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The primary structure of inhibitor of cysteine proteinases from potato

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The complete amino acid sequence of the cysteine proteinase inhibitor from potato tubers was determined. The inhibitor is a single-chain protein having 180 amino acid residues. Its primary structure was elucidated by automatic degradation of the intact protein and sequence analysis of peptides generated by CNBr, trypsin and glycyl endopeptidase. A search through the protein sequence database showed homology to other plant proteinase inhibitors of different specificities and non-inhibitory proteins of M_r around 20,000. On the basis of sequence homology, prediction of secondary structure and fold compatibility, based on a 3D–1D score to the three-dimensional profile of *Erythrina caffra* trypsin inhibitor, we suggest that the potato cysteine proteinase inhibitor belongs to the superfamily of proteins that have the same pattern of three-dimensional structure as soybean trypsin inhibitor. This superfamily would therefore include proteins that inhibit three different classes of proteinases – serine, cysteine and aspartic proteinases.

Cysteine proteinase inhibitor; Amino acid sequence; *Solanum tuberosum*; Soybean trypsin inhibitor superfamily

1. INTRODUCTION

Protein inhibitors of cysteine proteinases (CPIs), with M_rs from 10,000 to 13,000, have been isolated from various human and animal tissues [1,2], as well as from plants [3,4]. Their characterization revealed that they are structurally related proteins, forming the cystatin superfamily. The general mode of action of this group of inhibitors has been proposed on the basis of the crystal structure of chicken cystatin and a docking model of its interaction with papain [5], and was confirmed by X-ray structure of human stefin B in complex with papain [6].

Recently, a group of a new type of CPI was isolated from potato tuber (PCPI) [7]. PCPIs are single-chain proteins with M_rs ranging from 22,000 to 25,000, with different isoelectric points (pI) and inhibitory properties [7]. N-terminal sequences of some PCPIs have shown that they are homologous proteins related to the Kunitz-type soybean trypsin inhibitor (STI) [8]. The inhibitory specificity of the PCPI 8.3, the structure of which is described in this article, towards several human and plant proteinases was determined and compared with that of chicken egg white cystatin. It was characterized as a potent inhibitor of lysosomal proteinase cathepsin L with a K_i 0.07 nM [9].

A protein inhibitor of cathepsin D was also isolated from potato and was shown to possess homology to STI [10–12]. Recently, three proteins, of so far unknown function, were purified from potato tuber [13]. Their N-terminal sequences were homologous to STI and matched the amino acid sequence deduced from the cDNA sequence of an abundant potato tuber protein [14].

In this work the complete primary structure of the major form of PCPI with pI 8.3 (PCPI 8.3) is reported and compared with the protein structure database.

2. MATERIALS AND METHODS

PCPI 8.3 was purified from potato tubers as described previously [7]. Sequentially pure inhibitor was reduced with β-mercaptoethanol and afterwards S-alkylated in two different ways with iodoacetic acid (Sigma, USA) and 4-vinylpyridine (Fluka, Switzerland) [15].

CNBr cleavage of alkylated PCPI 8.3 was made in 85% trifluoroacetic acid, with a 50-fold molar excess of the reagent (Pierce, USA), under reducing conditions. The reaction, which took place in the dark at room temperature for 24 h was quenched with a 10-fold dilution of the reaction mixture with distilled water and freeze dried.

Hydrolysis by β-trypsin (EC 3.4.21.1) was made on carboxymethyl-PCPI 8.3 (CM-PCPI 8.3) and on maleylated, pyridylethylated-PCPI 8.3 (PE-PCPI 8.3). Maleylation, as well as demaleylation, was accomplished according to the procedure of Butler and Hartley [16]. The cleavage conditions in both cases were 0.1 M *N*-methylmorpholine/acetate buffer, pH 8.00, 38°C and 2-h incubation with 2.5% (w/w) β-trypsin [17].

Glycine-specific papaya proteinase IV (PP IV) (glycyl endopeptidase; EC 3.4.22.25) [18] cleavage of PE-PCPI 8.3 was performed in 0.1 M sodium phosphate buffer, pH 6.52, containing 1 mM EDTA and 2 mM dithiothreitol. The reaction was carried out at room temperature for 20 h with 2% (w/w) proteinase.

Peptide mixtures were separated by HPLC (Milton Roy, USA) on

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Abbreviations: CPI, cysteine proteinase inhibitor; PCPI, potato cysteine proteinase inhibitor; MRC, miraculin; STI, soybean trypsin inhibitor; ETI, *Erythrina caffra* trypsin inhibitor; WASI, wheat α-amylase/proteinase K (subtilisin) inhibitor; NID, novel inhibitor of cathepsin D; PP IV, papaya proteinase IV; CNBr, cyanogen bromide; CM-, carboxymethyl-; PE-, pyridylethyl-; HPLC, high performance liquid chromatography; 3D, threedimensional.

Chrompack reverse-phase C_8 and C_{18} columns (100 × 3 mm) equilibrated with 0.1% trifluoroacetic acid in water and eluted by various linear gradients of 80% acetonitrile containing 0.1% trifluoroacetic acid. The flow rate was 1 ml/min. Also gel filtration chromatography on Sephacryl S-200 HR (Pharmacia, Sweden) was used for peptide purification. Column (150 × 0.5 cm) was eluted with 50% HCOOH at a flow rate 2.4 ml/h. Absorbance was monitored at 215 and 280 nm, respectively.

Amino acid analyses of peptide hydrolysates, obtained after 24-h hydrolysis in 6 M HCl at 110°C, were performed by HPLC (Milton Roy, USA), using an ODS 2 column and pre-column *o*-phthalaldehyde derivatization.

Applied Biosystems (USA) liquid-pulsed protein sequencer 475A, on-line connected to phenylthiohydantoin amino acid analyser 120A from the same manufacturer, was used for the amino acid sequence determination.

A search through the PIR (Protein Identification Resource), release 33 protein database of protein sequences was performed by the program SEARCH [19]. A database search employing the 3D profile was done using the set of programs of Bowie et al. [20]. The protein alignment was supported by running the package for multiple sequence alignment ClustalV [21]. Secondary structure was predicted according to the method of Robson and Garnier [22]. Alignment was presented using programs Maligned [23] and Boxshade (program by Kay Hofmann). Tertiary structures of *Erythrina caffra* trypsin inhibitor (1TIE) and human interleukin 1 β (1IlB) were obtained from the Brookhaven Protein structure database [24,25].

3. RESULTS AND DISCUSSION

The native PCPI 8.3 was subjected to N-terminal amino acid sequence analysis. A single sequence was obtained up to amino acid residue 41 (uncleaved molecule in Fig. 1).

PCPI 8.3 was reduced with β-mercaptoethanol and alkylated with either iodoacetic acid or 4-vinylpyridine.

PE-PCPI 8.3 was cleaved with CNBr and the cleavage products separated by gel-filtration chromatography. 50% HCOOH was used as eluent. The resulting four peptides were sequenced (CN1–CN4 in Fig. 1). In the case of CN3 the first two amino acid residues could not be identified, after the third residue the sequence was clear up to Lys^{113}. The yields of CN2 and CN3 were normal (more than 50%) in spite of the unfavourable Met^{50}-Ser^{51} and Met^{79}-Thr^{80} peptide bonds which preceded them.

The second set of peptides was prepared by trypsin cleavage of CM- and PE-PCPI 8.3 (T peptides in Fig. 1). As the alkylated molecule, in both cases, was only barely soluble in the digestion buffer, the precipitated protein was thoroughly suspended by sonication before the addition of the proteinase. Peptides in the soluble fraction of the digest were sequenced. T3 peptide provided the overlap between peptides CN1 and CN2. Peptides T10, T16 and T17 + 18 revealed new parts of the PCPI 8.3 structure. Peptide T17 + 18 represented the C-terminus of PCPI 8.3, as the C-terminal Ala residue would not have been a site of cleavage by β-trypsin.

In order to increase the solubility of the unfolded PCPI 8.3, PE-PCPI 8.3 was maleylated. Indeed, maleylated protein dissolved readily in the tryptic cleavage buffer. Following fragmentation by β-trypsin and separation, peptides were demaleylated and subjected to amino acid sequence analysis (R peptides in Fig. 1). Peptide R5 gave the structure of the very hydrophobic core of the inhibitor. It provided the overlap between peptides CN2 and CN3 and positioned the peptide T10. R6 peptide confirmed the structure of the C-terminus of PCPI 8.3.

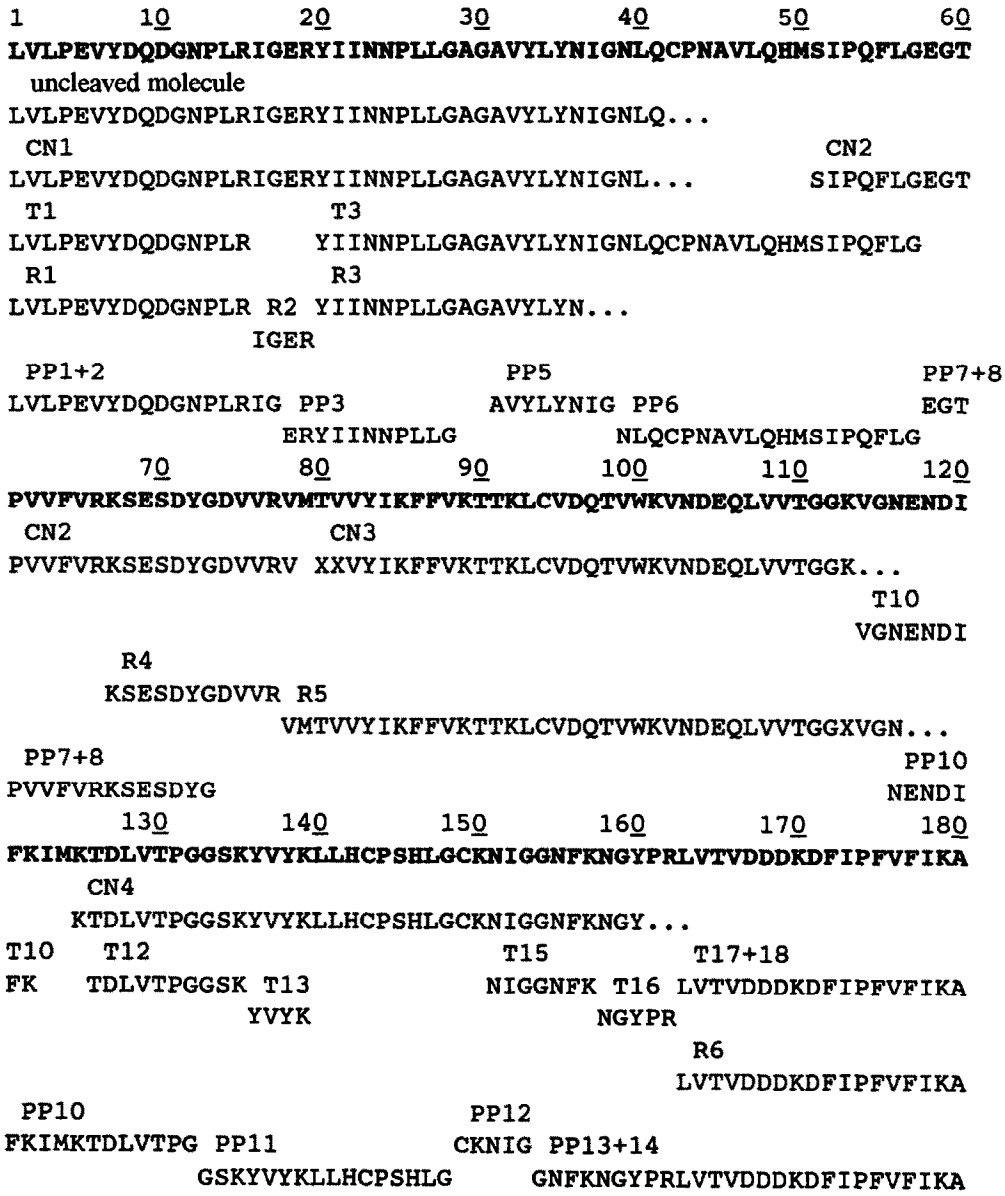
The last set of PCPI 8.3 peptides was obtained with glycyl endopeptidase (PP peptides in Fig. 1). The peptide from the central part of the molecule was not found in the soluble part of the reaction mixture. The last overlaps were provided with the peptides PP10 (between T10, T12 and CN4) and PP13 + 14 (between T16 and T17 + 18) which also represented the C-terminal PP peptide.

PCPI 8.3 is a single-chain protein, comprising 180 amino acid residues. Its calculated M_r is 20,085 (assuming 2 disulphide bonds).

The primary structure of PCPI 8.3 differs only in one amino acid residue from the published cDNA sequence encoding a 22-kDa potato tuber protein (Gly replacing Arg in position 132, Fig. 1), which was thought to be a putative inhibitor of the serine proteinases [14]. This and previous work [9], however, established that PCPI 8.3 is an inhibitor of the cysteine proteinases. In addition it was also known that PCPI 8.3 does not inhibit trypsin and chymotrypsin [8].

A search through the protein sequence database pointed to a homology to proteins related to Kunitz type trypsin inhibitors. Homology of about 20% was found with trypsin inhibitors of the soybean trypsin inhibitor family [26], aspartic proteinase inhibitors from potato [27,10] and non-inhibitory proteins miraculin (MRC) [28] and plant albumin [29]. Up to now, the tertiary structures of three proteins of this superfamily have been determined – STI [30], *Erythrina caffra* trypsin inhibitor (ETI) [31] and wheat α-amylase/proteinase K (subtilisin) inhibitor (WASI) [32]. Prediction of secondary structure has shown that PCPI 8.3 is predominantly a β-type protein having 58% of β-structure, which is in accordance with the percentage of β-structure deduced from the tertiary structures of STI, ETI and WASI (Fig. 2).

Since the primary structure revealed homology in the 'twilight zone' we employed a three-dimensional profile search to identify possible three-dimensional homology. A search through the database was performed using the 3D profile of ETI. A profile was constructed from the tertiary structure by classifying each residue into one of 18 classes, depending on its accessibility to the solvent, polarity of its surroundings and secondary structure [20]. A search through the primary structure database using such a 3D–1D profile has a high probability of identifying protein sequences that can fold into the same three-dimensional structure, i.e. which are compatible with the same structural framework. The *Z* score of



Ala A 4	Gln Q 6	Leu L 16	Ser S 5
Arg R 5	Glu E 6	Lys K 14	Thr T 9
Asn N 12	Gly G 17	Met M 3	Trp W 1
Asp D 12	His H 3	Phe F 9	Tyr Y 9
Cys C 4	Ile I 11	Pro P 10	Val V 24

Total: 180 residues, M_r 20085

Fig. 1. The amino acid sequence and composition of PCPI 8.3 and the strategy of its determination. Peptides designated as CN were obtained with CNBr cleavage. T and R peptides resulted from tryptic fragmentation of PCPI 8.3 and maleylated PCPI 8.3, respectively. PP peptides are from glycyl endopeptidase (PP IV) hydrolysis of the inhibitor molecule. The residues which were not directly identified are marked (X).

PCPI 8.3, based on the 3D–1D profile of 1TIE was 6.84. It was resolved together with a group of trypsin inhibitors (Z scores 5–11), aspartic proteinase inhibitors from potato (Z scores 4.6–5.8) and some other, homologous, non-inhibitory proteins, as MRC or plant albumin, from the rest of proteins in the database.

It was found out earlier that the structure of interleukin-1α and β, as well as fibroblast growth factor, have the same fold as STI, despite the lack of any detectable sequence homology [31,33]. A scan of the database with the 3D–1D profile of interleukin 1β assigned to PCPI 8.3 a Z score of 4.5, and 4.1–4.6 to aspartic pro-

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PCPI      LVLPE.VYDQDGNPLRIGERYIINNPLLGA..GAVYLYNIGNLQCPNAVLQHMSIPQFLGEGT
pred.SS   -----.-->>>****----------**--..--------->>--->-XXX---------*>**
3D ETI         ***>>>>>**>>>>------*>>.>>>>*----*>>>>>***----**>.>>>****-


PCPI      PVVFVRKSESDYGDVVRVMTVVYIKF.FVKTTKLCVDQ.TVWKVNDEQLVV..TGGKVGNENDI
pred.SS   -------*>>>>>----------XX.XXXX>>X----.----*XXXX---..--*****XXXX
3D ETI    ----****.....***>>>>*-----***>>...>>****-.---*.******----*****>>


PCPI      FKIMKTDLVTPGGSKYVYKLLHCPSHL.GCKNIGG..NFKNGYPRLVTVDDDKDFIPFVFIKA
pred.SS   XXXX------*>>*----------->>>.>----->..>>>>**-------X>XXX-XXXXXXX
3D ETI    >>**-------->>>>.-------*>>>>*--------->>>.>*-----**********-.-****
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Fig. 2. Comparison of predicted secondary structure of PCPI (pred. SS) with aligned secondary structure elements deduced from tertiary structure of ETI. Symbols used in the semi-graphical representation: helical conformation, X; extended, -; turn, > ; coil, *; insertion in the sequence, *.

teinase inhibitors from potato, thereby further supporting the hypothesis of a structural relationship between PCPI 8.3 and the Kunitz type trypsin inhibitors.

Alignment of protein sequences (Fig. 3) was produced by aligning the structures of PCPI 8.3, NID (novel inhibitor of cathepsin D) and MRC against the sequence profile based on tertiary structure alignment of three inhibitors with known crystal structure, according to the algorithm of Gribskov et al. [34], as implemented by the program ClustalV [21]. Manual intervention was applied to include information from the secondary structure prediction in the sequence alignment. The homology of alignment of PCPI with STI, ETI, WASI, NID from potato and MRC was 23, 20, 23, 21 and 22%, respectively.

On the basis of sequence homology, prediction of secondary structure and fold compatibility based on a 3D–1D score of the three-dimensional profile of ETI,

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PCPI   LVLPE.VYDQDGNPLRIGERYIINNPLLGA..GAVYLYNI..GNLQCPNAVLQHMSIPQ
STI         DFVLDNEGNPLENGGTYYILSDIT.AFGG.IRAAPT..GNERCPLTVVQSRNE.L
ETI          VLLDGNGEVVQNGGTYYLLPQVW.AQGGGVQLAKT..GEETCPLTVVQSPNE.L
WASI      DPPPVHDTDGNELRADANYYVLPANR.AHGGGLTMAPG..HGRRCPLFVSQEADG.Q
NID    ESPLPKPVLDTNGKELNPNSSYRIISIGRGALGGDVYLGKSPNSDAPCPDGVFRY....N
MRC    DS.APNPVLDIDGEKLRTGTNYYIVPVLRDHGGGLTVSATTPNGTFVCPPRVVQTRKEVD

                          *
PCPI   FLGEGTPVVFVRKSESDYGDVVRVMTVVYIKF.FVKTTKLCVD..QTVWKVNDEQLVV..
STI    DKGIGTIISSPYRIR.....FIAEGHPLSLKFDSFAVIMLCVG.IPTEWSV.VEDLPEGP
ETI    SDGKPIRIESRLRSA.....FIPDDDKVRIGFAYAPK...CAP.SP.WWTV.VEDEQEGL
WASI   RDGLPVRIAPHGGAPSD..KIIRLSTDVRISFRAYTT...CV..QSTEWHI.DSELVSGR
NID    SD..VGPSGTPVRFIPLSGG.IFEDQLLNIQFN.IPTVKLCV..SYTIWKVGNLNAYFRT
MRC    HD.RPLAFFPENPKE....DVVRVSTDLNINFSAFMP...CRWTSSTVSRLDKYDESTGQ


PCPI   TGGKVGNENDI.....FKIMKTDLVTPGGSKYVYKLLHCPSHL.........GCKNIGG
STI    AVKIGENKDA......MDGWFRLERVSDDEFNNYKLVFCPQQA.......EDDKCGDIGI
ETI    SVKLSEDEST.....QFDYPFKFEQVSDQ.LHSYKLLYCEGKH.........EKCASIGI
WASI   RHVITGPVRD.PSPSGRENAFRIEKYSGAEVHEYKLMACG...........DSCQDLGV
NID    MLLETGGT...IGQADNSY.FKIVKLSN...FGYNLLSCPFTSIICLRCPEDQFCAKVGV
MRC    YFVTIGGVKGNPGPETISSWFKIEE.FCGSGF.YKLVFCP.T..VCGSCKV..KCGDVGI


PCPI   ..NFKNGYPRLVTVDDDKDFIPFVFIKA
STI    SIDHDDGTRRLVVSKNKPLVVQFQKLDKESL
ETI    NRDQK.GYRRLVVTEDYPLTVVLKK.DESS
WASI   FRDLKGGAWFLGATEPYHV.VVFKKAPPA
NID    VIQ..NGKRRLALVNENPLDVLFQEV
MRC    YIDQK.GRRRLALSDKPFAFEFNKTVYF
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Fig. 3. Alignment of the primary structures of PCPI 8.3, soybean trypsin inhibitor (STI), *Erythrina caffra* trypsin inhibitor (ETI), wheat α-amylase/subtilisin inhibitor (WASI), novel inhibitor of cathepsin D (NID) and miraculin (MRC). The P1 position of the scissile bond of STI, ETI and WASI is marked by *.

we suggest that PCPI 8.3 belongs to the superfamily of proteins that have the same pattern of three-dimensional structure as STI.

The superfamily of STI would thus comprise proteins which have inhibitory activity against serine proteinases (trypsin and subtilisin family), aspartic proteinases (inhibitors active against cathepsin D), cysteine proteinases (inhibitors to papain family) and proteins with some other activity or function, like MRC with its sweet taste-modifying activity, or plant albumin, presumably functioning as a storage protein. Some of the members have multiple activities, like inhibition of serine and aspartic proteinases (NID, [10]) or inhibition of α-amylase and subtilisin [32].

The active site of PCPI 8.3 against cysteine proteinases cannot be determined based on the information available. According to the alignment, instead of arginine at the P1 position of the scissile bond (marked by * in Fig. 3) in STI and ETI, lysine is positioned in PCPI 8.3. In the case of WASI this is also the site, located at the active site of proteinase K [32]. Conformation of the reactive loop in STI-type of trypsin inhibitors is conserved by a hydrogen bonding network [31]. Topology of the loop in PCPI 8.3 is probably disturbed, due to neighboring insertions, which can explain why PCPI 8.3 does not inhibit serine proteinases.

There are at least 16 different families of inhibitors of the serine proteinases and most of them obey the 'canonical' mechanism of inhibition [35,36]. Inhibition of trypsin with STI follows this model. In contrast, all of the known inhibitors of cysteine proteinases of the papain family were grouped into one structurally related superfamily of proteins [2]. The mechanism of inhibition of cysteine proteinases by cystatins differs from the standard model. Cystatins do not bind in a substrate-like manner and are not cleaved by the enzyme but rather form a non-covalent complex with the enzyme through predominantly hydrophobic interactions, thereby sterically preventing the access of a substrate to the active center [6].

The primary structure of PCPI 8.3 is the first cysteine proteinase inhibitor structure not homologous to the structure of cystatin. Whether the inhibition of PCPI 8.3 follows the inhibition mechanism of cystatins, by some other parts of the structure substituting for contact regions found in cystatins, or accomplished by some other mechanism, remains to be established.

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