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The Most Abundant Protease Inhibitor in Potato Tuber (Cv. Elkana) Is a Serine Protease Inhibitor from the Kunitz Family

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The gene of the most abundant protease inhibitor in potato cv. Elkana was isolated and sequenced. The deduced amino acid sequence of this gene showed 98% identity with potato serine protease inhibitor (PSPI), a member of the Kunitz family. Therefore, the most abundant protease inhibitor was considered to be one of the isoforms of PSPI. The PSPI group represents ∼22% of the total amount of proteins in potato cv. Elkana and is composed of seven different isoforms that slightly differ in isoelectric point. Antibodies were raised against the two most abundant isoforms of PSPI. The binding of these antibodies to PSPI isoforms and protease inhibitors from different groups of protease inhibitor in potato showed that ∼70% of the protease inhibitors present in potato juice belong to the Kunitz family.

KEYWORDS: Potato; protease inhibitor; potato serine protease inhibitor; Kunitz family

INTRODUCTION

Protease inhibitors are ubiquitously abundant in tubers and plant seeds (*1*). In higher plants, several gene families of protease inhibitors have been characterized, particularly the serine protease inhibitors from Leguminosae, Solanaceae, and Graminae (*2*).

Protease inhibitors in plants are generally considered to be storage proteins (source of nitrogen) and a defense mechanism (*3*). They were shown to be involved with the wound-induced defense response of plants against herbivores and pathogens (*4*) and have been shown to have an antinutrient activity on *Spodoptera exigua* larvae (*5*). They also accumulate in potato leaves in response to wounding and UV irradiation (*4*, *6*, *7*).

In recent years, protease inhibitors have received new interest because of their potent activity in preventing carcinogenesis in a wide variety of in vivo and in vitro systems (*8*). Serine protease inhibitors have been reported to have inhibitory effects on tumor cell growth (*9*, *10*). In addition, by increasing the level of cholecystokinin via the inhibition of trypsin, serine protease inhibitors can also be used to reduce food intake in man (*11*).

In potatoes, a wide range of protease inhibitors is expressed (*12*). Potato tuber contains ∼1.5% (w/w) protein on a fresh weight basis (*12*). In cv. Elkana, protease inhibitors represent ∼50% of the total amount of soluble protein present in the tuber (*13*). According to Cleveland et al. (*14*), potato inhibitor 2 (PI-2) is the most abundant protease inhibitor, representing 5% of the total amount of soluble proteins in the tuber, showing activity against serine proteases such as trypsin and chymotrypsin. According to Bryant et al. (*15*), PI-2 is a dimeric protein with two identical, as determined by amino acid analysis, subunits of 10 kDa. However, according to McManus et al. (*16*), PI-2 is a dimeric protein with two subunits that differ in size as determined by SDS-PAGE (15 kDa for the larger subunit and 6 kDa for the smaller one).

Although Pouvreau et al. (*13*) found that the most abundant group of protease inhibitors also consists of a dimeric protein, with two subunits held together by a disulfide bridge, preliminary data obtained subsequently in our laboratory cast doubt on the identification of this group of proteins as PI-2. This protein group represents ∼22% of the total amount of protein in potato and was thus called PI-2 on the basis of its abundance (*14*), dimeric structure (*16*), and inhibitory activity (*15*). PI-2 6.1 and PI-2 6.5 are the two most abundant isoforms of the PI-2 group in potato juice (*13*) and therefore were used for further characterization.

The present study was, therefore, undertaken to investigate the nature of the most abundant protease inhibitor in potato (cv. Elkana) by identification and sequencing of its gene and by comparison of the binding of various protease inhibitors to polyclonal antibodies against this protease inhibitor.

MATERIALS AND METHODS

Materials. Potatoes of cv. Elkana (Avebe b.a., Veendam, The Netherlands) were stored at 4 °C in the dark at a relative humidity of 95−100% for a maximum period of 6 months.

Methods. *Preparation of Potato Juice.* Potatoes were chopped into large pieces (maximum 8 × 2.5 cm) and subsequently mixed in the

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presence of sodium bisulfite at a dosage of 0.5 g/kg of potatoes to prevent oxidation of phenolic compounds. Potato juice was prepared as described previously (*13*).

Purification of PI-2 Isoforms. The ÄKTA explorer protein chromatography system and the columns used for the protein purification were obtained from Amersham Biosciences (Uppsala, Sweden). The absorbance of the eluates was monitored at 280 and 320 nm.

PI-2 6.1 and 6.5 were purified according to the method of Pouvreau et al. (*13*). An additional chromatofocusing purification step was included, using a Polybuffer Exchanger 74 column (60 × 1.6 cm). The column was equilibrated with 0.025 M imidazole−HCl buffer, pH 7.4. The fractions corresponding to PI-2 6.1 and 6.5 (*13*) were loaded onto the column. The protein was eluted using Polybuffer 74−HCl, pH 5.0 (dilution factor 1:8) (Amersham Biosciences, Uppsala, Sweden). Fractions were collected and pooled. The Polybuffer was removed by hydrophobic interaction chromatography using an HP phenyl-Sepharose column (10 × 2.6 cm) (*Chromatofocusing Handbook*, Amersham Biosciences, Uppsala, Sweden). The chromatofocusing step resulted in the removal of some minor contaminants. After purification, the purified PI-2 6.1 and 6.5 were dialyzed at 4 °C against 7 mM sodium phosphate buffer, pH 7.5 (ionic strength of 15 mM). After dialysis, the samples were frozen in small volumes and stored until use at a concentration of 1 mg/mL.

Protein Purity. SDS-PAGE, in the presence and absence of 2-mercaptoethanol, was performed with a Pharmacia PhastSystem (Amersham Biosciences) according to the instructions of the manufacturer using gradient 8−25% gels and Coomassie brilliant blue R-250 staining.

Mass Spectrometry. MALDI-TOF MS analysis in the linear mode was performed using a Voyager DE RP instrument (Perseptive Biosystems, Framingham, MA) as described previously (*13*).

Polyclonal Antibody Production against PI-2 Purified from Cv. Elkana. Polyclonal antibodies were raised against PI-2 6.1 and PI-2 6.5 (*13*) in rabbits. A standard immunization protocol was carried out for 3 months at the Sequence Laboratories Göttingen GmbH (Göttingen, Germany).

ELISA. The antisera were diluted $20-10^5$ times for determination of the affinity for the two PI-2 isoforms. For the cross-reactivity, the antisera were diluted 5000 times. This dilution (for both antibodies) showed, for the two PI-2 isoforms, still a maximum absorbance (2.5−3) in the assay. Higher dilutions resulted in a lower absorbency. Poly(vinyl chloride) microtiter plates (Dynatech, Chantilly, VA) were coated overnight with 100 μL of 5 μg/mL protein in 0.07 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl (PBS). Hereafter, plates were washed three times with PBS containing 0.05% (w/w) Tween 20 (PBST). Antibodies against PI-2 6.1 and PI-2 6.5 were diluted 5000 times in PBST, and 100 μL of the appropriate antibody was added to the wells. After 1.5 h of incubation, the plates were washed three times with PBST. To each well was added 100 μL of 0.025% (v/v) anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO) in PBST containing 1% (w/w) bovine serum albumin. After 1.5 h of incubation, the plates were washed three times with PBST. Color development was performed by adding 100 μL of 1 M diethanolamine buffer, pH 9.8, containing 0.5 mM $MgCl_2$ and 1 g/L *p*-nitrophenyl phosphate. The reaction was stopped after 15 min by adding 100 μL of 3 M NaOH, and the absorbance was measured at 400 nm. All incubations were performed at ~20−22 °C. Each ELISA experiment was performed in duplicate, and the results for PI-2 6.1 and PI-2 6.5 were corrected for nonspecific reactivity by subtracting absorbances obtained using nonimmune sera.

N-Terminal Amino Acid Sequence Determination. For protein sequencing, the subunits of PI-2 6.1 were separated by SDS-PAGE (18% acrylamide) in the presence of 2-mercaptoethanol and transferred onto a poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad, Hercules, CA) using a Mini Trans-Blot apparatus (Bio-Rad, Hercules, CA) with 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol as transfer buffer. The membrane was thoroughly washed with water and subsequently with 100% methanol. The blot was stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol containing 5% (v/v) acetic acid. After destaining with 50% (v/v) methanol containing 10% (v/v) acetic acid, the bands were excised. The N-terminal amino acid sequence was determined by the Sequence Centre of Utrecht University (Utrecht, The Netherlands).

Table 1. Primers Used for PCR Amplification

name	sequence (5′–3′)
ST	ATGAAGTGTTTATTTTTGTTATG
FA	CTACCTAGTGATGCTACTCCA
FB	CTACCCAGTGATGCTACTCCA
RF	CAAACGAGGATCAAGTTCTTTAC
RE	TTA(C/T)TGGACTTGTTTGAAGGAGA

DNA Sequencing and Sequence Analysis. The N-terminal amino acid sequences for both subunits of PI-2 6.1 were used for a homology search. An identity of 100% was obtained with potato serine protease inhibitor (PSPI), which was purified and sequenced by Valueva et al. (*17*). The gene encoding for PI-2 6.1 and fragments of the gene were amplified by PCR using pairs of degenerate primers (**Table 1**) based on the N-terminal sequence and the DNA sequence from identical potato proteins from the GenBank database.

To clone the gene of interest, RNA was extracted from the potato tuber according to the method of Kuipers et al. (*18*). The first centrifugation step in the presence of phenol and RNA extraction buffer [50 mM Tris pH 9.0, 10 mM EDTA, 2% (w/v) SDS] was extended to 1 h to obtain a clear top phase.

After synthesis of the first-strand cDNA using a RevertedAid TM H Minus First Strand cDNA synthesis kit (MBI Fermentas GmbH, St Leon-Rot, Germany), a nested PCR amplification of the gene or part of the gene was performed using the deduced primers (**Table 1**). PCR products were ligated with a pGEM-T easy vector according to the instructions of the manufacturer (Progema Corp., Madison, WI). Transformation of the ligated vector into *Escherichia coli* XL1 Blue MRF′ cells was achieved by electroporation. Cells were subsequently grown on solid S−Gal plates (Sigma, St. Louis, MO) supplemented with ampicillin (50 μg/mL) at 37 °C. Colonies containing an insert (white colonies) were grown overnight in Luria−Bertina (LB) medium containing ampicillin (50 μg/mL) at 37 °C. Plasmids from these overnight cultures were isolated and purified using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and sequenced with an automated DNA Sequencer 373 (Applied Biosystems, Foster City, CA). The DNA sequence data were submitted to the GenBank Nucleotide Databases under the accession number AY166690. The BLAST2 program (*19*) was used for searching sequence homologies.

RESULTS AND DISCUSSION

Protein Quaternary Structure. To define its quaternary structure, the molecular weight of PI-2 purified from cv. Elkana was investigated using two different methods: SDS-PAGE and MALDI-TOF MS.

SDS-PAGE of PI-2 6.1 and PI-2 6.5 under nonreducing conditions showed for both isoforms one single band at ±20 kDa and under reducing conditions two bands of ≈15 kDa and ≈6 kDa, respectively (**Figure 1**), confirming the data of McManus et al. (*16*). This pattern also shows that both subunits are linked by one or more disulfide bridges.

MALDI-TOF MS experiments were carried out under the same conditions. The MALDI-TOF MS spectrum of PI-2 6.1 showed two peaks at 10136 and 20273 Da, which correspond to the doubly and singly charged ion of the same protein, respectively (**Figure 2A**). To determine the M_w of the subunits, the cysteinyl residues were blocked using iodoacetamide (*20*). The MALDI-TOF MS spectrum showed one single peak at 4229 Da, which could be assigned to the small subunit (**Figure 2B**). The peak corresponding to the large subunit could not be detected. However, from the combined results of MALDI-TOF MS and SDS-PAGE, it can be concluded that the PI-2 from cv. Elkana (*13*) was not the one described by Bryant et al. (*15*) because the masses of the two subunits are not identical. Also,

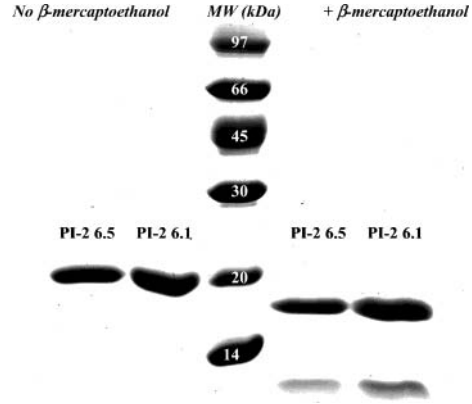

Figure 1. SDS-PAGE of PI-2 isoforms of cv. Elkana in absence and in the presence of 2-mercaptoethanol.

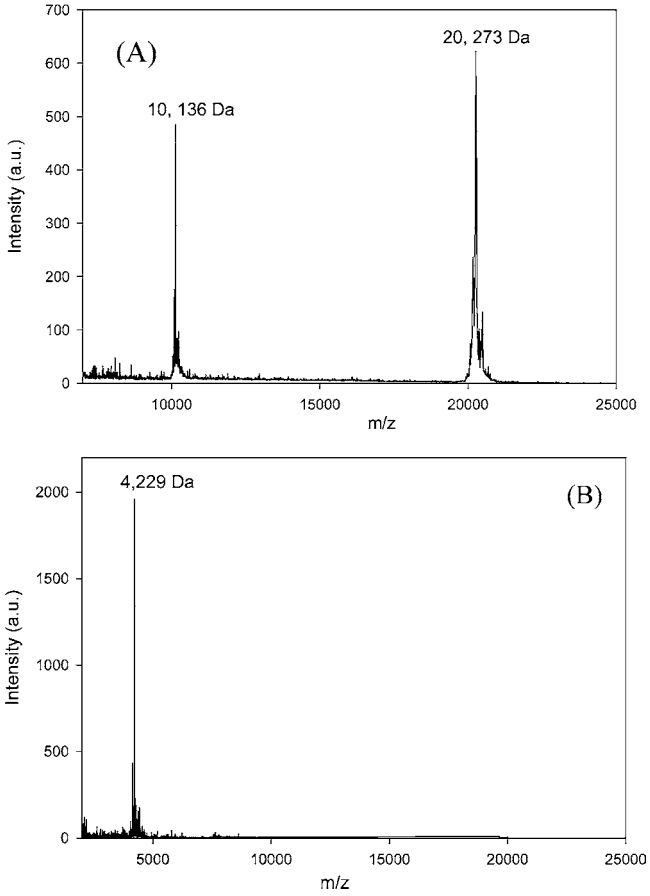

Figure 2. MALDI-TOF MS spectra of PI-2 6.1: (A) in native state; (B) with cysteinyl residues blocked.

the small subunit, determined by MALDI-TOF MS, is smaller as described for PI-2 by McManus et al. (*16*).

N-Terminal Sequencing. The large and small subunits of PI-2 6.1 had N-terminal amino acid sequences of LPSDATPVLDVTGKELDSRL and SSDDQFCLKVGVVHQNGKRRLALVKD, respectively. Both sequences were used for a homology search and showed 100% identity to a protein purified and sequenced by Valueva et al. (*17*), which was called potato serine protease inhibitor (PSPI). According to Valueva at al. (*17*), PSPI is a dimeric protein that belongs to the superfamily of Kunitz protease inhibitors. It therefore appears that the protein from cv. Elkana (*13*) designated PI-2 is apparently a member of the Kunitz family.

We have previously reported the presence of another dimeric protease inhibitor (*13*), which contains two subunits with different masses that are also disulfide linked. This protein showed inhibitory activity against trypsin, chymotrypsin, and elastase but represented only <1% of the total amount of protein in potato. Further characterization will be necessary to determine if this protein from cv. Elkana is PI-2 as described by McManus et al. (*16*).

DNA Sequence Analysis. To obtain the full-length gene of PI-2 from cv. Elkana, cDNA was prepared from mRNA isolated from potatoes. Subsequently, using PCR, a gene of 666 nucleotides was amplified. **Figure 3** shows a comparison of the deduced amino acid sequence of PI-2 with amino acid sequences of other protease inhibitors from potato tuber and with a protease inhibitor from winged bean (all belonging to the Kunitz family). Members of the Kunitz family have in their primary structure some conserved residues, such as the four cysteinyl residues forming the two indispensable disulfide bridges present in all Kunitz protease inhibitors. **Figure 3** shows that several characteristic amino acids of the Kunitz superfamily are conserved in PI-2. The amino acid sequences of PSPI (*17*), a Kunitz inhibitor (PKI) (*21*), and two aspartic protease inhibitors (PIG and NDI) (*22*, *23*) showed 98, 95, 96, and 74% homology with PI-2 from cv. Elkana, respectively. The homology with a chymotrypsin inhibitor from winged bean (*24*), often used as representative of the Kunitz family, was only 32%. Although these proteins show high homology with PI-2 from cv. Elkana and PSPI (*17*), they do not consist of two polypeptide chains in their mature form and also differ considerably in their enzyme specificities. PKI shows specificity only for trypsin, whereas PI-2 and PSPI also show activity against human leukocyte elastase and chymotrypsin. In contrast, PIG and NDI inhibit only aspartic proteases. PI-2 6.1 from cv. Elkana (*13*), due to its high homology (98%) with PSPI and its inhibitory specificity, can be considered as one of the isoforms of PSPI present in potato tuber. Therefore, the proteins, which were assumed to be members of the PI-2 group, should be designated PSPI. PI-2 6.1 and PI-2 6.5 will thus be called PSPI 6.1 and PSPI 6.5, respectively. It can be concluded that in cv. Elkana, not PI-2 but PSPI is the most abundant group of protease inhibitors.

On the basis of its sequence, PSPI appeared to be expressed in potato as a single polypeptide chain. Subsequently, due to post-translational processing or during the folding stage of the protein, the protein obtains its mature form. According to the N-terminal sequence and the molecular weight obtained by MALDI-TOF MS and by comparison with the protein purified by Valueva et al. (*17*), the small subunit starts at position 157 (**Figure 3**). The six preceding amino acids (151−156) are probably deleted during the post-translational process. This processing may also explain the existence of seven different isoforms of PSPI in cv. Elkana, differing in molecular weight and p*I*, because length and site of deletion may slightly differ from one isoform to another.

To calculate the mass increase due to the acetylation, the number of cysteinyl residues in the protein has to be determined. Therefore, from the protein sequence, the number of cysteinyl groups was calculated. The molecular mass of the small subunit, containing only one cysteinyl residue, is 4229 Da (as determined by MALDI-TOF MS) minus 57 Da of the blocking agent, which results in a mass of 4172 Da. By deduction, the molecular mass of the large subunit is 16100 Da for PSPI 6.1. This result is in agreement with the calculated molecular mass from the amino acid sequence deduced from the gene (16073 and 4169 Da for the large and small subunits, respectively).

Binding of PSPI 6.1 and PSPI 6.5 Polyclonal Antibodies against Protease Inhibitor Groups from Cv. Elkana. Poly-

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          1          10         20         30         40        *50         60
1     LPSDAT---PVLDVTGKELDSRLSYRIISTFWGALGGDVYLGKSPNSDAPCANGVF--RYNSDVG
2     LPSDAT---PVLDVTGKELDSRLSYRIISTFWGALGGDVYLGKSPNSDAPCANGVF--RYNSDVG
3     LPSDAT---PVLDVTGKELDPRLSYHIISTFWGALGGDVYLGKSPNSDAPCANGIF--RYNSDVG
4     LPSDAT---PVLDVAGKELDSRLSYRIISTFWGALGGDVYLGKSPNSDAPCANGIF--RYNSDVG
5     LPSESPLPKPVLDTNGKELNPDSSYRIISIGRGALGGDVYLGKSPNSDAPCPDGVF--RYNSDVG
6     LPSSTADDDLV-DAEGNLVENGGTYYLLPHIW-AHGGGIETAKTGN-E-PCPLTVVRSPNE--VS

          70         80         90        *100        110        120
1     PSGTPVRFIGSSSHFGQGIFEDELLNIQFAISTSKMCVSYTIWKVGDYDASLGTMLLETGGTIGQ
2     PSGTPVRFIGSSSHFGQGIFENELLNIQFAISTSKLCVSYTIWKVGDYDASLGTMLLETGGTIGQ
3     PSGTPVRFIGSSSHFGQGIFENELLNIQFAISTSKLCVSYTIWKVGDYDASLGTMLLETGGTIGQ
4     PSGTPVRF----SHFGQGIFENELLNIQFAISTSKLCVSYTIWKVGDYDASLGTMLLETGGTIGQ
5     PSGTPVRF----IPLSGGIFEDQLLNIQFNIPTVKLCVSYTIWKVGNLNAYFRTMLLETGGTIGQ
6     -KGEPIRI--SSQFLSLFIPRGSLVALGFANPPS--CAASPWWTVVDSPQGPAVKLSQQKLPEKD

        130        140        ** 150        160 **     170        180
1     ADSSWFKIVKSSQFG---YNLLYCPVTTTMTLPFSSDDQFCLKVGVVH--QNGKRRLALVKDNPL
2     ADSSWFKIVKSSQLG---YNLLYCPVTS------SSDDQFCLKVGVVH--QNGKRRLALVKDNPL
3     ADSSWFKIVQSSQFG---YNLLYCPVTSTMSCPFSSDDQFCLKVGVVH--QNGKRRLALVKDNPL
4     ADSSWFKIVKSSQFG---YNLLYCPVTSTMSCPFSSDDQFCLKVGVVH--QNGKRRLALVKDNPL
5     ADSSYFKIVKLSNFG---YNLLYCPITPPFLCPFCRDDNFCAKVGVVI--QNGKRRLALVNENPL
6     ILV--FKFEKVSHSNIHVYKLLYCQHDEE--------DVKCDQYIGIHRDRNGNRRLVVTEENPL

          190
1     DVSF---KQVQ
2     DISF---KQVQ
3     DVSF---KQVQ
4     DVSF---KQVQ
5     DVLF----QEV
6     ELVLLKAKSETASSH
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Figure 3. Alignment of amino acid sequences of (1) PI-2 (AY166690) from potato tuber cv. Elkana with other proteins of the Kunitz inhibitor family: (2) PSPI, a serine protease inhibitor from potato (*17*); (3) PKI (U30388), a protease inhibitor from potato (*21*); (4) PIG, an aspartate protease inhibitor (S66277) from potato (*22*); (5) NDI, an aspartate protease inhibitor (P17979) from potato (*23*); (6) WCI, a chymotrypsin inhibitor (P10822) from winged bean (*24*). Amino acid residues are numbered according to the sequence of PI-2 from potato tuber: in box, sequence from the N-terminal sequence; gray shading, the amino acids that are cleaved during the post-translational process in plant; conserved amino acid are slashed; * and **, disulfide bonds Cys48–Cys97 and Cys146–Cys157; arrows mark the P1 residues of the active sites of the inhibitor.

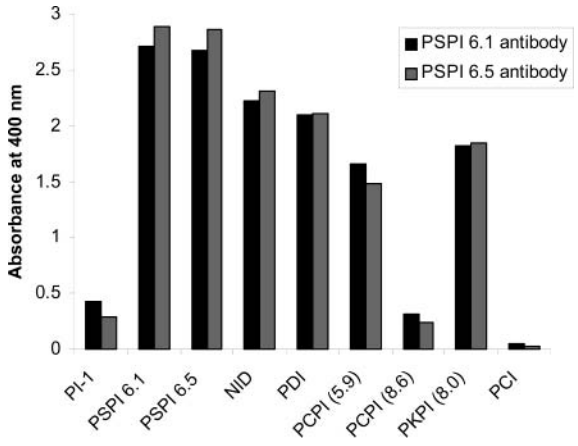

Figure 4. Binding of PSPI 6.5 and 6.1 antibodies against PSPI isoforms and potato protease inhibitors from different families (*13*): PI-1, potato inhibitor I; PI-2, potato inhibitor II; NID, novel inhibitor of cathepsin D; PDI, potato cathepsin D inhibitor; PCPI, potato cysteine protease inhibitor; PKPI, potato Kunitz protease inhibitor; PCI, potato carboxypeptidase inhibitor.

clonal antibodies were raised against PSPI 6.1 and 6.5. These antibodies showed the same affinity for both isoforms of PSPI (data not shown). To study the structure homology between the different families of protease inhibitors, the cross-reactivity of these antibodies was tested for purified representative protease inhibitors from potato cv. Elkana (*13*) (**Figure 4**).

Both antibodies showed a high binding for novel cathepsin D inhibitor (NID), potato cathepsin D inhibitor (PID), PCPI 5.9 (a potato cysteine protease inhibitor), and PKPI 8.0 (potato Kunitz protease inhibitor) but a low binding for PCPI 8.6, PI-1 (potato inhibitor 1), and potato carboxypeptidase inhibitor (PCI) (*13*). According to the literature (*25*−*28*), NID, PDI, PCPI 5.9, and PKPI 8.0 are indeed also members of the Kunitz family. The antibodies raised against PSPI therefore seem to show a high binding for members of the Kunitz family and only a low binding for other proteins. With these results, it appears that ∼70% of the total amount of protease inhibitors in potato are members of the Kunitz family.

PCPI 8.6, which was purified by Pouvreau et al. (*13*), was described as a new protease inhibitor in potato juice. This protease inhibitor did not bind to the antibodies and is therefore probably not a member of the Kunitz family.

Conclusion. The research described in this paper, combined with our previous work (*13*) leads to the conclusion that the most abundant protease inhibitor in cv. Elkana, and presumably in potato in general, is PSPI. PSPI is a group of proteins that, on the basis of its gene sequence, is expressed as one polypeptide chain and subsequently processed by removal of about six amino acids.

The binding of antibodies raised against PSPI isoforms to potato protease inhibitors shows that most of the protease inhibitors in potato tuber belong to the Kunitz-type family and represent ∼70% of the total amount of protease inhibitor in potato juice.

LITERATURE CITED

(1) Ryan, C. A. Proteolytic enzymes and their inhibitors in plant. *Annu. Rev. Plant Physiol.* **1977**, *24*, 173−196.

(2) Garcia-Olmeda, F.; Sakedo, G.; Sanchez-Monge, R.; Gomez, L.; Royo, J.; Carbonero, P. Plant proteinaceous inhibitors of proteinases. *Oxf. Surv. Plant Cell Mol. Biol.* **1987**, *4*, 275−334.

(3) Putzai, A.; Grant, G.; Stewart, J. C.; Watt, W. B. Isolation of soybean trypsin inhibitors by affinity chromatography on anhydrotrypsin-Sepharose 4B. *Anal. Biochem.* **1988**, *172*, 108−112.

(4) Bergey, D. R.; Howe, G. A.; Ryan, C. A. Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12053−12058.

(5) Jongsma, M. A.; Bakker, P. L.; Peters, J.; Bosch, D.; Stiekema, W. J. Adaptation of *Spedoptera exigua* larvae to plant proteinase inhibitors by induction of proteinase activity insensitive of inhibition. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 8041−8045.

(6) Conconi, A.; Smerdson, M. J.; Howe, G. A.; Ryan, C. A. The octadecanoid signalling pathway in plants mediates a response to ultraviolet radiation. *Nature* **1996**, *383*, 826−829.

(7) Graham, J. S.; Pearce, G.; Merryweather, J.; Titani, K.; Ericsson, L. H.; Ryan, C. A. Wound-induced proteinase inhibitors from tomato leaves. II. The cDNA-deduced primary structure of the pre-inhibitor II. *J. Biol. Chem.* **1985**, *260*, 6561−6564.

(8) Kennedy, A. R. Chemopreventive agents: protease inhibitors. *Pharmacol. Ther.* **1998**, *78*, 167−209.

(9) Kennedy, A. R. The Bowman−Birk inhibitor from soybeans as an anticarcinogenic agent. *Am. J. Clin. Nutr.* **1998**, *68*, 1406S−1412S.

(10) Huang, C.; Ma, W.-Y.; Ryan, C. A.; Dong, Z. Proteinase inhibitors I and II from potatoes specifically block UV-induced activator protein-1 activation through a pathway that is independent of extracellular signal-regulated kinases, c-jun N-terminal kinases, and P38 kinase. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11957−11962.

(11) Hill, A. J.; Peikin, S. R.; Ryan, C. A.; Blundell, J. E. Oral administration of proteinase inhibitor II from potatoes reduces energy intake in man. *Physiol. Behav.* **1990**, *48*, 241−246.

(12) Lisinska, G.; Leszczynski, W. *Potato Science and Technology*; Elsevier Applied Science: London, U.K., 1989.

(13) Pouvreau, L.; Gruppen, H.; Piersma, S. R.; van den Broek, L. A. M.; van Koningsveld, G. A.; Voragen, A. G. J. Relative abundance and inhibitory distribution of protease inhibitors in potato juice from cv. Elkana. *J. Agric. Food Chem.* **2001**, *49*, 2864−2874.

(14) Cleveland, T. E.; Thornburg, R. W.; Ryan, C. A. Molecular characterization of a wound-inducible inhibitor I gene from potato and the processing of its mRNA and protein. *Plant Mol. Biol.* **1987**, *8*, 199−207.

(15) Bryant, J.; Green, T. R.; Gurusaddaiah, T.; Ryan, C. A. Proteinase inhibitor II from potatoes: isolation and characterization of its protomers components. *Biochemistry* **1976**, *15*, 3418−3423.

(16) McManus, M. T.; Laing, W. A.; Christeller, J. T.; White, D. W. R. Posttranslational modification of an isoinhibitor from the potato proteinase inhibitor II gene family in transgenic tobacco yields a peptide with homology to potato chymotrypsin inhibitor I. *Plant Physiol.* **1994**, *106*, 771−777.

(17) Valueva, T. A.; Revina, T. A.; Mosolov, V. V.; Mentele, R. Primary structure of potato Kunitz-type serine proteinase inhibitor. *Biol. Chem. Hoppe-Seyler* **2000**, *381*, 1215−1221.

(18) Kuipers, A. G. J.; Jacobsen, E.; Visser, R. G. F. Formation and deposition of amylose in the potato tuber starch granule are affected by the reduction of granule-bound starch synthase gene expression. *Plant Cell* **1994**, *6*, 43−52.

(19) Altschul, S. F.; Madden, T. I.; Schäffer, A. A.; Zhang, J.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI−BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389−3402.

(20) Gerwin, B. I. Properties of the single sulfhydryl group of streptococcal proteinase. A comparison of the rates of alkylation by chloroacetic acid and chloroacetamide. *J. Biol. Chem.* **1967**, *242*, 451−456.

(21) Banfalvi, Z.; Molnar, A.; Molnar, G.; Lakatos, L.; Szabo, L. Starch synthesis-, and tuber storage protein genes are differently expressed in *Solanum tuberosum* and in *Solanum brevidens*. *FEBS Lett.* **1996**, *383*, 159−164.

(22) Maganja, D. B.; Strukelj, B.; Pungercar, J.; Gubensek, F.; Turk, V.; Kregar, I. Isolation and sequence analysis of the genomic DNA fragment encoding an aspartic proteinase inhibitor homologue from potato (*Solanum tuberosum* L.). *Plant Mol. Biol.* **1992**, *20*, 311−313.

(23) Strukelj, B.; Pungercar, J.; Mesko, P.; Barlic-Maganja, D.; Gubensek, F.; Kregar, I.; Turk, V. Characterization of aspartic proteinase inhibitors from potato at the gene, cDNA and protein levels. *Biol. Chem. Hoppe-Seyler* **1992**, *373*, 477−482.

(24) Peyachoknagul, S.; Matsui, T.; Shibata, H.; Hara, S.; Ikenaka, T. Sequence and expression of the mRNA encoding the chymotrypsin inhibitor in winged bean (*Psophocarpus tetra gonolobus* (L.) DC). *J. Biochem. (Tokyo)* **1989**, *111*, 249−258.

(25) Ritonja, A.; Krizaj, I.; Mesko, P.; Kopitar, M.; Lucovnik, P.; Strukelj, B.; Pungercar, J.; Buttle, D. J.; Barrett, A. J.; Turk, V. The amino acid sequence of a novel inhibitor of cathepsin D from potato. *FEBS Lett.* **1990**, *267*, 13−15.

(26) Mares, M.; Meloun, B.; Pavlik, M.; Kostka, V.; Baudys, M. Primary structure of cathepsin D inhibitor from potatoes and its structure relationship to soybean trypsin inhibitor family. *FEBS Lett.* **1989**, *251*, 94−98.

(27) Brzin, J.; Popovic, T.; Drobnic-Kosorok, M.; Kotnik, M.; Turk, V. Inhibitors of cysteine proteinases from potato. *Biol. Chem. Hoppe-Seyler* **1988**, *369*, 233−238.

(28) Mitsumori, C.; Yamagishi, K.; Fujino, K.; Kikuta, Y. Detection of immunologically related Kunitz and Bowman−Birk proteinase inhibitors expressed during potato tuber development. *Plant Mol. Biol.* **1994**, *26*, 961−969.

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