the substrate was added. All assays were performed in duplicate.

Trypsin inhibition was estimated according to Smith et al. (*23*) in 50 mM Tris−HCl buffer pH 7.8 containing 100 mM $CaCl_2$ in the presence of 0.28 μM enzyme using DL-BAPA (360 μM) as the substrate.

Chymotrypsin inhibition was estimated according to Geiger (*24*) in 50 mM Tris−HCl buffer pH 7.8 containing 100 mM $CaCl_2$ in the presence of 0.011 μM enzyme using succinyl-Ala-Ala-Pro-Phe-*p*Na (224 μM) as the substrate.

Papain inhibition was estimated according to Mole et al. (*25*) in 0.1 M potassium phosphate buffer pH 6.5 containing 0.3 M KCl, 0.24 mM EDTA, and 16 mM L-cysteine in the presence of 0.255 μM enzyme using L-BAPA (100 μM) as the substrate.

Cathepsin D inhibition was estimated according to van Jaarsveld et al. (*26*) in 0.1 M sodium acetate buffer pH 3.5 in the presence of 0.05 μM enzyme using acid denaturated haemoglobin (10 g/L) as the substrate.

Elastase inhibition was estimated according to Valueva et al. (*27*) in 0.1 M Tris−HCl buffer pH 8.0 in the presence of 0.035 μM enzyme using Succinyl-Ala-Ala-Val-*p*Na (350 μM) as the substrate.

Carboxypeptidase A inhibition was estimated according to Riordan and Holmquist (*28*) in 50 mM Tris−HCl buffer pH 7.5 containing 1 M NaCl in the presence of 0.182 μM enzyme using *N*-furanacryloyl-L-Phe-L-Phe (100 μM) as the substrate.

For all protease inhibition assays the degree of inhibition was measured as a function of protein concentration. Subsequent inhibition experiments were conducted in the concentration range where inhibition is linear with protein concentration.

Calculation of Inhibiting Activity. The trypsin and chymotrypsin inhibitory activity (TIA and CIA, respectively) has been determined according to the Kakade method as modified by Smith et al. (*23*). The decrease in activity in the presence of inhibitor was measured and the inhibiting activity was calculated according to the formula

$$\mathrm{IA} = \frac{(A_S - A_I)}{1000 \times A_{TI}} \times \frac{1}{S}\ \text{mg active enzyme/g inhibitor}$$

where A_S is the increase in absorbance due to enzyme in absence of inhibitor, A_I is the increase in absorbance due to enzyme in the presence of inhibitor, A_{TI} is the change of absorbance due to 1 μg of active enzyme, and S is the amount of the sample (g).

The proportion of active enzyme for trypsin and chymotrypsin was calculated by enzyme titration, as explained above.

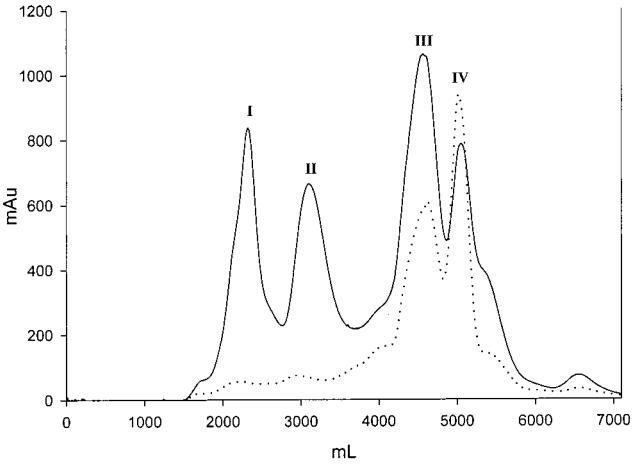

Figure 2. Gel filtration of potato juice on Superdex 75 prep grade (solid line, 280 nm; dotted line, 320 nm).

RESULTS AND DISCUSSION

Fractionation. As shown in Figure 1, gel filtration was used as a first fractionation step. The PJ was separated into four fractions: I, II, III, and IV (Figure 2). SDS−PAGE electrophoresis showed two major bands at 40 kDa and 8 kDa for fraction I. Fraction II contained a group of proteins with molecular weights between 20 and 25 kDa. No protein could be detected in fractions III and IV as judged from SDS−PAGE electrophoresis. However, the protease inhibitor assays indicated the presence of a carboxypeptidase inhibitor. The absorbance at 320 nm of fractions III and IV indicated the presence of polyphenols and oxidized polyphenols (*29*).

Fraction I. As determined by SDS−PAGE electrophoresis, the protein band of 8 kDa indicated the subunit form of PI-1 in fraction I (*30*). Because of the presence of patatin in fraction I, judged from the major band at 40 kDa (*8*), PI-1 was isolated according to the method of Melville and Ryan (*7*). The proteins in fraction I were heated at 80 °C during 5−6 min and filtrated through an 0.22 μm filter (Schleicher and Schüll, 301310). The presence of PI-1 (8 kDa) and the disappearance of almost all patatin (40 kDa) were confirmed by SDS−PAGE electrophoresis. The trypsin inhibition assay, performed before and after heating, showed similar inhibiting activity in both cases. The heat-treated fraction I was further fractionated by anion exchange chromatography into eight main subfractions denoted IN, Iag, and IA to IF, as shown in Figure 3A. The fractions IA to IF showed 1:1 stoichiometry for trypsin which is characteristic for PI-1 (*7*). Fraction Iag showed no protease inhibition activity and appeared as

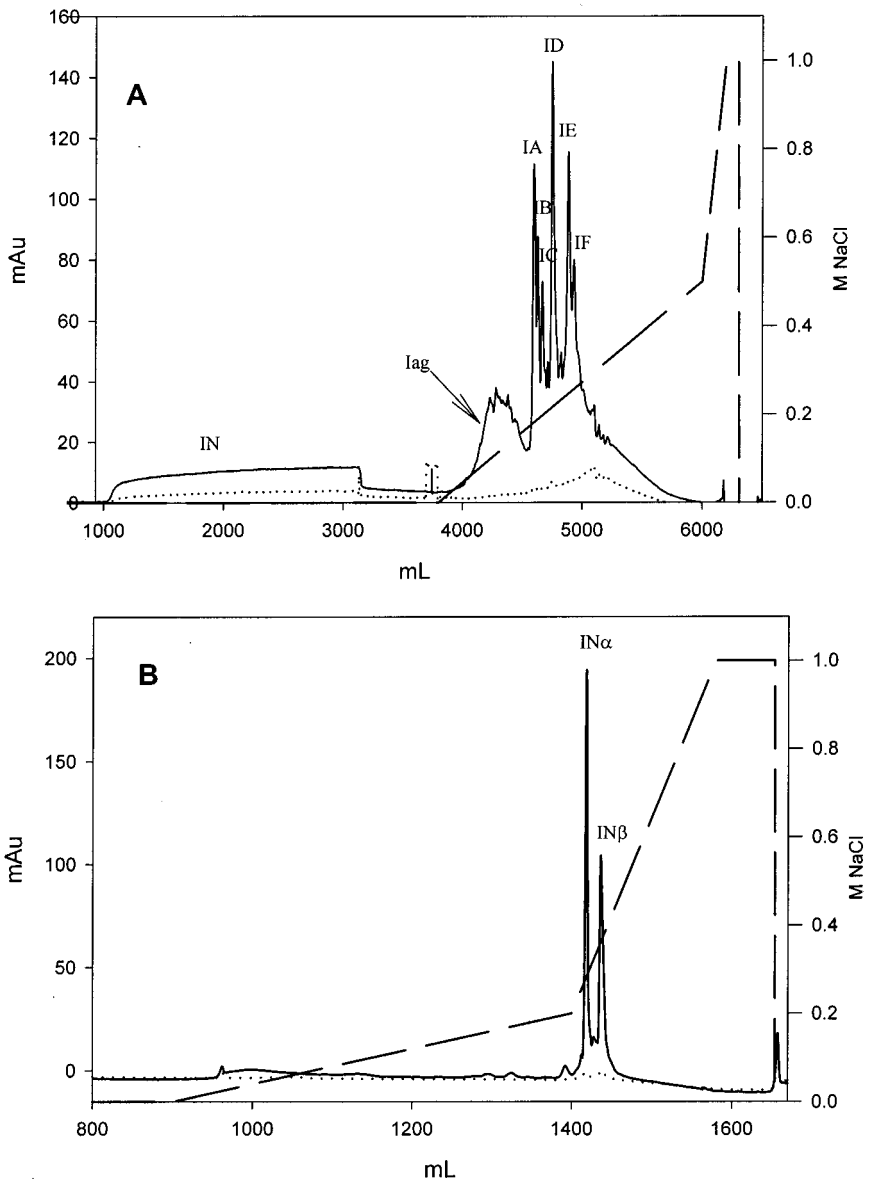

Figure 3. Fractionation of Superdex fraction I: **A**, anion-exchange chromatography of fraction I on source 15Q; **B**, cation-exchange chromatography of fraction IN on source 15S (solid line, 280 nm; dotted line, 320 nm; dashed line, salt gradient).

different protein bands, 40 and 80−100 kDa, on SDS−PAGE without β-mercaptoethanol. This fraction most likely contains patatin aggregates (*31*) and some phosphorylase isoenzymes (bands at 40 kDa and 100 kDa) (*32*) remaining after the heating step. The nonbound proteins (IN) were dialyzed against water and subsequently applied to a cation exchange column (Figure 3B). The fractions INα and IINβ were collected, and SDS−PAGE and the trypsin inhibiting activity confirmed the presence of PI-1 in both fractions. Summarizing, in fraction I we were able to identify, in addition to patatin, 8 isoforms of PI-1. They differ in isoelectric point: pH 5.6, 5.1, 5.8, 6.1, 6.5, and 6.3 for fractions IA to IG, respectively, and pH 7.2 and 7.8 for fractions INα and INβ, respectively, as estimated by isoelectrofocusing (no further data shown).

Fraction II. Fraction II was separated by anion exchange chromatography and the eluting proteins were collected. As shown in Figure 4A, the nonbound proteins (IIN) represent about 50% of the applied proteins. Five fractions were eluted by the salt gradient: 3 main fractions (IIB, IID, and IIE) and 2 minor fractions (IIA and IIC). Fraction IIF, eluted at high ionic strength, contained no protein as judged from SDS−PAGE, and the absorbance at 320 nm indicated the presence of polyphenols.

Nonreducing SDS−PAGE electrophoresis showed that the proteins in fractions IIB, IID, and IIE have molecular weights of 20.5 kDa. In the presence of β-mercaptoethanol, two bands appear with molecular weights of 14 and 7 kDa. SDS−PAGE electrophoresis of a commercial sample of potato PI-2 showed the same electrophoretic pattern as observed for fractions IIB, IID, and IIE. Also, a cloned potato PI-2 showed the same electrophoretic pattern (*33*). Therefore, these fractions could be identified as isoforms of PI-2. The isoelectric points were determined and two bands were observed for each fraction: pI 6.5 and 6.0 for fraction IIB, pI 6.1 and 5.7 for fraction IID, and pI 5.9 and 5.5 for fraction IIE.

To increase the purity of the PI-2 isoforms, hydrophobic interaction chromatography (HIC) was performed with fractions IIB, IID, and IIE. Each fraction contained one major and one minor protein. The HIC fractionation of fraction IIB is given as an example in

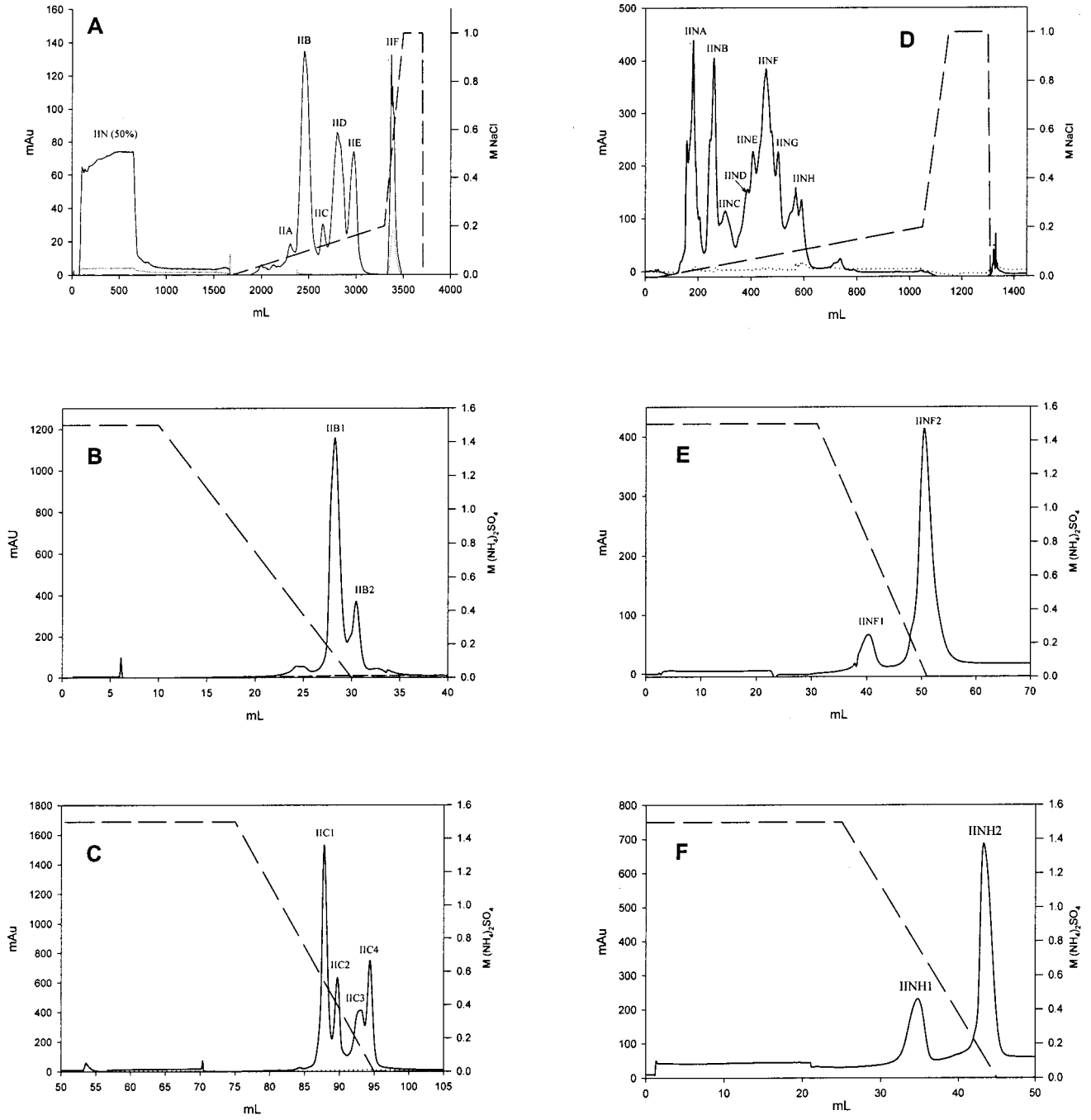

Figure 4. Fractionation of Superdex fraction II: **A**, anion-exchange chromatography of peak II on Source 15Q; hydrophobic interaction chromatography of fractions IIB **(B)** and IIC **(C)** on phenyl superose; **D**, cation-exchange chromatography of fraction IIN on source 15S; hydrophobic interaction chromatography of fraction IINF **(E)** and IINH **(F)** on phenyl superose (solid line, 280 nm; dotted line, 320 nm; dashed line, salt gradient).

Figure 4B. The major subfractions resulting from the fractions IIB, IID, and IIE were denoted IIB1 (6.5), IID3 (6.1), and IIE2 (5.9), respectively (between brackets the corresponding isoelectric point is given). The minor fractions were also collected and identified further.

The main proteins present in fractions IIA and IIC have a molecular mass of 22.7 kDa (as determined by MALDI−TOF MS and by reducing SDS−PAGE electrophoresis) and a pI around 6.7 and 6.6, respectively. Fractions IIA and IIC were further separated by HIC, resulting in a fraction containing the 22.7 kDa proteins (IIA1 and IIC1) (Figure 4C) and some minor fractions containing PI-2 and 20 kDa proteins. The minor fractions were also used for identification.

After dialysis against water, the nonbound proteins (IIN) were fractionated by cation exchange chromatography. In Figure 4D the elution profile is shown; the collected fractions are denoted IINA−IINH. The proteins showed a high pI between 7 and 9 (data not shown). The next chromatography step for all the fractions was HIC (For examples see Figure 4E and 4F). The main subfractions were denoted: IINA2 (6.9) and IINA3 (8.2); IINB2 (8.4 and 8.0); IINC1 (8.7) and IINC2 (7.5); IIND1 (8.6) and IIND2 (8.6); IINE1 (8.6) and IINE2 (8.6 and 8.8); IINF2 (8.3); IING1 (>9.0), IING2 (8.0), and IING3 (8.3), and IINH2 (8.0) (between brackets the corresponding isoelectric point is given). All proteins present showed a molecular mass of ap-

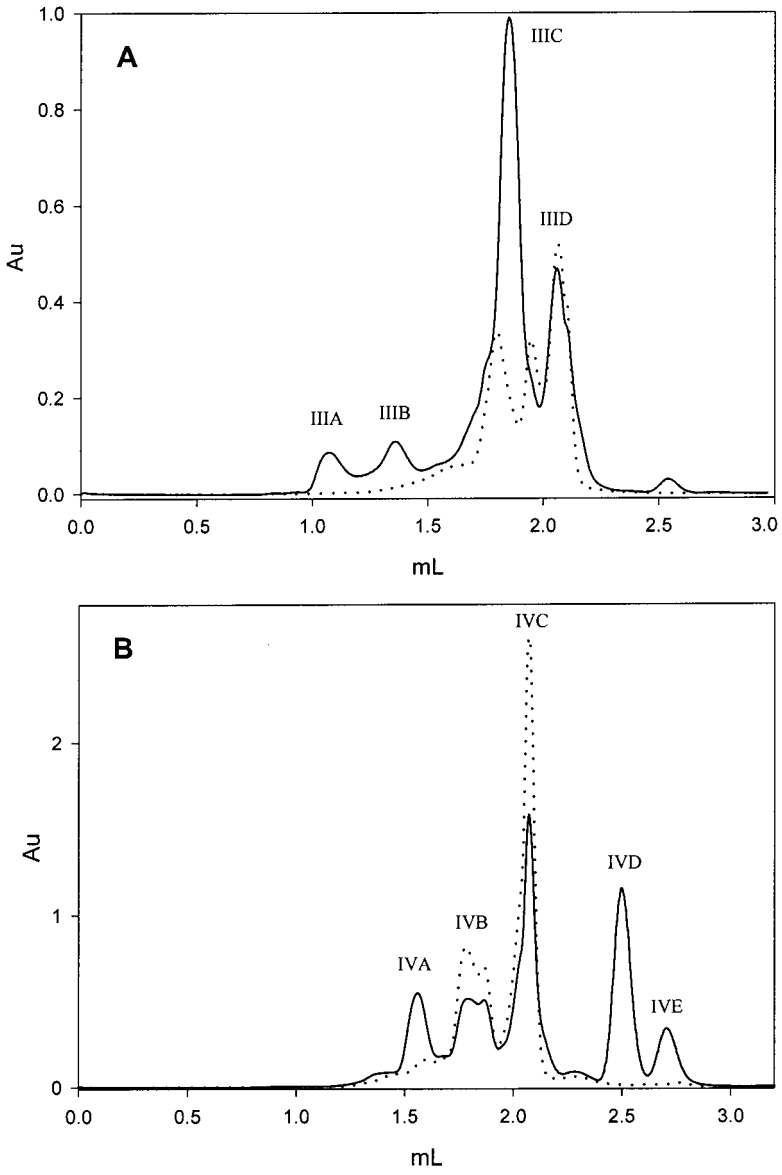

Figure 5. Fractionation of Superdex fractions III and IV: **A**, gel filtration of fraction III on Superdex peptide (solid line, 280 nm; dotted line, 320 nm); **B**, gel filtration of fraction IV on Superdex peptide (solid line, 280 nm; dotted line, 320 nm).

proximately 20 kDa and existed in a monomeric form, except for fractions IINA2, IINA3, and IINE2, as determined by SDS−PAGE with and without β-mercaptoethanol. All subfractions were used for protein quantification and for inhibition activity characterization.

Fractions III and IV. Fractions III and IV from the gel filtration step were freeze-dried, solubilized in water, and applied to a Superdex peptide gel filtration column. Four fractions were collected from fraction III and they were denoted IIIA to IIID (Figure 5A). The five fractions obtained from fraction IV were denoted IVA to IVE (Figure 5B). The carboxypeptidase inhibitor is the smallest inhibitor present in potato tuber with a molecular weight of 4.3 kDa (*34, 35*) and its presence was expected in fractions III and IV. The presence of proteins in fractions IIIA and IIIB was confirmed by the Bradford assay and by MALDI−TOF MS. Fraction IIIA contains proteins with a molecular weight of 20 kDa (exhibiting very low inhibiting activity against trypsin) and fraction IIIB proteins with a molecular weight of 4,274 Da. No protein was detected in the other sub-fractions of fractions III and IV. The molecular mass of 4.3 kDa, determined in fraction IIIB, is in accordance with the known molecular weight of the carboxypeptidase inhibitor. A carboxypeptidase assay was performed and fraction IIIB was identified as potato carboxypeptidase inhibitor (PCI).

Classification of Protease Inhibitors. The properties, collected by MALDI−TOF MS, SDS−PAGE, IEF, and inhibitory activity of the protease inhibitors separated in the various fractions, are given in Table 1. On the basis of comparing the properties and available literature data, the protease inhibitors of the protein fractions were identified and classified.

PI-1. On the basis of the nonreducing SDS−PAGE band pattern (one band at 7−8 kDa) and the data from MALDI−TOF MS (7,683−7,873 Da), the proteins in fractions IA to IF, INα to INβ, and IIC2 to IIC3 were identified as PI-1 isoforms.

PI-2. The proteins in fractions IIA2, IIB1, IIB2, IIC3, IID2, IID3, IIE2, IINA2, and IINA3 have many common characteristics, such as molecular weight (20.5 kDa), the same electrophoretic pattern on reducing SDS−PAGE, and their inhibitory activity. Therefore, we identified

Table 1. Characteristics and Protease Inhibition Activity of Proteins Obtained after Potato Juice Fractionation[a]

name	fraction	MW (± 5 Da)	pI	SU	Tryp[b]	Chym	Pap	CathD	HLE	CarboA
PI-1	I	7,683−7,873	5.1−6.3	5	+	+	−	−	+	−
	IN	7,683−7,873	7.2, 7.8	5	+	+	−	−	+	−
	IIC2, IIC3	7,683−7,873	5.1, 6.3	5	+	+	−	−	+	−
PI-2	IIA2, IIB1	20,279	6.5	2	+	+	−	−	+	−
	IIB2	20,023	6.0	2	+	+	−	−	−	−
	IIC3, IID3	20,273	6.1	2	+	+	−	−	+	−
	IIC4	20,674	5.8	2	−	+	−	−	+	−
	IID2	20,676	5.5	2	+	+	−	−	+	−
	IIE2	20,315	5.9	2	+	+	−	−	+	−
	IINA2, IINA3	20,265	6.9	2	+	+	−	−	+	−
PIG	IIC4	19,987	6.2	1	−	+	−	+	+	−
NID	IINB2	20,039	8.4	1	+	+	−	+	+	−
PDI	IIND2, IINE2	22,025	8.6	1	+	+	−	+	+	−
PI-8	IINC1	19,878	8.7	1	+	−	−	+	+	−
PI-13	IINC2	20,141	7.5	1	+	+	−	+	−	−
PAPI-8.15	IINA3	19,883	8.2	1	+	+	−	+	−	−
PCPI-23 kDa	IIA1	22,755	6.7	1	−	+	+	−	+	−
PCPI-6.6	IIC1	22,769	6.6	1	+	+	+	−	−	−
PCPI-5.9	IID1	22,674	5.8	1	−	+	+	−	−	−
PCPI-7.1	IINA1	22,773	7.1	1	+	+	+	−	−	−
PCPI-8.0	IINB2	20,096	8.0	1	+	+	+	−	+	−
PCPI-8.6	IIND1, IINE1	20,127	8.6	1	+	−	+	−	−	−
PCPI-9.4	IINF1, IING1	20,134	>9.0	1	+	−	+	−	+	−
PCPI-8.3	IINF2, IING2, IIIG3	20,433	8.3	1	+	+	+	−	−	−
PKPI-9.0	IINH1	20,237	>9.0	1	+	−	−	−	−	−
PKPI-8.0	IINH2, IING2	20,194	8.0	1	+	+	−	−	−	−
HLE inh.	IINE2	21,025	8.8	2	+	+	−	−	+	−
22 kDa inh.	IINA2, IINA3	21,804	7.5	1	+	+	−	−	+	−
PCI	IIIB	4,274	nd[c]	1	−	−	−	−	−	+

[a] SU, subunit; Tryp, Trypsin; Chym, Chymotrypsin; Pap, Papain; CathD, Cathepsin D; HLE, human leukocyte elastase; CarboA, Carboxypeptidase A; PI-1, potato inhibitor I; PI-2, potato inhibitor II; PAPI, potato aspartate protease inhibitor; PCPI, potato cysteine protease inhibitor; PKPI, potato Kunitz-type protease inhibitor; PCI, potato carboxypeptidase inhibitor. [b] +, inhibiting activity present; −, no inhibiting activity present. [c] nd, not determined.

these proteins as PI-2 isoforms that vary slightly in pI and MW (*11*, *36*).

Aspartate Protease Inhibitors. The major protein in fraction IIC4 has a molecular weight of 19,987 Da, a pI of 6.2, and inhibits aspartate proteases. This protein was identified as a potato aspartate protease inhibitor (PAPI), described by Strukelj et al. (*37*) and denoted PIG. The proteins present in fractions IINC1 and IINC2 were identified, according to their isoelectric points and their inhibiting activities, as PI-8 and PI-13, respectively (*37*). The main protein in fraction IINB2 with a MW of 20,039 Da and a pI of 8.4 was identified as a PAPI, denoted NID (novel inhibitor of cathepsin D) as reported by Ritonja et al. (*38*). The protein present in fraction IIND2 and IINE2 is slightly larger (MW 22,025 Da) and also inhibits aspartate proteases. It was identified as a monomeric PAPI as reported by Mares et al. (*18*) and denoted PDI (potato cathepsin D inhibitor).

To our knowledge no literature is published to date on the potato aspartate protease inhibitor present in fraction IINA3. We denoted this protein PAPI-8.2 according to its pI. The amino acid sequence of this new identified PAPI will have to be determined.

Cysteine Protease Inhibitors. According to its pI (5.7−5.8) and its inhibiting activity, the protein in fraction IID1 is a potato cysteine protease inhibitor (PCPI) and can be identified as PCPI-5.9 (*17*). The protein in fraction IIC1 can be identified according to its pI (6.6) and its inhibiting activity as PCPI-6.6 (*17*) and the proteins in fractions IING1 and IINF2 as PCPI-9.4 and PCPI-8.3, respectively (*17*, *39*). However, all these cysteine protease inhibitors have lower molecular weights, as estimated by MALDI−TOF MS, than those identified by Brzin et al. (*17*) and Krizaj et al. (*39*); in these studies, the molecular weights were determined by SDS−PAGE only. Fraction IIC1 shows a band at ±25 kDa on SDS−PAGE, whereas the MALDI−TOF MS spectrum gave 22,762 Da (the same comparison could be made for all other fractions containing PCPI). Thus, the migration on Tris−HCl gels seems disturbed. Slight differences in molecular weight were observed for the other inhibitor families, but the differences were not as large as those for the PCPI family. According to its inhibiting activity against papain and its molecular weight of 22,755 Da, the protein in fraction IIA1 can be identified as the protein described by Valueva et al. (*40*), which was named PCPI-23 kDa.

To our knowledge no literature is available describing the potato cysteine protease inhibitors present in fraction IINA1, IINB2, IIND1, and IINE1. We denoted these proteins PCPI-7.1 (22,773 Da), PCPI-8.0 (20,096 Da), and PCPI-8.6 (20,127 Da) according to their pI. The amino acid sequences of these newly identified PCPIs will have to be determined to know the degree of homology between these proteins and the others members of the PCPI family.

Kunitz-Type Protease Inhibitors. The fraction IINH1 mainly contains a monomeric 20,237 Da protein that shows a low inhibiting activity against trypsin. This inhibitor was identified as a potato Kunitz-type protease inhibitor (PKPI), named PKPI-9.0 (*16*, *41*). Also, the monomeric protein in fraction IINH2 was characterized as a serine protease inhibitor with inhibiting activity against trypsin and chymotrypsin. This inhibitor is also classified in the PKPI family according to Mitsumori et al. (*41*), and named PKPI-8.0.

Table 2. Relative Mass (w/w) Distribution of Protease Inhibitor Families in Potato Juice

	Superdex fraction	group	proportion in PJ
	I (48.0%)	patatin	37.5
		PI-1	4.5
	II (48.0%)	PI-2	22.3
		PCPI family	11.9
		PAPI family	5.9
		PKPI family	3.6
		OSPI	1.5
	III (2.0%)	PCI	0.9
recovery 100%	98.0%		88.1%

Other Serine Protease Inhibitors (OSPI). The main protein present in fraction IINE2 is a dimeric protein. The subunits must be disulfide linked because the SDS−PAGE pattern changes in the presence of β-mercaptoethanol. This 21,025 Da protein showed inhibiting activity against trypsin, chymotrypsin, and elastase. Except for PI-2, only one other dimeric protein has been described in potato: a serine protease inhibitor with two subunits of 16.5 and 4.5 kDa, respectively. Therefore, the protein in fraction IINE2 can be identified as the human leukocyte elastase (HLE) inhibitor described by Valueva et al. (*27*).

One of the proteins present in fraction IINA2 and IINA3 is a monomeric protein showing inhibiting activity against trypsin and chymotrypsin and has a molecular weight of 21,804 Da. This protein can be identified as the 22 kDa serine protease inhibitor described by Suh et al. (*42, 43*).

Carboxypeptidase Inhibitor. The monomeric protein in fraction IIIB was identified as the carboxypeptidase inhibitor, according to its molecular weight of 4.3 kDa and inhibiting activity against carboxypeptidase A (*34, 35*).

Protein and Protease Inhibiting Activity Distribution in Potato Juice. *Protein Distribution.* The distribution of the different protein families with respect to the total amount of protein present in PJ has been determined and the results are summarized in Table 2.

Prior to this study, soluble proteins present in potato tuber have been divided into three different groups: patatin, representing ±40% of the potato proteins (*8*), the group of protease inhibitors, representing 20−30% of total protein (*7*), and a group of other proteins (mainly enzymes such as kinases and enzymes involved in starch synthesis), representing 20−30% of the total protein (*44, 45*). In the PJ of the investigated cultivar the protease inhibitors represent, however, ±50% of the total protein (Table 2).

Patatin represents 80.0% of the protein in fraction I, whereas PI-1 represents 10.0%. Therefore, patatin and PI-1 represent 37.5% and 4.5% of the total proteins in PJ, respectively. This latter value is higher than expected from the literature in which PI-1 represents only 2.0% of total protein (*30, 46*). All the different isoforms of PI-2 represent 22.3% of total protein in potato juice, which again is higher than the value of 5% previously reported (*30*).

The PCPI, PAPI, and PKPI families belong to the previously denoted family of 20-kDa proteins (*10*) and together they represent 22.9% of the total protein in PJ. The PCPI family contains 8 different inhibitors, which differ in molecular weight and pI, and represents 11.9% of the total protein in PJ. The most abundant potato cysteine protease inhibitor is PCPI-8.3, which represents 3.6% of the total amount of protein. The PAPI family contains 6 different inhibitors, which also differ in molecular weight and pI, and represents 5.9% of the total amount of protein. The main proteins in this group are NID and PDI, which each represent 1.4% of the total protein. The PKPI family represents only 3.6% of the total amount of protein in PJ. PKPI-8.0 is the main representative of this family and accounts for 2.7% of the total soluble protein. The OSPI and PCI families represent only 1.5 and 0.9%, respectively, of total protein in PJ.

Differences in the preparation of PJ, such as the use of different cultivars (*46*), extent of tuber development, peeling of potatoes, and/or the technique to make PJ (by mixing or pressing), could account for some variations in protein content. Also, the method for the determination of protein concentration can influence the distribution results. Some studies used the Lowry method, the nitrogen determination by the Kjeldahl method, spectrophometrically at 280 nm, or an immunological method (*8, 47*). However, all studies published so far have been focused on a particular protease inhibitor and no global data have been presented about other proteins present. In this study, for the first time, an overview is given of all the proteins present in PJ with emphasis on the distribution of potato protease inhibitors. Although the authors realize that in the present study only one cultivar was used and no variation in grow conditions and storage (*48*) was investigated, the results indicate that the amount of protease inhibitors in potato may have up to now been underestimated. We have shown that, in one cultivar (Elkana), protease inhibitors represent up to 50% of the soluble proteins in PJ. In this cultivar patatin (*49*) and other proteins represent 37.5% and 10−12% of total soluble proteins, respectively.

Most of the other proteins in PJ have molecular weights of 40 kDa and higher (*32*), thus they are mainly collected in fraction I. They most likely coprecipitate with patatin during the heating step and are possibly enzymes involved in starch synthesis (*47, 50*). In addition, the potato multicystatin inhibitor (PMC; 85 kDa) should be present in a small amount in fraction I (*51*). Also, minor losses of proteins during the purification procedure (Figure 1) contribute to the calculated amount (11.9%) of nonrecovered protein.

Inhibiting Activity Distribution. Table 3 gives an overview of the distribution of enzyme inhibition activities over the seven inhibitor families. The inhibiting activity of papain, cathepsin D, and carboxypeptidase A is due to a single family for each protease: PCPI, PAPI, and PCI, respectively. One remarkable conclusion is that 82% of the trypsin inhibiting activity can be attributed to PI-2. The remaining 18% is distributed over the other families except for PCI. The chymotrypsin inhibition activity is also distributed over the different families, except PCI. PI-1 and PI-2 are the most important chymotrypsin inhibitors, representing 19 and 50% of the total inhibition activity, respectively.

Subsequently, the distribution of trypsin and chymotrypsin inhibiting activity (TIA and CIA, respectively) within each inhibitor family was determined (Table 4). The TIA values vary between 10 and 485 mg of trypsin inhibited per gram of inhibitor (a factor of 48) whereas the CIA values vary by a factor of 24 (between 0.5 and

Table 3. Distribution of Inhibiting Activity over Potato Inhibitor Families

	distribution of inhibiting activity in %							
enzyme	PI-1	PI-2	PCPI	PAPI	PKPI	OSPI	PCI	total
trypsin	2	82	10	2	2	2		100
chymotrypsin	19	50	16	9	3	3		100
papain			100					100
cathepsin D				100				100
carboxypeptidase A							100	100

Table 4. Inhibiting Activity against Trypsin and Chymotrypsin and Quantitative Distribution of Trypsin and Chymotrypsin Inhibition over Inhibitor Fractions

family		PI prop[a]	TIA[b] (mg/g)	TIA dist[c]	CIA[d] (mg/g)	CIA dist[c]
PI-1						
	PI-1 (5.1)	0.9	40	0.2	10.7	1.9
	PI-1 (5.6)	0.9	70	0.3	10.2	2.2
	PI-1 (5.8)	0.9	50	0.2	10.1	2.0
	PI-1 (6.1)	1.2	75	0.5	12.2	3.3
	PI-1 (6.3)	0.9	65	0.3	10.1	2.1
	PI-1 (6.5)	0.9	60	0.3	9.8	2.1
	PI-1 (7.2)	1.6	80	0.6	10.9	3.4
	PI-1 (7.8)	1.5	50	0.4	9.5	3.7
PI-2						
	PI-2 (5.5)	2.6	60	0.7	5.5	2.7
	PI-2 (5.8)	0.6	45	0.1	3.5	0.4
	PI-2 (5.9)	6.9	480	16.9	7.0	11.9
	PI-2 (6.0)	4.5	280	4.9	4.5	3.7
	PI-2 (6.1)	11.3	480	22.8	7.5	16.7
	PI-2 (6.5)	14.5	485	28.7	4.1	11.1
	PI-2 (6.9)	3.7	425	8.5	5.5	5.2
PCPI						
	PCPI-5.9	2.6	10	0.1	2.5	1.1
	PCPI-6.6	1.6	0	0.0	3.5	1.0
	PCPI-7.1	1.5	35	0.2	7.5	2.1
	PCPI-8.0	2.6	55	0.5	0.5	0.2
	PCPI-8.3	7.2	145	4.2	6.0	8.1
	PCPI-8.6	4.1	40	0.6	0.0	0.0
	PCPI-9.4	3.1	90	1.1	0.0	0.0
	PCPI-23 kDa	0.9	0	0.0	4.1	0.6
PAPI						
	PI-8	2.6	90	1.0	4.2	2.1
	PAPI-8.15	0.8	110	0.3	1.5	0.2
	PI-13	2.4	100	1.1	4.9	2.8
	PIG	0.2	45	0.1	3.5	0.3
	NID	2.8	90	1.3	1.1	0.7
	PDI	2.8	50	0.5	3.0	1.5
PKPI						
	PKPI-8.0	5.3	80	1.6	3.1	2.8
	PKPI-9.0	1.8	15	0.2	0.0	0.0
OSPI						
	HLE inh.	1.3	230	0.2	8.2	1.9
	22 kDa inh.	1.7	100	1.8	2.9	0.9
PCI		1.8	0	0.0	0.0	0.0
total		100		100		100

[a] PI Prop, proportion of inhibitor expressed as weight percentage of the total amount of protease inhibitors present in PJ. [b] TIA, trypsin inhibiting activity. [c] TIA or CIA dist, expressed as percentage of TIA or CIA distribution in PJ. [d] CIA, chymotrypsin inhibiting activity.

12.2 mg of chymotrypsin inhibited per gram of inhibitor) over the different inhibitors. In general, the highest values of TIA were found for PI-2 isoforms.

PI-2 (6.5) and PI-2 (6.1), the most abundant PI-2 isoforms (representing 14.5 and 11.3% of the total amount of protease inhibitors in PJ), show the highest TIA values. PI-2 (6.1) also shows the highest CIA value of the PI-2 family. PI-2 is a dimeric protein and can have four different subunits (A, B, C, and D) in potato tuber (*11*). Each monomer has active sites for trypsin and chymotrypsin and shows different affinity constants for these enzymes (*33*). Thus, depending on its subunit composition, the dimeric protein can show different affinities for trypsin or chymotrypsin (Table 4).

Whereas PI-2 family has a high variation between the TIA values, the different isoforms of PI-1 show similar TIA, and also CIA, values. However, PI-1 seems to have the highest affinity for chymotrypsin. PI-1 is a pentameric protein (*10*) and each subunit differs in amino acid sequence and in reactivity against trypsin and chymotrypsin (*7*). PI-1 has a binding stoichiometry of 1:1 for trypsin and a stoichiometry of 2:1 or 3:1 for chymotrypsin (*7*). PI-1 (7.2), the most abundant representative of the PI-1 family (1.6% of the total amount of protease inhibitors in PJ), shows the highest TIA value and one of the highest CIA values in the PI-1 family.

In general, PCPI family members show lower TIA and CIA values than those of members of PI-2 and PI-1 and are responsible for 16% of the chymotrypsin inhibition. Also in the PCPI family, the most abundant protein, PCPI-8.3 representing 7.1% of the total protease inhibitors in PJ, is the most active inhibitor against trypsin and has one of the highest activities against chymotrypsin within this family.

PAPI family members show values of TIA similar to those of PI-1 members and values of CIA similar to those of PI-2 members. The PAPI family is responsible for 9% of the total chymotrypsin inhibition.

In conclusion, the amount of protease inhibitors in PJ (50%) may have been underestimated previously. PI-2 and PCPI are the most abundant protease inhibitor families in the potato tubers investigated in this study. The most abundant protease inhibitors for each of the seven families generally also display the highest trypsin and chymotrypsin inhibiting activities within their family.

ABBREVIATIONS USED

PJ, Potato juice; PI-1, potato inhibitor I; PI-2, potato inhibitor II; PCPI, potato cysteine protease inhibitor; PAPI, potato aspartate protease inhibitor; PKPI, potato Kunitz-type protease inhibitor; OSPI, other serine protease inhibitor; PCI, potato carboxypeptidase inhibitor; TIA, trypsininhibiting activity; CIA, chymotrypsin inhibiting activity, MALDI−TOF MS, matrix-assisted laser desorption/ionization−time of flight mass spectrometry.

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Received for review January 29, 2001. Revised manuscript received April 12, 2001. Accepted April 12, 2001.

JF010126V