Occurrence and physico-chemical properties of protease inhibitors from potato tuber

(*Solanum tuberosum*)

Promotor:

Prof. dr. Ir. A. G. J. Voragen

Hoogleraar in de levensmiddelenchemie

Co-promotores:

Dr. Ir. H. Gruppen

Universitair hoofdocent, leerstoelgroep Levensmiddelenchemie

Dr. G. A. van Koningsveld

Onderzoeker, leerstoelgroep Levensmiddelenchemie

Promotiecommissie:

Prof. dr. R. G. F. Visser

Wageningen Universiteit

Prof. dr. S.C. de Vries

Wageningen Universiteit

Prof. dr. B. Strukelj

University of Lubjana, Lubjana, Slovenia

Dr. A. A. van Dijk

DSM Food Specialities, Delft

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Occurrence and physico-chemical properties of protease inhibitors from potato tuber

(*Solanum tuberosum*)

Laurice Pouvreau

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ABSTRACT

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Protease inhibitors represent approximately 50% of the total amount of proteins in potato juice (cv. *Elkana*). Potato protease inhibitors have a broad spectrum of inhibitory activity, showing inhibition against serine proteases, cysteine proteases, aspartate proteases and metallo-proteases. They can be classified in 7 different groups based on the class of enzymes they inhibit and additionally on their molecular mass, their protein architecture and their isoelectric pH. Using an antibody assay it was shown that approximately 70% of the protease inhibitors in potato presumably belongs to the Kunitz-type inhibitors.

The most abundant group of protease inhibitors, denoted Potato Serine Protease Inhibitor (PSPI), is a Kunitz-type serine protease inhibitor, and represents approximately 44% of the total amount of protease inhibitors in potato. PSPI isoforms represent 80% and 50% of the total trypsin and chymotrypsin inhibiting activity, respectively. Based on its structural characteristics, PSPI was classified as a β-II protein; *i.e.* a protein in which most of the residues are involved in irregular and short β-sheets. PSPI shows no changes in secondary and tertiary structural characteristics in the pH range 3.0 to 7.5, at ambient temperature. Also, the heat induced unfolding behaviour does not change within this pH range. Unfolding studies showed that the thermal as well as the guanidinium-induced unfolding of PSPI occurs via a non-two state mechanism in which at least two parts of the protein unfold more or less independently. Aggregation, upon heating, seems to occur via a specific mechanism in which the end product is a tetrameric form of PSPI.

Isoforms of the Potato Cysteine Protease Inhibitor (PCPI) group showed very similar structural characteristics as those of PSPI, and were, therefore, also classified as β-II proteins. At pH 4.0, isoforms of PCPI unfold at approximately 67°C. Similar to PSPI, PCPI isoforms (especially for PCPI 8.3) do not unfold via a two state mechanism and at least one intermediate is present. Upon heating, PCPI aggregates are formed, but the size of the aggregates (>100kDa) differs from those of PSPI.

Recombinant Potato Inhibitor I (PI-1) showed very similar structural characteristics to those of PI-1 purified from potato, as well as a similar transition temperature (88°C). Using recombinant PI-1, it was suggested that PI-1 is a hexameric protein rather than a pentamer, as previously described in literature. Differential scanning calorimetry analysis showed that PI-1 unfolds having a dimer instead of a monomer as a cooperative unit.

SYMBOLS AND ABBREVIATIONS

PI(s)	Protease inhibitor(s)
PI-1	Potato Inhibitor I
PI-2	Potato Inhibitor II
PSPI	Potato Serine Protease Inhibitor
PCPI	Potato Cysteine Protease Inhibitor
PAPI	Potato Aspartate Protease Inhibitor
PKPI	Potato Kunitz-type Protease Inhibitor
OSPI	Other Serine Protease Inhibitor
PCI	Potato Carboxypeptidase A Inhibitor
NID	Novel Inhibitor of Cathepsin D
PDI	Potato Cathepsin D Inhibitor
BPTI	Bovine Pancreatic Trypsin Inhibitor
BBI	Bowman-Birk Inhibitor
SSI	Streptomyces Subtilisin Inhibitor
STI	Soybean Trypsin Inhibitor
PJ	Potato Juice
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
CD	Circular dichroism
DSC	Different scanning calorimetry
ΔH_{cal}	Calorimetric enthalpy
ΔH_{vH}	Van't Hoff enthalpy

CONTENTS

Chapter 1

General Introduction

1 NATURALLY OCCURRING PROTEINACEOUS PROTEASE INHIBITORS

Proteinaceous protease inhibitors are ubiquitously abundant in tubers and plant seeds (Ryan, 1977). In higher plants, several gene families of these protease inhibitors have been characterised, particularly the serine protease inhibitors from *Leguminosae*, *Solanaceae* and *Graminae* (Garcia-Olmeda et al., 1987). The classification of proteinaceous protease inhibitors (PIs) remains, however, unclear.

The confusion in classification is resulting from the fact that PIs are often named after their first discovered biological origin (e.g: Potato Inhibitor II), the last name of the discoverer (e.g. Kunitz inhibitor from soybean) and/or the enzyme they inhibit (e.g. Serpins) (Birk, 2003a).

Proteolytic enzymes catalyse the cleavage of peptide bonds in proteins. Proteases are mostly classified according to the main catalytic amino acid residue in their active site: (1) serine proteinases, with a serine and a histidine: (2) cysteine proteinases, with a cysteine; (3) aspartic proteinases, with an aspartate group and (4) metalloproteinases, with a metallic ion (Zn^{2+}, Ca^{2+}, or Mn^{2+}) (Neurath, 1984), in their active site.

Proteolysis is a key process in all living organisms and must be carefully controlled in order not to be hazardous to the organism itself. It is, therefore, not surprising that a large number of naturally occurring proteinaceous protease inhibitors have been found in animals, plants and micro organisms.

Naturally occurring proteinaceous PIs are primarily classified based on the type(s) of enzyme they inhibit (Ryan, 1990; Bode and Huber, 2000) into classes of protease inhibitors, such as e.g. serine protease inhibitors. Some PIs are double-headed, meaning that they have two distinct active sites in their structure and, therefore, can inhibit two different enzymes. This criterion is, however, not of use classifying PIs in a family since PIs from the same family can be single or double headed. Next to this criterion, classification of PIs is also performed based on sequence homology, such as is done for the Kunitz-type inhibitors. A further classification can be done on the basis of their molecular mass, their protein architecture (monomeric or multimeric), the number of disulfide bridges present and their isoelectric points. These criteria determine in which family a protease inhibitor can be classified. Table 1 provides an overview of the known classes and families of PIs in animals and plants, classified according to these criteria.

1.1 Serine protease inhibitors

The serine protease inhibitors are by far the largest class of protease inhibitors when looking at the number of families. Serine protease inhibitors can be classified into 13 structurally distinct families, based on their origin.

1.1.1 Serine protease inhibitors from mammalian or microbial origin.

Six different families of mammalian or microbial origin are known.

- *Hirudin family*: PIs from this family consist of one polypeptide chain of 65-66 amino acids with 3 S-S bridges (Grutter et al., 1990). The main member, Hirudin, was isolated from

the saliva of leeches. Hirudin is an efficient PI that is entirely specific for thrombin, a serine protease involved in the blood coagulation process (Walsmann and Markwardt, 1981).

- *Bovine pancreatic trypsin inhibitor (BPTI) family*. This family was named based on its main member: BPTI. BPTI is a highly stable, 58 residues protein with a structure stabilised by 3 S-S bridges (Yu et al., 1995). BPTI is a well-known inhibitor and the X-ray structure has been determined for free BPTI (Deisenhofer and Steigemann, 1975). The native structure of BPTI consists of an α-helix on the N-terminus, a highly twisted anti-parallel β-sheet and an α-helix near the C-terminus.
- *The Kazal family* consists of a group of proteins with a molecular weight of approximately 9 kDa containing 3 S-S bridges. They are double-headed inhibitors, and can inhibit simultaneously *e.g.* trypsin and chymotrypsin (Mistry et al., 1997).
- *The Chelonianin family* consists of a small group of PIs, of which the most familiar members are secretory leukocyte protease inhibitor (SLPI) and R-Elafin (Francart et al., 1997). SLPI has a molecular mass of 12 kDa, contains 7 S-S bridges and is a double-headed PI inhibiting trypsin and elastase (Fritz, 1988). R-Elafin, a 57 amino acid protein, inhibits only elastase, but shows a high sequence homology with the C-terminus of SLPI, explaining why they are classified in the same family (Francart et al., 1997).
- *The Streptomyces subtilisin inhibitor (SSI) family.* Members of this family consist of a stable dimer composed of 2 identical subunits of 11.5 kDa (Mitsui et al., 1979). SSI strongly and specifically inhibits alkaline proteases such as subtilisin, although it also weakly inhibits trypsin and α-chymotrypsin.
- *Serpins family*. Although the name serpins is the acronym for serine protease inhibitors, it is specifically used for a family within the serine protease inhibitor class. The serpins family now consists of over 60 members, which widely occur in higher organisms. Examples of serpins are ovalbumin, α-antitrypsin and anti-thrombin (Janciauskiene, 2001). They are glycoproteins of one polypeptide chain of 40-45 kDa and contain 3 S-S bridges. They inhibit serine protease such as trypsin and their inhibition is irreversible, making them so-called suicidal inhibitors (irreversible binding) (Janciauskiene, 2001).

1.1.2 Serine protease inhibitors from plant origin

Serine protease inhibitors in plants can be divided into seven families and, as mentioned above, the first criterion for classification is the molecular weight, followed by the number of disulfide bridges.

- *The Cucurbit family* was named following the discovery of *Momordica charantia* inhibitor 3 (MCI-3), a trypsin inhibitor from a Cucurbitaceae (Zeng et al., 1988). MCI-3 is a monomeric protein of 3-4 kDa, containing no cysteine residues. It shows some sequence homology with Potato Inhibitor I (PI-1) and, therefore, is sometimes classified in the PI-1 family. PI-1 and members of the cucurbit family, however, differ significantly in molecular weight, and only the cucurbit members show activity towards the "Hageman factor" (a protease involved in the blood coagulation).

Table 1: Protease inhibitors classification.

Family	Molecular weight (kDa)	Nb subunit	Nb cys/sub	Nb active site	Inhibited enzymes										Origin	References
SERINE PROTEASE INHIBITOR CLASS																
Animal/Microbial																
Hirudin	7	1	6	1						Th					Leech	(Folkers et al., 1989)
Bovine Pancreatic Trypsin Inhibitor	7	1	6	1	T	C									Bovine	(Bode et al., 1984) (Deisenhofer and Steigemann, 1975)
Kazal	9	1	6	2	T		E								Bovine Porcine Human	(Sommerhoff et al., 1994) (Mistry et al., 1997)
Chelonianin	12 6	1 1	14 8	2 1	T		E E								Human	(Grutter et al., 1988) (Francart et al., 1997)
Steptomyces Subtilisin Inhibitor	23	2	4	1				S							Streptomyces	(Mitsui et al., 1979)
Serpins	40-45	1	6	1	T	C									Ovine Human	(Stein et al., 1990)
Plant																
Cucurbit	3-4	1	6	1	T				H						Cucurbitaceae	(Holak et al., 1989)
Bowman-Birk Inhibitor	6-8	1	14	2	T	C	E								Leguminoseae Gramineae	(Ferrasson et al., 1997) (Werner and Wemmer, 1991)
Cereal superfamily	12-13	1	8-10	2	T T T				H		A				Gramineae	(Kashlan and Richardson, 1981)
Potato Inhibitor II	20-21	2	16	2	T	C	E								Solanaceae	(McManus et al., 1994) (Taylor et al., 1993)
Thaumatin	21-22	1	16	2	T						A				Gramineae	(Richardson et al., 1987)
Kunitz-type	21-23	1, 2	4	1 2	T T T	C		S			A				Leguminoseae Gramineae Araceae Alistaceae Solanaceae	(Sweet et al., 1974) (Suh et al., 1990) (Zemke et al., 1991)

Family	Molecular weight (kDa)	Nb subunit	Nb cys/sub	Nb active site	Inhibited enzymes										Origin	References
SERINE PROTEASE INHIBITOR CLASS (continued)																
Potato Inhibitor I	40-45	5	2	2	(T)	C		S							Leguminoseae Solanaceae Gramineae Cucurbitaceae Polygonaceae	(Cleveland et al., 1987) (Plunkett and Ryan, 1980)
CYSTEINE PROTEASE INHIBITOR CLASS																
Animal																
Stefin	11	1	0	1								CP			Human Rat	(Lenarnic et al., 1986) (Katumuna and Kominami, 1983)
Cystatin	13	1	4	1								CP			Human Ovine	(Brzin et al., 1984) (Turk and Bode, 1991)
Kininogen	120 68	1 1	0 0	1								CP CP			Human	(Muller-Esterl et al., 1985)
Plant																
Stefin	12	1	0	1								CP			Gramineae	(Abe et al., 1991)
Multicystatin	85	1	0	8								CP			Solanaceae	(Walsh and Strickland, 1993)
Kunitz-type	20-21	1	4	1 2	T							CP CP			Solanaceae	(Krizaj et al., 1993) (Brzin et al., 1988) (Gruden et al., 1997)
Clitocypin	34	2	0	1								CP			Fungi	(Kidric et al., 2002)
ASPARTATE PROTEASE INHIBITOR CLASS																
Kunitz-type	20-22	1	4	1 2	T								CD CD		Solanaceae	(Mares et al., 1989)
METALLOPROTEASE INHIBITOR CLASS																
Potato Carboxypeptidase Inhibitor	4	1	6	1										CaP	Solanaceae	(Clore et al., 1987)

Inhibited enzymes: Th: Thrombin; T: Trypsin, C: α-chymotrypsin; S: Subtilisin; A: α-amylase; E: Elastase; H: Hageman factor; CP: Cysteine Proteases; CD: Cathepsin D; CaP: Carboxypeptidase.

- *The Bowman-Birk family* is composed of proteins with molecular weights varying from 6-8 kDa, which contain 7 S-S bridges (Werner and Wemmer, 1991). They are typically found in legume seeds such as soybean and pea (Ferrasson et al., 1997). They are double-headed, binding simultaneously to two serine proteases, such as trypsin and α-chymotrypsin (Bode and Huber, 1992).
- *The "Cereal superfamily" family* is a small group of PIs extracted from cereals such as wheat, barley, maize and ragi (Kashlan and Richardson, 1981; Campos and Richardson, 1983). They are proteins of 12-13 kDa containing 4-5 S-S bridges. They show inhibition against trypsin and Hageman factor, but more interestingly some members show also inhibition against α-amylase, an enzyme involved in starch hydrolysis.
- *The Potato inhibitor II (PI-2) family* consists of a group of proteins showing a high degree of homology with inhibitor II from potato (Bryant et al., 1976). PI-2 was also purified from tomato (Taylor et al., 1993). PI-2 members are double-headed inhibitors and showed inhibition against trypsin, α-chymotrypsin and elastase. They are dimeric proteins of 22 kDa, containing 16 cysteine residues per subunit, of which only 6 form intramolecular disulfide bridges.
- *The Thaumatin family* is a group of monomeric proteins with a molecular mass of 21-22 kDa, containing 8 S-S bridges (Franco et al., 2002). They inhibit specifically α-amylase but in some cases also trypsin, and can therefore be regarded as protease inhibitor.
- *The Kunitz-type family* is a large group of proteins sharing common criteria. Kunitz-type inhibitors are mostly monomeric. Dimeric members, in which the subunits are linked by a disulfide bridge, have also been described (Richardson, 1991). Kunitz-type inhibitors are proteins with 170-190 amino acids, which usually contain 4 cysteine residues that form two disulfide bridges. Kunitz-type inhibitors mostly contain one active site (single headed) that is located in the loop formed by the S-S bridge close to the N-terminus (Richardson, 1991), although double headed Kunitz-type inhibitors also exist. The inhibitor acts as a pseudo-substrate with the amino acid in position P1 defining its specificity, e.g. arginine in P1 against trypsin. Being a member of the Kunitz-type of inhibitor means that all PIs in this family have a high degree of sequence homology with STI, the first described member of the Kunitz family (Sweet et al., 1974).
- *The Potato inhibitor I (PI-1) family*: PI-1, which was first described by Ryan and Balls (1962), is a multimeric protein with a molecular weight of 40-45 kDa (Melville and Ryan, 1972). Each subunit (8 kDa) contains 1 S-S bridge and is double-headed, against trypsin and α-chymotrypsin. In its multimeric form PI-1 members, however, show a much higher affinity for α-chymotrypsin than for trypsin.

1.2 Cysteine protease inhibitors

The class of cysteine protease inhibitors is also known as the “Cystatin superfamily”. This superfamily excludes the members belonging to the Kunitz-type inhibitor and the clitocypin family. Cysteine protease inhibitors are present mainly in plants, but are also present in mammalian organisms (Barret, 1987). All members of Cystatin superfamily are monomeric proteins and contain a highly conserved region of 5 amino acids: Gln-X-Val-X-

Gly, always located in position 57 (according to the human stefin B sequence) (Ishikawa et al., 1994a).

1.2.1 Cysteine protease inhibitors in animals

Cysteine protease inhibitors in animals can be divided in three families according to their molecular weight and the presence or absence of cysteine residues. A large number of cysteine proteases are present in animals, microorganisms and plants, such as e.g. papain, bromelain, cathepsin B, H, and L and also several lysosomal cysteine proteases.

- *The Stefin family* is a group of monomeric proteins with a molecular mass of 12 kDa without cysteine residues (Barret, 1987). Stefins are potent reversible competitive inhibitors of cysteine protease such as papain, bromelain and cathepsin H (Katumuna and Kominami, 1983).
- *The Cystatin family*. The main characteristic of this family is the presence of 2 S-S bridges located towards the C-terminal end of the molecule (Brzin et al., 1984). They have a molecular mass of 13 kDa (Turk and Bode, 1991).
- *The Kininogen family* can be subdivided into 3 groups according firstly to their molecular weight and secondly to their affinity towards cysteine proteases (Turk and Bode, 1991) into the groups H-kininogen (120kDa), L-kininogen (68 kDa) and T-kininogen (68 kDa). They are multi-domain monomeric proteins.

1.2.2 Cysteine protease inhibitors from plant origin

Cysteine protease inhibitors in plants are known as phytocystatins (5-87 kDa) and show characteristics similar to those found in the Stefin and Cystatin families described above (Turk et al., 1997; Arai et al., 1998). Phytocystatins have been identified in a wide variety of monocotyl and dicotyl species, such as maize, rice, potato, soybean and apple (Abe et al., 1991; Gruden et al., 1997; Ryan et al., 1998). They are divided in four different families.

- *The Stefin family*. The proteins of this family contain one domain of approximately 12 kDa (Pernas et al., 1998) and shows sequence homology with the Stefin family in animals.
- *The Multicystatin family*. The second family has multiple domains, such as the multicystatins found in potato tubers (85 kDa), tomato leaves and sunflower seeds (Walsh and Strickland, 1993; Kouzuma et al., 2000; Wu and Haard, 2000). They show homology with the cystatin family in animals.
- *The Kunitz-type family*. The third family of cysteine protease inhibitors in plants shows sequence homology with the Kunitz-type serine protease inhibitors. An example of this third family is that of the potato cysteine protease inhibitors (Krizaj et al., 1993). They do not contain the conserved region (Gln-X-Val-X-Gly) that characterises the other families of the cysteine protease inhibitor class. They inhibit papain, and in some cases also trypsin.
- *The Clitocypin family.* The fourth family consists so far only of one member: clitocypin (Kidric et al., 2002). Clitocypin is a recently discovered cysteine protease inhibitor isolated from the mushroom *Clitocybe neburalis.* This PI does not show any sequence homology with the cystatin, the stefin and the Kunitz-type inhibitor families. Clitocypin is a homodimeric protein of 34 kDa lacking disulfide bonds. It shows inhibition against papain, cathepsin L and B and bromelain but is inactive against trypsin.

1.3 Aspartate protease inhibitors

In contrast to the widespread distribution of the aspartate proteases, the aspartate protease inhibitors are relatively uncommon. The aspartate protease inhibitors from Solanaceae are the most well known and well characterised in plant (Mares et al., 1989). Until now, only one family has been described. This family comprises protease inhibitors with a molecular mass of 20-22 kDa, and that contain two S-S bridges. They show sequence homology with the Kunitz-type inhibitor and are, therefore, classified as members of the Kunitz-type family. They all inhibit cathepsin D and in some cases also trypsin.

1.4 Metallo protease inhibitors

Only one family of the class of metallo protease inhibitors has so far been discovered: the potato carboxypeptidase inhibitor (PCI) from *Solanum tuberosum* (Hass et al., 1975; Molina et al., 1994). PCI is a small protein of 38-39 amino acid residues, containing 3 S-S bridges, and is known to be highly thermostable (Huang et al., 1981).

2 ROLE OF PROTEASE INHIBITORS IN PLANTS

As PIs are naturally present in many tubers and plant seeds (Ryan, 1977), they have been proposed to be storage proteins, and/or to control endogenous proteases and/or to act as a defence mechanism. The proportion of PIs has been observed to increase in response to wounding in the vicinity of the wound (local) and in the whole plant (systemic). These three proposed roles will be discussed below.

2.1 Protease Inhibitors as a defence mechanism

2.1.1 Induction of protease inhibitors in plants

Plants react to wounding, insect and pathogen attack by activating a set of genes, among which those of protease inhibitors and polyphenol oxydase. Most of the proteins, encoded by these genes, play a role in wound healing and prevention of a subsequent pathogen invasion (Peña-Cortés et al., 1995). Some of these genes are only expressed in the vicinity of the wound site (Bergey et al., 1996), while others are also systematically activated in the non-damaged parts of the plant (Koiwa et al., 1997). Members of the PI-2 family gene families are the best-studied examples of genes that are systematically activated upon mechanical damage (Kim et al., 1991; Kim et al., 1992; Herde et al., 1996). The known and presumed compounds of the signal transduction pathway mediated by insect attack, pathogens or wounding are shown in **figure 1.** As can be seen from this figure, oligosaccharides are released from the cell wall by endogalacturonases in the vicinity of the wound site. These oligosaccharides are presumed to act as extracellular elicitors of the signal pathway leading to PI gene expression (Doares et al., 1995; Reymond et al., 1995; Bergey et al., 1996). These oligosaccharide elicitors do not appear to be mobile within the plant. They mediate the local induction of PI gene expression (**figure 1**) via abscisic acid (ABA) and the activation of a protein kinase.

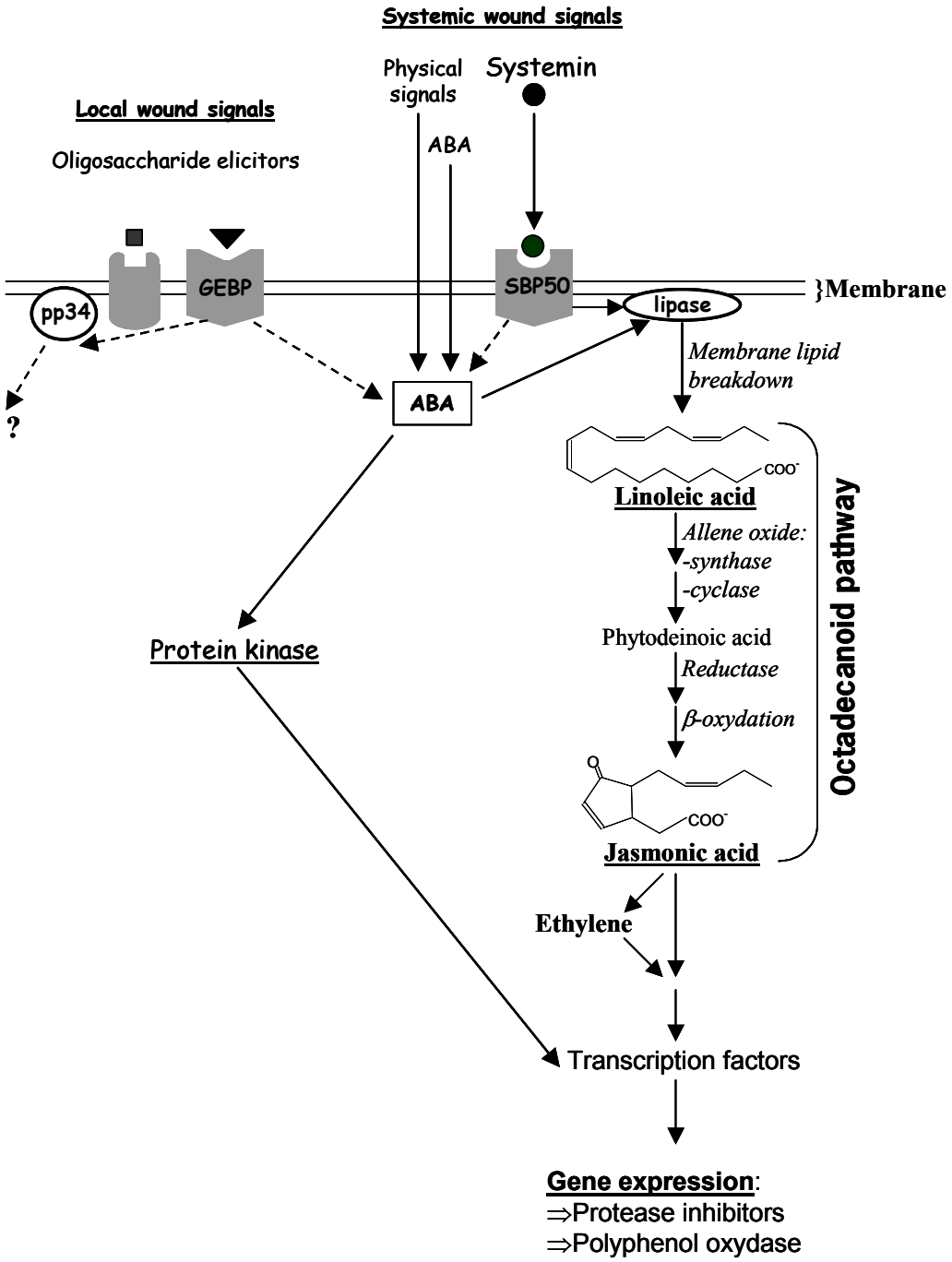

Figure 1: Proposed intracellular wound signal transduction pathway leading to the induction of protease inhibitor and polyphenol oxydases (Bergey et al., 1996; Koiwa et al., 1997). Filled arrows illustrate portions of the pathway proposed from direct evidence. Broken arrows represent inferred pathways or interactions. ABA: Abscisic acid; GEBP: β-glucan-elicitor-binding protein; pp34: phosphoprotein of 34 kDa; SBP50: systemin-binding protein of 50 kDa.

ABA and ethylene are also factors in the mix of signals that modulate wound-induced gene activation in Solanaceous plants (Herde et al., 1996). The ABA involvement in this process was suggested by studies with ABA-deficient tomato and potato mutants in which the wound- and systemin-induced levels of PI-2 in leaves were markedly reduced compared to wild-type plants (Peña-Cortés, 1996). Ethylene, a well-known product of wounded plant tissue, can positively regulate jasmonate levels in the plant (O'Donnell et al., 1996).

Systemin is released during wounding and is capable of systemic movement within the plant. Current models place systemin as an initiator of a cascade that triggers the release of linoleate from membrane lipids and the synthesis of jasmonic acid to activate genes encoding PIs and other anti-herbivore defence-associated factors (Schaller and Ryan, 1995; Bergey et al., 1996; Koiwa et al., 1997). Systemin is synthesised as a 200 amino acid precursor protein, the prosystemin, and processed into an 18 amino acid long peptide by a mechanism, which still remains unclear. The octadecanoid pathway (**figure 1**) leading to jasmonic acid biosynthesis has been studied extensively in relation to the wound-induced systemic induction of protease inhibitors and plant resistance to insect herbivores. The jasmonates are members of a large class of oxygenated lipids generated by the action of lipoxygenases on polyunsaturated fatty acids. Jasmonic acid and its volatile ester methyl jasmonate are potent inducers of protease inhibitors (Ryan, 1990; Farmer et al., 1992; Kim et al., 1992) and also of other defence related factors, such as polyphenol oxydase and lipoxygenase (Stout et al., 1994; Duffey and Stout, 1996), aimed at protecting plants from insect attacks from different feeding guilds (Thaler et al., 1996). Systemin also seems to induce protease inhibitor gene expression via the activation of a protein kinase.

2.1.2 Effect of protease inhibitors on pathogen growth

Studies on the effects of PIs on the development of insects were first reported by Lipke and coworkers (Lipke et al., 1954), who observed that the worm *Tribolium confusum* failed to develop on raw soybeans. They showed that a protein fraction from soybeans inhibited growth as well as *in vitro* proteolytic activity of the larvae. Most of the PIs relevant to plant defence are members of the serine protease inhibitor class while some of them are cysteine protease inhibitors (Ryan, 1990; Peña-Cortés et al., 1995; Bergey et al., 1996; Botella et al., 1996). Little information is available on the effect of aspartic- and metallo-protease inhibitors on the inhibition of insect larvae (Ishikawa et al., 1994b; Wu and Haard, 2000).

Johnson and coworkers (Johnson et al., 1989) showed that the growth of *Manduca Sexta* larvae (tobacco hornworms) feeding on leaves of transgenic tobacco containing PI-2, a powerful inhibitor of trypsin and chymotrypsin, was significantly retarded compared to the growth of these larvae fed on untransformed leaves. The presence of tomato inhibitor I, which belongs to the PI-1 family and is a potent inhibitor of chymotrypsin, in transgenic tobacco leaves had little effect on the growth of these larvae. These results, confirmed by other studies, indicate that the trypsin inhibitory activity is mainly responsible for the inhibition of larvae growth. Similar results were observed with the growth of the black field cricket (*Teleogryllus commodus*) after ingestion of PI-2 (Sutherland et al., 2002).

Fabrick and coworkers (Fabrick et al., 2002) showed that the main protease in the midgut of the southern corn rootworm, *Diabrotica undecimpunctata howardi*, is a cysteine

protease. Furthermore, they showed that a cysteine protease inhibitor from potato (Kunitz-type family) is a potent inhibitor of this protease. The incorporation of this inhibitor in the diet of the larvae resulted in an increase of mortality and in substantial growth inhibition.

2.2 Protease inhibitors as storage proteins

Since PIs (*e.g.* in potato tuber) are present in relatively large amounts without any wound induction, they are presumed to play another role than only a defence mechanism. The proposed role for protease inhibitors as storage proteins was suggested first by Pusztai (1972), who showed that during the germination of kidney bean the period of maximum protease inhibitor content coincided with the period of maximum proteolysis. In general, a protein can be considered as a storage protein when this protein is present in amounts of 5 % and more of the total protein content (Derbyshire et al., 1976). However, since PIs in tubers and plant seeds belongs to different classes and families and that their concentration varies during the maturation, it would not fit the definition of a storage protein.

Pusztai (1972) proposed that PIs may serve as a source of important sulphur-containing amino acids for the germination of the seed. PIs are in general considered to be proteins rich in cysteine residues (Jongsma, 1995). In table 1, however, it can be observed that this postulate is true mainly for PIs with a small molecular weight (3-13 kDa) which exhibit a proportion of cysteine residues >20%. For PIs with a higher molecular weight (>13 kDa), only Thaumatin and PI-2 families (20 kDa) contain a high number of cysteine residues (lower than 10%). Knowing that most of the PIs in plants belong to the Kunitz-type of inhibitors, in which the cysteine residues consist of approximately 2%, it can be concluded that the proportion of cysteine residues is rather small.

The potency of PIs as retarding insect proteinases is clear, but the assessment of PIs as storage proteins is complicated by the fact that maturation is accompanied by the expression of different PIs (McManus et al., 1999).

2.3 Protease inhibitors as control of endogenous proteases

The evidence that protease inhibitors may be active against endogenous proteinases came initially from studies on the interaction between trypsin-like proteases and PIs in lettuce seeds (Shain and Mayer, 1965). Morita and coworkers (Morita et al., 1996) showed that Kunitz-type and Bowman-Birk inhibitors, which are the two most abundant serine PIs in soybean, inhibited a purified serine protease from soybean. Using specific antibodies, it was shown that the PIs are localised in the protein bodies of the cotyledon, in the cytoplasm and in the cell wall (McManus et al., 1995). Valeski and coworkers (Valeski et al., 1991) suggested that cysteine protease inhibitors, which are present in lower amounts than the serine protease inhibitors in seeds and tuber, were involved in the regulation of physiological/metabolic processes. Abe and coworkers (Abe et al., 1987) showed that in maize the expression of cysteine protease inhibitors occurs parallel to that of glutelin accumulation. They, therefore, proposed that the inhibitor may be involved in protection against hydrolysis of this storage protein.

3 INTEREST OF PROTEASE INHIBITORS

Westfall and Hague (1948) showed that the nutritive quality of soybean flour heated at various temperatures increased proportionally to the inactivation of the trypsin inhibitors, leading to the conclusion that the trypsin inhibitors were the major source of the poor utilisation of the protein from raw soybeans. It was also shown that chicks and rats fed with raw soybean meal developed a remarkable hypertrophy of the pancreas (Lyman and Lepkovshy, 1957). However, the evidence that PIs constitute a hazard to human health should be placed in perspective, since most of the *in vivo* research has been done with small animals given a diet containing large amounts of PIs over a large period of time (a situation almost unfeasible in eating patterns of humans) (Birk, 2003b).

Although PIs have long been considered only as antinutritional factors, they have regained interest in recent years because of their possible anticarcinogenic (Kennedy, 1998) and positive dietary effects (Hill et al., 1990).

3.1 Anticarcinogenic agent

PIs are well established as a class of cancer chemopreventive agents (Kennedy, 1998). While PIs from different families have been shown to prevent the carcinogenic process, the most potent of the known anticarcinogenic PIs are those with the ability to inhibit chymotrypsin-like proteases. The Bowman-Birk inhibitor (BBI) from soybean is until now the PI that has been most studied as an anticarcinogenic agent. However, also potato protease inhibitors have been shown to be potent anticarcinogenic agents, especially PI-1 and PI-2 (Frenkel et al., 1987; Billings et al., 1989; Huang et al., 1997). The interest for the other classes of potato PIs, *e.g.* PIs active against cysteine proteases and carboxypeptidase has grown as well (Billings et al., 1989; Blanco-Aparicio et al., 1998; Laurent-Matha et al., 1998).

3.1.1 Potato Inhibitor I and II

Solar UV irradiation is the causal factor for the increasing incidence of human skin carcinomas. The activation of the transcription factor activator protein-1 (AP-1) has been shown to be responsible for the tumor promoter action of UV light in mammalian cells. Huang and coworkers (Huang et al., 1997) demonstrated that PI-1 and PI-2 from potato tubers, when applied to mouse epidermal cells, block UV-induced AP-1 activation. Moreover, the inhibition is specific for the UV-induced signal transduction for AP-1 activation.

In response to invasion of bacteria, phagocytic cells are stimulated and generate active oxygen species, which are known to contribute to inflammatory diseases, necrosis of surrounding tissues, mutagenicity and carcinogenicity (Weitzman et al., 1985). Frenkel and coworkers (Frenkel et al., 1987) showed that chymotrypsin inhibitors, especially Potato Inhibitor I, are capable of inhibiting formation of these active oxygen species during the oxidative burst of stimulated human polymorphonuclear leukocytes.

3.1.2 Potato Carboxypeptidase Inhibitor (PCI)

Epidermal growth factor (EGF) and its receptor (EGFR) are involved in several aspects of the development of carcinomas, including tumor cell growth, vascularisation,

invasiveness and metastasis (Nelson, 1991). Since EGFR was found to be over-expressed in many tumors of epithelial origin, it is a potential target for antitumor therapy. PCI is the only known natural antagonist of human EGF (Blanco-Aparicio et al., 1998). It competes with EGF for binding to the EGFR and thus inhibits EGFR activation and concomitant cell proliferation induced by this growth factor. PCI suppressed the growth of several human pancreatic adenocarcinoma cell lines, both *in vitro* and *in vivo* (nude mice). PCI has a special disulfide scaffold, called a T-knot, which is also present in many growth factors, including EGF and transforming growth factor α. These structural similarities with these factors may explain the antagonist action of PCI and makes it a potent anticarcinogenic agent.

3.2 Satiety agent

PIs have been reported to influence satiety. **Figure 2** shows the role played by cholecystokinin (CCK), a 33 amino acid peptide, in the mechanism of satiety.

CCK, which is secreted by the small intestine in response to a meal, stimulates gall bladder contraction and secretion of pancreatic trypsinogen, which is converted to active trypsin in the duodenum (Ivy and Oldberg, 1928). By a mechanism, which still remains unclear in human (Owyang et al., 1986), trypsin subsequently inhibits the release of CCK by a negative feed back regulation mechanism.

High levels of circulating CCK were shown to reduce food intake in humans, and, therefore, CCK has been studied as a satiety agent (Smith and Gibbs, 1987). The main effect of CCK administration before a meal is that a person stops to eat earlier (Pi-Sunyer et al., 1982) and, interestingly, persons claimed to be satisfied (Kissileff et al., 1981; Pi-Sunyer et al., 1982). The first experiments on CCK were done by administration of CCK intravenously.

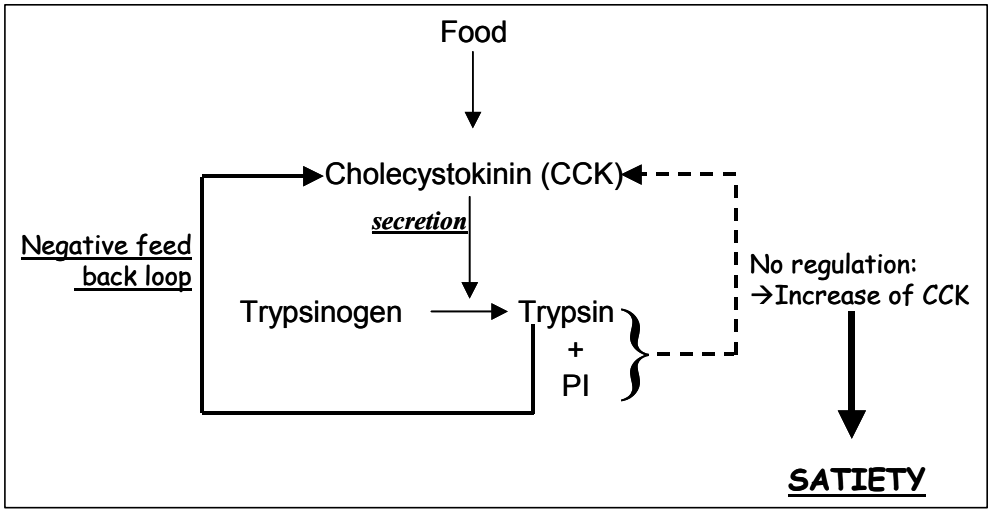

Figure 2: Role of CCK in satiety.

However, these techniques were reported to be accompanied by negative side effects, such as nausea, in a few individuals (Smith and Gibbs, 1987). Serine protease inhibitors have been observed to cause the maintenance of high levels of circulating CCK (Owyang et al.,

1986). This indicates that the proteolytic activity of trypsin is required in the negative feed back regulation of CCK. Hill and coworkers (Hill et al., 1990) showed that by the use of PI-2 as a supplement in food the levels of CCK increased and that the food intake was concomitantly reduced. Moreover, the side effects observed with intravenous CCK were completely absent.

3.3 Medical application in dermatitis

In order to degrade proteins in ingested foods, the stomach, the pancreas and the small intestine secrete several classes of proteases. In normal cases, this proteolytic activity is highly controlled (by lowering the pH and controlling the level of water present) and reduced during the stay in the intestine. In cases of infection or surgical intervention, the proteolytic juice passes the colon almost undiluted and unretarded. The high levels of proteolytic activity in the faeces may cause peri-anal dermatitis (Rafii et al., 1999). In order to prevent this form of dermatitis PIs with a broad range of inhibitory activities are needed. Potato PIs, showing inhibiting activity against serine proteases but also against cysteine, aspartate and metallo-proteases, are a plant source of PIs that can fulfil this requirement.

Another application can be the treatment of skin wounds. After disruption of the skin barrier, proteolytic activity in the epidermis increased within 1-2 hours. It was shown that topical application of trypsin-like serine protease inhibitors inhibited this increase and accelerated the barrier recovery (Denda et al., 1997). Therefore, the protease balance might be important for the barrier homeostasis and skin pathology.

4 PROTEASE INHIBITORS IN POTATO TUBERS

Soluble potato tuber proteins have so far been classified in three different groups: 1) a group consisting of all protease inhibitors present in potato juice; 2) the major protein in potato tuber: patatin; 3) all the other proteins present in the tuber. Although it has been reported that PIs constitute up to 30% of all proteins (Melville and Ryan, 1972), the exact proportion in which each of these groups occurs in potato remains unknown since so far no overall quantitative data about the composition has been established.

4.1 Protease inhibitors

Until now, potato protease inhibitors have been classified in three different classes and groups (Richardson, 1991) rather than in families. The first class consists of potato inhibitor I, a pentameric serine protease inhibitor, composed of five isoinhibitor protomer species (Richardson and Cossins, 1974). The second class consists of potato inhibitor II, a class of dimeric serine protease inhibitors in which a disulfide bridge connects the N termini of each protomer. The protein behaves as a single domain protein (Lee et al., 1999; Beekwilder et al., 2000). The third class consists of 20-22kDa proteins and is subdivided in four different groups: 1) Kunitz protease inhibibitors, showing inhibition towards serine proteases (Walsh and Twitchell, 1991); 2) Cysteine protease inhibitors exhibiting activity towards papain and cathepsin L (Brzin et al., 1988; Krizaj et al., 1993); 3) Aspartic protease inhibitors showing

Table 2: Protease Inhibitors in potato tuber.

Family	Molecular weight (kDa)	Subunit	Cys/sub	Inhibited enzymes	Class + group in potato PI classification	References
Serine Protease Inhibitor Class						
Kunitz-type	21-23	1	4	T C	III, 1	(Walsh and Twitchell, 1991) (Mitsumori et al., 1994)
Potato Inhibitor I	40	4-5	2	T C S	I	(Richardson, 1974)
Potato Inhibitor II	20-21	2	14	T C E	II	(Bryant et al., 1976) (Sanchez-Serrano et al., 1986)
Cysteine Protease Inhibitor Class						
Multicystatins	85	1	0	P		(Walsh and Strickland, 1993)
Kunitz-type	20-21	1	4	P CB CL	III, 2	(Brzin et al., 1988)
				T P CB CL		(Krizaj et al., 1993) (Valueva et al., 1997)
Aspartate Protease Inhibitor Class						
Kunitz-type	20-22	1	4	CD	III, 3	(Strukelj et al., 1992)
				T CD		(Ritonja et al., 1990) (Mares et al., 1989)
Metalloprotease Inhibitor Class						
Potato Carboxypeptidase Inhibitor	4	1	6	CaP	III, 4	(Ryan et al., 1974) (Hass et al., 1976)

Inhibited enzymes: T: Trypsin, C: α-chymotrypsin; S: Subtilisin; E: Elastase; P: Papain; CB: Cathepsin B; CL: Cathepsin L; CD: Cathepsin D; CaP: Carboxypeptidase.

activity towards trypsin and cathepsin D (Mares et al., 1989; Ritonja et al., 1990; Strukelj et al., 1992); and 4) Carboxypeptidase inhibitor showing activity against Carboxypeptidase A (Hass et al., 1975; Hass et al., 1976). Table 2 shows an overview of all the protease inhibitors from potato that have been described in literature up to now, classified according to the 'potato PI' classification as well as to the generic PI classification (Table 1).

4.2 Other proteins present in potato

Patatin was given its trivial name by Racusen and Foote (Racusen and Foote, 1980). Patatin represents 40 % of the soluble protein in potato tuber and consists of a family of 40-43 kDa glycoproteins with pI's between 4.5 and 5.2 (Racusen and Foote, 1980). Its apparent molecular weight in non-dissociating media, where it appears as a dimeric protein, is 80 kDa (Racusen and Weller, 1984). Patatin has a lipid acyl hydrolase (LAH) activity for both lipid deacylation and wax ester formation (Galliard and Dennis, 1974; Andrews et al., 1988). It has been suggested that this LAH-activity may play a role in the plant defense mechanism.

Other proteins, present in potato, have molecular weights higher than 40 kDa, such as lectins (Allen et al., 1996), polyphenoloxidases (Partington and Bolwell, 1996), enzymes involved in starch synthesis (Marshall et al., 1996) and phosphorylase isozymes (Gerbrandy and Doorgeest, 1972).

5 PROTEINS PROPERTIES

The classification of PIs does not take in account elements of secondary and tertiary structure. However, it is this structure, which determines their mode of action, stability and application, and is of interest when processing of foods and/or agricultural material containing PIs is involved.

5.1 Protein structure

Four levels of structure are commonly used in discussions on protein architecture (Creighton, 1996). The *primary structure* is determined by the amino acid sequence. The *secondary structure* refers to the spatial arrangements of amino acid residues, which are near to each other in the linear sequence. The α-helix and β-sheet are elements of the secondary structure. The α-helix is a rodlike structure. The tightly coiled polypeptide forms the inner part of the rod and the side chains extend outward in a helical array. The structure is stabilised by hydrogen bonds between the NH and CO of the peptide bonds in the main chain. The β-strand is an extended structure, which is on itself not stable and is only observed in the form of β-sheets. β-Sheets are aggregates of multiple β-strands, running in the same (parallel) or opposite (antiparallel) directions. When the chain is forming anti-parallel β-sheets, the turning part is called β-turn. The *tertiary structure* refers to the spatial arrangement of amino acids far apart in the linear sequence. Proteins, which contain more than one polypeptide chain, have an additional level of structural organisation, called the *quaternary structure*.

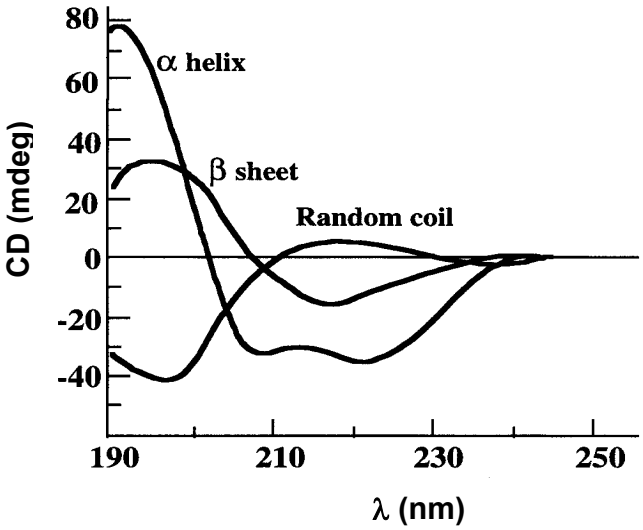

Figure 3: CD spectra of poly-L-lysine in various conformations (Greenfield and Fasman, 1969).

Protein structure and stability can be followed by methods in which conformational changes in structure can be easily seen, such as fluorescence, far, near ultra violet circular dichroism (far and near UV CD), and differential scanning calorimetry (DSC). The far UV CD spectra of poly-L-lysine in various conformations (**figure 3**) show that different conformations give distinctive spectra (Greenfield and Fasman, 1969) and therefore, far UV CD can be used to predict the secondary structure.

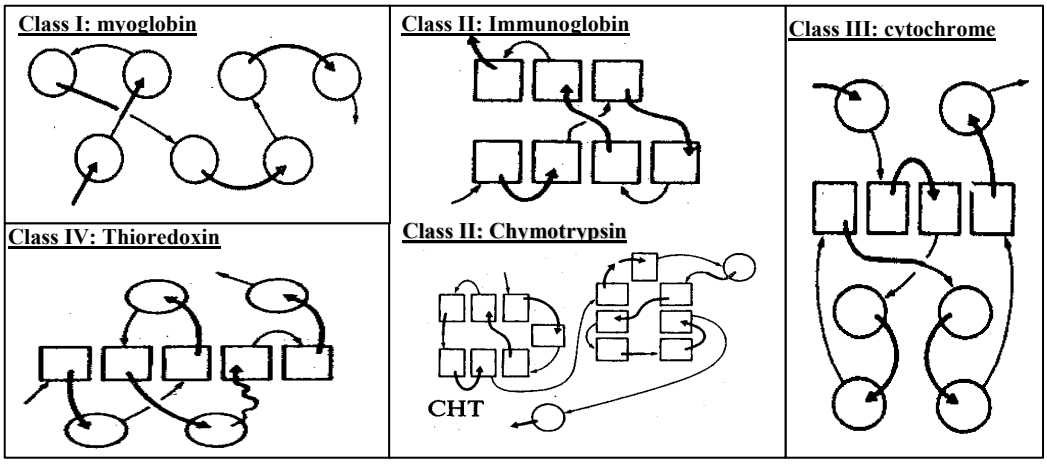

Figure 4: Examples of structure belonging to the 4 different classes (□ = β-strand and O = α-helix) (Lewitt and Chothia, 1976).

General protein folds: Based on the presence and abundance of secondary structure elements, four classes of proteins can be roughly distinguished: Class I or all-α proteins, Class II or all-β proteins, Class III or α+β proteins and Class IV or α/β proteins (Lewitt and Chothia, 1976).

- Class I proteins: In the all-α proteins more than 60% of the amino acid residues are involved in α-helices. Myoglobin is a typical example of an all α-protein (**figure 4**).
- Class II proteins: The all-β proteins are built from β-sheets stacked to form a layered structure. Although the number of β-strands varies from 2 to 7, there are always two layers of β-sheet. All the class II proteins, except chymotrypsin, have only one domain. In chymotrypsin the two domains are identical but the orientation differs (**figure 4**).
- Class III proteins: The α+β proteins consist of a mixture of all-α helix and all-β-sheet within the same polypeptide chain. Often the β-sheet, formed by anti-parallel β-strands, is surrounded by a cluster of α-helices at one or both ends of the β-sheet. Cytochrome b_5 is a typical example of α+β proteins (**figure 4**).

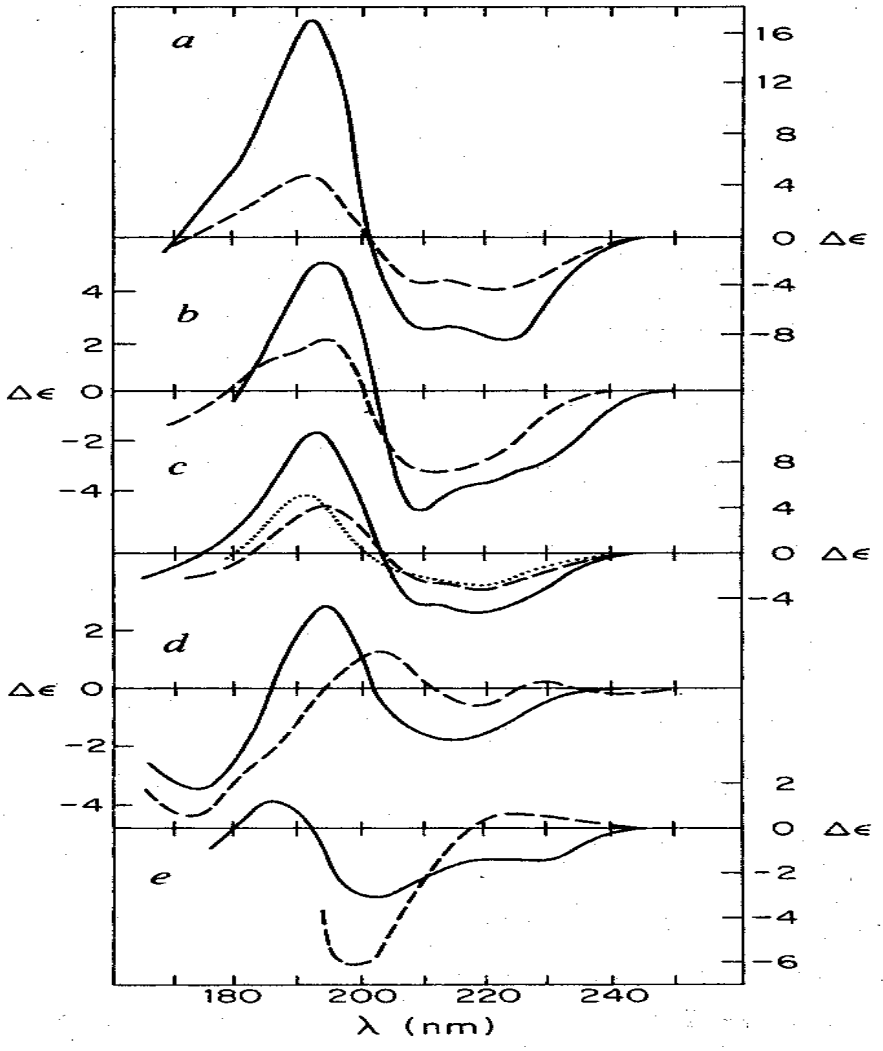

Figure 5: Examples representing the extremes in CD spectra for the tertiary structures classes. a) All α-proteins: myoglobin (solid) and cytochrome c (dashed); b) α+β proteins: lysozyme (solid) and ribonuclease A (dashed); c) α/β proteins: triose phosphate isomerase (solid), flavodoxin (dashed) and subtilisin BPN' (dotted); d) All β-proteins: prealbumin (solid) and Bence-Jones protein (dashed); e) All β-proteins: α-chymotrypsin (solid) and soybean trypsin inhibitor (dash). (Manavalan and Johnson, 1983).

➢ Class IV proteins: The α/β proteins have α-helices and β-strands that occur one after the other, in such a way that most α-helices are separated by β-strands along the sequence and visa versa. Most of these proteins have a single sheet with α-helices in between, but in some of the larger proteins there are extra, smaller β-sheets. Thioredoxin is a typical example of α/β proteins (**figure 4**).

This classification is, however, just a simplification as in reality the structure of proteins is much more complex, and overlap between the classes may occur. **Figure 5** shows some typical examples of far ultra-violet circular dichroism spectra for each class of proteins (Manavalan and Johnson, 1983). Even though proteins belong to the same class, their CD spectra may be completely different due to different topological arrangements of secondary structure elements within the protein.

5.2 Conformational stability and unfolding

The conformation of globular proteins results from an equilibrium between forces, such as hydrophobic interactions, hydrogen bonds, Van der Waals and ionic interactions, and the conformational entropy (Alber, 1989). This equilibrium may be easily disturbed, by e.g. increasing temperature or by changing solvent conditions. If this occurs, unfolding of the protein ensues.

Upon unfolding, the polypeptide chain becomes less compact, more solvated, and much more flexible (Tanford, 1968). Intrinsic viscosity measurements made at identical temperatures suggest that the unfolded chains produced by heat and guanidinium hydrochloride are almost random coils (Privalov, 1979). Unfolding of a protein can be easily performed by using extreme pHs, denaturants and by increase of temperature. Many proteins unfold at pH values lower than 5 or higher than 10. Unfolding at such pHs occurs by ionisation of the non-ionised groups buried inside the protein. Also electrostatic repulsion between charged groups at the surface and effects on salt bridges may contribute to pH-induced unfolding (Darby and Creighton, 1993). Denaturants, like urea and guanidinium hydrochloride, are known to affect protein stability. The temperatures at which proteins unfold vary greatly. All of them unfold at elevated temperatures and some also at low temperatures (cold denaturation). Many proteins unfold at temperatures only a few degrees higher than those at which they function best.

5.3 Aggregation

Aggregates of proteins are formed through intermolecular interactions. These interactions are the same as those involved in intramolecular structure. Two distinct mechanisms of irreversible aggregation have been identified (Lin et al., 1989). Diffusion-limited aggregation occurs when there is negligible repulsive force between the particles, so that the rate of aggregation is limited solely by the time it takes for particles to encounter each other by diffusion. Reaction-limited protein aggregation occurs when there is still a substantial repulsive force between the particles, so that the aggregation rate is limited by the time it takes for two particles to overcome this repulsive barrier.

Protein aggregation is of importance in food industry since aggregation often results in a complete loss of solubility and thus also of other functional properties and, therefore, affects the quality of the final product.

6 INDUSTRIAL RELEVANCE OF POTATO PROTEASE INHIBITORS

For potatoes, it has been reported that PIs represent about 30% of the total tuber protein (Melville and Ryan, 1972). In industrial processes, potato proteins are recovered as a by-product of potato starch production (Knorr et al., 1977), and, therefore, can only be used in low-value applications such as feed or fertiliser (Ahldén and Trägårdh, 1992).

6.1 Potato (*Solanum tuberosum*)

Cultivated potatoes all belong to one botanical species, *Solanum tuberosum*, but this species alone includes about 2800 species that vary in size, shape, color, and other sensory characteristics.

Nowadays, potato is a major world crop with 307 million tons (Mt) produced annually (FAO, 2002). Only wheat (572 Mt), rice (576 Mt) and maize (602 Mt) are produced in larger amounts for human consumption (FAO, 2002). Potato is a major crop in the Netherlands also. It contributes about 5 % to the total value of the Dutch agricultural production and resulted in 1999 in a total production of 8.2 million tons of potatoes. About 2.8 million tons (34 %) were used in the starch industry in 1999 in the Netherlands (CBS, 1999). The potatoes used in the starch industry are from special varieties and may contain up to 22 % (w/w) starch as dry matter (ISI, 1999) and generally contain about 1.5 % (w/w) protein on fresh weight basis (Lisinska and Leszczynski, 1989).

6.2 Potato starch production

In **figure 6**, the industrial process for production of potato starch is schematically shown. The process starts by washing the potatoes followed by grinding in a rasping machine. The rasped potatoes are then passed through rotating sieves. The fibres are retained and are discharged as potato pulp (potato fibres). The remaining starch slurry contains soluble compounds (sugars, proteins, acids, salts) and fine fibres. These are separated by further treatment through continuous centrifugal separators (hydrocyclones) and fine sieves. The purified starch slurry is used for the production of potato starch derivatives or is dewatered and dried. The by-products that remain after starch manufacture are the (wet) fibres and the so-called potato juice. Potato juice has a pH of approximately 5.6 and contains 5 % (w/w) dry matter (Plieger, 1986), which constitutes for about 35 % of proteins and amino acids, 35 % sugars, 20 % minerals, 4 % organic acids and 6 % other compounds such as phenolic compounds (Knorr et al., 1977). The potato proteins are recovered by an acidic-heat treatment of the potato juice. This results in a completely irreversible precipitation of the proteins, with a complete loss of functionality for food application, as well as in terms of inhibitory activity (Knorr et al., 1977). This explains that so far potato proteins can only be used in low-value applications such as feed.

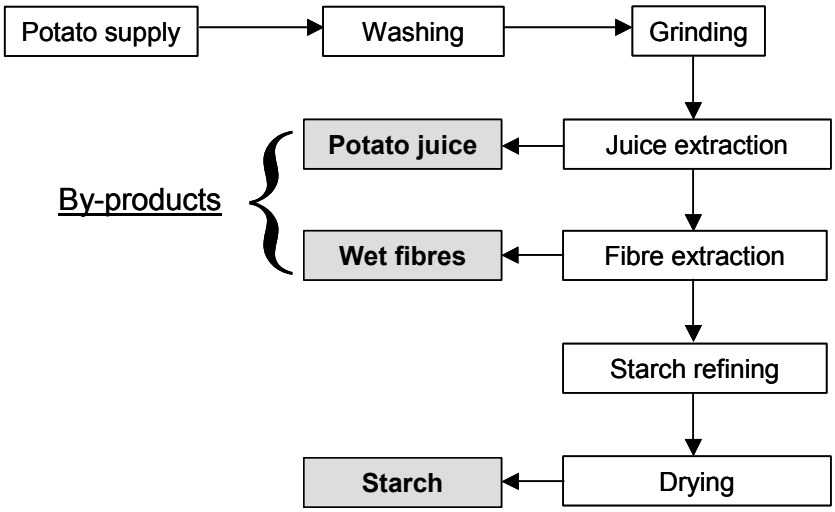

Figure 6: Potato starch manufacturing process (Adapted from Swinkels (Swinkels, 1990)).

Potato proteins are known to have the capacity to form and stabilise emulsions and foams (van Koningsveld, 2001; van Koningsveld et al., 2002). Furthermore, potato protein has a high nutritional value, derived from its amino acid composition (Liedl et al., 1987), with a relatively high lysine content (7.5 %), when compared to other plant proteins. For these reasons, potatoes are a promising source of plant proteins for human consumption.

Van Koningsveld and coworkers (van Koningsveld et al., 2001) showed that potato proteins unfold between 55 and 75°C and that increasing the ionic strength generally causes an increase in denaturation temperature. Potato proteins were shown to be soluble at neutral and acidic pH. The structure of patatin is perturbed by exposure to a pH lower than pH 5, whereas the PIs seem unchanged. Thermal unfolding of patatin is almost completely irreversible (with a complete loss of enzymatic activity) and depending on the conditions may result in the formation of large aggregates (Pots et al., 1998; Pots et al., 1999).

Whereas the thermal behaviour of patatin has been investigated, information on the thermal unfolding of PIs is lacking. Moreover, the relative abundance of the different PIs present in potato tuber is completely absent.

7 AIM

The aim of this research was to investigate and understand the thermal unfolding behaviour of potato protease inhibitors. This was done by determining the relative abundance and inhibitory activity of protease inhibitors present in potato tuber. Subsequently, the stability of the most abundant and representative protease inhibitors in potato juice as a function of temperature and pH was investigated. This information maybe of help to understand the mechanism of the irreversible precipitation occurring in industrial processes, thereby creating possibilities to obtain soluble potato proteins that can be used in food and pharmaceutical applications.

8 OUTLINE

Chapter 2 describes a general scheme to purify the PIs present in potato juice in significant quantities. The PIs are identified on the basis of their inhibitory activity, their molecular weight, their protein architecture and their isoelectric pH. Subsequently they are classified in seven different families using the criteria mentioned above and literature data available. The relative abundance of each protein is determined as well. In chapter 3, the gene sequence of Potato Serine Protease Inhibitor 6.1 (prominent member of the most abundant family) is determined and its identification is performed by sequence homology. In chapter 4, the assignment of PSPI as a member of the β-II protein subclass is done and its pH stability is studied. In Chapter 5, the unfolding of PSPI with temperature and denaturant is studied in order to define a possible unfolding mechanism. In Chapter 6, the conformational stability of 3 isoforms of Potato Cysteine Protease Inhibitor is studied at 20°C together with the thermal unfolding behaviour of these proteins. Chapter 7 is dealing with the expression of Potato Inhibitor I (cv. *Bintje*) in *Pichia pastoris* and its characterisation by means of molecular weight, inhibitory activity against trypsin/α-chymotrypsin and thermal unfolding behaviour. Chapter 8 presents a general discussion focussing on the main results of the previous chapters.

LITERATURE CITED

Abe, K.; Emori, Y.; Kondo, H.; Suzuki, K.; Arai, S. Molecular cloning of a cysteine proteinase inhibitor of rice (oryzacystatin). *J. Biol. Chem.* **1987**, 262, 16793-16797.

Abe, K.; Kondo, H.; Watanabe, H.; Emori, Y.; Arai, S. Oryzacystatins as the first well-defined cystatins of plant origin and their target proteinases in rice seeds. *Biomed. Biochim. Acta* **1991**, 50, 637-641.

Ahldén, I.; Trägårdh, G. A. A study of soluble proteins from four potato varieties used in the Swedish starch industry. *Food Chem.* **1992**, 44, 113-118.

Alber, T. Mutational effects on protein stability. *Annu. Rev. Biochem.* **1989**, 58, 765-798.

Allen, A. K.; Bolwell, G. P.; Brown, D. S.; Sidebottom, C.; Slabas, A. R. Potato lectin: a three-domain glycoprotein with a novel hydroxyproline-containing sequences and sequence similarities to wheat-germ agglutinin. *Int. J. Biochem. Cell Biol.* **1996**, 28, 1285-1291.

Andrews, D. L.; Beames, B.; Summers, M. D.; Park, W. D. Characterization of the lipid acyl hydrolase activity of the major potato (*Solanum tuberosum*) tuber protein, patatin, by cloning and abundant expression in a baculovirus vector. *Biochem. J.* **1988**, 252, 199-206.

Arai, S.; Matsumoto, I.; Abe, K. Phytocystatins and their target enzymes: From molecular biology to practical application: A review. *J. Food Biochem.* **1998**, 22, 287-299.

Barret, A. J. The cystatins: a new class of peptidase inhibitors. *TIBS* **1987**, 12, 193-196.

Beekwilder, J.; Schipper, B.; Bakker, P.; Bosch, D.; Jongsma, M. Characterization of potato proteinase II reactive site mutants. *Eur. J. Biochem.* **2000**, 267, 1-11.

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.

Billings, P. C.; Morrow, A. R.; Ryan, C. A.; Kennedy, A. R. Inhibition of radiation-induced transformation of C3H/10T1/2 cells by carboxypeptidase inhibitor 1 and inhibitor II from potatoes. *Carcinogenesis* **1989**, 10, 687-691.

Birk, Y. Inhibitors families of plant origin: Classification and characterisation. In *Plant protease inhibitors*; Springer, eds; Verlag Berlin Heidelberg New-York, **2003a**; 11-74.

Birk, Y. Plant protease inhibitors in nutrition. In *Plant protease inhibitors*; Springer, eds; Verlag Berlin Heidelberg New-York, **2003b**; 75-84.

Blanco-Aparicio, C.; Molina, M. A.; Frenandez-Salas, E.; Frazier, M. L.; Mas, J. M.; Quero, E.; Aviles, F. X.; de Llorens, R. Potato carboxypeptidase inhibitor, a T-knot protein, is an epidermal growth factor antagonist that inhibits tumor cell growth. *J. Biol. Chem.* **1998**, 273, 12370-12377.

Bode, W.; Huber, R. Natural protein proteinase inhibitors and their interaction with proteinases. *Eur. J. Biochem.* **1992**, 204, 433-451.

Bode, W.; Huber, R. Structural basis of the endoproteinase-protein inhibitor interaction. *Biochim. Biophys. Acta* **2000**, 1477, 241-252.

Bode, W.; Walter, J.; Huber, R.; Wenzel, H. R.; Tschesche, H. The refined 2.2 A (0.22 nm) X-ray crystal structure of the ternary complex formed by bovine trypsinogen, valine-valine and the Arg^{15} analogue of bovine pancreatic trypsin inhibitor. *Eur. J. Biochem.* **1984**, 144, 185-190.

Botella, M. A.; Xu, Y.; Prabha, T. N.; Zhao, Y.; Narasimhan, M. L.; Wilson, K. A.; Nielsen, S. S.; Bressan, R. A.; Hasegawa, P. M. Differential expression of soybean cysteine proteinase inhibitor genes during development and in response to wounding and methyl jasmonate. *Plant Physiol.* **1996**, 112, 1201-1210.

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

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.

Brzin, J.; Popovic, T.; Turk, V.; Borchart, U.; Machleidt, W. Human cystatin, a new protein inhibitor of cysteine proteinases. *Biochem. Biophys. Res. Commun.* **1984**, 118, 103-109.

Campos, F. A. P.; Richardson, M. The complete amino acid sequence of the bifunctional a-amylase/trypsin inhibitor from seeds of ragi (Indian finger millet, *Eleusine coracana* Gaertn.). *FEBS Lett.* **1983**, 152, 300-304.

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.

Clore, G. M.; Gronenborn, A. M.; Nilges, M.; Ryan, C. A. Three-dimensional structure of potato carboxypeptidase inhibitor in solution. A study using nuclear magnetic resonance, distance geometry, and restrained molecular dynamics. *Biochemistry* **1987**, 26, 8012-8023.

Creighton, T. E. *Proteins: strutures and molecular properties*, W. H. Freeman, New-York, **1996**.

Darby, N. J.; Creighton, T. E. *Protein structure*. Oxford University Press: Oxford, **1993**.

Deisenhofer, J.; Steigemann, W. Crystallographic refinement of the structure of bovine pancreatic trypsin inhibitor at 1.5 A resolution. *Acta Crystallogr.* **1975**, B31, 238-250.

Denda, M.; Kitamura, K.; Elias, P. M.; Feingold, K. R. Trans-4-(aminomethyl)cyclohexane carboxilic acid (t-AMCHA), an anti-fibrinolytic agent, accelerates barrier recovery and prevents the epidermal hyperplasia induced by epidermal injury in hairless mice and humans. *J. Invest. Dermatol.* **1997**, 109, 84-90.

Derbyshire, E.; Wright, D. J.; Boulter, D. Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry* **1976**, 15, 3-24.

Doares, S. H.; Syrovets, T.; Weiler, E. W.; Ryan, C. A. Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, 92, 4095-4098.

Duffey, S. S.; Stout, M. J. Antinutritive and toxic components of plant defense against insects. *Arch. Insect Biochem. Physiol.* **1996**, 32, 3-37.

Fabrick, J.; Behnke, C.; Czapla, T.; Bala, K.; Rao, A. G.; Kramer, K. J.; Reeck, G. R. Effects of a potato cysteine proteinase inhibitor on midgut proteolytic enzyme activity and growth of the southern corn rootworm, *Diabrotica undecimpunctata howardi* (Cloeoptera: Chrysomelidae). *Insect Biochem.* **2002**, 32, 405-415.

Farmer, E. E.; Johnson, R. R.; Ryan, C. A. Regulation of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiol.* **1992**, 98, 995-1002.

Ferrasson, E.; Quillien, L.; Gueguen, J. Proteinase inhibitors from pea seeds: purification and characterization. *J. Agric. Food Chem.* **1997**, 45, 137-131.

Folkers, P. J. M.; Clore, G. M.; Driscoll, P. C.; Dodt, P. C.; Kohler, S.; Gronenborn, A. M. Solution structure of recombinant hirudin and the Lys-47----Glu mutant: a nuclear magnetic resonance and hybrid distance geometry-dynamical simulated annealing study. *Biochemistry* **1989**, 28, 2601-2617.

Francart, C.; Dauchez, M.; Alix, A. J. P.; Lippens, G. Solution structure of R-elafin, a specific inhibitor of elastase. *J. Mol. Biol.* **1997**, 268, 666-667.

Franco, O. L.; Rigden, D. J.; Melo, F. R.; Grossi-de-Sa, M. F. Plant a-amylase inhibitors and their interaction with a-amyases - Structure, function and potential for crop protection. *Eur. J. Biochem.* **2002**, 269, 397-412.

Frenkel, K.; Chrzan, K.; Ryan, C. A.; Wiesner, R.; Troll, W. Chymotrypsin-specific protease inhibitors decrease H_2O_2 formation by activated human polymorphonuclear leukocytes. *Carcinogenesis* **1987**, 8, 1207-1212.

Fritz, H. Human mucus proteinase inhibitor (human MPI). Human seminal inhibitor I (HUSI-I), antileukoprotease (ALP), secretory leukocyte protease inhibitor (SLPI). *Bio. Chem. Hoppe-Seyler* **1988**, 369, 79-82.

Galliard, T.; Dennis, S. Isoenzymes of lipolytic acyl hydrolase and esterase in potato tuber. *Phytochemistry* **1974**, 13, 2463-2468.

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.

Gerbrandy, S. J.; Doorgeest, A. Potato phosphorylase isoenzymes. *Phytochemistry* **1972**, 11, 2403-2407.

Greenfield, N. J.; Fasman, G. D. Computed circular dichroism spectra for evaluation of protein conformation. *Biochemistry* **1969**, 8, 4108-4116.

Gruden, K.; Strukelj, B.; Ravnikar, M.; Poljsak-Prijatelj, M.; Mavric, I.; Brzin, J.; Pungercar, J.; Kregar, I. Potato cysteine proteinase inhibitor gene family: molecular cloning, characterisation and immunocytochemical localisation studies. *Plant Mol. Biol.* **1997**, 34, 317-323.

Grutter, M. G.; Fendrich, G.; Huber, R.; Bode, W. The 2.5 A X-ray crystal structure of the acid stable proteinase inhibitor from human mucous secretions analysed in its complex with alpha-chymotrypsin. *EMBO J.* **1988**, 7, 345-351.

Grutter, M. G.; Priestle, J. P.; Rahuel, J.; Grossenbacher, H.; Bide, W.; Hofsteenge, J.; Stone, S. R. Crystal structure of the thrombin-hirudin complex: a novel mode of serine protease inhibition. *EMBO J.* **1990**, 9, 2361-2365.

Hass, G. M.; Ako, H.; Grahn, D. T.; Neurath, H. Carboxypeptidase inhibitor from potatoes. The effects of chemical modifications on inhibitory activity. *Biochemistry* **1976**, 15, 93-100.

Hass, G. M.; Nau, H.; Biemann, K.; Grahn, D. T.; Ericsson, L. H.; Neurath, H. The amino acid sequence of a carboxypeptidase inhibitor from potatoes. *Biochemistry* **1975**, 14, 1334-1342.

Herde, O.; Atzorn, R.; Fisahn, J.; Wasternack, C.; Willmitzer, L.; Peña-Cortés, H. Localized wounding by heat initiates the accumulation of proteinase inhibitor II in abscisic acid-deficient plants by triggering jasmonic acid biosythesis. *Plant Physiol.* **1996**, 112, 853-860.

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.

Holak, T. A.; Bode, W.; Huber, R.; Otlewski, J.; Wilusz, T. Nuclear magnetic resonance solution and X-ray structure of squash trypsin inhibitor exhibits the same conformation of the proteinase binding loop. *J. Mol. Biol.* **1989**, 210, 649-654.

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.

Huang, D. Y.; Swanson, B. G.; Ryan, C. A. Stability of proteinase inhibitors in potato tubers during cooking. *J. Food Sci.* **1981**, 46, 287-290.

Ishikawa, A.; Ohta, S.; Matsuoka, K.; Hattori, T.; Nakamura, K. A family of potato genes that encode Kunitz-type proteinase inhibitors: structural comparisons and differential expression. *Plant Cell Physiol.* **1994a**,

Ishikawa, A.; Yoshihara, T.; Nakamura, K. Jasmonate-inducible expression of a potato cathepsin D inhibitor-GUS gene fusion in tobacco cells. *Plant Mol. Biol.* **1994b**, 26, 403-414.

Ivy, A. C.; Oldberg, E. Hormone mechanism for gall bladder contraction. *Am. J. Physiol.* **1928**, 85, 381-383.

Janciauskiene, S. Conformational properties of serine proteinase inhibitor (serpins) confer multiple pathophysiological roles. *Biochim. Biophys. Acta* **2001**, 1535, 221-235.

Johnson, R.; Narvaez, J.; An, G.; Ryan, C. Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. *Proc. Natl. Acad. Sci. U. S. A.* **1989**, 86, 9871-9875.

Jongsma, M. A. The resistance of insects to plant proteinase inhibitors. *Center for Plant Breeding and Reproduction Research*. Wageningen. Wageningen university **1995**,

Kashlan, N.; Richardson, M. The complete amino acid sequence of a major wheat protein inhibitor of a-amylase. *Phytochemistry* **1981**, 20, 1781-1784.

Katumuna, N.; Kominami, E. Structures and functions of lysosomal thiol proteinases and their endogenous inhibitor. *Curr. Top. Cell. Regul.* **1983**, 22, 71-101.

Kennedy, A. R. Chemopreventive agents: protease inhibitors. *Pharmacol. Ther.* **1998**, 78, 167-209.

Kidric, M.; Fabian, H.; Brzin, J.; Popovic, T.; Pain, R. H. Folding, stability, and secondary structure of a new cysteine dimeric proteinase inhibitor. *Biochem. Biophys. Res. Commun.* **2002**, 297, 962-967.

Kim, S.-R.; Choi, J.-L.; Costa, M. C.; An, G. Identification of G-box sequence as an essential element for methyl jasmonate response of potato proteinase II protomer. *Plant Physiol.* **1992**, 99, 627-631.

Kim, S.-R.; Costa, M. A.; An, G. Sugar response element enhances wound response of potato proteinase inhibitor II promoter in transgenic tobacco. *Plant Mol. Biol.* **1991**, 17, 973-983.

Kissileff, H. R.; Pi-Sunyer, F. X.; Thornton, J.; Smith, G. P. Cholecystokinin-octapeptide (CCK-8) decreases food intake in man. *Am. J. Clin. Nutr.* **1981**, 34, 154-160.

Knorr, D.; Kohler, G. O.; Betschart, A. A. Potato protein concentrates: the influence of various methods of recovery upon yield, compositional and functional characteristics. *J. Food Process. Preserv.* **1977**, 1, 235-247.

Koiwa, H.; Bressan, R. A.; Hasegawa, P. M. Regulation of protease inhibitors and plant defense. *Trends in plant science* **1997**, 2, 379-384.

Kouzuma, Y.; Inanaga, H.; Doi-Kawano, K.; Yamasaki, N.; Kimura, M. Molecular cloning and functional expression of cDNA encoding the cysteine proteinase inhibitor with three cystatin domains from sunflower seeds. *J. Biochem. (Tokyo).* **2000**, 128, 161-166.

Krizaj, I.; Drobnic-Kosorok, M.; Brzin, J.; Jerala, R.; Turk, V. The primary stucture of inhibitor of cysteine proteinases from potato. *FEBS Lett.* **1993**, 333, 15-20.

Laurent-Matha, V.; Farnoud, M. R.; Lucas, A.; Rougeot, C.; Garcia, M.; Rochefort, H. Endocytosis of pro-cathepsin D into breast cancer cells is mostly independent of mannose-6-phosphate receptors. *J. Cell Sci.* **1998**, 111, 2539-2549.

Lee, M. C. S.; Scanlon, M. J.; Craik, D. J.; Anderson, M. A. A novel two-chain proteinase inhibitor generated by circularization of a multidomain precursor protein. *Nat. Struct. Biol.* **1999**, 6, 526-530.

Lenarnic, B.; Ritonja, A.; Sali, A.; Kotnic, M.; Turk, V.; Machleidt, W. In *Cysteine proteinases and their inhibitors*; Turk V, eds; Walter de Gruyter: Berlin, **1986**; 473-487.

Lewitt, M.; Chothia, C. Structural patterns in globular proteins. *Nature* **1976**, 261, 552-558.

Liedl, B. E.; Kosier, T.; Desborough, S. L. HPLC isolation and nutritional value of a major potato tuber protein. *Am. Potato J.* **1987**, 64, 545-557.

Lin, M. Y.; Lindsay, H. M.; Weitz, D. A.; Ball, R. C.; Klein, R.; Meakin, P. Universality in colloid aggregation. *Nature* **1989**, 339, 360-362.

Lipke, H.; S., F. G.; Liener, I. E. Effect of soybean inhibitors on growth of *Tribolium confusum*. *Food Chem.* **1954**, 2, 410-414.

Lisinska, G.; Leszczynski, W. *Potato science and technology*, Elsevier Applied Science, London, **1989**.

Lyman, R. L.; Lepkovshy, S. The effect of raw soybean meal and trypsin inhibitor diets on pancreatic enzyme secretion in the rat. *J. Nutr.* **1957**, 62, 269-284.

Manavalan, P.; Johnson, C. J. Sensitivity of circular dichroism to protein tertiary structure class. *Nature* **1983**, 305, 831-832.

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.

Marshall, J.; Sidebottom, C.; Debet, M.; Martin, C.; Smith, A. M.; Edwards, A. Identification of the major starch synthase in the soluble fraction of potato tubers. *Plant Cell* **1996**, 8, 1121-113.

McManus, M. T.; Corner, R.; Garthwaite, I. Characterisation of monoclonal antibodies that recognise the soybean (Kunitz) trypsin inhibitor: Binding to the inhibitor interrupts the formation of the trypsin inhibitor complex. *J. Plant Physiol.* **1995**, 146, 243-248.

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.

McManus, M. T.; Ryan, S.; Laing, W. A. **1999.** *The functions of proteinase inhibitors in seeds*. Seed symposium, 3-13.

Melville, J. C.; Ryan, C. A. Chymotrypsin inhibitor I from potatoes: Large scale preparation and characterization of its subunit components. *J. Biol. Chem.* **1972**, 247, 3443-3453.

Mistry, R.; Snashall, P. D.; Totty, N.; Briskin, S.; Guz, A.; Tetley, T. D. Purification and characterization of a novel-type serine proteinase inhibitor of neutrophil elastase from sheep lung. *Biochim. Biophys. Acta* **1997**, 1342, 51-61.

Mitsui, Y.; Satow, Y.; Watanabe, Y.; Hirono, S.; Iitaka, Y. Crystal structures of *Streptomyces* subtilisin inhibitor and its complex with subtilisin BPN'. *Nature* **1979**, 277, 447-452.

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.

Molina, M. G.; Marino, C.; Oliva, B.; Aviles, F. X.; Querol, E. C-tail valine is a key residue for stabilization of complex between potato inhibitor and carboxypeptidase A. *J. Biol. Chem.* **1994**, 269, 21467-21472.

Morita, S.; Fukase, M.; Hoshino, K.; Fukuda, Y.; Yamaguchi, M.; Morita, Y. Partial purification and characterization of a novel soybean protease which is inhibited by Kunitz and Bowman-Birk trypsin inhibitors. *J. Biochem. (Tokyo)*. **1996**, 119, 711-718.

Muller-Esterl, W.; Fritz, H.; Kellermann, J.; Lottspeich, F.; Machleidt, W.; Turk, V. Genealogy of mammalian cysteine proteinase inhibitors - Common evolutionary origin of stefins, cystatins and kininogens. *FEBS Lett.* **1985**, 191, 221-226.

Neurath, H. Evolution of proteolytic enzymes. *Science* **1984**, 224,

O'Donnell, P. J.; Calvert, C.; Leyser, H. M. O.; Bowles, D. J. Ethylene as a signal mediating the wound response of tomato plants. *Science* **1996**, 274, 1914-1917.

Owyang, C.; Louie, D. S.; Tatum, D. Feedback regulation of pancreatic enzyme secretion - Suppression of cholecystokin release by trypsin. *J. Clin. Invest.* **1986**, 77, 2042-2047.

Partington, J. C.; Bolwell, G. P. Purification of polyphenol oxidase free of the storage protein patatin from potato tuber. *Phytochemistry* **1996**, 42, 1499-1502.

Peña-Cortés, H. Abscisic acid-deficient plants do not accumulate proteinase inhibitor II following systemin treatment. *Planta* **1996**, 198, 447-451.

Peña-Cortés, H.; Fisahn, J.; Willmitzer, L. Signals involved in wound-induced protease inhibitor II gene expression in tomato and potato plants. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, 92, 4106-4113.

Pernas, M.; Sanchez-Monge, R.; Gomez, L.; Salcedo, G. A chestnut seed cystatin differentially effective against cysteine proteinases from closely related pests. *Plant Mol. Biol.* **1998**, 38, 1235-1242.

Pi-Sunyer, X.; Kissileff, H. G.; Thornton, J.; Smith, G. P. C-terminal octapeptide of cholecystokinin decreases food intake in obese man. *Physiol. Behav.* **1982**, 29, 627-630.

Plieger, P. *The composition of potato juice. A literature review.* Dutch institute for carbohydrate Research Reportnr. PI 86-3, Groningen, **1986**.

Plunkett, G.; Ryan, C. A. Reduction and carboxyamidomethylation of the single disulfide bond of proteinase inhibitor I from potato tubers. *J. Biol. Chem.* **1980**, 255, 2752-2755.

Pots, A. M.; de Jongh, H. H. J.; Gruppen, H.; Hamer, R. J.; Voragen, A. G. J. Heat-induced conformational changes of patatin, the major potato tuber protein. *Eur. J. Biochem.* **1998**, 252, 66-72.

Pots, A. M.; ten Grotenhuis, E.; Gruppen, H.; Voragen, A. G. J.; de Kruif, K. G. Thermal aggregation of patatin *in situ*. *J. Agric. Food Chem.* **1999**, 47, 4600-4605.

Privalov, P. L. Stability of proteins. Small globular proteins. *Adv. Protein Chem.* **1979**, 33, 167-241.

Pusztai, A. Metabolism of trypsin-inhibitory proteins in the germination seeds of kidney bean (*Phaesolus vulgaris*). *Planta* **1972**, 107, 121-129.

Racusen, D.; Foote, M. A major soluble glycoprotein of potato tubers. *J. Food Biochem.* **1980**, 1, 13-52.

Racusen, D.; Weller, D. L. Molecular weight of patatin, a major potato tuber protein. *J. Food Biochem.* **1984**, 8, 103-107.

Rafii, F.; Ruseler-van Embden, J. G. H.; van Lieshout, L. M. C. Changes in bacterial enzymes and PCR profiles of fecal bacteria from a patient with ulcerative colitis before and after antimicrobial treatments. *Dig. Dis. Sci.* **1999**, 44, 637-642.

Reymond, P.; Grünberger, S.; Paul, K.; Müller, M.; Farmer, E. E. Oligogalacturonide defense signals in plants: large fragments interact with the plasma membrane *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, 92, 4145-4149.

Richardson, M. Chymotrypsin inhibitor I from potatoes: The amino acid sequence of subunit A. *Biochem. J.* **1974**, 137, 101-112.

Richardson, M. Seed Storage proteins: the enzyme inhibitors. *Methods in Plant Biochemistry* **1991**, 5, 259-305.

Richardson, M.;Cossins, L. Chymotrypsic inhibitor I from potatoes: The amino acid sequences of subunits B, C and D. *FEBS Lett.* **1974**, 45, 11-13.

Richardson, M.; Valdes-Rodriguez, S.; Blanco-Labra, A. A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. *Nature* **1987**, 327, 432-434.

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.

Ryan, C. A. Proteolytic enzymes and their inhibitors in plant. *Ann. Rev. Plant Physiol.* **1977**, 24, 173-196.

Ryan, C. A. Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. *Annu. Rev. Phytopathol.* **1990**, 28, 425-449.

Ryan, C. A.; Balls, A. K. An inhibitor of chymotrypsin from *Solanum tuberosum* and its behavior toward trypsin. *Proc. Natl. Acad. Sci. U. S. A.* **1962**, 48, 1839-1844.

Ryan, C. A.; Hass, G. M.; Kuhn, R. W. Purification and properties of a carboxypeptidase inhibitor from potatoes. *J. Biol. Chem.* **1974**, 249, 5495-5499.

Ryan, C. A.; Laing, W. A.;McManus, M. T. A cysteine proteinase inhibitor purified from apple fruit. *Phytochemistry* **1998**, 49, 957-963.

Sanchez-Serrano, J. J.; Schmidt, R.; Schell, J.; Willmitzer, L. Nucleotide sequence of proteinase inhibitor II encoding cDNA of potato (*Solanum tuberosum*) and its mode of expression. *Mol. Gen. Genet.* **1986**, 203, 15-20.

Schaller, A.; Ryan, C. A. Systemin - a polypeptide defense signal in plants. *BioEssays* **1995**, 18, 27-33.

Shain, Y.; Mayer, A. M. Proteolytic enzymes and endogenous trypsin inhibitor in germinating lettuce seeds. *Physiol. Plant.* **1965**, 18, 853-859.

Smith, G. P.; Gibbs, J. The effect of gut peptides on hunger, satiety and food intake in humans. *Ann. N. Y. Acad. Sci.* **1987**, 499, 132-136.

Sommerhoff, C. P.; Sollner, C.; Mentele, R.; Piechotta, G. P.; Auerswald, E. A.; Fritz, H. A Kazal-type inhibitor of human mast cell tryptase: isolation from the medical leech *Hirudo medicinalis*, characterization, and sequence analysis. *Biol. Chem. Hoppe. Seyler* **1994**, 375, 685-694.

Stein, P. E.; Leslie, A. G. W.; Finch, J. T.; Turnell, W. G.; McLaughlin, P. J.; Carrell, R. W. Crystal structure of ovalbumin as a model for the reaction center of serpins. *Nature* **1990**, 347, 99-102.

Stout, M. J.; Workman, J.; Duffey, S. S. Differential induction of tomato foliar proteins by arthropod herbivores. *J. Chem. Ecol.* **1994**, 20, 2575-2594.

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.

Suh, S.-G.; Peterson, J. E.; Stiekema, W. J.; Hannapel, D. J. Purification and characterization of the 22-kilodalton potato tuber proteins. *Plant Physiol.* **1990**, 94, 40-45.

Sutherland, P. W.; Burgess, E. P. J.; Philip, B. A.; McManus, M. T.; Watson, L.; Christeller, J. T. Ultrastructural changes to the midgut of the black field cricket (*Teleogryllus commodus*) following ingestion of potato protease inhibitor II. *J. Insect Physiol.* **2002**, 48, 327-336.

Sweet, R. M.; Wright, H. T.; Janin, J.; Chothia, C. H.; Blow, D. M. Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6-A resolution. *Biochemistry* **1974**, 13, 4212-4228.

Swinkels, J. J. M. *Industrial starch chemistry: Properties, modifications and applications of starch*, AVEBE b.a. International Marketing: Veendam; Product information ref. nb: 05.00.02.006 EF, **1990**.

Tanford, C. Protein denaturation. *Adv. Protein Chem.* **1968**, 23, 122-282.

Taylor, B. H.; Young, R. J.;Scheuring, C. F. Induction of a proteinase II-class gene by auxin in tomato roots. *Plant Mol. Biol.* **1993**, 23, 1005-1014.

Thaler, J. S.; Stout, M. J.; Duffey, S. S.; Karban, R. Exogenous jasmonates stimulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *J. Chem. Ecol.* **1996**, 22, 1767-1781.

Turk, B.; Turk, V.; Turk, D. Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biol. Chem.* **1997**, 378, 141-150.

Turk, V.; Bode, W. The cystatins: protein inhibitors of cysteine proteinases. *FEBS Lett.* **1991**, 285, 213-219.

Valeski, K.; Fernandes, S.; Campos, F. A. P.; Do Val, R. R.; Xavier-Filho, J. The expression of papain inhibitor during development of cowpea seeds. *Plant Sci.* **1991**, 74, 179-184.

Valueva, T. A.; Revina, T. A.;Mosolov, V. V. Potato tuber protein proteinase inhibitors belonging to the Kunitz soybean inhibitor family. *Biochemistry (Moscow)* **1997**, 62, 1367-1374.

van Koningsveld, G. Physico-chemical and functional properties of potato proteins. *PhD thesis*. Wageningen. Wageningen University **2001**,

van Koningsveld, G. A.; Gruppen, H.; de Jongh, H. H. J.; Wijngaards, G.; van Boekel, M. A. J. S.; Walstra, P.; Voragen, A. G. J. The effects of pH and heat treatment on the structure and solubility of potato proteins in different preparations. *J. Agric. Food Chem.* **2001**, 49, 4889-4897.

van Koningsveld, G. A.; Walstra, P.; Gruppen, H.; Wijngaards, G.; van Boekel, M. A. J. S.; Voragen, A. G. J. Formation and stability of foam made with various potato protein preparations. *J. Agric. Food Chem.* **2002**, 50, 7651-7659.

Walsh, T. A.; Strickland, J. A. Proteolysis of the 85-kilodalton crystalline cysteine proteinase inhibitor from potato releases functional cystatin domains. *Plant Physiol.* **1993**, 103, 1227-1234.

Walsh, T. A.; Twitchell, W. P. Two Kunitz-type proteinase inhibitors from potato tubers. *Plant Physiol.* **1991**, 97, 15-18.

Walsmann, P.; Markwardt, F. Biochemical and pharmacological aspects of the thrombin inhibitor hirudin. *Pharmazie* **1981**, 36, 633-660.

Weitzman, S. A.; Weitberg, A. B.; Clark, E. P.; Stossel, T. P. Phagocytes as carcinogens: malignant transformation produced by human neutrophils. *Science* **1985**,

Werner, M. H.; Wemmer, D. E. ^{1}H assignments and secondary structure determination of the soybean trypsin/chymotrypsin Bowman-Birk inhibitor. *Biochemistry* **1991**, 30, 3356-3364.

Westfall, E. J.; Hague, S. M. The nutritive quality and trypsin inhibitor content of soybean flour heated at various temperatures. *J. Nutr.* **1948**, 35, 374-380.

Wu, J.; Haard, N. F. Purification and characterization of a cystatin from the leaves of methyl jasmonate treated tomato plants. *Comp. Biochem. Physiol.* **2000**, 127, 20-220.

Yu, M.-H.; Weissman, J. S.; Kim, P. S. Contribution of individual side-chains to the stability of BPTI examined by alanine-scanning mutagenesis. *J. Mol. Biol.* **1995**, 249, 388-397.

Zemke, K. J.; Muller-Fahrnow, A.; Jany, K.-D.; Pal, G. P.; Saenger, W. The three-dimensional structure of the bifunctional proteinase K/a-amylase inhibitor from wheat (PKI3) at 2.5 A resolution. *FEBS Lett.* **1991**, 279, 240-242.

Zeng, F.-Y.; Qian, R.-R.;Wang, Y. The amino acid sequence of a trypsin inhibitor from the seeds of *Momordica charantia* Linn, cucurbitaceae. *FEBS Lett.* **1988**, 234, 35-38.

Chapter 2

Relative abundance and inhibitory distribution of protease inhibitors in potato juice from cv. *Elkana*

ABSTRACT

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 in seven different groups: Potato Inhibitor I, Potato Inhibitor II (PI-2), Potato Cysteine Protease Inhibitor (PCPI), Potato Aspartate Protease Inhibitor, Potato Kunitz-type Protease Inhibitor, Potato Carboxypeptidase Inhibitor (PCI) and 'other serine protease inhibitors' (OSPI) groups. The most abundant groups were the PI-2 and PCPI groups, representing 22 and 12 % of all proteins in potato juice, respectively. Potato protease inhibitors show a broad spectrum of enzyme inhibition. All the groups (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 group and between protease inhibitor groups.

Based on:

L. Pouvreau, H. Gruppen, S. R. Piersma, L. A. M. van den Broek, G. A. van Koningsveld and A. G. J. Voragen. Relative abundance and inhibitory distribution of protease inhibitors in potato juice from cv. *Elkana*. *Journal of Agricultural and Food Chemistry*, 2001, 49, 2864-2874.

1 INTRODUCTION

Although protease inhibitors have been long considered only as antinutritional factors, they have regained interest in recent years because of their possible anticarcinogenic (Kennedy, 1998) and positive dietary effects (Hill et al., 1990). Potato tuber protease inhibitors have been reported to act as anticarcinogenic agent by interfering in tumor-cell proliferation (Blanco-Aparicio et al., 1998), H_2O_2 formation (Frenkel et al., 1987) and processes resulting from solar-UV irradiation (Huang et al., 1997). In addition, by intervening with cholecystokinin (Hill et al., 1990), 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 (Lisinska and Leszczynski, 1989). It has been reported that protease inhibitors represent about 30% of the total tuber protein (Melville and Ryan, 1972). In contrast to patatin, the major potato tuber protein (Racusen and Foote, 1980), 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 potato inhibitor I (PI-1), potato 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) (Richardson and Cossins, 1974; Ralet and Gueguen, 1999). PI-2 is a dimeric serine protease inhibitor composed of two 10.2 kDa subunits (Bryant et al., 1976). A disulfide bridge links the subunits and the protein behaves like a single domain protein (Lee et al., 1999). The 4.3 kDa PCI is the smallest inhibitor present in potato tuber and is a single subunit peptide (Hass et al., 1975) and is remarkably thermo-stable (Huang et al., 1981; Oliva et al., 1991). 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-type inhibitors (Walsh and Twitchell, 1991), cysteine protease inhibitors (Brzin et al., 1988), and cathepsin D inhibitors (Mares et al., 1989).

In industrial processes, potato proteins are recovered as a by-product of potato starch production (Knorr et al., 1977). 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 (Knorr et al., 1977). In order 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 groups will be presented.

2 MATERIAL AND METHODS

2.1 Materials

Porcine pancreas trypsin (T-0134, lot 100H0658), bovine chymotrypsin (C-4129, lot 58H7001), papaya latex papain (P-9886, lot 66H7130), bovine pancreas carboxypeptidase 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 from Sigma Chemical Co.

Potato PI-2 (lot B14718), purified by affinity chromatography on immobilised 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.

2.2 Preparation of potato juice (PJ)

Potatoes were chopped in large pieces (max. 8 x 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 (10,000 x *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).

2.3 Protein purification

Akta Purifier and Explorer protein chromatography systems and the columns used for the protein purification were from Amersham Biosciences (Uppsala, Sweden). 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 PJ was applied to a Superdex 75 prep grade gel filtration column (65 x 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 x 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 x 2.6 cm). For fraction IN, the column was equilibrated with 10 mM sodium

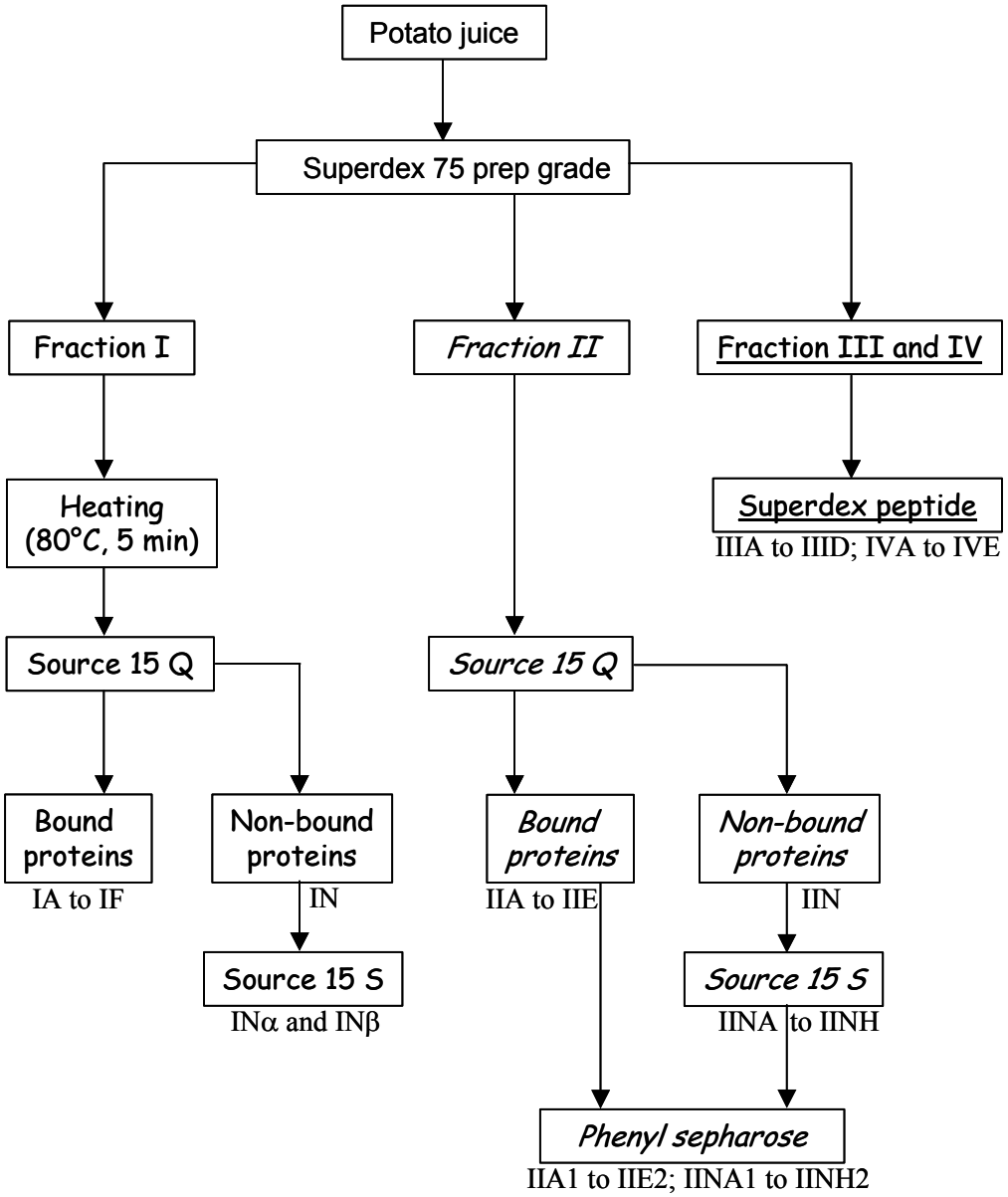

Figure 1: Schematic representation of the fractionation procedure.

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 sulphate in the same buffer.

A Superdex peptide column (30 x 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.

2.4 Protein purity

SDS-PAGE, with and without β-mercaptoethanol, and IEF electrophoresis were performed with a Pharmacia PhastSystem (Amersham Biosciences, Uppsala, Sweden) 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.

2.5 Protein quantification

The protein concentration for each of the intermediate and purified fractions was determined using the Bradford assay (Bradford, 1976), using bovine serum albumin (Sigma) as a standard. For PJ, patatin and for at least one of the isoforms within each protease inhibitor group, the nitrogen content was also determined using the combustion (Dumas) method on the NA 2100 Nitrogen and Protein Analyser (Intersciences) according to the instructions of the manufacturer. For PJ, a distinction was made between protein nitrogen and non-protein 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 groups 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, based on the protein content, determined by the Dumas method, calibration curves were made for patatin and representative inhibitors of each protease inhibitor groups 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 groups as standards. No major differences in Bradford response within each protease inhibitor group were expected and/or detected. All the assays were performed at least in duplicate.

2.6 Mass spectrometry

MALDI-TOF MS analysis in the linear mode was performed using a Voyager DE RP instrument (Perseptive Biosystems, Framingham, MA, USA). Protease inhibitor fractions were dialysed against nanopure 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 where externally calibrated using thioredoxin, insulin and apo-myoglobin (Perseptive Biosystems) as standards.

2.7 Enzyme titration

The concentration of active enzyme was determined by titrating their active sites: trypsin with *p*-nitrophenyl-*p'*-guanidine benzoate (Chase Jr and Shaw, 1970) and α-

chymotrypsin with N-trans-cinnamoylimidazole (Schonbaum et al., 1961). The titration showed 75 and 25 % activity for trypsin and α-chymotrypsin, respectively.

2.8 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 pre-incubated during 15 minutes with inhibitor, before the substrate was added. All assays were performed in duplicate.

Trypsin inhibition was estimated according to Smith and coworkers (Smith et al., 1980) 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 (1984) 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 and Horton (1973) 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 and corworkers (van Jaarsveld et al., 1997) 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 and coworkers (Valueva et al., 1999) 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 (1984) 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.

2.9 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 and coworkers (Smith et al., 1980). The decrease in activity in the presence of inhibitor was measured and the inhibiting activity was calculated according to the following formula:

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

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 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.

3 RESULTS AND DISCUSSION

3.1 Fractionation

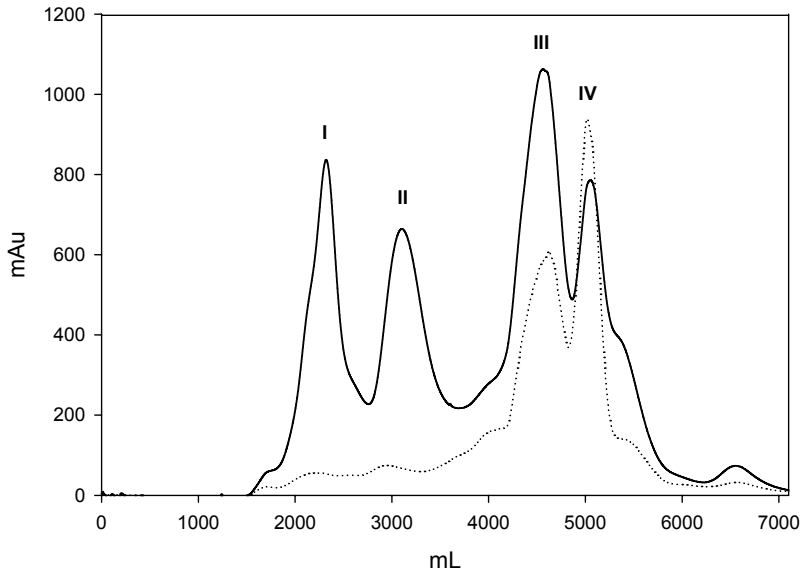

Figure 2: Gel filtration of potato juice on superdex 75 prep grade (solid line: 280 nm; dotted line: 320 nm).

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 20-25 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 oxidised polyphenols (Muralidhara and Prakash, 1995).

Fraction I

As determined by SDS-PAGE electrophoresis the protein band of 8 kDa indicated the subunit form of PI-1 in fraction I (Cleveland et al., 1987). Because of the presence of patatin in fraction I, judged from the major band at 40 kDa (Racusen and Foote, 1980), PI-1 was isolated according to the method of Melville and Ryan (1972). The proteins in fraction I were heated at 80°C during 5-6 minutes and filtrated through an 0.22 μm filter (Schleicher and

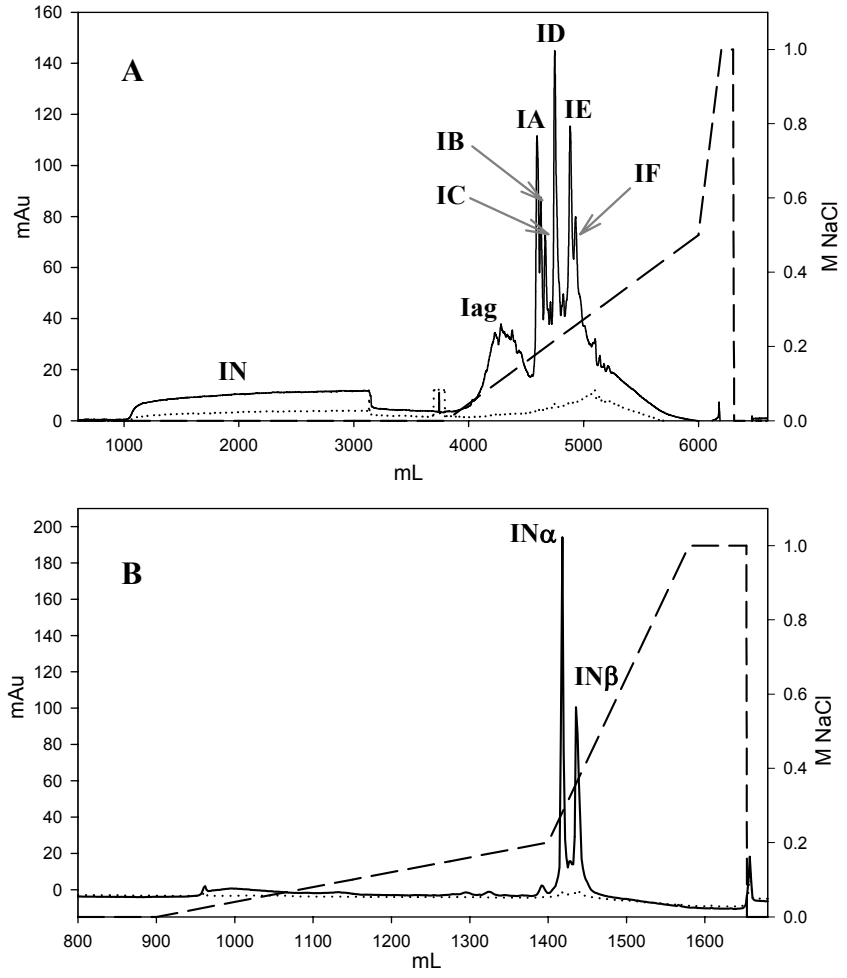

Figure 3: Fractionation of superdex fraction I; A: Anion exchange chromatography of fraction I on source 15Q; B: Cation exchange chromatography of fraction IN on source 15S (solid line: 280 nm; dotted line: 320 nm; dash line: salt gradient).

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 an 1:1 stoichiometry for trypsin which is characteristic for PI-1 (Melville and Ryan, 1972). Fraction Iag showed no protease inhibition activity and appeared as different protein bands, 40 and 80-100 kDa, on SDS-PAGE without β-mercaptoethanol. This fraction most likely contains patatin aggregates (Pots et al., 1999c) and some phosphorylase isoenzymes (bands at 40 kDa and 100 kDa) (Shiravam, 1976) remaining after the heating step. The non-bound proteins (IN) were dialysed 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. Summarising, in fraction I we were able to identify, in addition to patatin, 8 isoforms of PI-1.

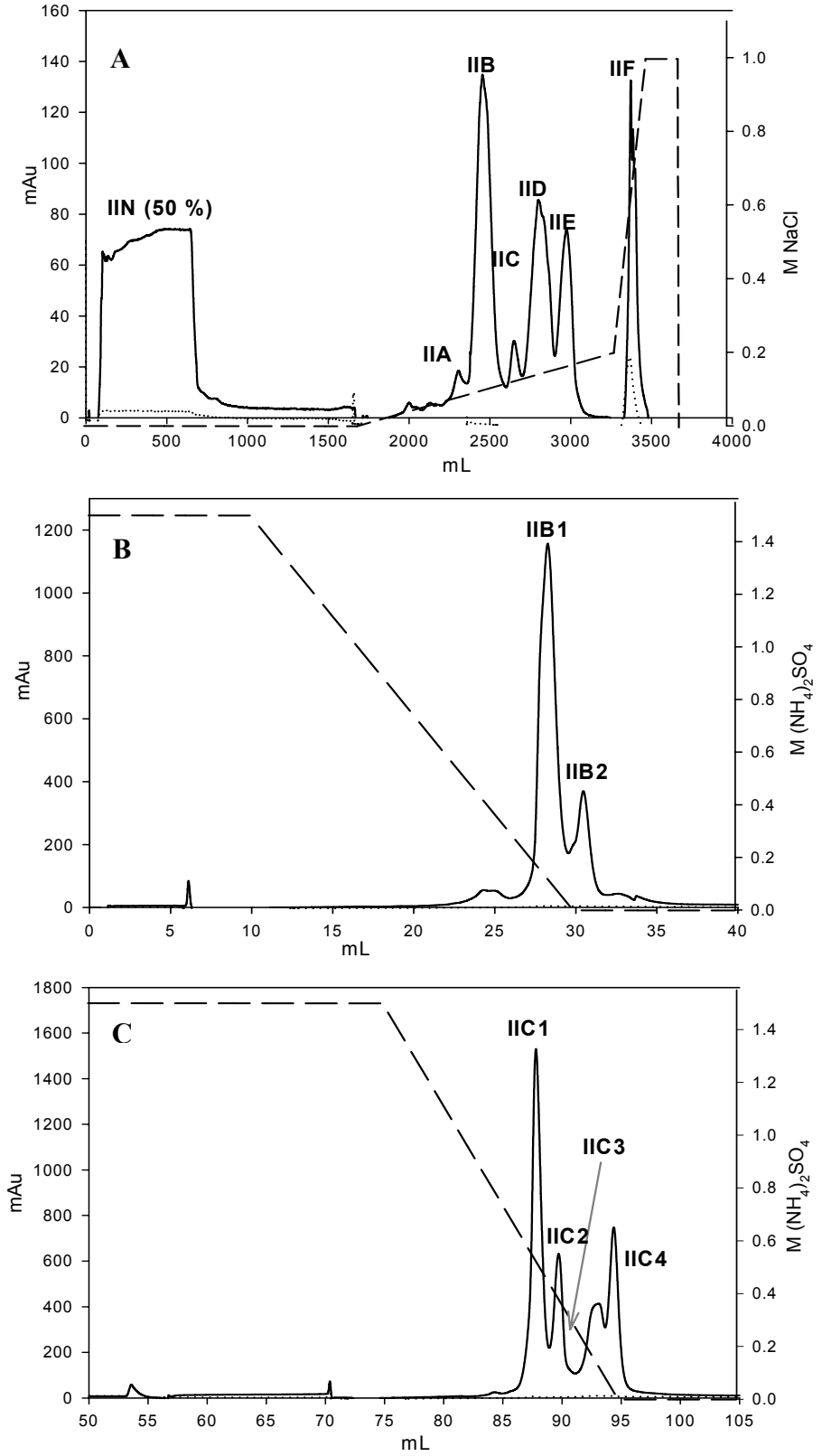

Figure 4: Fractionation of superdex fraction II; A: Anion exchange chromatography of peak II on source 15Q; Hydrophobic interaction chromatography of fractions IIB (B) and IIC (C) on phenyl superose (solid line: 280 nm; dotted line: 320 nm; dashed line: salt gradient).

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 non-bound 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.

Non-reducing SDS-PAGE electrophoresis showed that the proteins in fractions IIB, IID and IIE have molecular weights of approximately 20 kDa. In the presence of β-mercaptoethanol, two bands appear with a molecular weight 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 (Beekwilder et al., 2000). 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.

In order 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 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 containingthe 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 non-bound proteins (IIN) were fractionated by cation exchange chromatography. In **Figure 5A** 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 5B and 5C**). 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 approximately 20 kDa and existed in a monomeric form, except for fraction 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 characterisation.

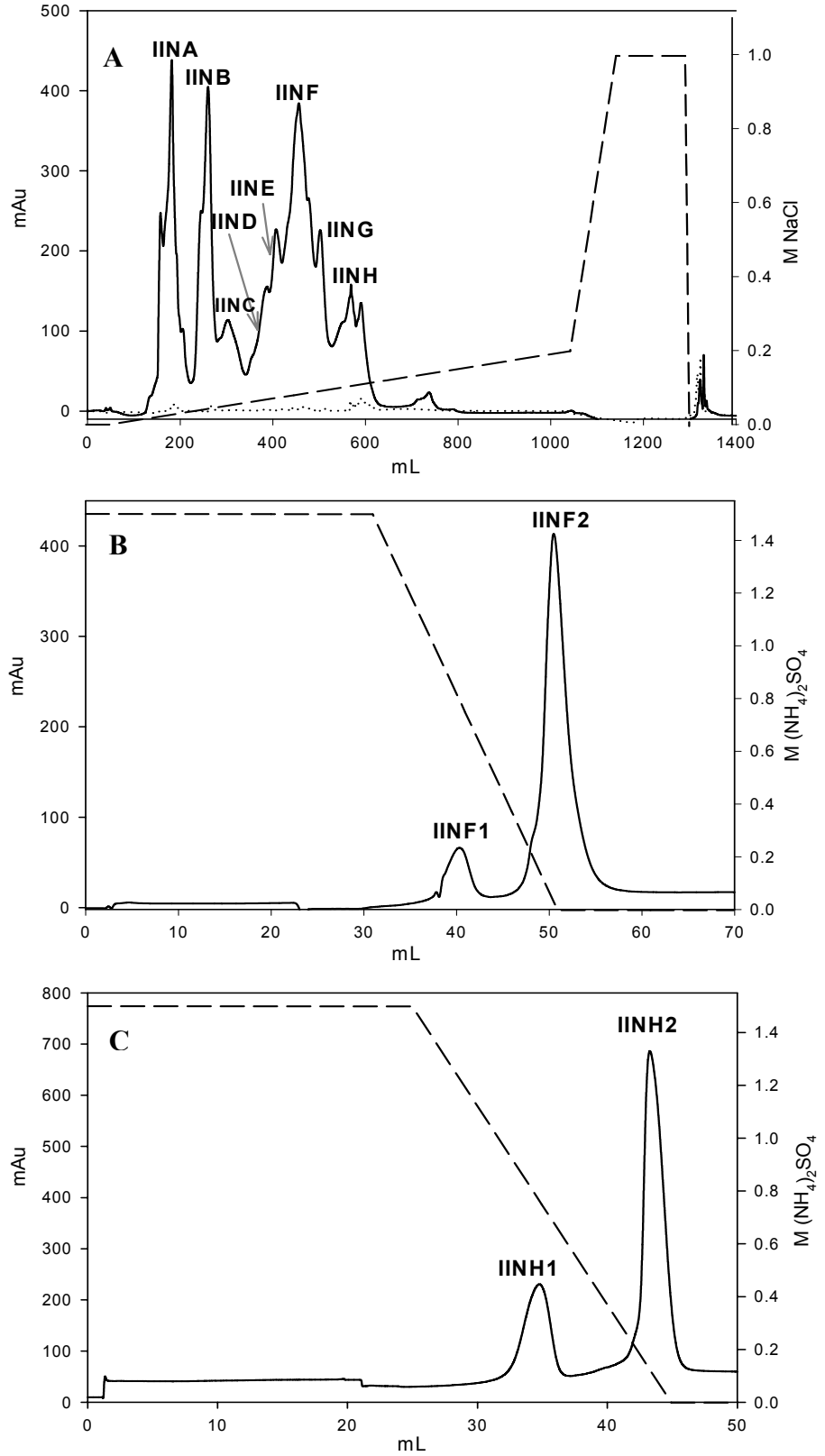

Figure 5: Fractionation of superdex fraction II; A: Cation exchange chromatography of fraction II on source 15S; Hydrophobic interaction of fraction IINF (B) and IINH (C) on phenyl superose (solid line: 280 nm; dotted line: 320 nm; dashed line: salt gradient).

Fractions III/IV

Fractions III and IV from the gel filtration step were freeze-dried, solubilised 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 6A).** The five fractions obtained from fraction IV were denoted IVA to IVE (**Figure 6B)**. The carboxypeptidase inhibitor is the smallest inhibitor present in potato tuber with a molecular weight of 4.3 kDa (Ryan et al., 1974; Hass et al., 1976) 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).

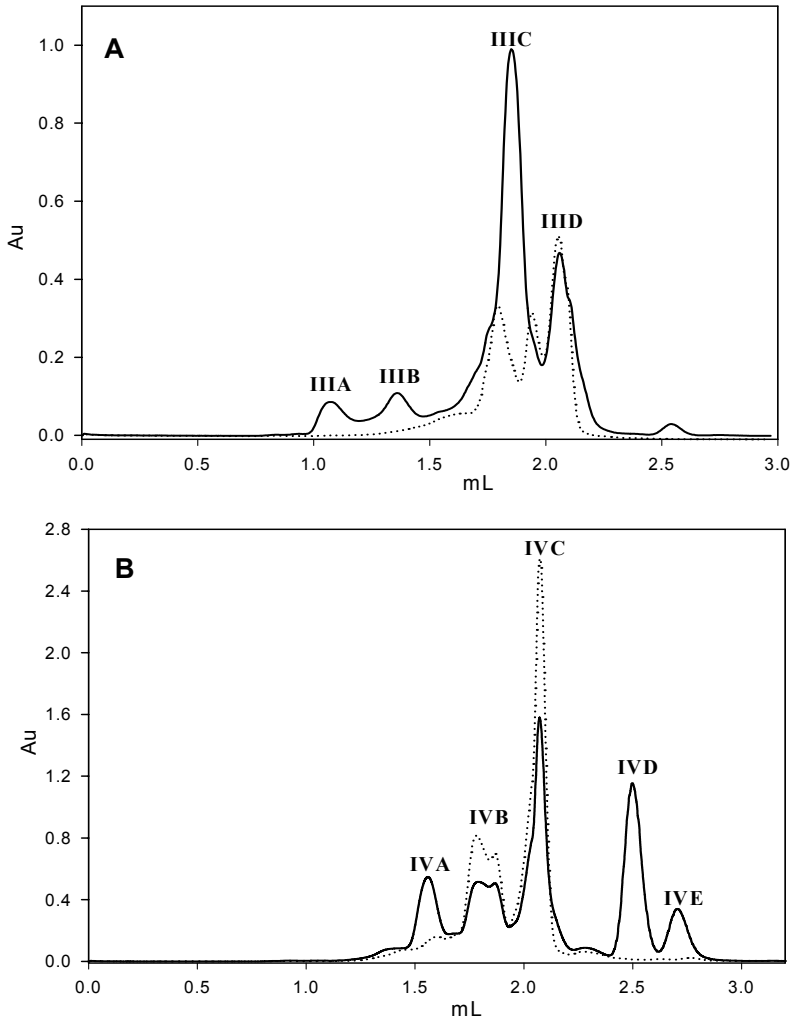

Figure 6: Fractionation of superdex fractions III and IV; A: Gel filtration of fraction III on superdex peptide; B: Gel filtration of fraction IV on superdex peptide (solid line: 280 nm; dotted line: 320 nm).

3.2 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**. Based on comparison of the properties and available literature data the protease inhibitors of the protein fractions were identified and classified.

PI-1

Based on the non-reducing 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, like molecular weight (20.5 kDa), the same electrophoretic pattern on reducing SDS-PAGE and their inhibitory activity. Therefore, we identified these proteins as PI-2 isoforms that vary slightly in pI and Mw (Bryant et al., 1976; Sanchez-Serrano et al., 1986).

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 Maganja and coworkers (Maganja et al., 1992) and denoted PIG. The protein present in fractions IINC1 was identified according to its isoelectric point and its inhibiting activity, as PI-13 (Strukelj et al., 1992). The protein present in fraction IINC2 was identified as a PAPI according to its isoelectric point and its inhibitory activity, described by Herbers and coworkers (Herbers et al., 1994), and denoted PI-8. 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 and coworkers (Ritonja et al., 1990). 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 and coworkers (Mares et al., 1989) 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 (Brzin et al., 1988). The protein in fraction IIC1 can be identified according to its pI (6.6) and its inhibiting activity as PCPI-6.6 (Brzin et al., 1988) and the proteins in fractions IING1 and IINF2 as PCPI-9.4 and PCPI-8.3, respectively (Brzin et al., 1988; Krizaj et al., 1993). However, all

Table 1: Characteristics and protease inhibition activity of proteins obtained after potato juice fractionation.

Name	Fraction	Mw (± 5 Da)	PI	SU	Tryp	Chym	Pap	CathD	HLE	CarboA
	I	7,683-7,873	5.1-6.3	5	+	+	-	-	+	-
PI-1	IN	7,683-7,873	7.2, 7.8	5	+	+	-	-	+	-
	IIC2, IIC3	7,683-7,873	5.1, 6.3	5	+	+	-	-	+	-
	IIA2, IIB1	20,279	6.5	2	+	+	-	-	+	-
	IIB2	20,023	6.0	2	+	+	-	-	-	-
	IIC3, IID3	20,273	6.1	2	+	+	-	-	+	-
PI-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-13	IINC1	19,878	8.7	1	+	-	-	+	+	-
PI-8	IINC2	20,141	7.5	1	+	+	-	+	-	-
PAPI-8.2	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	1	-	-	-	-	-	+

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.
+: inhibiting activity present, -: no inhibiting activity present.
nd: not determined.

these cysteine protease inhibitors have a lower molecular weight, as estimated by MALDI-TOF MS, than those identified by Brzin and coworkers (Brzin et al., 1988) and Krizaj and coworkers (Krizaj et al., 1993); in these studies, the molecular weight was 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 groups but not as large as those for the PCPI group. 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 and coworkers (Valueva et al., 1997), 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 PCPI will have to be determined to know the degree of homology between these proteins and the others members of the PCPI group.

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 (Mitsumori et al., 1994; Walsh and Twitchell, 1991). Also, the monomeric protein in fraction IINH2 was characterised as a serine protease inhibitor with inhibiting activity against trypsin and chymotrypsin. This inhibitor is also classified in the PKPI group according to Mitsumori and coworkers (Mitsumori et al., 1994), and named PKPI-8.0.

Other serine protease inhibitors (OSPI)

The main protein present in fraction IINE2 is a dimeric protein. The subunits must be disulfide linked since 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 and coworkers (Valueva et al., 1999).

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 and coworkers (Suh et al., 1990; Suh et al., 1991).

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 (Ryan et al., 1974; Hass et al., 1976).

3.3 Protein and protease inhibiting activity distribution in PJ

Protein distribution

The distribution of the different protein groups with respect to the total amount of protein present in PJ has been determined and the results are summarised in **Table 2**.

Table 2: Relative mass (w/w) distribution of protease inhibitor groups in potato juice.

	Superdex fraction	**Group**	**Proportion in PFJ**
	I (48.0 %)	Patatin	37.5
		PI-1	4.5
	II (48.0 %)	PI-2	22.3
		PCPI	11.5
		PAPI	6.1
		PKPI	3.6
		OSPI	1.5
	III (2.0 %)	PCI	0.9
Recovery			
100 %	98.0 %		88.1 %

Prior to this study, soluble proteins present in potato tuber have been divided in three different groups: patatin, representing ± 40 % of the potato proteins (Racusen and Foote, 1980), the group of protease inhibitors, representing 20-30 % of total protein (Melville and Ryan, 1972), and a group of other proteins (mainly enzymes like kinases and enzymes involved in starch synthesis), representing 20-30 % of the total protein (Man et al., 1997; Marshall et al., 1996). In the PJ of the investigated cultivar the protease inhibitors represent, however, ± 50 % of the total protein (**Table 2**).

Patatin represents approximately 80.0 % of the protein in fraction I, whereas PI-1 represents approximately 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 (Ryan et al., 1976; Cleveland et al., 1987). 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 (Cleveland et al., 1987).

The PCPI, PAPI and PKPI groups belong to the previously denoted class of 20 kDa proteins (Ralet and Gueguen, 1999) and together they represent 22.9 % of the total protein in PJ. The PCPI group contains 8 different inhibitors, which differ in molecular weight and pI, and represents 11.5 % 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 group contains 6 different inhibitors, which also differ in molecular weight and pI, and represents 6.1 % of the total amount of protein. The main proteins in this group are NID and PDI, which represent 1.7 and 1.4 % of the total protein, respectively. The PKPI group represents only 3.6 % of the total amount of protein in PJ. PKPI-8.0 is the main representative of this group and

accounts for 2.7 % of the total soluble protein. The OSPI and PCI groups 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 (Ryan et al., 1976), 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 (Venkaiah and Kumar, 1995); (Racusen and Foote, 1980). However, all studies published so far have been focussed 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 realise that in the present study only one cultivar was used and no variation in grow conditions and storage (Pots et al., 1999b) 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 (Pots et al., 1999a) and other proteins represent 37.5 % and 10-12 % of total soluble proteins, respectively.

Most of the other proteins in PJ have a molecular weight of 40 kDa and higher (Shiravam, 1976), thus they are mainly collected in fraction I. They most likely co-precipitate with patatin during the heating step and are possibly enzymes involved in starch synthesis (Gerbrandy and Doorgeest, 1972; Venkaiah and Kumar, 1995). In addition, the potato multicystatin inhibitor (PMC; 85 kDa) should be present in a small amount in fraction I (Walsh and Strickland, 1993). Also, minor losses of proteins during the purification procedure (see **Figure 1**) contribute to the calculated amount (11.9 %) of non-recovered protein.

Inhibiting activity distribution

Table 3 gives an overview of the distribution of enzyme inhibition activities over the seven inhibitor groups. The inhibiting activity of papain, cathepsin D and carboxypeptidase A

Table 3: Distribution of inhibiting activity over the different potato inhibitor groups.

Enzyme	Distribution of inhibiting activity in %							
	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.

Group	PI Prop	TIA (mg/g)	TIA Dist	CIA (mg/g)	CIA Dist
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	2.0	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-8.2	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	3.4	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

PI Prop: Proportion of inhibitor expressed as weight percentage of the total amount of protease inhibitors present in PJ, TIA: Trypsin Inhibiting Activity, CIA: Chymotrypsin Inhibiting Activity, TIA or CIA Dist: expressed as percentage of TIA or CIA distribution in PJ.

is due to a single group 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 groups except for PCI. The chymotrypsin inhibition activity is also distributed over the different groups, 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 group 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 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 group. PI-2 is a dimeric protein and can have four different subunits (A, B, C and D) in potato tuber (Bryant et al., 1976). Each monomer has active sites for trypsin and chymotrypsin and shows different affinity constant for these enzymes (Beekwilder et al., 2000). Thus, depending on its subunit composition, the dimeric protein can show different affinities for trypsin or chymotrypsin (**Table 4**).

Whereas PI-2 group 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 (Ralet and Gueguen, 1999) and each subunit differs in amino acid sequence and in reactivity against trypsin and chymotrypsin (Melville and Ryan, 1972). PI-1 has a binding stoichiometry of 1:1 for trypsin and a stoichiometry of 2:1 or 3:1 for chymotrypsin (Melville and Ryan, 1972). PI-1 (7.2), the most abundant representative of the PI-1 group (1.6 % of the total amount of protease inhibitors in PJ), shows the highest TIA value and one of the highest CIA values in PI-1 group.

In general, PCPI group members show lower TIA and CIA values than PI-2 and PI-1 and are responsible for 16 % of the chymotrypsin inhibition. Also in the PCPI group 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 group values of CIA as PI-2 members. The PAPI group is responsible for 9 % of the total chymotrypsin inhibition.

4 CONCLUSION

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

LITERATURE CITED

Beekwilder, J.; Schipper, B.; Bakker, P.; Bosch, D.; Jongsma, M. Characterization of potato proteinase II reactive site mutants. *Eur. J. Biochem.* **2000**, 267, 1-11.

Blanco-Aparicio, C.; Molina, M. A.; Frenandez-Salas, E.; Frazier, M. L.; Mas, J. M.; Quero, E.; Aviles, F. X.; de Llorens, R. Potato carboxypeptidase inhibitor, a T-knot protein, is an epidermal growth factor antagonist that inhibits tumor cell growth. *J. Biol. Chem.* **1998**, 273, 12370-12377.

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, 72, 248-254.

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

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.

Chase Jr, T.; Shaw, E. Titration of trypsin, plasmin, and thrombin with *p*-nitrophenyl *p'*-guanidinobenzoate HCl. *Methods Enzymol.* **1970**, 19, 20-27.

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.

Frenkel, K.; Chrzan, K.; Ryan, C. A.; Wiesner, R.; Troll, W. Chymotrypsin-specific protease inhibitors decrease H_2O_2 formation by activated human polymorphonuclear leukocytes. *Carcinogenesis* **1987**, 8, 1207-1212.

Geiger, R. In *Methods of enzymatic analysis*; Bergemeyer HU, Bergemeyer J and Grass M, eds; **1984**;

Gerbrandy, S. J.;Doorgeest, A. Potato phosphorylase isoenzymes. *Phytochemistry* **1972**, 11, 2403-2407.

Hass, G. M.; Ako, H.; Grahn, D. T.; Neurath, H. Carboxypeptidase inhibitor from potatoes. The effects of chemical modifications on inhibitory activity. *Biochemistry* **1976**, 15, 93-100.

Hass, G. M.; Nau, H.; Biemann, K.; Grahn, D. T.; Ericsson, L. H.; Neurath, H. The amino acid sequence of a carboxypeptidase inhibitor from potatoes. *Biochemistry* **1975**, 14, 1334-1342.

Herbers, K.; Prat, S.; Willmitzer, L. Cloning and characterization of a cathepsin D inhibitor gene from *Solanum tuberosum* L. *Plant Mol. Biol.* **1994**, 26, 73-83.

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.

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.

Huang, D. Y.; Swanson, B. G.; Ryan, C. A. Stability of proteinase inhibitors in potato tubers during cooking. *J. Food Sci.* **1981**, 46, 287-290.

Kennedy, A. R. Chemopreventive agents: protease inhibitors. *Pharmacol. Ther.* **1998**, 78, 167-209.

Knorr, D.; Kohler, G. O.; Betschart, A. A. Potato protein concentrates: the influence of various methods of recovery upon yield, compositional and functional characteristics. *J. Food Process. Preserv.* **1977**, 1, 235-247.

Krizaj, I.; Drobnic-Kosorok, M.; Brzin, J.; Jerala, R.; Turk, V. The primary stucture of inhibitor of cysteine proteinases from potato. *FEBS Lett.* **1993**, 333, 15-20.

Lee, M. C. S.; Scanlon, M. J.; Craik, D. J.; Anderson, M. A. A novel two-chain proteinase inhibitor generated by circularization of a multidomain precursor protein. *Nat. Struct. Biol.* **1999**, 6, 526-530.

Lisinska, G.; Leszczynski, W. *Potato science and technology*, Elsevier Applied Science, London, **1989**.

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.

Man, A. L.; Purcell, P. C.; Hannapel, U.; Halford, N. G. Potato SNF1-related protein kinase: molecular cloning, expression analysis and peptide kinase activity measurements. *Plant Mol. Biol.* **1997**, 34, 31-43.

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.

Marshall, J.; Sidebottom, C.; Debet, M.; Martin, C.; Smith, A. M.; Edwards, A. Identification of the major starch synthase in the soluble fraction of potato tubers. *Plant Cell* **1996**, 8, 1121-113.

Melville, J. C.; Ryan, C. A. Chymotrypsin inhibitor I from potatoes: Large scale preparation and characterization of its subunit components. *J. Biol. Chem.* **1972**, 247, 3443-3453.

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.

Mole, J. E.; Horton, H. R. Kinetics of papain-catalyzed hydrolysis of a-*N*-benzoyl-L-arginine-*p*-nitroanilide. *Biochemistry* **1973**, 12, 816-822.

Muralidhara, B. K.; Prakash, V. Interaction of 3'-O-caffeoyl D-quinic acid with human serum albumin. *Int. J. Pept. Protein Res.* **1995**, 46, 1-8.

Oliva, B.; Wastlund, M.; Nilsson, O.; Cardenas, R.; Querol, E.; Aviles, F. X.; Tapia, O. Stability and fluctuations of the potato carboxypeptidase A protein inhbitor fold: a molecular dynamics study. *Biochem. Biophys. Res. Commun.* **1991**, 176, 616-621.

Pots, A. M.; Gruppen, H.; Hessing, M.; van Boekel, M. A. J. S.; Voragen, A. G. J. Isolation and characterisation of patatin isoforms. *J. Agric. Food Chem.* **1999a**, 47, 4587-4592.

Pots, A. M.; Gruppen, H.; van Diepenbeek, R.; van der Lee, J. J.; van Boekel, M. A. J. S.; Wijngaards, G.; Voragen, A. G. J. The effect of storage of whole potatoes of three cultivars on the patatin and protease inhibitor content; a study using capillary electrophoresis and MALDI-TOF mass spectrometry. *J. Sci. Food Agric.* **1999b**, 79, 1557-1564.

Pots, A. M.; ten Grotenhuis, E.; Gruppen, H.; Voragen, A. G. J.; de Kruif, K. G. Thermal aggregation of patatin *in situ*. *J. Agric. Food Chem.* **1999c**, 47, 4600-4605.

Racusen, D.; Foote, M. A major soluble glycoprotein of potato tubers. *J. Food Biochem.* **1980**, 1, 13-52.

Ralet, M.-C.; Gueguen, J. Les proteines de pomme de terre: composition, isolement et proprietes fonctionnelles. *Science des Aliments* **1999**, 19, 147-165.

Richardson, M.; Cossins, L. Chymotrypsic inhibitor I from potatoes: The amino acid sequences of subunits B, C and D. *FEBS Lett.* **1974**, 45, 11-13.

Riordan, J. F.; Holmquist, B. In Bergemeyer HU, Bergemeyer J and Grassl M, eds; **1984**;

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.

Ryan, C. A.; Hass, G. M.; Kuhn, R. W. Purification and properties of a carboxypeptidase inhibitor from potatoes. *J. Biol. Chem.* **1974**, 249, 5495-5499.

Ryan, C. A.; Kuo, T.; Pearce, G.; Kunkel, R. Variability in the concentration of three heat stable proteinase inhibitor proteins in potato tubers. *Am. Potatp J.* **1976**, 53, 443-455.

Sanchez-Serrano, J. J.; Schmidt, R.; Schell, J.; Willmitzer, L. Nucleotide sequence of proteinase inhibitor II encoding cDNA of potato (*Solanum tuberosum*) and its mode of expression. *Mol. Gen. Genet.* **1986**, 203, 15-20.

Schonbaum, G. R.; Zerner, B.; Bender, M. L. The spectrophotometric determination of the operational normality of an a-chymotrypsin solution. *J. Biol. Chem.* **1961**, 236, 2930-2935.

Shiravam, K. N. Purification and properties of potato phosphorylase isozymes. *Z. Naturforsch., C: Biosci.* **1976**, 31c, 424-432.

Smith, C.; Van Megen, W.; Twaalfhoven, L.; Hitchcock, C. The determination of trypsin inhibitor levels in foodstuffs. *J. Sci. Food Agric.* **1980**, 31, 341-350.

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.

Suh, S.-G.; Peterson, J. E.; Stiekema, W. J.; Hannapel, D. J. Purification and characterization of the 22-kilodalton potato tuber proteins. *Plant Physiol.* **1990**, 94, 40-45.

Suh, S.-G.; Stiekema, W. J.; Hannapel, D. J. Proteinase-inhibitor activity and wound-inducible gene expression of the 22-kDa potato-tuber proteins. *Planta* **1991**, 184, 423-430.

Valueva, T. A.; Revina, T. A.; Kladnitskaya, G. V.; Mosolov, V. V.; Mentele, R. Primary structure of a 21-kD protein from potato tubers. *Biochemistry (Moscow)* **1999**, 11, 1258-1265.

Valueva, T. A.; Revina, T. A.; Mosolov, V. V. Potato tuber protein proteinase inhibitors belonging to the Kunitz soybean inhibitor family. *Biochemistry (Moscow)* **1997**, 62, 1367-1374.

van Jaarsveld, F. P.; Naude, R. J.; Oelofsen, W. Optimisation of calcium-dependent protease and cathepsin D assays in ostrich muscle and the effect of chemical and physical dry-curing parameters. *Meat Science* **1997**, 47, 287-299.

Venkaiah, B.; Kumar, A. A process for the recovery and immobilization of starch phosphorylase from starch-based industrial wastewater. *Biotechnol. Appl. Biochem.* **1995**, 21, 77-85.

Walsh, T. A.; Strickland, J. A. Proteolysis of the 85-kilodalton crystalline cysteine proteinase inhibitor from potato releases functional cystatin domains. *Plant Physiol.* **1993**, 103, 1227-1234.

Walsh, T. A.; Twitchell, W. P. Two Kunitz-type proteinase inhibitors from potato tubers. *Plant Physiol.* **1991**, 97, 15-18.

Chapter 3

The most abundant protease inhibitor group in potato tuber (cv. *Elkana*) is a serine protease inhibitor from the Kunitz-type

ABSTRACT

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-type inhibitor. Therefore, the most abundant protease inhibitor was considered to be one of the isoforms of PSPI. The PSPI group represents approximately 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 group of protease inhibitor in potato showed that approximately 70% of the protease inhibitors present in potato juice belongs to the Kunitz-type inhibitor.

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1 INTRODUCTION

Protease inhibitors are ubiquitously abundant in tubers and plant seeds (Ryan, 1977). In higher plants, several gene families of protease inhibitors have been characterised, particularly the serine protease inhibitors from *Leguminosae*, *Solanaceae* and *Graminae* (Garcia-Olmeda et al., 1987).

Protease inhibitors in plants are generally considered to be storage proteins (source of nitrogen) and as a defence mechanism (Pusztai et al., 1988). They were shown to be involved with the wound-induced defence response of plants against herbivores and pathogens (Bergey et al., 1996) and have been shown to have an antinutrient activity on *Spodoptera exigua* larvae (Jongsma et al., 1995). They also accumulate in potato leaves in response to wounding and UV-irradiation (Graham et al., 1985; Bergey et al., 1996; Conconi et al., 1996).

In recent years, protease inhibitors have received new interest because of their potent activity in preventing carcinogenesis in a wide variety of *in vitro* and *in vivo* systems (Kennedy, 1998b). Serine protease inhibitors have been reported to have inhibitory effects on tumor cell growth (Huang et al., 1997; Kennedy, 1998a). 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 (Hill et al., 1990).

In potatoes, a wide range of protease inhibitors is expressed (Lisinska and Leszczynski, 1989). Potato tuber contains approximately 1.5 % (w/w) protein on fresh weight basis (Lisinska and Leszczynski, 1989). In cv. *Elkana*, protease inhibitors represent approximately 50 % of the total amount of soluble protein present in the tuber (chapter 2). According to Cleveland and coworkers (Cleveland et al., 1987), 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 like trypsin and chymotrypsin. According to Bryant and coworkers (Bryant et al., 1976), PI-2 is a dimeric protein with two identical, as determined by amino acid analysis, subunits of 10 kDa. However, according to Mc Manus and coworkers (McManus et al., 1994), PI-2 is a dimeric protein with two subunits that differ in size as determined by SDS PAGE (15 kDa for the large subunit and 6 kDa for the small one).

Although it was found that the most abundant group of protease inhibitors also consists of a dimeric protein (chapter 2), with two subunits held together by a disulfide bridge, preliminary data obtained subsequently in our laboratory cast doubt on identification of this group of proteins as PI-2. This protein group represents approximately 22 % of the total amount of protein in potato, and was thus called PI-2 based on its abundance (Cleveland et al., 1987), dimeric structure (McManus et al., 1994) and inhibitory activity (Bryant et al., 1976). PI-2 6.1 and PI-2 6.5 are the two most abundant isoforms of PI-2 group in potato juice (chapter 2) and therefore were used for further characterisation.

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 comparison of the binding of various protease inhibitors to polyclonal antibodies against this protease inhibitor.

2 MATERIAL AND METHODS

2.1 Material

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 maximum period of 6 months.

2.2 Preparation of potato juice

Potatoes were chopped in large pieces (max. 8 x 2.5 cm) and subsequently mixed in the presence of sodium bisulfite at a dosage of 0.5 g/kg potatoes to prevent oxidation of phenolic compounds. Potato juice was prepared as described previously (chapter 2).

2.3 Purification of PI-2 isoforms

The ÄKTA explorer protein chromatography system and the columns used for the protein purification were 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 as described previously (chapter 2). An additional chromatofocusing purification step was included, using a Polybuffer Exchanger 74 column (60 x 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 (chapter 2) were loaded onto the column. The protein was eluted using a 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 a HP phenyl sepharose column (10 x 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 dialysed at 4°C against 7 mM sodium phosphate buffer pH 7.5 (ionic strength 15 mM). After dialysis, the samples were frozen in small volumes and stored until use at a concentration of 1 mg/mL.

2.4 Protein purity

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

2.5 Mass spectrometry

MALDI-TOF MS analysis in the linear mode was performed using a Voyager DE RP instrument (Perseptive Biosystems, Framingham, MA, USA) as described previously (chapter 2).

2.6 Polyclonal antibody production against PI-2 purified from cv. *Elkana*

Polyclonal antibodies were raised against PI-2 6.1 and PI-2 6.5 (chapter 2) in rabbits. A standard-immunisation protocol for three months was carried out at the Sequence Laboratories Göttingen GmbH (Göttingen, Germany).

2.7 ELISA

The antisera were diluted 20 to 10^5 times for determination of the affinity for the two PI-2 isoforms. For the cross-reactivity, the antisera were diluted 5,000 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 absorbancy. Polyvinyl chloride microtitre plates (Dynatech, Chantilly, VA, USA) were coated overnight with 100 µL 5 µg/mL protein in 0.07 M sodium phosphate buffer pH 7.0 containing 0.15 M NaCl (PBS). Hereafter, plates were washed 3 times with PBS containing 0.05 % (w/w) Tween 20 (PBST). Antibodies against PI-2 6.1 and PI-2 6.5 were diluted 5,000 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 3 times with PBST. To each well 100 µL 0.025% (v/v) anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St Louis, MO) in PBST containing 1 % (w/w) bovine serum albumin was added. After 1.5 h of incubation, the plates were washed 3 times with PBST. The 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 minutes by adding 100 µL 3 M NaOH and the absorbance was measured at 400 nm. All incubations were performed at about 20-22^{o}C. Each ELISA experiment was performed in duplicate and the results for PI-2 6.1 and PI-2 6.5 were corrected for non-specific reactivity by subtracting absorbances obtained using non- immune sera.

2.8 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 β-mercaptoethanol and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) using a Mini Trans-Blot apparatus (Bio-Rad, Hercules, CA, USA) 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).

2.9 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 and coworkers (Valueva et al., 2000). 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 potato tuber according to Kuipers and coworkers (Kuipers et al., 1994). 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 one hour, to obtain a clear top phase.

Table 1: Primers used for PCR amplification.

Name	Sequence (5'-3')
ST	ATGAAGTGTTTATTTTTGTTATG
FA	CTACCTAGTGATGCTACTCCA
FB	CTACCCAGTGATGCTACTCCA
RF	CAAACGAGGATCAAGTTCTTTAC
RE	TTA(C/T)TGGACTTGTTTGAAGGAGA

After synthesis of the first strand cDNA using 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 Corporation, Madison, WI, USA). 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, USA) 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, USA) and sequenced with an automated DNA Sequencer 373 (Applied Biosystems, Foster City, CA, USA). The DNA sequence data were submitted to the GenBank Nucleotide Databases under the accession number AY166690. The BLAST2 program (Altschul et al., 1997) was used for searching sequence homologies.

3 RESULTS AND DISCUSSION

3.1 Protein quaternary structure

In order 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 non-reducing 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 Mc Manus and coworkers (McManus et al., 1994). This pattern also shows that both subunits are linked by one or more disulfide bridges.

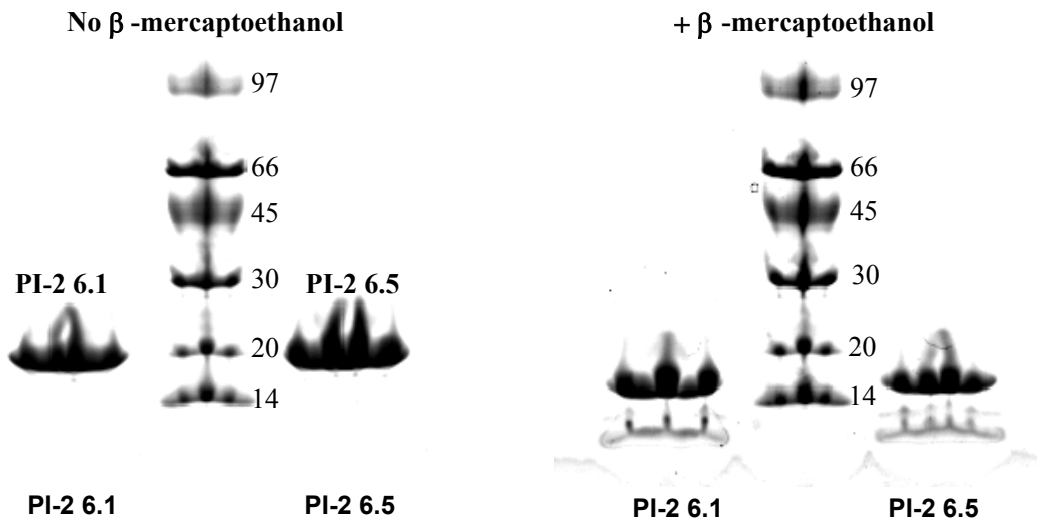

Figure 1: SDS PAGE of PI-2 isoforms of cv. *Elkana* in absence and in presence of β-mercaptoethanol.

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 10,136 and 20,273 Da which correspond to the doubly and singly charged ion of the same protein, respectively (**figure 2A**). To determine the Mw of the subunits, the cysteinyl residues were blocked using iodoacetamide (Gerwin, 1967). The MALDI-TOF MS spectrum showed one single peak at 4,229 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* (chapter 2) was not the one described by Bryant and coworkers (Bryant et al., 1976) because the masses of the two subunits are not identical. Also, the small subunit, determined by MALDI-TOF MS, is smaller as described for PI-2 by Mc Manus and coworkers (McManus et al., 1994).

3.2 N-terminal sequencing

The large and small subunits of PI-2 6.1 had an N-terminal amino acid sequence of LPSDATPVLDVTGKELDSRL and SDDQFCLKVGVVHQNGKRRLALVKD, respectively. Both sequences were used for a homology search and showed 100 % identity to a protein purified and sequenced by Valueva and coworkers (Valueva et al., 2000), which was called Potato Serine Protease Inhibitor (PSPI). According to Valueva and coworkers (Valueva et al., 2000), PSPI is a dimeric protein that belongs to kunitz-type of protease inhibitors. It therefore appears that the protein from cv. Elkana (chapter 2) designated as PI-2 is apparently a member of the Kunitz-type inhibitor.

We have previously reported the presence of another dimeric protease inhibitor (chapter 2), which contains two subunits with a different mass that are also disulfide linked. This protein showed inhibitory activity against trypsin, chymotrypsin and elastase but only represented less than 1 % of the total amount of protein in potato. Further characterisation will be necessary to determine if this protein from cv. *Elkana* is PI-2 as described by Mc Manus and coworkers (McManus et al., 1994).

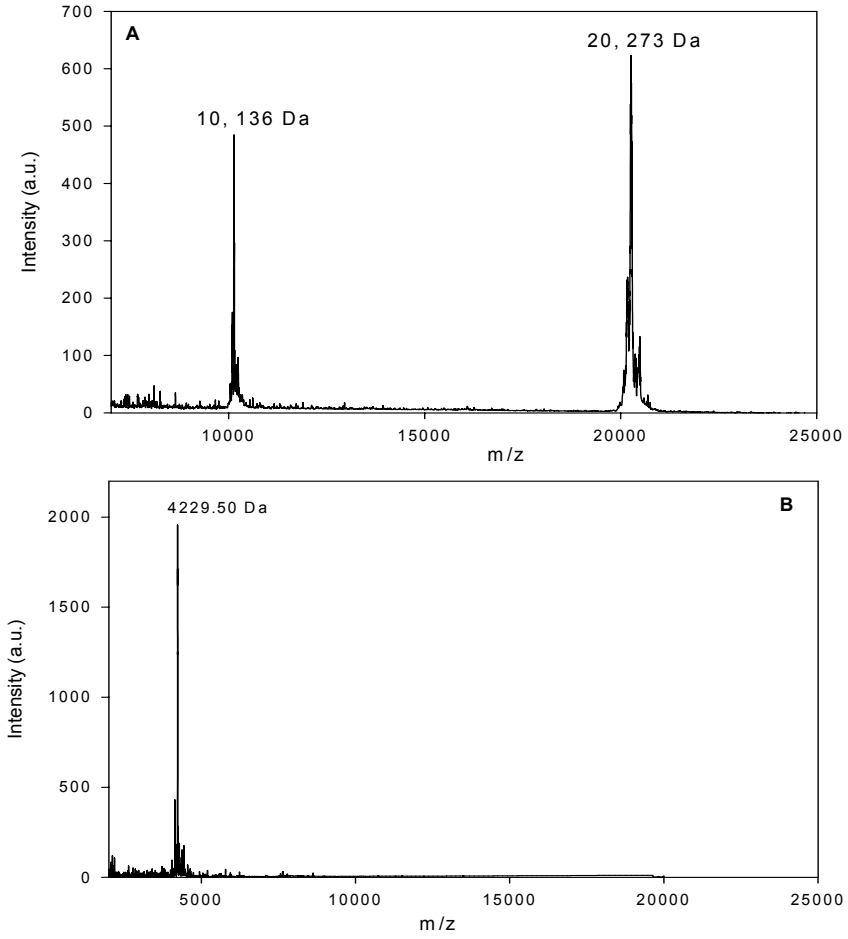

Figure 2: MALDI-TOF MS spectra of PI-2 6.1: (A) in native state and (B) with cysteinyl residues blocked.

3.3 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-type inhibitor). Members of the Kunitz-type of inhibitor have in their primary structure some conserved residues, such as e.g. 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-type inhibitors are conserved in "PI-2". The amino acids sequences of PSPI (Valueva et al., 2000), a Kunitz-type inhibitor (PKI) (Banfalvi et al., 1996) and two aspartic protease inhibitors (Strukelj et al., 1990; Maganja et al., 1992) showed 98, 95, 96 and 74 % homology with "PI-2" from cv. *Elkana,* respectively. The homology with a chymotrypsin inhibitor from winged bean (Peyachoknagul et al., 1989), often used as representative of the Kunitz–type inhibitor, was only 32 %. Although these proteins show high homology with "PI-2" from cv. *Elkana* and PSPI (Valueva et al., 2000), they do not consist of two polypeptide chains in their

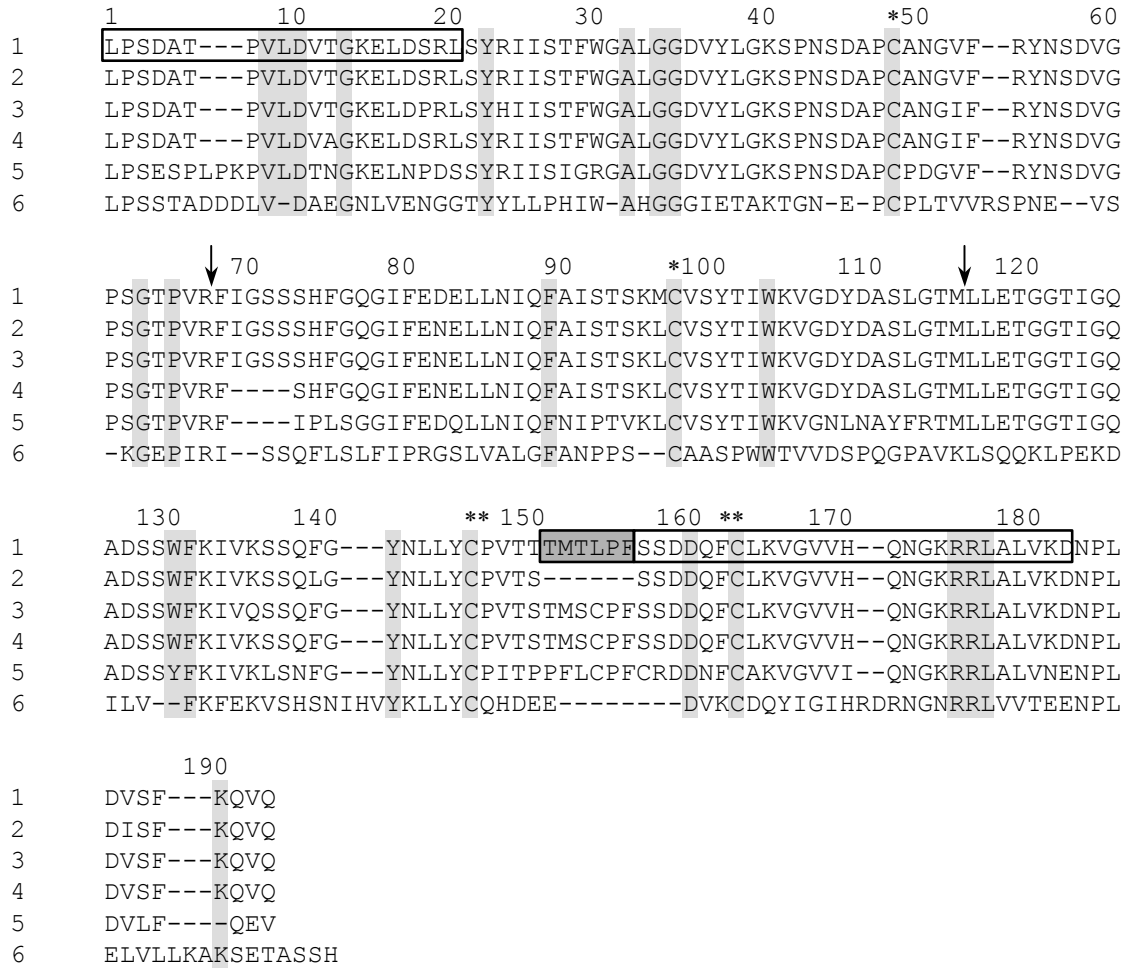

Figure 3: Alignment of amino acid sequences of (1) "PI-2" (AY166690) from potato tuber cv. *Elkana* with other proteins of the Kunitz-type inhibitor: (2) PSPI, a serine protease inhibitor from potato (Valueva et al., 2000); (3) PKI (U30388), a protease inhibitor from potato (Banfalvi et al., 1996); (4) PIG, an aspartate protease inhibitor (S66277) from potato (Maganja et al., 1992); (5) an aspartate protease inhibitor (P17979) from potato (Strukelj et al., 1990); (6) WCI, a chymotrypsin inhibitor (P10822) from winged bean (Peyachoknagul et al., 1989).

Amino acid residues are numbered according to the sequence of "PI-2" from potato tuber; in box, sequence from the N-terminal sequence; in grey box, the amino acids, which are cleaved during the post-translational process in plant; conserved amino acid are striped; * and **, disulfide bonds Cys48-Cys97 and Cys146-Cys157; the arrows marked the P1 residues of the active sites of the inhibitor.

mature form and also differ considerably in their enzyme specificity. PKI shows specificity only for trypsin, while "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* (chapter 2), 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 as PSPI. PI-2 6.1 and PI-2 6.5 will thus be called PSPI 6.1 and 6.5, respectively. It can be concluded that in cv. *Elkana*, not PI-2 but PSPI is the most abundant group of protease inhibitors.

Based on 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, the molecular weight obtained by MALDI-TOF MS, and by comparison with the protein purified by Valueva and coworkers (Valueva et al., 2000), the small subunit starts at position 157 (**figure 3**). The 6 preceding amino acids (151-156) are probably deleted during the post-translational process. This processing may also explain the existence of 7 different isoforms of PSPI in cv. *Elkana*, differing in molecular weight and pI, because length and site of deletion may slightly differ from one isoform to another.

In order 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 4,229 Da (as determined by MALDI-TOF MS) minus 57 Da of the blocking agent, which results in a mass of 4,172 Da. By deduction, the molecular mass of the large subunit is 16,100 Da for PSPI 6.1. This result is in agreement with the calculated molecular mass from the amino acid sequence deduced from the gene (16,073 and 4,169 Da for the large and small subunit, respectively).

3.4 Binding of PSPI 6.1 and 6.5 polyclonal antibodies against protease inhibitors groups from cv. Elkana

Polyclonal antibodies were raised against PSPI 6.1 and 6.5. These antibodies showed the same affinity for both isoforms of PSPI (data not shown). In order to study the structure homology between the different groups of protease inhibitors, the cross-reactivity of these antibodies was tested for purified representative protease inhibitors from potato *cv. Elkana* (chapter 2) (**figure 4**).

Both antibodies showed a high binding for NID (Novel Cathepsin D Inhibitor), PDI (Potato Cathepsin D Inhibitor), PCPI 5.9 (member of the of 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 PCI (Potato Carboxypeptidase Inhibitor) (chapter 2). According to literature (Brzin et al., 1988; Mares et al., 1989; Ritonja et al., 1990; Mitsumori et al., 1994), NID, PDI, PCPI 5.9 and PKPI 8.0 are indeed also members of the Kunitz-type inhibitor. The antibodies raised against PSPI therefore seem to show a high binding for members of the Kunitz-type of inhibitor, and only a low binding for other proteins. With these results, it appears that approximately 70 % of the total amount of protease inhibitors in potato are probably members of the Kunitz-type inhibitor.

PCPI 8.6 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-type.

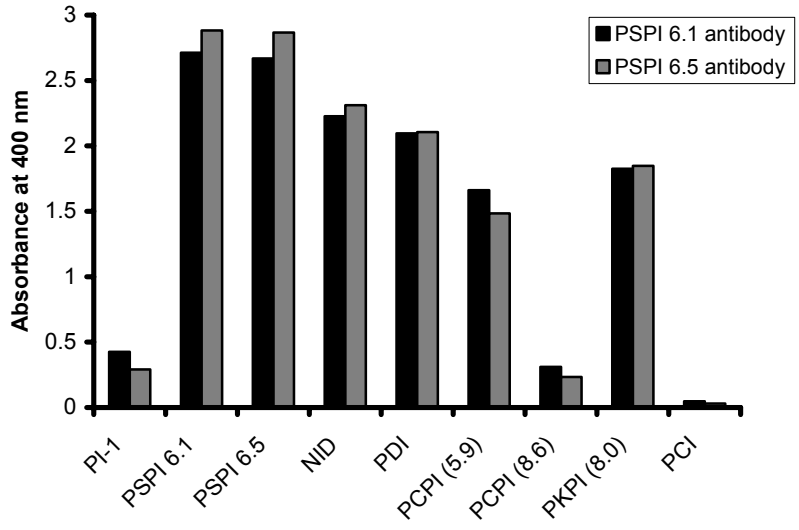

Figure 4: Binding of PSPI 6.5 and 6.1 antibodies against PSPI isoforms and potato protease inhibitors from different groups (chapter 2).

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.

4. CONCLUSION

The research described in this paper, combined with our previous work (chapter 2) 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, based on its gene sequence, is expressed as one polypeptide chain and subsequently processed by removal of about 6 amino acids.

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

LITERATURE CITED

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.

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.

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.

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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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

Kennedy, A. R. Chemopreventive agents: protease inhibitors. *Pharmacol. Ther.* **1998b**, 78, 167-209.

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.

Lisinska, G.; Leszczynski, W. *Potato science and technology*, Elsevier Applied Science, London, **1989**.

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.

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.

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.

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.

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.

Pusztai, 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.

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.

Ryan, C. A. Proteolytic enzymes and their inhibitors in plant. *Ann. Rev. Plant Physiol.* **1977**, 24, 173-196.

Strukelj, B.; Pungercar, J.; Ritonja, A.; Krizaj, I.; Gubensek, F.; Kregar, I.; Turk, V. Nucleotide and deduced sequence of an aspartic proteinase inhibitor homologue from potato tubers (*Solanum tuberosum* L.). *Nucleic Acids Res.* **1990**, 18, 4605.

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

Chapter 4

Tentative assignment of the Potato Serine Protease Inhibitor group as β-II proteins based on their spectroscopic characteristics

Abstract

Potato Serine Protease Inhibitor (PSPI) is the most abundant protease inhibitor group in potato tuber. The investigated PSPI isoforms have a highly similar structure at both the secondary and tertiary level. From the results described, PSPI is classified as a β-II protein based on: (1) the presence in the near UV spectra of sharp peaks, indicating a rigid and compact protein, (2) the sharp transition from the native to the unfolded state upon heating (only 6°C) and (3) the similarity in secondary structure to soybean trypsin inhibitor, a known β-II protein, as indicated by a similar far UV CD spectrum and a similar amide I band in the IR spectrum. The conformation of PSPI was shown also to be stable at ambient temperature in the pH range 4 to 7.5. Upon lowering the pH to 3.0, some minor changes in the protein core occur, as observed from the increase of the intensity of the phenylalanine peak in the near UV CD spectrum.

Based on:

L. Pouvreau, H. Gruppen, G. A. van Koningsveld, L. A. M. van den Broek and A. G. J. Voragen. Tentative assignment of the Potato Serine Protease Inhibitor group as β-II proteins based on their spectroscopic characteristics. *Submitted for publication*.

1 INTRODUCTION

Protease inhibitors are abundant in tubers and plant seeds (Ryan et al., 1974). In higher plants, several gene families have been characterised, particularly those constituting the serine protease inhibitors from the Leguminosae, Solanaceae and Graminae (Garcia-Olmeda et al., 1987).

Protease inhibitors in plants seem to be involved in various processes (McManus et al., 1999). They have been shown to play a protectant role against insect attack and virus infection (Bergey et al., 1996). They have also been proposed to be used as storage proteins in seeds, deduced from the fact that the maximum level of protease inhibitors coincides with the maximum level of proteolysis during germination (Ambekar et al., 1996). They also are considered to participate in the activity regulation of endogenous proteases (Morita et al., 1996).

In recent years, protease inhibitors have regained interest because of their potential activity in preventing carcinogenesis in a wide range of *in vitro* and *in vivo* systems (Kennedy, 1998b). For example, serine protease inhibitors have been reported to have inhibitory effects on tumor cell growth (Huang et al., 1997; Kennedy, 1998a). In addition, by increasing the level of cholecystokinin via the inhibition of trypsin, serine protease inhibitors may also be used to reduce food intake in man (Hill et al., 1990).

In potato, a wide range of protease inhibitors is expressed. Potato tubers contain approximately 1.5 % of protein on a fresh weight basis (Lisinska and Leszczynski, 1989), of which, in cv. *Elkana*, protease inhibitors represent up to 50 % (chapter 2). Potato Serine Protease Inhibitor (PSPI) was first described by Valueva and coworkers (Valueva et al., 1997) as a dimeric serine protease inhibitor containing two active sites, one against trypsin and one against α-chymotrypsin/elastase. PSPI is the most abundant group of protease inhibitors in potato tuber (cv. *Elkana*) accounting for 44% of the total amount of protease inhibitors (chapter 2). The PSPI group consists of a group of proteins that is expressed as one polypeptide chain, that subsequently undergoes post-translational processing, during which ≈ 6 amino acids are cleaved off (chapter 3). As a result, a dimeric protein of ≈ 20.2 kDa is obtained with the two sub-units held together by a disulfide bridge. Seven different isoforms of PSPI, with isoelectric pHs varying from 5.5 to 6.9, have been identified in potato juice (cv. *Elkana*) (chapter 2).

Protease inhibitors are important tools of nature for regulating the proteolytic activity of endogenous as well as exogenous proteases. It is, therefore, of great interest to determine which characteristic structural properties confer inhibitory activity to these proteins. Until now, the 3D structures of only a limited amount of protease inhibitors have been determined (Bode and Huber, 1992). In this study, we determine the structural characteristics of PSPI using far and near UV circular dichroism, fluorescence and FTIR spectroscopy. By comparing these structural characteristics to those of more extensively studied protease inhibitors, we attempt to classify PSPI in a (sub)structural class of proteins. Proteins involved in defence mechanism, such as protease inhibitors and lectins, are known to be heat stable (Moses and Hinz, 1983; Ingram, 1985; Ceciliani et al., 1994). Therefore, using the mentioned techniques

as well as differential scanning calorimetry, the conformational stability of PSPI upon heating and at various pHs is studied.

2 MATERIAL AND METHODS

2.1 Preparation of PSPI isoform solutions

PSPI isoforms 6.1 and 6.5 were purified as described previously (chapter 2 and 3) from potato juice (cv. *Elkana*). After purification, PSPI 6.1 and 6.5 were dialysed at 4°C against buffers of various pH (3.0, 4.0, 5.0, 6.0, 7.0 and 7.5) all having a calculated ionic strength of 15 mM. The buffers used were 18 mM sodium phosphate buffer (pH 3.0), 95 mM sodium acetate buffer (pH 4.0), 24 mM sodium acetate buffer (pH 5.0), 20 mM piperazine buffer (pH 6.0), 9 mM sodium phosphate buffer (pH 7.0), and 7 mM sodium phosphate buffer (pH 7.5). These buffers are denoted Buffer pH 3, pH 4, pH 5, pH 6, pH 7 and pH 7.5, respectively. Subsequently, the samples were frozen in small volumes at a concentration of 0.8 mg/mL and stored at -20°C until use.

Soybean trypsin inhibitor (STI) was purchased from Fluka (art. no.: 93618). STI (1 mg/mL) was dissolved in 9 mM sodium phosphate buffer (pH 7.0) and dialysed against the same buffer overnight at 4°C.

2.2 Spectroscopic measurements

All samples were filtered through a 0.22 μm filter before spectroscopic measurements.

Far-ultra violet CD

Far-ultra violet circular dichroism (far-UV CD) spectra of 0.2 mg/mL of PSPI in Buffer pH 3, pH 4, pH 5, pH 6, pH 7 and pH 7.5 were recorded on a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) at temperatures ranging from 20 to 85°C with intervals of approximately 5 degrees, using a heating rate of 30°C/hour. The temperature in the sample was measured using a thermocouple wire. Starting from 20°C, the proteins were heated to the desired temperature and equilibrated for 3 minutes at this temperature before the wavelength-scans were recorded. Quartz cells with an optical path length of 0.1 cm were used. The scan range was 260-190 nm, the scan speed was 50 nm/min, the data interval 0.2 nm, the bandwidth 1.0 nm, the sensitivity 20 mdeg and the response time was 0.125 seconds. Spectra were recorded in 10-fold and averaged. Spectra were corrected by subtracting the spectrum of a protein free sample, obtained under identical conditions. Noise reduction was applied using the Jasco software. The spectra were analysed from 240 to 190 nm to estimate the secondary structure content of the protein, using a non-linear regression procedure (De Jongh et al., 1994). Spectra were fitted using the reference spectra of poly-lysine in the α-helix, β-strand and random coil conformation (Greenfield and Fasman, 1969), and the spectrum of β-turn structures, extracted from 24 proteins with known X-ray structure (Chang et al., 1978). Changes in secondary structure of PSPI during heating were also monitored by measuring the ellipticity at 222 nm.

Near-ultra violet CD

Near-ultra violet circular dichroism (Near-UV CD) spectra of 0.8 mg/mL PSPI, in Buffer pH 3, pH 4, pH 5, pH 6, pH 7, and pH 7.5, were recorded on a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) at various temperatures ranging from 20 to 85°C, using a heating rate of 30°C/hour. Starting from 20°C, the proteins were heated to the desired temperature and equilibrated for 6 min at this temperature before the wavelength-scans were recorded. Spectra were recorded 30-fold and averaged. Spectra were corrected by subtracting the spectrum of a protein free sample, obtained under identical conditions. A quartz cell with an optical path length of 1.0 cm was used. The scan interval was 250-350 nm, the scan speed 100 nm/min, the data interval 0.2 nm, the bandwidth 1.0 nm, the sensitivity 20 mdeg and the response time was 0.125 seconds.

Fluorescence spectroscopy

Fluorescence spectra of 0.1 mg/mL PSPI in Buffer pH 3, pH 4, pH 5, pH 6, pH 7, and pH 7.5 were recorded on a Perkin Elmer Luminescence Spectrophotometer LS 50 B (Perkin Elmer Corp., Boston, MA, USA) with a pulsed Xenon source. Excitation was done at 295 nm and the resulting emission spectrum was recorded from 305 to 405 nm, using a scan speed of 100 nm/min. Both the excitation and the emission slit were set at 3.5 nm. Spectra were recorded 3-fold and averaged. Spectra were corrected by subtracting the spectrum of a protein free sample, obtained under identical conditions.

Fourier Transformed Infrared (FTIR) spectroscopy

Attenuated total reflection infrared (ATR-IR) spectra were recorded on a Biorad FTS 6000 spectrometer equipped with a DTGS detector (Bio Rad Laboratories Inc., Cambridge, MA, USA). Typically, 50 µl of a 0.8 mg/mL PSPI sample in 9 mM phosphate-buffer (pH 7.0) were transferred onto a germanium crystal (1x8 cm) and dried under air to remove excess water. Next, the crystal was placed such in the light beam that 6 total reflections were obtained. Spectra were accumulated at ambient temperature in the spectral region of 4000-800 cm^{-1} with a spectral resolution of 0.5 cm^{-1} prior to zero-filling and Fourier transformation, using a speed of 5 kHz and a filter of 1.2 kHz. Typically 100 spectra were accumulated and subsequently averaged. A spectrum representing atmospheric water was subtracted from the sample-spectra. All samples were prepared and analysed at least in duplicate. Spectra were deconvoluted in order to analyse the underlying absorption bands using K = 2.4 and a full width at half height (FWHH) of 24 cm^{-1}.

2.3 Differential scanning calorimetry

DSC measurements were performed on a VP-DSC Microcalorimeter (MicroCal Inc., Northampton MA, USA). Solutions containing 0.6 mg/mL PSPI in Buffer pH 3, pH 4, pH 5, pH 6, pH 7, and pH 7.5 were heated from 20 to 85 °C with a scan rate of 30°C/hour.

3 RESULTS

3.1 Structural properties

PSPI 6.1 and 6.5 are the two most abundant isoforms of the PSPI group (chapter 2). To study possible differences in conformation between the two isoforms of PSPI at various pH's, far-UV CD, near-UV CD, fluorescence and ATR-IR spectra of both isoforms were recorded at 20°C.

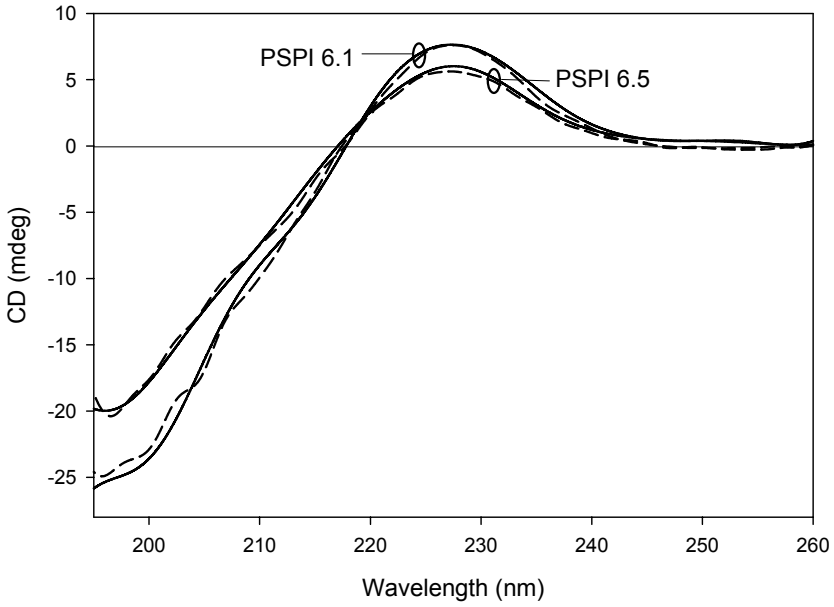

Figure 1: Far UV CD spectra of PSPI 6.1 and 6.5 at pH 3.0 (solid line) and at pH 7.0 (dashed line), at 20°C.

As a typical example, **figure 1** shows far UV CD spectra of PSPI 6.1 and 6.5 at pH 3 and pH 7 at 20°C. All spectra show a very similar pattern with a zero crossing at about 217 nm, a minimum at 197 nm and a maximum at 228 nm. The large similarity between the spectra indicates that the isoforms have a highly similar secondary structure in the pH range from 3 to 7.5. An estimation of the secondary structure content of PSPI isoforms, obtained by curve-fitting analysis, showed that PSPI seems to consist not only of the familiar α-helix and/or β-sheet elements, as the spectra can not be fitted with the normal reference spectra. Especially the presence of the positive maximum at 228 nm, which can not be ascribed to any of the known elements of secondary structure, impairs this curve fitting analysis.

Near UV CD spectra can be used for estimating interactions of aromatic side chains with other groups such as side chain amide and carboxylate groups and peptide main chain bonds and, therefore, are applicable as a measure for the local tertiary structure of a protein (Hennessey and Johnson, 1981). As a typical example, **figure 2** shows the near UV CD spectra of PSPI 6.1 and 6.5 at pH 3 and 7. The spectra for both PSPI isoforms show extremes at 292, 285,and around 268 nm. The remarkable sharpness of the peaks at 285 and 292 nm

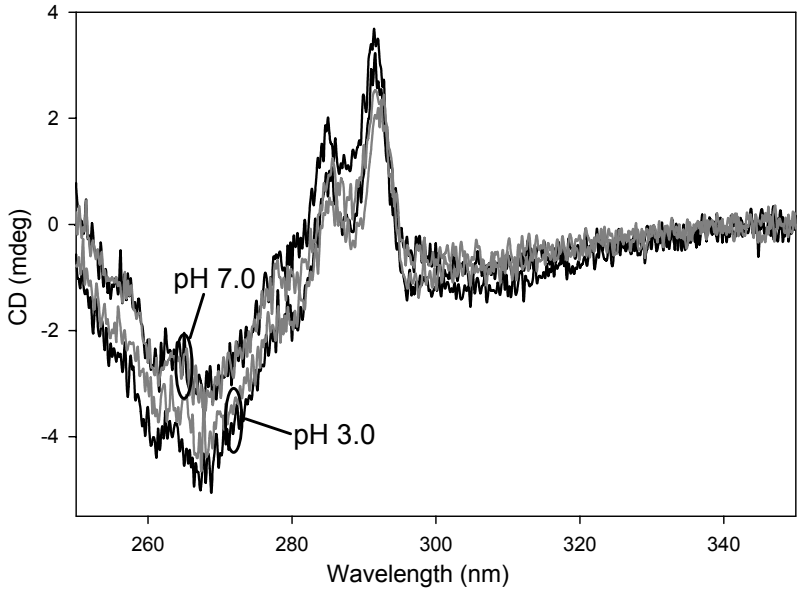

Figure 2: Near UV CD spectra of PSPI 6.1 (black) and 6.5 (gray), at pH 3.0 and pH 7.0 at 20°C.

indicates a compact rigid protein structure. The peak at 292 nm points to the presence of tryptophan residues, whereas the peaks at 285 and 268 nm indicate the presence of tyrosyl and phenylalanyl residues, respectively. These results are in accordance with the amino acid sequence of PSPI (chapter 3), which shows the presence of 3 tryptophan, 7 tyrosyl and 10 phenylalanyl residues. No changes in the wavelength and the intensity of the peaks could be observed between pH 4 and pH 7.5 for both PSPI 6.5 and 6.1 (data not shown), indicating the absence of significant changes in the tertiary structure in this pH range. At pH 3 (**figure 2**), a higher absolute intensity at 268 nm can be observed for both isoforms when compared to the spectra at higher pH's. This could indicate that the protein core, where the phenylalanyl residues are most frequently located, has become more compact.

Fluorescence spectroscopy provides information about the polarity of the environment of tryptophan and tyrosine residues, *i.e.* about the solvent accessibility of these residues. Fluorescence spectroscopy is, therefore, sensitive to local conformational changes at a tertiary level of folding (Pace et al., 1989). As typical examples, **figure 3** shows the emission spectra at pH 3 and 7 for both PSPI isoforms. Within the pH range 4 to 7.5 the emission spectra for both isoforms remained unchanged and showed a maximum at 347 nm. This emission maximum wavelength indicates that the tryptophan residues are in a non-polar environment. The form and intensity of the emission spectra of both isoforms remained identical at pH 3, but the emission maximum had shifted 4 nm to a lower wavelength compared to the spectra at pH ≥ 4. This indicates that at pH 3 the environment of at least one tryptophan residue present in PSPI (chapter 3) has become even less polar than at pH 4 to 7.5, and that it thus seems that the protein has become more compact.

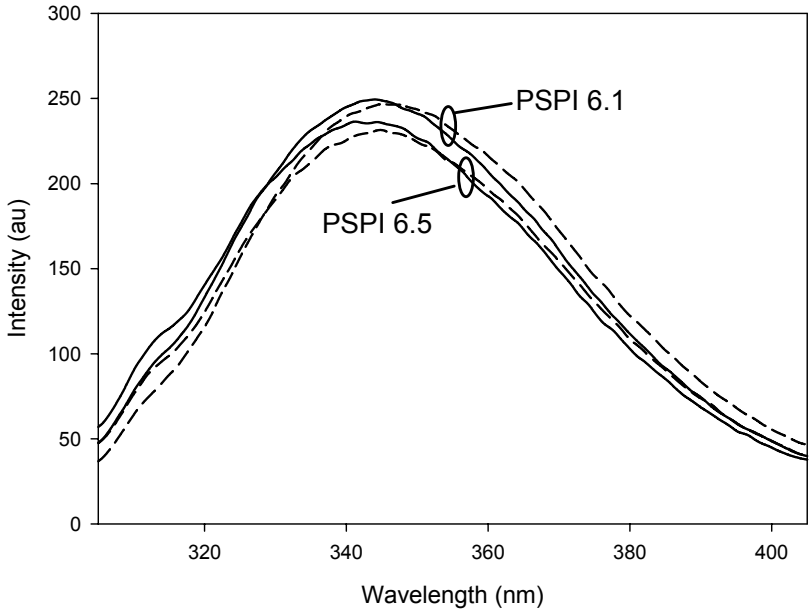

Figure 3: Tryptophan fluorescence spectra of PSPI 6.1 and 6.5 at pH 3.0 (solid line) and at pH 7.0 (dashed line) at 20°C.

Infrared spectroscopy is another method to investigate protein secondary structure and is based on molecular vibration of specific bonds, such as the C=O vibrations in the amide I band (1600-1700 cm^{-1}). Therefore, FTIR spectroscopycan give information on the secondary structure (Haris and Severcanb, 1999).

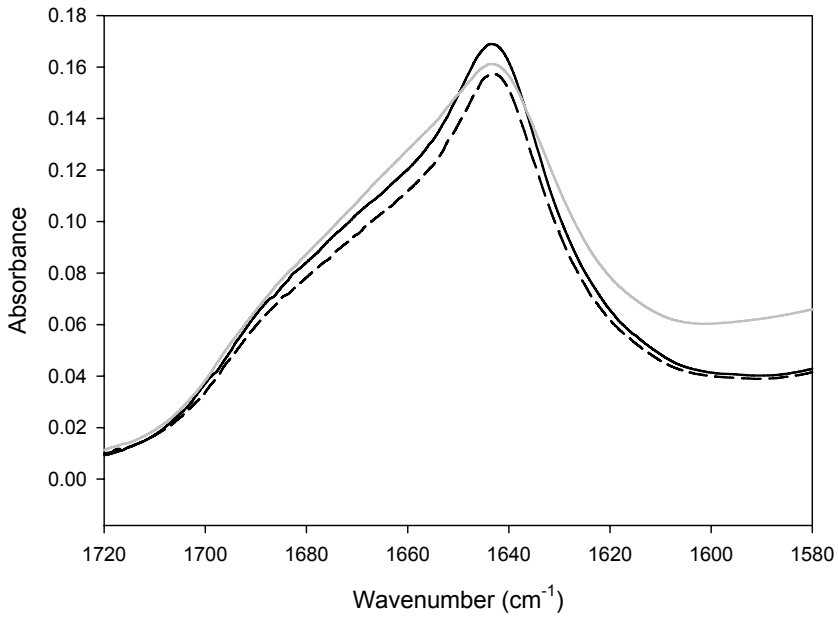

Figure 4: Amide I band of the ATR-IR spectra of PSPI 6.1 (solid line), PSPI 6.5 (dash line) and STI (gray line) at pH 7.0 at 20°C.

Figure 4 shows the Amide I band of the infrared spectra of both PSPI isoforms and the Kunitz type soybean trypsin inhibitor (STI), of which the X-ray structure has revealed that it contains approximately 2 % α-helix, 38 % β-sheet, 23 % β-turn and 37 % unordered structure (De Meester et al., 1998; Song and Suh, 1998). The spectra indicate that there is a high degree of similarity in secondary structure between STI and the PSPI isoforms. Deconvolution of the ATR-IR spectra revealed the presence of a major band at 1642 cm^{-1}, which indicates the presence of both unordered structure and short β-sheets (Goormaghtigh et al., 1994). Other deconvoluted bands at 1689 and 1624 cm-1, and at 1672 and1662 cm^{-1} can be observed indicating the presence of β-sheets, and turns, respectively (Goormaghtigh et al., 1994). The intensity of these bands shows that STI is somewhat richer in β-sheet and especially β-turns than PSPI.

3.2 Thermal stability

3.2.1 Secondary structure

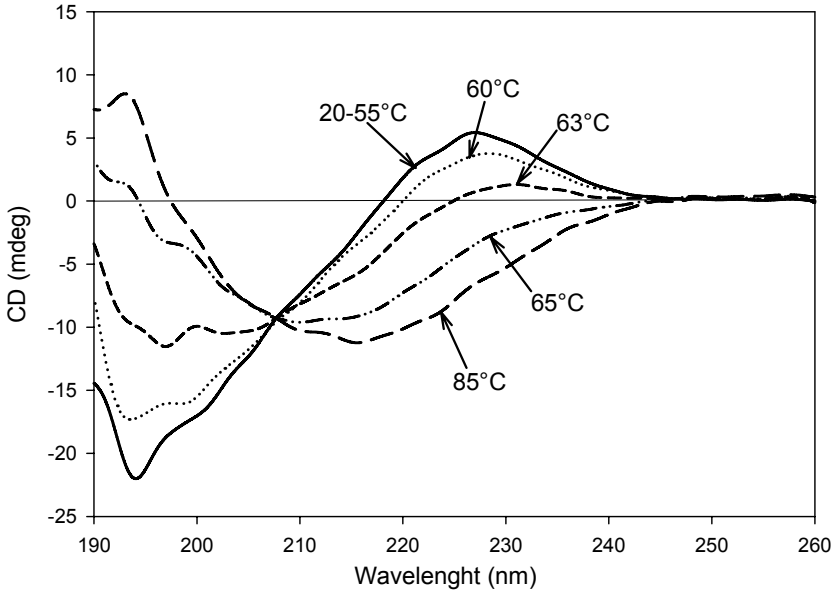

Figure 5: Far UV CD spectra of PSPI 6.5 at pH 3.0 at various temperatures.

As a typical example, far UV CD spectra of PSPI 6.5 at pH 4 at various temperatures are shown in **figure 5**. Similar results were obtained at the other pH values studied, for both isoforms upon heating. No changes in intensity occurred up to 55°C. With increasing temperature above 55°C, the absolute intensities at 196 and at 228 nm decreased and the intensities were inverted. The spectral changes in **figure 5** occurred with a clear isodichroic point at 208 nm. The existence of this point suggests that this transition may proceed essentially as a two state process (Tamura et al., 1991). The isodichroic point was present at all pHs and seemed not to vary with pH (207.5-208.5 nm, data not shown).

Figure 6 shows the ellipticity of PSPI 6.1 at 222 nm as a function of temperature at pH 3, 4, 5 and 7. Similar results were observed for PSPI 6.5 (data not shown). It can be seen that the shape of the thermal unfolding curves is very similar. At pH 5 and pH 6, the thermal unfolding curves showed smaller transition amplitudes than at the other pH values. These differences can be explained by the fact that at these pH values aggregation and/or precipitation may have taken place, making the estimation of the midpoint unreliable. Nevertheless, it can be observed that at these pH values the changes in ellipticity at 222 nm occur at higher temperatures than at the other pH's. It thus seems that PSPI 6.1 and 6.5 are more thermostable close to their isoelectric pH than at other pH values. The ellipticity at 222 nm for PSPI 6.1 at *e.g.* pH 4 showed changes between 61.5 and 67.2°C with a mid point at 64.2°C, whereas, in the case of PSPI 6.5, the changes occurred between 65.3 and 71.1°C with a mid point at 67.8°C. From these data, it can be concluded that PSPI 6.5 is at all pH's more heat stable than PSPI 6.1, and that the difference in transition midpoint remains constant (3 and 3.5°C). It is remarkable that the temperature range in which the changes in secondary structure in PSPI take place is very narrow (only about 6°C), in the pH range 3-7.5.

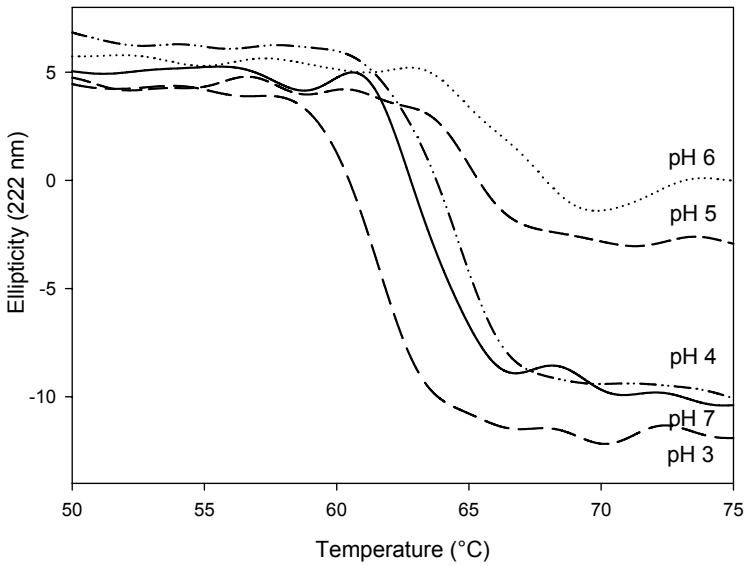

Figure 6: Thermal unfolding of curves of PSPI 6.1 at various pH monitored by the CD signal at 222 nm.

3.2.2 Tertiary structure

In **figure 7,** near UV CD spectra of PSPI 6.1 at pH 4 at various temperatures are shown, as typical examples. Increasing the temperature from 20 to 50°C did not cause significant changes. Between 50 and 55°C, a clear decrease of the peaks at 285 and 292 nm was observed. The peak at 268 nm showed an increase in absolute intensity between 50 and 55°C, probably indicating that the core of the protein had become more compact in this temperature range. Between 60°C and 62°C, a decrease in the absolute intensity at 268 nm was observed, while at 85°C all three peaks had disappeared. These results imply that the

surroundings of the tryptophan and tyrosine residues are more heat sensitive than those of the phenylalanyl residues. The tryptophan and tyrosine residues are thus likely to be located more at the outside of the protein than the phenylalanyl residues. It can be noticed that, although at pH 3 (20°C) the near UV CD spectrum of PSPI was different from that at pH≥4, PSPI unfolds at pH 3 in a similar way as at pH 4. Therefore, no effect of pH on the thermal unfolding of the tertiary structure of PSPI was observed in the pH range 3 to 7.5.

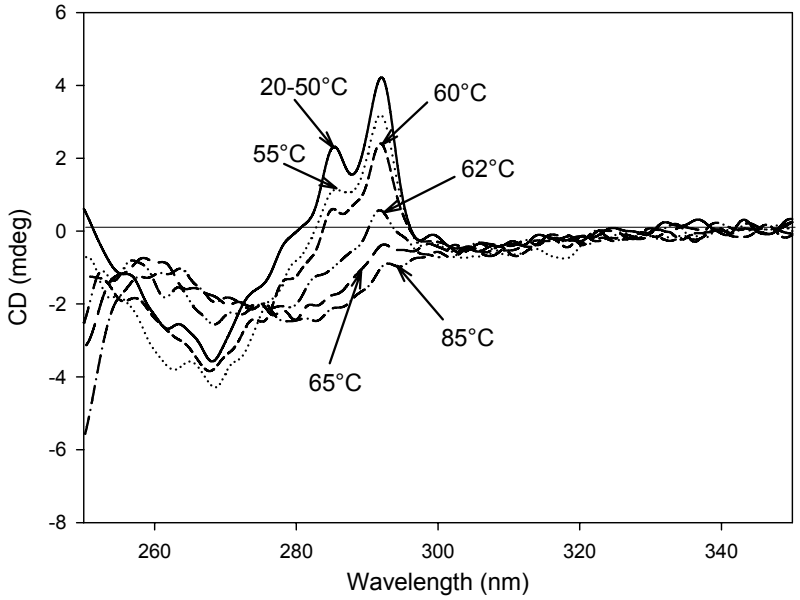

Figure 7: Near UV CD spectra of PSPI at pH 4.0 and at various temperatures.

DSC is an additional way to study the thermal unfolding of proteins (Boye et al., 1997). The DSC profiles of PSPI 6.1 and 6.5 showed one symmetric peak at all pHs (**figure 8**), except at pH 3 for PSPI 6.5 where an asymmetric peak was observed. The variation in transition temperature with pH is shown in **figure 9.** The endothermic peak of PSPI 6.5 is always smaller in amplitude and broader than that of PSPI 6.1 (figure 7). The denaturation enthalpy of PSPI 6.5 is higher than that of PSPI 6.1 in the pH range of 3 to 5, but becomes smaller than that of PSPI 6.1 at ≥ pH 7 (**figure 9**). The calorimetric enthalpies of unfolding of PSPI 6.1 and 6.5 vary between 360 and 300 kJ/mol in the pH range 3-7.5. Similar to the data obtained from far UV CD experiments, the transition temperatures of both PSPI 6.1 and 6.5 are highest around pH 6, and decrease when deviating from this pH (**figure 9**). The transition temperature of PSPI 6.5 is always higher than that of PSPI 6.1 (**figure 9**). Transition temperatures for PSPI 6.1 vary between 62°C (pH 3) and 65°C (pH 6), whereas those for PSPI 6.5 vary between 64°C (pH 3) and 69°C (pH 5 and 6). The differences in transition temperatures as observed with CD and DSC at pH 3, 5 and 6 can be explained by the fact that at these pH values aggregation and/or precipitation may have taken place, making the estimation of the midpoint, obtained from the CD thermal unfolding curves, unreliable.

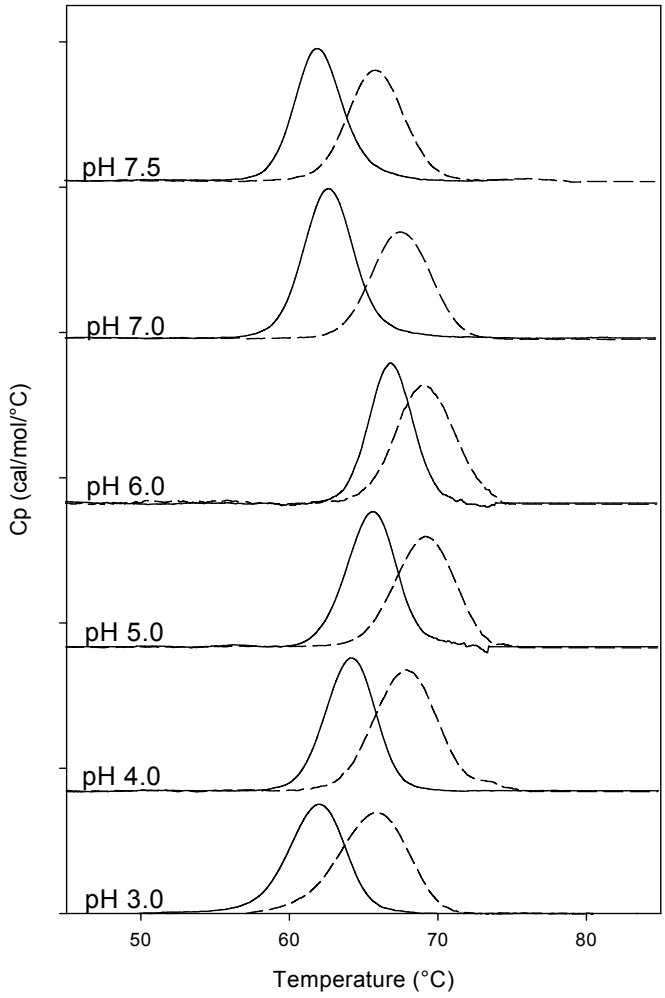

Figure 8: DSC thermograms of PSPI 6.1 (solid lines) and PSPI 6.5 (dashed lines).

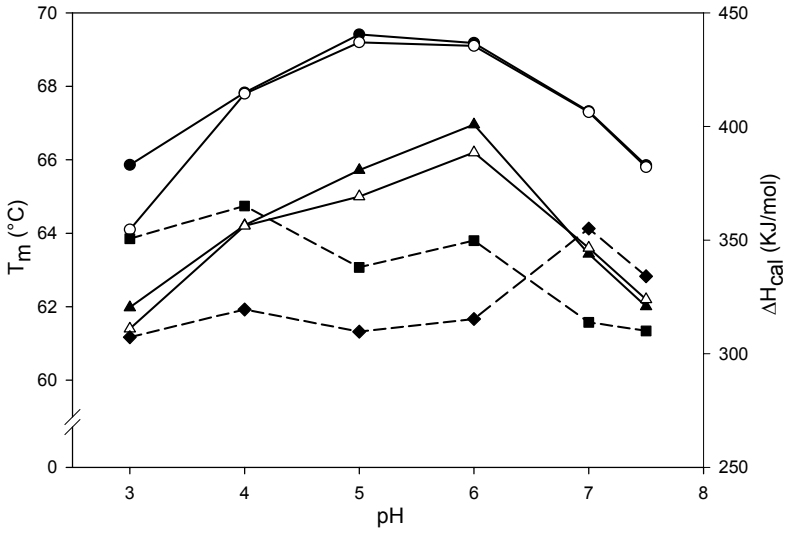

Figure 9: Transition temperatures from far UV CD (white) and DSC (black) measurements for PSPI 6.1 (▲) and PSPI 6.5 (●), and ΔH_{cal} for PSPI 6.1 (♦) and PSPI 6.5 (■), as a function of pH.

4 DISCUSSION

4.1 Effect of pH on the thermal stability of PSPI

Due to the presence of a constant isodichroic point for both isoforms at all pH's (**figure 4**) and the similarity of the thermal unfolding curves (**figure 5**), it can be concluded that the PSPI isoforms are almost identical and unfold via a highly similar pathway.

PSPI, at ambient temperature, did not show any variations in secondary structure in the pH range 3 to 7.5. At pH 3, in comparison to pH 4-7.5, small changes in the tertiary structure of PSPI did occur, which indicate that the core of the protein is somewhat more compact at this pH. Therefore, the structure of PSPI seems to be very stable against variation in pH. Furthermore, the calorimetric enthalpy (ΔH_{cal}) of PSPI 6.1 and 6.5 varies only between 300 and 350 kJ/mol (approximately 15 J/g) in the pH range 3-7.5. Experiments performed with a mixture of potato protease inhibitors showed a similar calorimetric enthalpy (approximately 19 J/g) (van Koningsveld et al., 2001). The thermodynamic stability of globular proteins is usually significantly affected by the pH (Privalov, 1979). Bovine serum albumin showed similar variations in transition temperature as PSPI in the pH range 3-7.5. However, its ΔH_{cal} varied from 155 to 850 kJ/mol in the same pH range (Yamasaki et al., 1990). Contrastingly, the α-amylase inhibitor tendimast, showed a constant ΔH_{cal} in the pH range 3-7.5, whereas the variation in its transition temperature was > 15°C (Graziano et al., 2000). For PSPI, this variation is only of 4°C over the same pH range. The results obtained and the comparison made above clearly show the high conformational stability of PSPI in a wide range of pH.

4.2 Assignment of PSPI as a β-II protein

The far UV CD spectra of PSPI isoforms are very unusual showing extremes at 197 nm and 228 nm. A minimum around 200 nm has been observed in two different classes of proteins: β-II proteins and unstructured proteins (Venyaminov and Yang, 1996). The β-II proteins are a sub-class of the all-β proteins (Manavalan and Johnson, 1987), indicating that most of the amino acid residues are predominantly involved in β-sheets and/or β-turns. In β-II proteins, most amino acid residues are involved in irregular β-sheets. The β-sheet does not have a plane extended structure but is distorted.

The far UV CD spectra of β-II proteins resemble those of unfolded proteins in showing a minimum around 200 nm. The β-II proteins have two features that are absent for unstructured proteins. These two features can, therefore, be a tool to distinguish them. First, β-II proteins show sharp peaks in the near UV region due to the compact structure of these proteins (Wu et al., 1992), even though the far UV CD spectra resemble those of unordered protein. Second, the CD ellipticities, both in the far and in the near UV region, of these compact and rigid proteins encompass a sharp transition upon thermal denaturation, whereas those of an unordered form usually change linearly with increasing temperature (Wu et al., 1992). PSPI shows, at ambient temperature, a far UV CD spectrum resembling that of unordered proteins, with a minimum at 197 nm, and a near UV CD spectrum with sharp peaks. Furthermore, the CD thermal unfolding curves show a sharp transition, suggesting that

the changes in secondary structure occurred in a small temperature range (6°C) (Kidric et al., 2002). These results strongly indicate that the PSPI group is a β-II protein.

Soybean Trypsin Inhibitor (STI) is a well-known example of a β-II protein (Sweet et al., 1974). Similar to PSPI, STI belongs to the Kunitz-type of protease inhibitors. STI and PSPI have a number of conserved amino acid residues in common (≈30 % sequence homology) and they have a similar molecular mass (≈ 20 kDa). The far UV CD spectrum of STI also shows a minimum at 200 nm, characteristic of the β-II proteins. Moreover, the far UV CD spectrum of STI shows a maximum at 228 nm (Wu et al., 1992). STI does not contain helical parts and most of its amino acid residues are involved in irregular β-sheet parts (Sweet et al., 1974). The high similarity between the far UV CD and ATR-IR spectra of PSPI and those of STI (Tetenbaum and Miller, 2001) are additional indications that the PSPI group belongs to the β-II protein sub-class.

The positive maximum at 220 – 230 nm in the far UV CD spectra of STI and PSPI has also been observed for many other proteases inhibitors (Menegatti et al., 1985; Bonsager et al., 2003), such as e.g. sporamin, α-amylase/subtilisin inhibitor, both Kunitz-type inhibitors (Lin and Chen, 1980; Vallée et al., 1998) and *Erythrina* Trypsin Inhibitor, a known β-II protein (Collen and Lijnen, 1995; Venhudova et al., 2001). Therefore, it seems that the maximum at 220 – 230 nm in the far UV CD spectrum is a characteristic of protease inhibitors of the β-II subclass.

Interestingly, many protease inhibitors of which the 3D structure is known (small or large molecular weight) belong to the all-β protein class, of which the β-II proteins are a subclass. This may indicate that the presence of β-sheets is of importance for the stability of these proteins and allows the presence of stable loops at the outside of these proteins.

LITERATURE CITED

Ambekar, S. S.; Patil, S. C.; Giri, A. P.; Kachole, M. S. Proteinaceous inhibitors of trypsin and of amylases in developing and germinating seeds of pigeon pea (*Cajanus caja* (L) Millsp). *J. Sci. Food Agric.* **1996**, 72, 57-62.

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.

Bode, W.; Huber, R. Natural protein proteinase inhibitors and their interaction with proteinases. *Eur. J. Biochem.* **1992**, 204, 433-451.

Bonsager, B. C.; Praetorius-Ibba, M.; Nielsen, P. K.; Svensson, B. Purification and characterization of the β-trefoil protein barley α-amylase/subtilisin inhibitor overexpressed in *Escherichia coli*. *Protein Expr. Purif.* **2003**, 30, 185-193.

Boye, J. I.; Alli, I.; Ismail, A. A. Use of differential scanning calorimetry and infrared spectroscopy in the study of thermal and structural stability of α-lactalbumin. *J. Agric. Food Chem.* **1997**, 45, 1116-1125.

Ceciliani, F.; Bortolotti, F.; Menegatti, E.; Ronchi, S.; Ascenzi, P.; Palmieri, S. Purification, inhibitory properties, amino acid sequence and identification of the reactive site of a new serine proteinase inhibitor from oil-rape (*Brassica napus*) seed. *FEBS Lett.* **1994**, 342, 221-224.

Chang, C. T.; Wu, C.-S. C.; Yang, J. T. Circular dichroic analysis of protein conformation: Inclusion of the β-turns. *Anal. Biochem.* **1978**, 91, 13-31.

Collen, D.; Lijnen, H. R. Molecular basis of fibrinolysis, as relevant for thrombolytic therapy. *Thromb. Haemost.* **1995**, 74, 167-71.

De Jongh, H. H. J.; Goormaghtigh, E.; Killian, A. Analysis of circular dichroism spectra of oriented protein-lipid complexes: Toward a general application. *Biochemistry* **1994**, 33, 14521-14528.

De Meester, P.; Brick, P.; Lloyd, L. F.; Blow, D. M.; Onesti, S. Structure of the Kunitz-type soybean trypsin inhibitor (STI): implication for the interactions between members of the STI family and tissue-plasminogen activator. *Acta Crystallogr. D. Biol. Crystallogr.* **1998**, 54, 589-597.

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.

Goormaghtigh, E.; Cabiaux, V.; Ruysschaert, J.-M. Determination of soluble and membrane protein structure by Fourier transform infrared spectroscopy. III. Secondary structures. *Subcell. Biochem.* **1994**, 23, 405-450.

Graziano, G.; Catanzano, F.; Barone, G. On the pH dependence of thermodynamic stability of α-maylase inhibitor tendamistat. *Thermochim. Acta* **2000**, 345, 59-66.

Greenfield, N. J.; Fasman, G. D. Computed circular dichroism spectra for evaluation of protein conformation. *Biochemistry* **1969**, 8, 4108-4116.

Haris, P. V.; Severcanb, F. FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media. *J. Mol. Catal.* **1999**, 7, 207-221.

Hennessey, J. P.; Johnson, W. C. Information content in the circular dichroism of proteins. *Biochemistry* **1981**, 20, 1085-1094.

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.

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.

Ingram, G. A. Lectins and lectin-like molecules in lower plants - I. Marine algae. *Dev. Comp. Immunol.* **1985**, 9, 1-10.

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

Kennedy, A. R. Chemopreventive agents: protease inhibitors. *Pharmacol. Ther.* **1998b**, 78, 167-209.

Kidric, M.; Fabian, H.; Brzin, J.; Popovic, T.; Pain, R. H. Folding, stability, and secondary structure of a new cysteine dimeric proteinase inhibitor. *Biochem. Biophys. Res. Commun.* **2002**, 297, 962-967.

Lin, Y. H.; Chen, H. L. Level and heat stability of trypsin inhibitor activity among sweet potato (Ipomoea batatas LAm.) varieties. *Bot. Bull. Acad. Sin.* **1980**, 21, 1-13.

Lisinska, G.; Leszczynski, W. *Potato science and technology*, Elsevier Applied Science, London, **1989**.

Manavalan, P.; Johnson, C. J. Variable selection method improves the prediction of protein secondary structure from circular dichroism spectra. *Anal. Biochem.* **1987**, 167, 76-85.

McManus, M. T.; Ryan, S.; Laing, W. A. **1999.** *The functions of proteinase inhibitors in seeds*. Seed symposium, 3-13.

Menegatti, E.; Palmieri, S.; Walde, P.; Luisi, P. L. Isolation and characterization of a trypsin inhibitor from white mustard (*Sinapis alba* L.). *J. Agric. Food Chem.* **1985**, 33, 784-789.

Morita, S.; Fukase, M.; Hoshino, K.; Fukuda, Y.; Yamaguchi, M.; Morita, Y. Partial purification and characterization of a novel soybean protease which is inhibited by Kunitz and Bowman-Birk trypsin inhibitors. *J. Biochem. (Tokyo).* **1996**, 119, 711-718.

Moses, E.; Hinz, H., -J. Basic pancreatic trypsin inhibitor has unusual thermodynamic stability parameters. *J. Mol. Biol.* **1983**, 170, 765-776.

Pace, C., N.; Shirley, B. A.; Thomson, J. A. Measuring the conformational stability of a protein. In *Protein structure; a practical approach*; Creighton, T. E. eds; IRL Press: Oxford: **1989**;

Privalov, P. L. Stability of proteins. Small globular proteins. *Adv. Protein Chem.* **1979**, 33, 167-241.

Ryan, C. A.; Hass, G. M.; Kuhn, R. W. Purification and properties of a carboxypeptidase inhibitor from potatoes. *J. Biol. Chem.* **1974**, 249, 5495-5499.

Song, H. K.; Suh, S. W. Kunitz-type soybean trypsin inhibitor revisited: refined structure of its complex with porcine trypsin reveals an insight into the interaction between a homologous inhibitor from *Erythrina caffra* and tissue-type plasminogen activator. *J. Mol. Biol.* **1998**, 275, 347-363.

Sweet, R. M.; Wright, H. T.; Janin, J.; Chothia, C. H.; Blow, D. M. Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6-A resolution. *Biochemistry* **1974**, 13, 4212-4228.

Tamura, A.; Kimura, K.; Takahara, H.; Akasaka, K. Cold denaturation and heat denaturation of Streptomyces subtilisin inhibitor. 1. CD and DSC studies. *Biochemistry* **1991**, 30, 11307-11313.

Tetenbaum, J.; Miller, L. M. A new spectroscopic approach to examining the role of disulfide bonds in the structure and unfolding of soybean trypsin inhibitor. *Biochemistry* **2001**, 40, 12215-12219.

Vallée, F.; Kadziola, A.; Bourne, Y.; Juy, M.; Rodenburg, K. W.; Svensson, B.; Haser, R. Barley alpha-amylase bound to its endogenous protein inhibitor BASI: crystal structure of the complex at 1.9 A resolution. *Structure* **1998**, 6, 649-659.

Valueva, T. A.; Revina, T. A.; Mosolov, V. V. Potato tuber protein proteinase inhibitors belonging to the Kunitz soybean inhibitor family. *Biochemistry (Moscow)* **1997**, 62, 1367-1374.

van Koningsveld, G. A.; Gruppen, H.; de Jongh, H. H. J.; Wijngaards, G.; van Boekel, M. A. J. S.; Walstra, P.; Voragen, A. G. J. The effects of pH and heat treatment on the structure and solubility of potato proteins in different preparations. *J. Agric. Food Chem.* **2001**, 49, 4889-4897.

Venhudova, G.; Canals, F.; Querol, E.; Aviles, F. X. Mutations in the N-and C-terminal tails of potato carboxypeptidase inhibitor influence its oxidative refolding process at the reshuffling stage. *J. Biol. Chem.* **2001**, 276, 11683-11690.

Venyaminov, S. Y.; Yang, J. T. Determination of protein secondary structure. In *Circular dichroism and the conformational analysis of biomolecules*; Fasman GD, eds; Plenum press: New York, **1996**; 69-109.

Wu, J.; Yang, J. T.; Wu, C.-S. C. β-II conformation of all-β proteins can be distinguished from unordered form by circular dichroism. *Anal. Biochem.* **1992**, 200, 359-364.

Yamasaki, M.; Yano, H.; Aoki, K. Differential scanning calorimetric studies on bovine serum albumin: I. Effects of pH and ionic strength. *Int. J. Biol. Macromol.* **1990**, 12, 263-268.

Chapter 5

Conformational stability of the Potato Serine Protease Inhibitor group (cv. *Elkana*)

ABSTRACT

The thermal unfolding of Potato Serine Protease Inhibitor (PSPI), the most abundant protease inhibitor group in potato tuber, was measured using far UV CD spectroscopy, fluorescence spectroscopy and DSC. The results indicate that the thermal as well as the guanidinium-induced unfolding of PSPI occurs via a non two state mechanism in which at least two parts of the protein unfold more or less independently. Additionally, the occurrence of aggregation, especially at low scan rates, increases the apparent cooperativity of the unfolding and makes the system kinetically rather than thermodynamically controlled. Aggregate formation seems to occur via a specific mechanism of which PSPI in a tetrameric form is the end product, and which may involve disulfide interchanges.

1 INTRODUCTION

Protease inhibitors are abundant in tubers and plant seeds (Ryan et al., 1974). In higher plants, several gene families have been characterised, particularly those constituting the serine protease inhibitors from Leguminosae, Solanaceae and Graminae (Garcia-Olmeda et al., 1987).

In recent years, protease inhibitors have regained interest because of their potent activity in preventing carcinogenis in a wide range of *in vivo* and *in vitro* systems (Kennedy, 1998b). Serine protease inhibitors have also been reported to have inhibitory effects on tumor cell growth (Huang et al., 1997; Kennedy, 1998a). In addition, by increasing the level of cholecystokinin via the inhibition of trypsin, serine protease inhibitors may also be used to reduce food intake in man (Hill et al., 1990).

In potato (*Solanum tuberosum*), a wide range of protease inhibitors is expressed, of which Potato Serine Protease Inhibitor (PSPI), a Kunitz-type inhibitor (chapter 3), is the most abundant group. It accounts for 22 % (w/w) of the soluble proteins (chapter 2). PSPI is a serine protease inhibitor showing activity against trypsin and chymotrypsin. It is a dimeric protein of 20.2 kDa, consisting of disulfide-linked subunits of 16.1 and 4.1 kDa (chapter 3). Based on its spectroscopic characteristics, PSPI has been classified as a β-II protein. The β-II proteins are a subclass of all-β proteins, in which most of the amino acids residues are involved in relatively short irregular β-sheets.

In industrial processing of potato starch, potato proteins are recovered as a by-product (Knorr et al., 1977). 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. In a previous study (chapter 4), it was shown that the thermal unfolding of PSPI shows an unusually sharp transition in its secondary structure from folded to unfolded state. Therefore, the aim of the present study was to get insight into the unfolding mechanism of PSPI at neutral pH (pH 7.5). To this end, using various techniques, changes at the secondary and tertiary structure level of the two main PSPI isoforms at pH 7.5 were studied as a function of both temperature and guanidinium chloride concentration.

2 MATERIAL AND METHODS

2.1 Material

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. Guanidinium chloride ultra pure (GndHCl) (G-7153; Lot: 60K5423) was from Sigma Chemical Co. All other chemicals used were of analytical grade.

2.2 Preparation of PSPI solutions

PSPI isoforms 6.1 and 6.5 were purified as described previously (chapter 2). After purification, PSPI 6.1 and 6.5 were dialysed at 4°C against 7 mM sodium phosphate buffer

(pH 7.5); ionic strength of 15 mM). Subsequently, the samples were frozen in small volumes at a concentration of 0.8 mg/mL and stored until use.

A GndHCl stock solution (8 M) was prepared gravimetrically in a volumetric flask. For each data point in the unfolding experiments, the protein sample, in 7 mM sodium phosphate buffer (pH 7.5), was diluted using the stock solution of GndHCl (8 M) to reach the appropriate concentration (3.5 and 6 M for the gel filtration experiments; 0 to 5 M for the CD experiments; 0 to 6.5 M for the fluorescence experiments). GndHCl-induced unfolding of PSPI was in equilibrium within 2h, as judged from CD and fluorescence spectra. All the samples were, therefore, equilibrated for 2h prior to measurement. All measurements were performed at 20°C.

Experiments in the presence of DTT were performed in 7 mM sodium phosphate buffer pH 7.5 containing 1 mM DTT. The samples were left at room temperature for 5 hours prior to measurement.

2.3 Gel filtration

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

A Superdex 75 HR column (30 x 0.32 cm) was used to determine if aggregation had taken place during heating and to estimate the size of possible aggregates. The column was equilibrated with 100 mM sodium phosphate buffer (pH 7.5) and operated at a flow rate of 0.1 mL/min. Proteins used for calibration were: ribonuclease A (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), BSA (67.0 kDa) and Blue dextran (2,000 kDa). PSPI 6.1 solutions, with concentrations varying from 0.2 to 0.8 mg/mL, were heated with a heating rate of 30°C/hour up to 85°C and cooled down to 20°C before applying them onto the column. Proteins were subsequently eluted, while the absorbance at 280 nm was monitored. The molecular weight of the aggregates was estimated from the calibration curve on the basis of elution volume.

Gel filtration was also performed in the presence of GndHCl. To this end, the column was equilibrated with 100 mM sodium phosphate (pH 7.5) containing 3.5 or 6 M GndHCl.

2.4 Spectroscopic measurements

All samples were filtered through 0.22 μm filter before spectroscopic measurements. Between two measurements, the cuvette was thoroughly cleaned with nanopure water and subsequently rinsed with ethanol.

Far-ultra violet CD

Far-ultra violet circular dichroism (far-UV CD) spectra of 0.2 mg/mL solutions of PSPI in 7 mM sodium phosphate buffer (pH 7.5) were recorded on a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) at temperatures ranging from 20 to 85°C with intervals of approximately 5 degrees, using a heating rate of 30°C/hour. The temperature in the sample was measured using a thermocouple wire. Starting from 20°C, the proteins were heated to the desired temperature and equilibrated for 3 minutes at this temperature before

wavelength-scans were recorded. Quartz cells with an optical path length of 0.1 cm were used. The scan range was 260-190 nm, the scan speed was 50 nm/min, the data interval 0.2 nm, bandwidth 1.0 nm, the sensitivity 20 mdeg and the response time was 0.125 seconds. Spectra were recorded 10-fold and averaged. Spectra were corrected by subtracting the spectrum of a protein free sample, obtained under identical conditions. Noise reduction was applied using the Jasco software. Changes in the secondary structure of PSPI during heating were also monitored by measuring the ellipticity at 222 nm as a function of temperature (20°C-85°C).

For GndHCl unfolding curves, the ellipticity at 222 nm was recorded by taking an average of 60 points in a 2 min trace at each concentration.

The effect of DTT was measured by monitoring the changes in secondary structure as a function of temperature via the ellipticity at 222 nm.

Fluorescence spectroscopy

Fluorescence spectra of 0.2 mg/mL PSPI in 7 mM sodium phosphate buffer (pH 7.5) were recorded on a Perkin Elmer Luminescence Spectrophotometer LS 50 B (Perkin Elmer Corp., Boston, MA, USA) with a pulsed Xenon source, at temperatures ranging from 20 to 85°C with intervals of approximately 5 degrees, using a heating rate of 30°C/hour. Excitation was done at 295 nm and the resulting emission spectrum was measured from 305 to 405 nm with a scan speed of 100 nm/min. Both the excitation and the emission slit were set at 3.5 nm. Spectra were recorded 3-fold and averaged. Spectra were corrected by subtracting the spectrum of a protein free sample, obtained under identical conditions. Starting from 20°C, the proteins were heated to the desired temperature and equilibrated for 6 minutes at this temperature before wavelength-scans were recorded. To check the reversibility of the conformational changes, the samples were cooled down to 20°C and allowed to equilibrate for 20 minutes prior to measurement.

In the GndHCl-induced unfolding studies, spectra were recorded on a Perkin Elmer Luminescence Spectrophotometer LS 50 B (Perkin Elmer Corp., Boston, MA, USA) using the same parameters as above. All data were corrected for the fluorescence emission observed for a series of blank GndHCl solutions.

Changes in the tertiary structure of PSPI during heating were also monitored by measuring changes in fluorescence intensity at 320 nm upon heating from 20 to 85°C using a heating rate of 30°C/hour, followed using a Varian Cary Fluorimeter (Varian Cary Inc., Palo Alto, CA, USA).

2.5 Differential scanning calorimetry

DSC measurements were performed on a VP-DSC Microcalorimeter (MicroCal Incorporated, Northampton, MA, USA). Solutions containing 0.6 mg/mL PSPI in 7 mM sodium phosphate buffer (pH 7.5) were heated from 20 to 85 °C with a scan rate of 30°C/hour.

To investigate the reversibility of unfolding, the samples were heated with a scan rate of 30°C/hour to a temperature just after the transition was complete, subsequently cooled to 20°C and re-heated to 85°C with the same scan rate.

To investigate the effect of the scan rate on the transition temperature, the protein samples were heated with scan rates varying between 2°C/hour and 60°C/hour. To investigate the influence of the protein concentration on the transition temperature, PSPI 6.1 concentrations from 0.1 to 1.6 mg/mL (pH 7.5) were used and the samples were heated with a scan rate of 30°C/hour.

3 RESULTS

3.1 Thermal stability

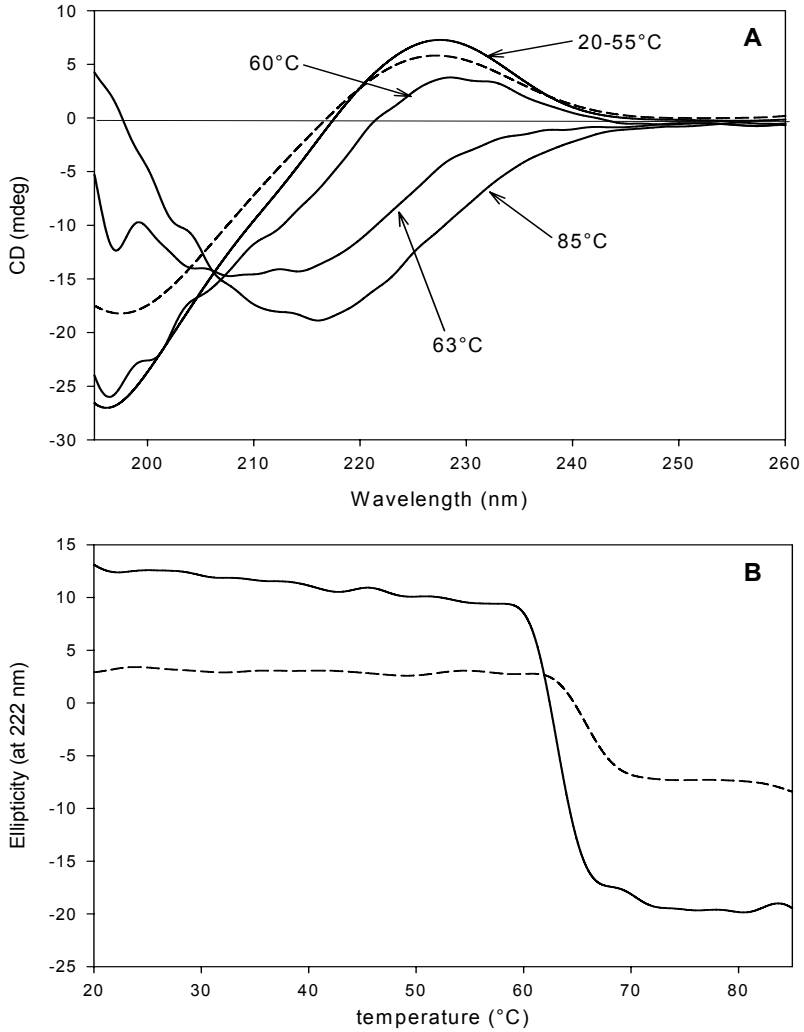

Figure 1: **A**: Far UV CD spectra at pH 7.5 of PSPI 6.1 (solid line) at various temperatures and 6.5 (dashed line) at 20°C; **B**: Thermal unfolding curves of PSPI 6.1 (solid line) and 6.5 (dashed line) at pH 7.5, monitored by the CD signal at 222 nm.

Figure 1A shows far UV CD spectra of PSPI 6.1 at various temperatures and that of PSPI 6.5 at 20°C. The spectra of the PSPI isoforms show a very similar pattern at 20°C with a

zero crossing at approximately 217 nm, a minimum at approximately 200 nm and a maximum at 228 nm. Upon heating, above 55°C, the absolute intensities of the extremes at 200 and 228 nm in the spectra of PSPI decreased and were inverted (**figure 1A**). **Figure 1B** shows the ellipticity of PSPI 6.1 and PSPI 6.5 at 222 nm as a function of temperature. It can be observed that for both isoforms the shape of the thermal unfolding curves are similar, with the highest transition mid-point for PSPI 6.5. The temperature range, in which the changes in secondary structure occur, is only about 6°C.

DSC is an additional technique to determine the transition temperature and to look at the energy content of the heat-induced conformational transitions of proteins (Boye et al., 1997).

The DSC profiles of PSPI 6.1 and 6.5, at pH 7.5, showed a symmetric peak with unfolding temperatures of 62.0 and 65.9°C, respectively (**figure 2**). The calorimetric enthalpies obtained for PSPI 6.1 and 6.5 are ≈ 325 and 300 kJ/mol, respectively.

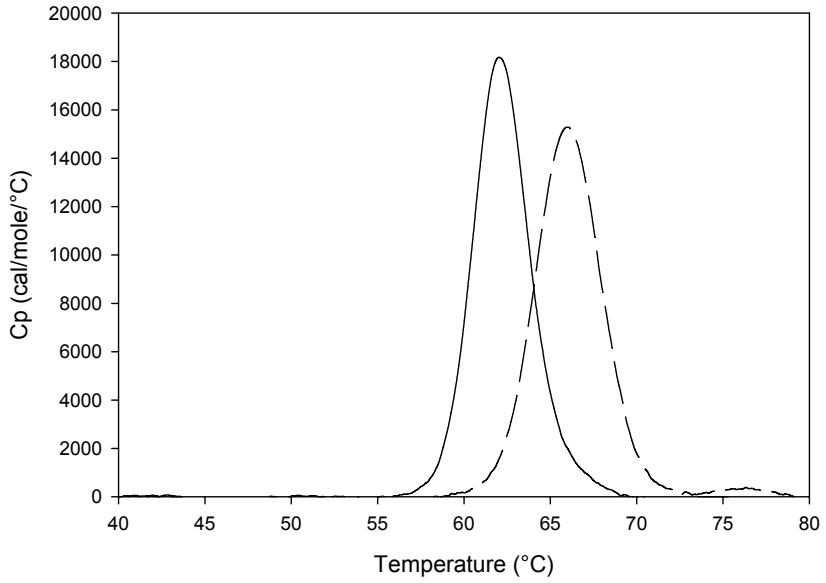

Figure 2: DSC thermograms of PSPI 6.1 (solid line) and 6.5 (dashed line).

In order to obtain thermodynamic data from the thermal unfolding CD curves of PSPI, the model given by Van Mierlo and coworkers (van Mierlo et al., 1998), based on thermodynamic equations (Becktel and Schellman, 1987; Pace et al., 1989) was used. The values of the Van't Hoff (ΔH_{vH}) enthalpy and the transition temperatures obtained from the CD-unfolding curve are shown in t**able 1,** together with the thermodynamic data obtained from DSC experiments.

The transition temperatures, obtained for both isoforms from CD and DSC, are similar (**table 1**). The values of ΔH_{vH}, obtained from CD thermal unfolding curves, are also comparable to those obtained from the DSC data. The ratios of the Van't Hoff enthalpy (ΔH_{vH}) obtained from CD as well as from DSC experiments and the calorimetric enthalpy (ΔH_{cal}) are also shown in **table 1.** They are similar for both isoforms and range from 2.92 to

Table 1: Thermodynamic data from fits of CD thermal unfolding curve (222 nm) and DSC profile of PSPI isoforms at pH 7.5.

	CD (222 nm)		DSC	
	PSPI 6.1	**PSPI 6.5**	**PSPI 6.1**	**PSPI 6.5**
Tm (°C)	62.2 ±0.05	65.8 ± 0.1	62.0 ± 0.07	65.9 ± 0.04
ΔH_{cal} (kJ/mol)			327.7± 0.4	302.1 ± 0.6
ΔH_{vH} (kJ/mol)	879.3 ± 3.6	895.4 ± 4.5	958.1 ± 1.5	840.0 ± 2.4
$\Delta H_{vH}/\Delta H_{cal}$	2.68	2.96	2.92	2.78

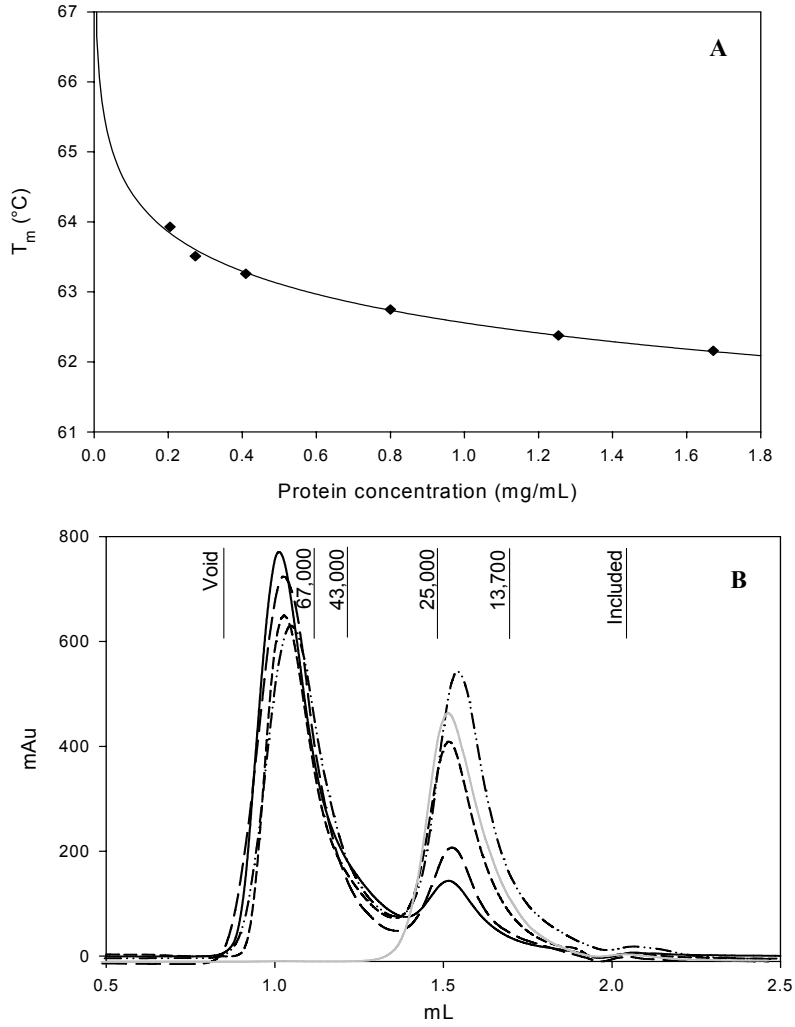

Figure 3: **A:** Transition temperature of PSPI as a function of protein concentration as determined using DSC; **B:** Gel filtration of PSPI after heating (20 to 85 °C; 30°C/hour) at different concentrations: 0.8 mg/mL (solid); 0.6 mg/mL (long dashes); 0.4 mg/mL (short dashes); 0.2 mg/mL (dash-dot). The gray curve represents PSPI without any heat treatment (0.4 mg/mL).

2.78. Such high ratios indicate that the thermal unfolding of both PSPI isoforms does not follow a two-state mechanism at the conditions used.

Also, rescanning of both PSPI isoforms using DSC resulted in less than 5 % of the original peak being recovered upon re-heating, indicating that the transition is almost completely irreversible (data not shown).

3.2 Concentration dependency

Figure 3A shows the transition temperature as a function of the protein concentration for PSPI 6.1, obtained using DSC. It can be clearly seen that the transition temperature decreases when the protein concentration increases. These results indicate that the unfolding of PSPI results in aggregation, which becomes more extensive with increasing protein concentration (even though the solution remains clear) (Verheul et al., 1998).

In order to examine this aggregation behaviour in more detail, PSPI samples with concentrations between 0.1 and 0.8 mg/ mL were studied by gel filtration after heating at 85°C. As shown in **figure 3B**, after heating, two peaks are present: one which corresponds to non-aggregated PSPI (Ve=1.6 mL) and one which corresponds the aggregates formed (Ve = 1.1 mL). Upon increasing the protein concentration, a decrease in the proportion of non-aggregated PSPI is observed. The results clearly show that heating leads to the formation of only one size of aggregates with an apparent molecular weight of 80 kDa, which is equivalent to 4 molecules of PSPI.

3.3 Scan rate dependency

In order to establish equilibrium between the native and the unfolded state at all temperatures, the scan rate should be much slower than the folding/unfolding rates (Yu et al., 1994). To verify if this condition is met for the
thermal unfolding of PSPI, the protein was heated at various scan rates ranging from 2°C/hour to 60°/hour.

Figure 4A shows typical thermograms of PSPI 6.1 at various scan rates. The peak remained symmetric within the scan-rate range from 5 to 60°C/hour, but the shape of the peak did change. With decreasing scan rate the transition temperature also decreased (**Figure 4B**) and the peaks became sharper. The decrease in transition temperature is most apparent at low scan rates (**Figure 4B**). The calorimetric enthalpy increased from 270 to 340 kJ/mole when increasing the scan rate from 5 to 60 °C. Similar to the observations made for the transition temperature, the changes in ΔH_{cal} are more apparent around a scan rate of 2°C/hour, when an apparent ΔH_{cal} value of only 150 kJ/mol is obtained. These results indicate that no equilibrium was reached, even at the lowest scan rate. It also indicates the dominancy of aggregation in the unfolding process at low scan rates, as shown by a decrease of the apparent ΔH_{cal}. Gel filtration chromatograms confirm that aggregation is favoured at low scan rates, as indicated by the disappearance of the peak corresponding to the non-aggregated PSPI (data not shown). The ratio $\Delta H_{vH}/\Delta H_{cal}$ increases with decreasing scan rate, and varies from 2.6 at a scan rate of 60°C/hour to 11.0 at a scan rate of 2°C/hour (no further data shown).

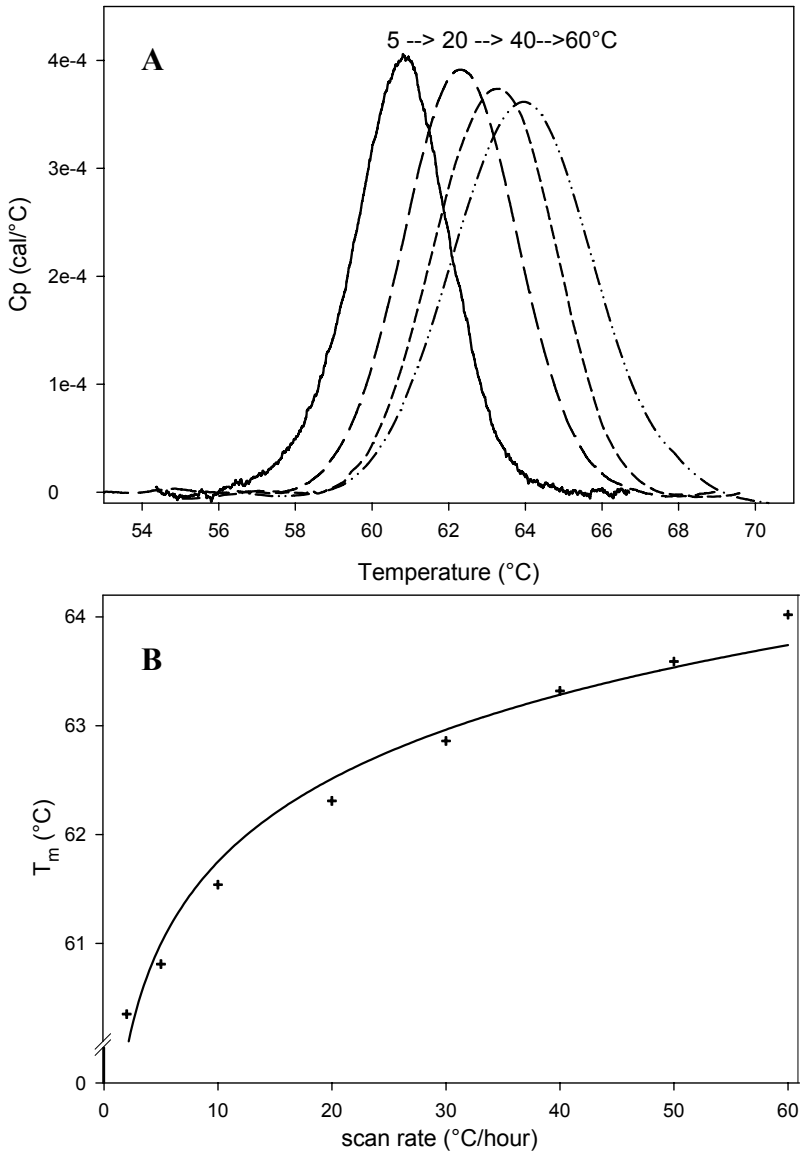

Figure 4: **A**: DSC thermograms of PSPI 6.1 (pH 7.5) at various scan rates; **B**: Transition temperatures of PSPI 6.1 (pH 7.5) as a function of scan rate

3.4 Thermal unfolding followed by fluorescence and far UV CD

The thermal unfolding curves of PSPI 6.1 as obtained using far UV CD (**figure 1B**) and fluorescence spectroscopy were expressed as the fraction of protein in the folded form (**figure 5A**). The fluorescence emission intensity at 320 nm gives information about possible alterations in the tertiary structure, whereas the ellipticity at 222 nm gives information on the changes in the secondary structure. Upon heating, the changes in secondary structure took place between 60 and 66 °C, whereas the changes in tertiary structure took place between 60 and 64°C. It thus seems that the unfolding of the secondary and tertiary structure start

simultaneously, but the tertiary structure has disappeared already at temperatures where part of the secondary structure is still intact.

3.5 GndHCl-induced equilibrium unfolding

The GndHCl-induced unfolding of PSPI 6.1 was monitored by CD and fluorescence spectroscopy (**Figure 5B**). The changes in secondary structure took place between 3.0 and 3.7 M of GndHCl, with a midpoint at 3.4 M, whereas the changes in tertiary structure took place between 3.0 and 4.9 M of GndHCl with a midpoint at 3.7 M. It seems that the unfolding of

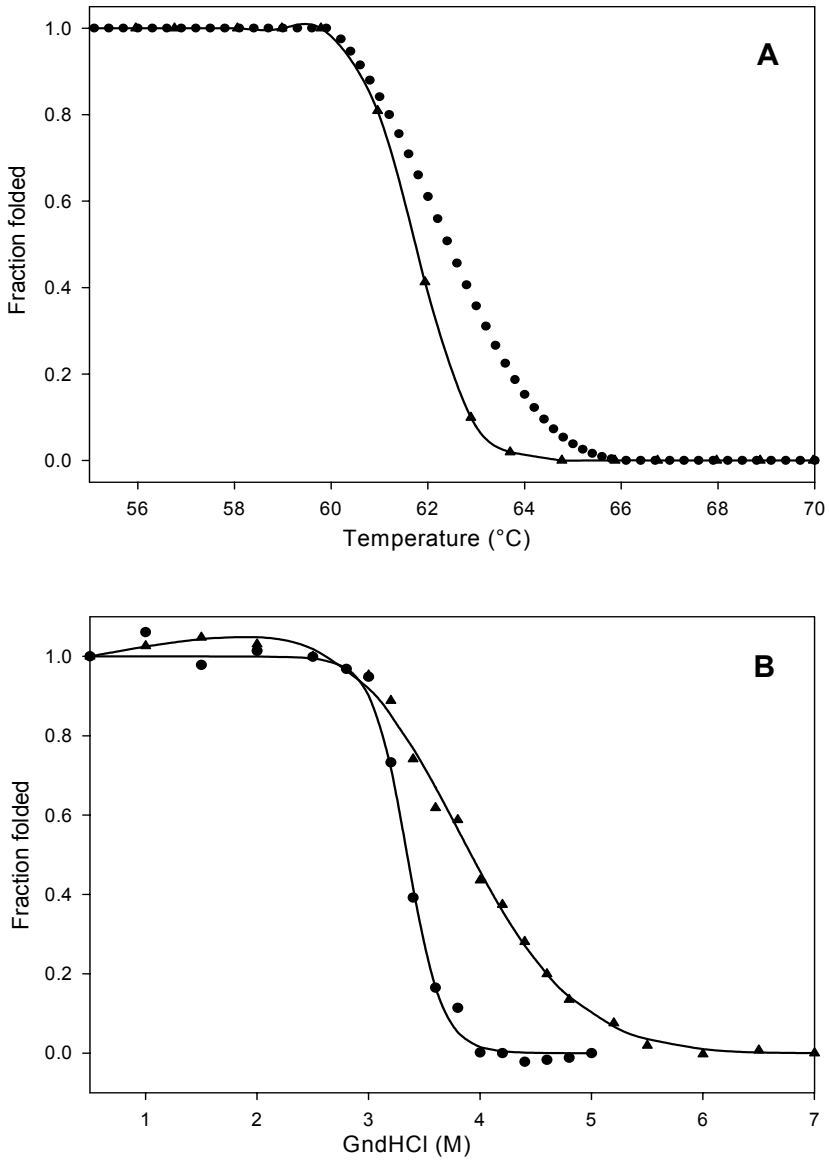

Figure 5: **A:** Thermal unfolding curves as monitored by the tryptophan fluorescence at 320 nm (▲) and the far UV CD signal (222 nm) (●); **B:** GndHCl-induced unfolding of PSPI 6.1 (pH 7.5) at 20°C, as monitored by the tryptophan fluorescence at 320 nm (▲) and the far UV CD signal (222 nm) (●).

the secondary and tertiary structure start simultaneously, but that the changes in tertiary structure take place over a larger range of GndHCl concentrations than the changes in secondary structure.

3.6 Effect of DTT

PSPI is known to possess two disulfide bridges (chapter 3). In the presence of DTT the importance of these disulfide bridges on the overall stability of the protein was examined. **Figure 6** shows the thermal unfolding curves of PSPI 6.1 at pH 7.5 in the absence and presence of 1 mM DTT. In the absence of DTT, PSPI showed changes between 60 and 66°C,

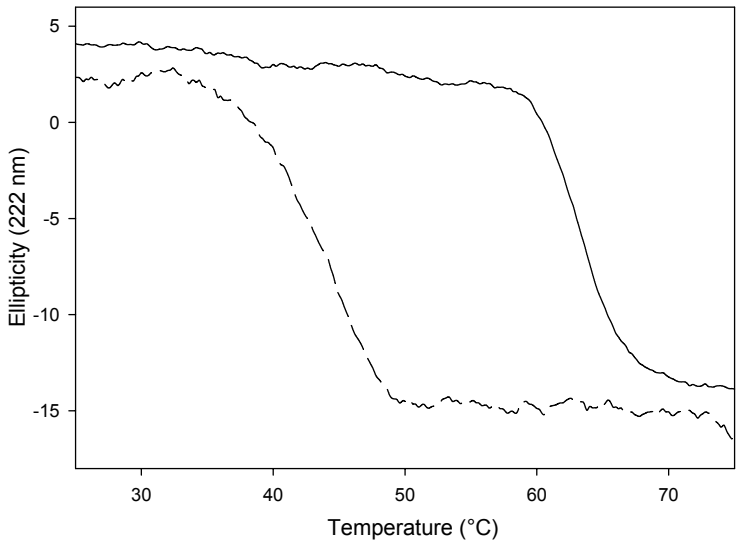

Figure 6: Thermal unfolding curves of PSPI 6.1 (pH 7.5) in the absence (solid line) and in the presence of 1 mM DTT (dashed line), monitored by the CD signal at 222 nm.

with a mid point at 62.2°C, whereas in the presence of 1 mM DTT PSPI showed changes between 36 and 50°C, with a midpoint at 45.2°C. Thus, the changes in secondary structure took place at a much lower temperature in a much broader temperature range (14°C instead of 6°C) when the disulfide bridges were broken.

4 DISCUSSION

4.1 Thermal unfolding

From the results obtained it can be seen that the thermal unfolding of PSPI is irreversible. Therefore, equilibrium thermodynamics cannot normally be applied in this case (Makhatadze, 1998). In some cases, however, it is still possible to use equilibrium thermodynamics for irreversibly unfolding proteins, if several conditions are met (Sanchez-Ruiz, 1992).

First, the unfolding should be independent of scan rate. Lowering the scan rate favours a more reliable characterisation of proteins showing a slow folding equilibrium. However, as shown by the sharpening of the peaks and the increase of $\Delta H_{vH}/\Delta H_{cal}$ at lower scan rates, aggregation becomes more dominant, thereby influencing the unfolding. Another indication that the thermal unfolding of PSPI is affected by aggregation is the observed concentration dependency. Since, at high scan rates and low concentration, aggregation can be reduced but not prevented and the unfolding does not reach equilibrium, there is no scan rate and concentration at which reliable thermodynamic data can be obtained for PSPI. Normally, the denaturation temperature is estimated by extrapolation of the transition temperature to zero scan rate (Yu et al., 1994; Hoffmann et al., 1997). Our results show that it would be virtually impossible to determine the "denaturation" temperature of PSPI in such a way. The denaturation temperatures given in this study should, therefore, be regarded as an indication of the unfolding temperature, and the unfolding enthalpies should be interpreted with even greater care. Comparison of the thermal unfolding of the secondary and tertiary structure of PSPI shows that at least one intermediate state is populated. It thus seems that PSPI consists of at least two parts that unfold more or less independently with increasing temperature.

4.2 Denaturant-induced unfolding

The maximum at 220 – 230 nm of the CD spectrum seems to be a characteristic of several β-II protease inhibitors such as PSPI, Soybean Trypsin Inhibitor and *Erythrina* Trypsin Inhibitor (Wu et al., 1992; Collen and Lijnen, 1995) (chapter 4). In order to investigate if this maximum, as monitored by the CD signal at 222 nm, is an indicator of local (disulfide bridges or aromatic side-chains) (Woody, 1994; Perczel and Hollosi, 1996) or global structure, additional experiments were performed. In a folded protein the fluorescence emission spectra upon excitation at 285 nm are dominated by photon transfer from tyrosine to tryptophan, as indicated by an emission maximum at approximately 335 nm. This maximum is absent in the unfolded state. If the maximum at 220 – 230 nm would be due to local interactions between tyrosine side-chains, then this photon transfer should be already almost absent at a GndHCl concentration of 3.5 M (see the 222 nm curve in Figure 5B). In the fluorescence emission spectra of PSPI at 3.5 M GndHCl the photon transfer can, however, still be observed, indicating that the maximum at 220 – 230 nm is not due to local tyrosine interactions (data not shown). Also, the intactness of the disulfide bridge connecting the subunits of PSPI in the presence of 6 M GndHCl was proven using gel filtration chromatography. It thus seems that the observed changes in this maximum indicate a global unfolding of PSPI.

Remarkably, the secondary structure of PSPI unfolds at a lower concentration of GndHCl than the tertiary structure. The conclusion that can be drawn from this is that at least one intermediate state is present. This may indicate that the structure of PSPI is composed of one more stable part, in which the aromatic residues are located (at least the Trp residues), and another part that is less stable.

The results presented indicate that the thermal as well as the GndHCl induced unfolding of PSPI occurs via a non two state mechanism in which at least two parts of the

protein unfold more or less independently. Interpretation of the thermal unfolding data is further complicated by the occurrence of aggregation. Especially at low scan rates, aggregation increases the apparent cooperativity of the unfolding and makes the unfolding mechanism kinetically rather than thermodynamically controlled. Aggregate formation seems to occur via a specific mechanism of which the PSPI tetramer is the end product and which may involve disulfide interchanges.

LITERATURE CITED

Becktel, W. J.; Schellman, J. A. Protein stability curves. *Biopolymers* **1987**, 26, 1859-1877.

Boye, J. I.; Alli, I.; Ismail, A. A. Use of differential scanning calorimetry and infrared spectroscopy in the study of thermal and structural stability of α-lactalbumin. *J. Agric. Food Chem.* **1997**, 45, 1116-1125.

Collen, D.; Lijnen, H. R. Molecular basis of fibrinolysis, as relevant for thrombolytic therapy. *Thromb. Haemost.* **1995**, 74, 167-71.

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.

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.

Hoffmann, M. A. M.; van Miltenburg, J. C.; van Mil, P. J. J. M. The suitability of scanning calorimetry to investigate slow irreversible protein denaturation. *Thermochim. Acta* **1997**, 306, 45-49.

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.

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

Kennedy, A. R. Chemopreventive agents: protease inhibitors. *Pharmacol. Ther.* **1998b**, 78, 167-209.

Knorr, D.; Kohler, G. O.; Betschart, A. A. Potato protein concentrates: the influence of various methods of recovery upon yield, compositional and functional characteristics. *J. Food Process. Preserv.* **1977**, 1, 235-247.

Makhatadze, G. I. Measuring protein thermostability by differential scanning calorimetry. In *Current protocols in protein science*; Coligan JE, Dunn BM, Ploegh HL, Speicher DW and Wingfield PT, eds; John Wiley and sons: New York, **1998**; 7.9.1-7.9.14.

Pace, C., N.; Shirley, B. A.; Thomson, J. A. Measuring the conformational stability of a protein. In *Protein structure; a practical approach*; Creighton, T. E. eds; IRL Press: Oxford: **1989**;

Perczel, A.; Hollosi, M. Turns. In *Circular dichroism and the conformational analysis of biomolecules*; Fasman GD, eds; Plenum press: New York, **1996**; 285-380.

Ryan, C. A.; Hass, G. M.;Kuhn, R. W. Purification and properties of a carboxypeptidase inhibitor from potatoes. *J. Biol. Chem.* **1974**, 249, 5495-5499.

Sanchez-Ruiz, J. M. Theoretical analysis of Lumry-Eyring models in differential scanning calorimetry. *Biophys. J.* **1992**, 61, 921-935.

van Mierlo, C. P. M.; van Dongen, W. M. A. M.; Vergeldt, F.; van Berkel, W. J. H.; Steensma, E. The equilibrium unfolding of Azotobacter vinelandii apoflavodoxin II occurs via a relatively stable folding intermediate. *Protein Sci.* **1998**, 7, 2331-2344.

Verheul, M.; Roefs, S. P. F. M.; de Kruif, K. G. Kinetics of heat-induced aggregation of β-lactoglobulin. *J. Agric. Food Chem.* **1998**, 46, 896-903.

Woody, R. W. Contributions of tryptophan side chains to the far-ultraviolet circular dichroism of proteins. *Eur. Biophys. J.* **1994**, 23, 253-262.

Wu, J.; Yang, J. T.; Wu, C.-S. C. β-II conformation of all-β proteins can be distinguished from unordered form by circular dichroism. *Anal. Biochem.* **1992**, 200, 359-364.

Yu, Y.; Makhatadze, C.; Pace, N.; Privalov, P. L. Energetics of ribonclease T1 structure. *Biochemistry* **1994**, 33, 3312-3319.

Chapter 6

Structure and stability of the Potato Cysteine Protease Inhibitor group (cv. *Elkana*)

ABSTRACT

The conformational stability of Potato Cysteine Protease Inhibitor (PCPI), the second most abundant protease inhibitor group in potato tuber, was investigated at ambient temperature and upon heating using far and near UV CD spectroscopy, fluorescence spectroscopy and DSC. The PCPI isoforms investigated have a highly similar structure at both the secondary and tertiary level. PCPI isoforms show structural properties similar to those of Potato Serine Protease Inhibitor group and the Kunitz-type soybean trypsin inhibitor, a known β-II protein. Therefore, PCPI isoforms are also classified as members of the β-II protein subclass. Results show that the thermal unfolding of PCPI isoforms does not follow a two-state mechanism, and that at least one intermediate is present. The occurrence of this intermediate is most apparent in the thermal unfolding of PCPI 8.3 as indicated by the presence of two peaks in the DSC thermogram. Additionally, the formation of aggregates (>100 kDa), especially at low scan rates, increases the apparent cooperativity of the unfolding and makes the system kinetically rather than thermodynamically controlled.

1 INTRODUCTION

Protease inhibitors are ubiquitously abundant in tubers and plant seeds (Ryan, 1977). In higher plants, several gene families of protease inhibitors have been characterised, particularly those constituting the serine protease inhibitors from *Leguminosae*, *Solanaceae* and *Graminae* (Garcia-Olmeda et al., 1987).

In potatoes, a wide range of protease inhibitors is expressed (Lisinska and Leszczynski, 1989). Potato tuber contains approximately 1.5 % (w/w) protein on fresh weight basis (Lisinska and Leszczynski, 1989). In cv. *Elkana*, a variety used for the industrial processing of potato starch, protease inhibitors represent approximately 50 % of the total amount of soluble protein present in the tuber (cv. *Elkana*) of which the Potato Cysteine Protease Inhibitor (PCPI) group represents approximately 12% (chapter 2).

Cysteine proteases are widely distributed among living organisms, the most abundant being the papain family. This family consists of papain and related plant proteases, such as chymopapain and bromelain. In humans, well-known cysteine proteases are cathepsins B, H and L. Cysteine proteases are involved in a variety of physiological processes such as protein degradation, antigen presentation, bone resorption and hormone processing (Turk et al., 2002). They also play a role in many pathological processes including tumor invasion and metastasis (Kos et al., 1997). Therefore, specific cysteine protease inhibitors, such as PCPI, may have considerable potential for diagnosis and treatment. In some cases, insects have adapted their digestive proteases to combat the plant endogenous inhibitors (Gruden et al., 1997). Therefore, for crop protection it may also be of interest to genetically modify plants with cysteine protease inhibitors genes in order to broaden their range of inhibitors.

PCPI isoforms are potent inhibitors of cysteine proteases such as papain, but show also activity again trypsin (chapter 2). PCPI's are monomeric proteins with a molecular mass varying from 20 to 22 kDa. Eight isoforms of PCPI, with isoelectric pHs varying from pH 5.8 to 9.4, have been characterised in potato juice (cv. *Elkana*) (chapter 2).

In industrial processes, potato proteins are recovered as a by-product (Knorr et al., 1977). This is done by an acidic heat-treatment of the potato protein containing liquid and results in irreversibly precipitated proteins (Knorr et al., 1977). Understanding the conformational changes of these potato proteins induced by temperature may help to understand the mechanism of the ensuing aggregation and precipitation. However, data about pH and heat stability of PCPIs is absent. The aim of the present study was, therefore, to investigate the structural properties and the thermal stability of the three most abundant isoforms of PCPI (chapter 2).

2 MATERIAL AND METHODS

2.1 Preparation of PCPI solutions

PCPI isoforms 8.3, 8.6 and 9.4 were purified from cv. *Elkana* as described previously (chapter 2). An additional chromatofocusing purification step was included, using a Polybuffer Exchanger 118 column (60 x 1.6 cm) (Amersham Biosciences, Uppsala, Sweden).

The column was equilibrated with 0.025 M diethanolamine-HCl buffer pH 9.4 for PCPI 8.3 and 8.6 and with 0.025 M triethanolamine-HCl, pH 11.0 for PCPI 9.4. The fractions corresponding to PCPI 8.3, 8.6 and 9.4 (chapter 2) were loaded onto the column. The protein was eluted using Polybuffer 96-HCl (pH 7.0) (dilution factor 1:10) for PCPI 8.3 and 8.6, and using Pharmylate-HCl (pH 8.0) (dilution 1:45) for PCPI 9.4 (Amersham Biosciences, Uppsala, Sweden). The absorbance of the eluates was monitored at 280 and 320 nm. Appropriate fractions (20 mL) were collected and pooled. The Polybuffer was removed by hydrophobic interaction chromatography using a HP Phenyl Sepharose column (10 x 2.6 cm) (Amersham Biosciences, Uppsala, Sweden). After purification, the purified PCPI 8.3, 8.6 and 9.4 were dialysed at 4°C against a 95 mM sodium acetate buffer (pH 4.0) (ionic strength 15 mM). After dialysis, the samples were frozen in small volumes and stored until use at a concentration of 1 mg/mL.

Soybean trypsin inhibitor (STI) was purchased from Fluka (art. no.: 93618). STI was dissolved in 9 mM sodium phosphate buffer (pH 7.0) and dialysed against the same buffer overnight at 4°C.

2.2 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. Gels were stained according to the Coomassie brilliant blue R-250 staining procedure provided by the manufacturer.

2.3 Protein quantification

Since the sequence of PCPI 8.3 is known, the protein concentration of PCPI 8.3 was determined by measuring its absorbance at 280 nm in the presence of 6 M GndHCl, using a theoretical extinction coefficient of 17210 $M^{-1}.cm^{-1}$ based on its sequence. The protein content of PCPI 8.3, 8.6 and 9.4 was also determined using the Bradford assay (Bradford, 1976). In order to be able to use BSA instead of PCPI 8.3 (being a representative PCPI) as a standard a normalisation was used to correct the difference in response between BSA and PCPI 8.3.

2.4 Mass spectrometry

MALDI-TOF MS analysis in the linear mode was performed using a Voyager DE RP instrument (Perseptive Biosystems, Framingham, MA, USA) as described previously (chapter 2).

2.5 Gel filtration

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

A Superdex 75 (30 x 0.32 cm) was used to determine if aggregation was taking place during heating and to estimate the size of possible aggregates. The column was equilibrated with 100 mM sodium phosphate buffer (pH 7.5) and operated at a flow rate of 0.5 mL/min. Proteins used for calibration were: ribonuclease A (13.7 kDa), chymotrypsinogen A (25.0

kDa), ovalbumin (43.0 kDa), BSA (67.0 kDa) and Blue dextran (2,000 kDa). The protein samples (0.2 to 0.8 mg/mL) were heated with a scan rate of 30°C/hour up to 85°C and cooled down to 20°C before applying them onto the column.

2.6 Spectroscopic measurements

All samples were filtered through a 0.22 μm filter before spectroscopic measurements. Between two measurements, the cuvette was thoroughly cleaned with nanopure water and subsequently rinsed with ethanol.

Far-ultra violet CD

Far-ultra violet circular dichroism (far-UV CD) spectra of 0.2 mg/mL of PCPI in 95 mM sodium acetate buffer (pH 4.0) were recorded on a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) at temperatures ranging from 20 to 85°C with intervals of approximately 5 degrees, with a heating rate of 30°C/hour. The temperature was measured in the sample using a thermocouple wire. Starting from 20°C, the proteins were heated to the desired temperature and equilibrated for 3 minutes at this temperature before the wavelength-scans were recorded. Quartz cells with an optical path length of 0.1 cm were used. The scan range was 260-190 nm, the scan speed was 50 nm/min, the data interval 0.2 nm, the bandwidth 1.0 nm, the sensitivity 20 mdeg and the response time was 0.125 seconds. Spectra were recorded in 10-fold and averaged. Spectra were corrected using a spectrum of a protein-free sample obtained under identical conditions. Noise reduction was applied using the Jasco software. The spectra were analysed from 240 to 190 nm to estimate the secondary structure content of the protein, using a non-linear regression procedure (de Jongh et al., 1994). Spectra were fitted using the reference spectra of poly-lysine in the α-helix, β-strand and random coil conformation (Greenfield and Fasman, 1969) and the spectrum of β-turn structures, extracted from 24 proteins with known X-ray structure (Chang et al., 1978). Changes in secondary structure of PCPI during heating were also monitored by measuring the ellipticity at 222 nm as a function of temperature.

Near-ultra violet CD

Near-ultra violet circular dichroism (near-UV CD) spectra of 0.6 mg/mL PCPI in 95 mM sodium acetate buffer (pH 4.0) were recorded on a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) at 20°C. Spectra were recorded 30-fold and averaged. Spectra were corrected using a spectrum of a protein-free sample obtained under identical conditions. A quartz cell with an optical path length of 1.0 cm was used. The scan interval was 250-350 nm, the scan speed 100 nm/min, the data interval 0.2 nm, the bandwidth 1.0 nm, the sensitivity 20 mdeg and the response time was 0.125 seconds.

Fluorescence spectroscopy

Fluorescence spectra of 0.2 mg/mL solutions of PCPI in 95 mM sodium acetate buffer (pH 4.0) at 20°C were recorded on a Perkin Elmer Luminescence Spectrophotometer LS 50 B (Perkin Elmer Corp., Boston, MA, USA). Excitation was done at 285 nm and the resulting emission was measured from 290 to 405 nm with a scan speed of 100 nm/min. Both the

excitation and the emission slit were set at 3.5 nm. Spectra were recorded 3-fold and averaged. Spectra were corrected using a spectrum of a protein-free sample obtained under identical conditions.

Changes in the tertiary structure of PCPI during heating were also monitored by measuring the changes in fluorescence intensity at 300 nm as a function of temperature using a Varian Cary fluorimeter (Varian Cary Inc. Palo Alto, CA, USA).

Fourier Transformed Infrared (FTIR) spectroscopy

Attenuated total reflection infrared (ATR-IR) spectra were recorded on a Biorad FTS 6000 spectrometer equipped with a DTGS detector (Bio Rad Laboratories Inc., Cambridge, MA, USA). Typically, 50 µl of a 0.8 mg/mL PCPI samples in 9 mM phosphate-buffer (pH 7.0) were transferred onto a germanium crystal (1x8 cm) and dried under air to remove excess water. Next, the crystal was placed such in the light beam that 6 total reflections were obtained. Spectra were accumulated at ambient temperature in the spectral region of 4000-800 cm^{-1} with a spectral resolution of 0.5 cm^{-1} prior to zero-filling and Fourier transformation, using a speed of 5 kHz and a filter of 1.2 kHz. Typically 100 spectra were accumulated and subsequently averaged. A spectrum representing atmospheric water was subtracted from the sample-spectra. All samples were prepared and analysed at least in duplicate. Spectra were deconvoluted in order to analyse the underlying absorption bands using K = 2.4 and a full width at half height (FWHH) of 24 cm^{-1}.

2.7 Differential scanning calorimetry

DSC measurements were performed on a VP-DSC Microcalorimeter (MicroCal Inc., Northampton MA, USA). Solutions containing 0.6 mg/mL PCPI in 95 mM sodium acetate buffer (pH 4.0) were heated from 20 to 85 °C with a scan rate of 30°C/hour.

To investigate the reversibility of the unfolding, the samples were heated with a scan rate of 30°C/hour to a temperature just after the transition was complete. The sample was subsequently cooled to 20°C and re-heated to 85°C with the same scan rate.

3 RESULTS

3.1 Protein architecture

The purity and the molecular mass of the three main isoforms of PCPI were studied using SDS-PAGE and MALDI-TOF MS. SDS-PAGE under reducing conditions showed a single band at approximately 20 kDa for each isoform (data not shown).

In order to determine the molecular mass of these three PCPI isoforms more precisely, MALDI-TOF MS experiments were carried out **(figure 1)**. The MALDI-TOF MS spectra showed two peaks at 10,216 and 20,433 Da for PCPI 8.3, at 10,064 and 20,128 Da for PCPI 8.6 and at 10,066 and 20,132 Da for PCPI 9.4, respectively. The two peaks correspond to the doubly and singly charged ion of the same protein. The similarity in molecular mass between the isoforms is in accordance with previous results (chapter 2).

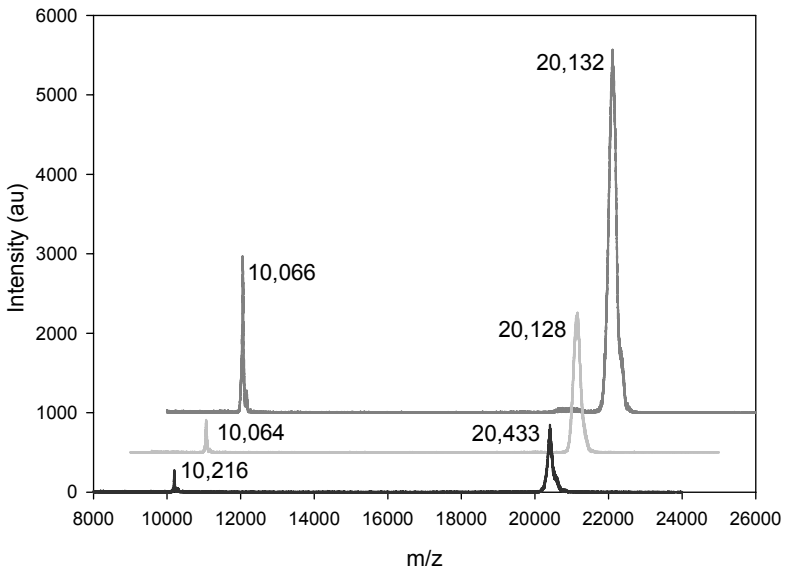

Figure 1: MALDI TOF MS spectra of PCPI 8.3 (black), PCPI 8.6 (clear gray) and PCPI 9.4 (dark gray).

3.2 Structural properties at ambient temperature

To study the structural properties of the three isoforms of PCPI at pH 4.0, far UV CD, near UV CD, fluorescence and ATR-IR spectra of all PCPI isoforms were recorded.

Figure 2 shows far UV CD spectra of the three isoforms of PCPI at pH 4.0. The spectra for PCPI 8.6 and 9.4 have similar characteristics with a zero crossing at 214.5 nm, a minimum around 197 nm and a maximum at 228 nm. The spectrum of PCPI 8.3 is slightly deviating from the two others by the fact that it shows a zero crossing at a somewhat higher wavelength (≈217 nm). The large similarities between the spectra indicate that the isoforms have a highly similar structure. Similar to the spectra for Potato Serine Protease Inhibitor (PSPI) (chapter 4), no secondary structure elements can be assigned to PCPI isoforms based on the fitting of the spectra using reference spectra for α-helix, β-sheet, β-turn and random coil.

Near UV CD spectra give an indication of the interactions of aromatic side-chains with other side-chain groups and peptide bonds, reflecting the tertiary structure of a protein (Kelly and Price, 1997). **Figure 3** shows the near UV CD spectra of the three isoforms of PCPI at pH 4.0 (20°C). All spectra show extremes at 292, 285 and 268 nm, allthough the peak at 285 nm for PCPI 8.3 is very weak compared to those for PCPI 8.6 and 9.4. The remarkable sharpness of the peaks at 285 and 292 nm indicates a compact and rigid protein structure. The peak at 292 nm points to the presence of tryptophan residues, whereas the peaks at 285 and 268 nm indicate the presence of tyrosyl and phenylalanine residues, respectively. In comparison to the near UV CD spectra of PSPI (chapter 4), the peaks of PCPI isoforms are sharper and more distinct. In the case of PCPI 8.3, the presence of these three peaks is in accordance with its amino acid sequence ((Krizaj et al., 1993); SwissProt: O24383), which shows the presence of 1 tryptophan, 7 tyrosyl and 9 phenylalanyl residues. PCPI 9.4 has been

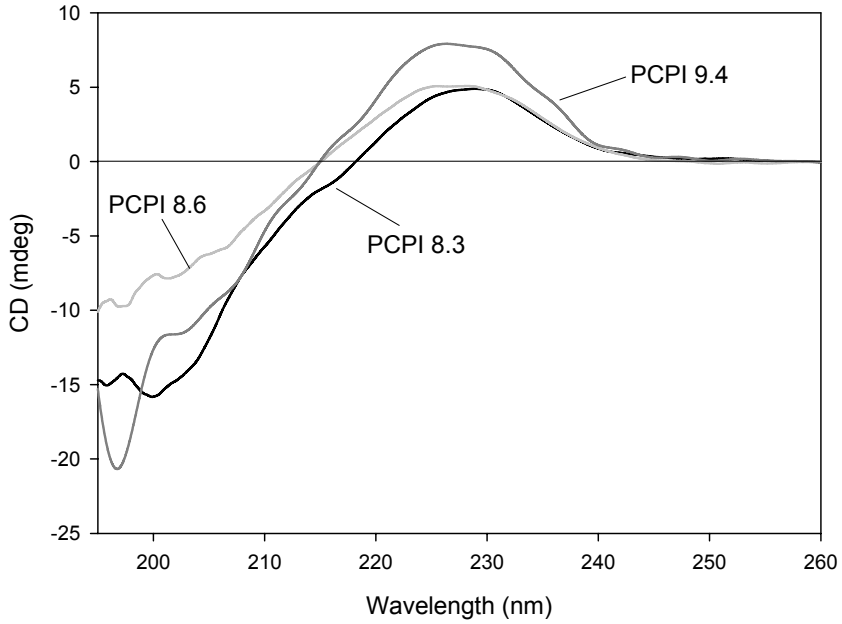

Figure 2: Far UV CD spectra of PCPI isoforms (pH 4.0) at 20°C.

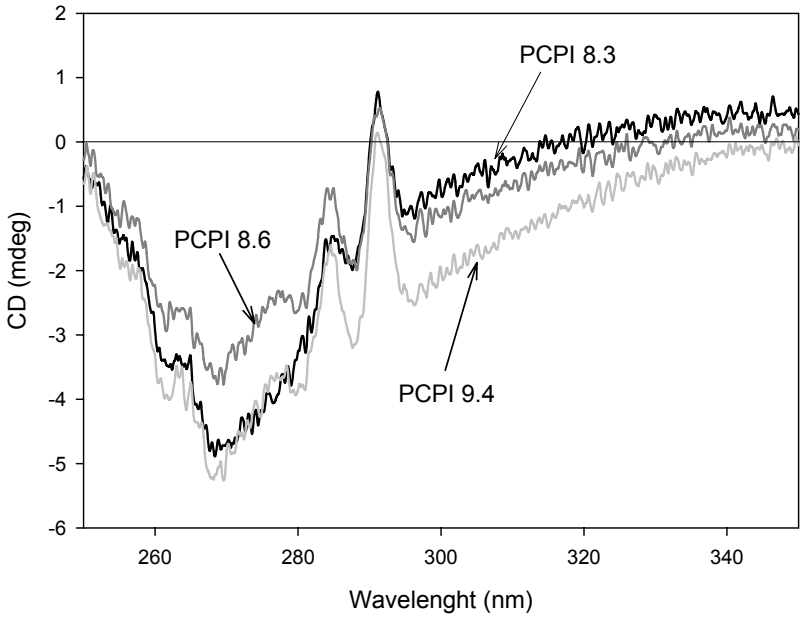

Figure 3: Near UV CD spectra of PCPI isoforms (pH 4.0) at 20°C.

previously described (Brzin et al., 1988), but no amino acid sequence was given. PCPI 8.6 has been described as a new cysteine protease inhibitor in chapter 2, and also of this protein no amino acid sequence is available. From the near UV CD spectra, it is, however, clear that both PCPI 8.6 and PCPI 9.4 contain at least one tryptophan residue, and several tyrosyl residues.

Fluorescence spectroscopy can give information about the solvent accessibility of the chromophores (tryptophan, tyrosine and phenylalanine). Therefore, the fluorescence spectrum is sensitive to local changes in the tertiary structure of a protein (Pace et al., 1989).

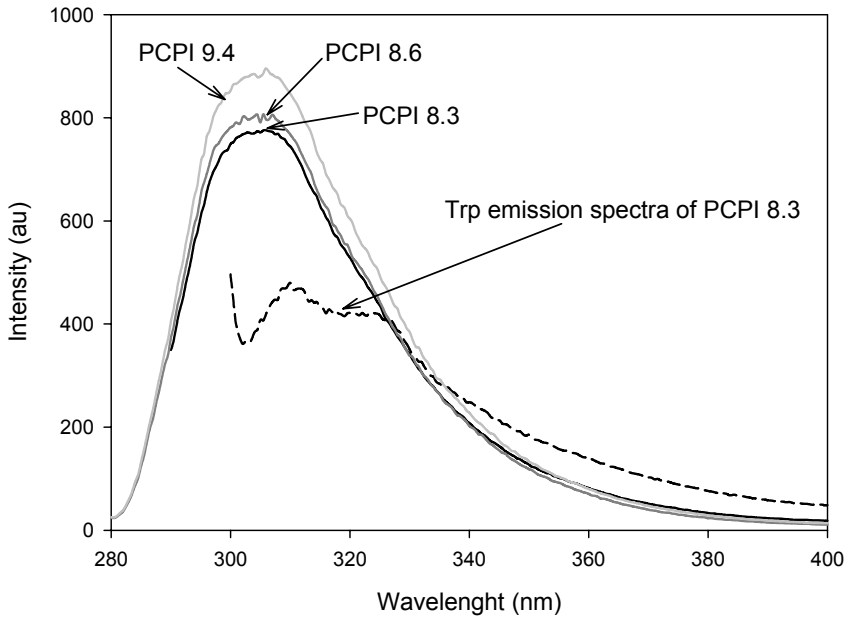

Figure 4: Tyrosine fluorescence emission spectra of PCPI isoforms (pH 4.0) at 20°C.

Because the near UV CD spectra of PCPI isoforms all showed the presence of tryptophan residues, tryptophan fluorescence emission spectra were recorded. Surprisingly, no tryptophan emission maxima were observed for all three isoforms (**figure 4**). The quenching in tryptophan emission may be explained by the presence of strong hydrogen bonds between the side chains of the tryptophan with that of phenylalanine. Therefore, tyrosine emission spectra were recorded. These spectra (**figure 4**) showed, for all three isoforms, a maximum at 305 nm, indicating that some of the tyrosyl residues are located on the periphery of the protein and that they are not near the tryptophan residue, since no photon transfer is occurring from the tyrosyl to the tryptophan residue.

Infrared spectroscopy is another method to investigate protein secondary structure based on molecular vibration of specific bonds , such as the C=O vibrations in the Amide I band (1600-1700 cm^{-1}). FTIR spectroscopy, therefore, can give information on the secondary structure (Haris and Severcanb, 1999).Figure 5 shows the Amide I band of the infrared spectra of both PCPI isoforms and the Kunitz type soybean trypsin inhibitor (STI), of which the X-ray structure has revealed that it contains approximately 2 % α-helix, 38 % β-sheet, 23 % β-turn and 37 % unordered structure (De Meester et al., 1998; Song and Suh, 1998). The spectra indicate that there is a very high degree of similarity between STI and PCPI isoforms. Deconvolution of the spectra revealed the presence of a major band at 1642 cm^{-1}, which indicates the presence of both unordered structure and short β-sheets (Goormaghtigh et al., 1994). Other deconvoluted bands at 1689 and 1624 cm-1, and at 1672 and 1662 cm^{-1} can be

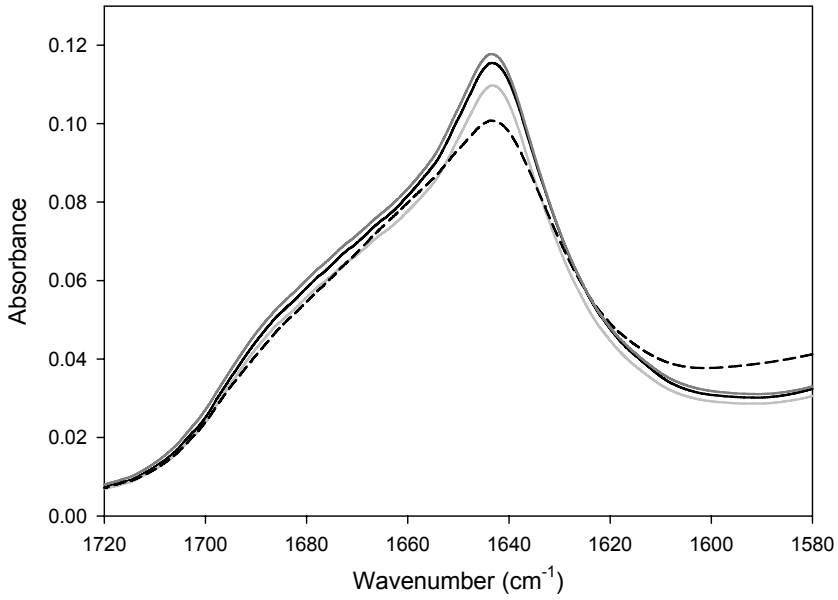

Figure 5: Amide I region of the ATR-IR spectra of PCPI 8.3 (black solid line), PCPI 8.6 (clear gray), PCPI 9.4 (dark gray) and STI (dashed line).

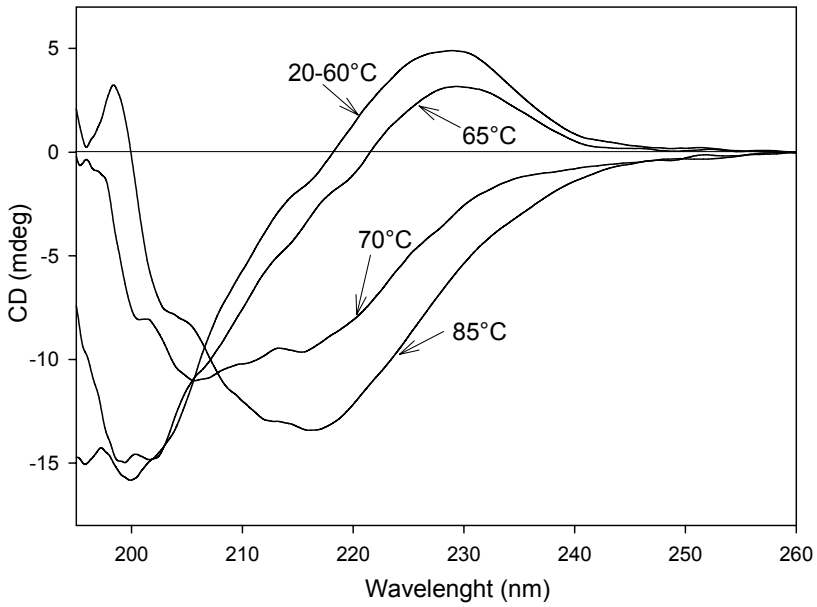

Figure 6: Far UV CD spectra of PCPI 8.3 (pH 4.0) at various temperatures.

observed, indicating the presence of β-sheets, and turns, respectively (Goormaghtigh et al., 1994). The intensity of these bands shows that STI is somewhat richer in β-sheet and β-turns than PCPI isoforms. In comparison to Potato Serine Protease Inhibitor (PSPI) (chapter 4),

PCPI isoforms seem even more similar to STI (Tetenbaum and Miller, 2001) than the PSPI group members.

3.3 Thermal stability

The thermal stability of PCPI was investigated using far and near UV CD spectroscopy, fluorescence spectroscopy, and DSC. As a typical example, far UV CD spectra of PCPI 8.3 at various temperatures (pH 4.0) are shown in **figure 6**. Similar results were also obtained for PCPI 8.6 and PCPI 9.4. No changes in intensity occurred up to 60°C. With increasing temperature above 60°C, the absolute intensities at 200 and 228 nm decreased and were inverted.

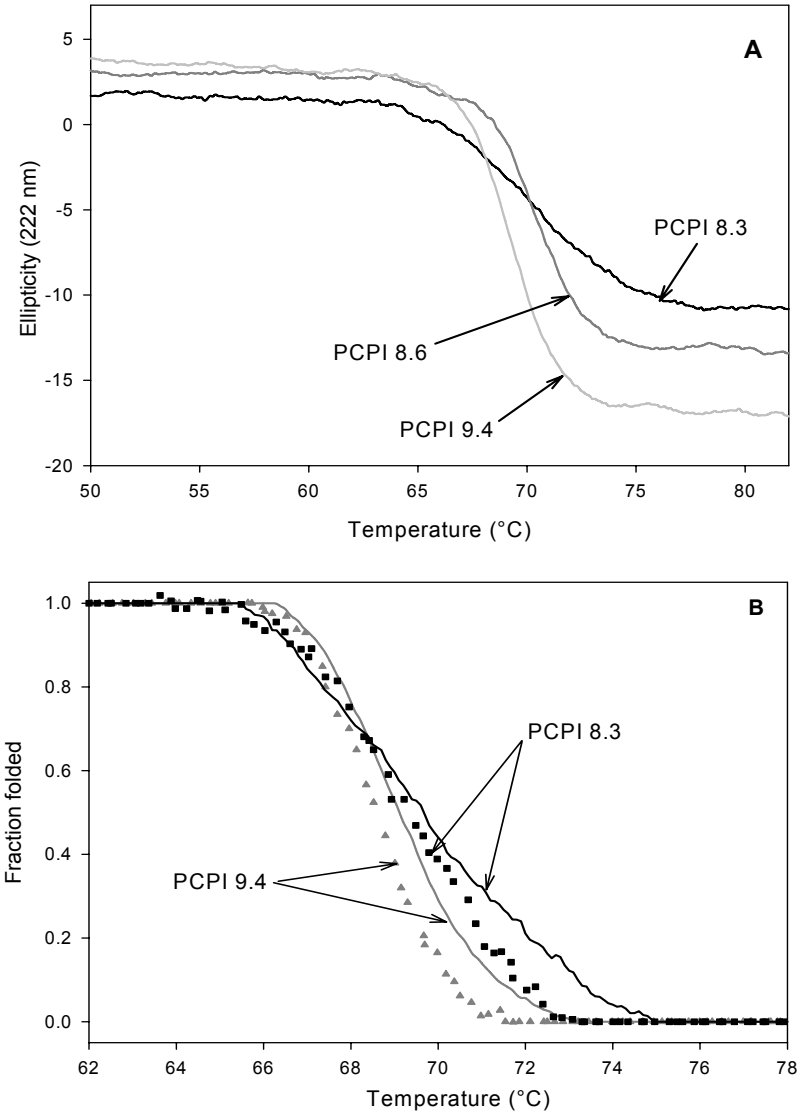

Figure 7: (A) Thermal unfolding curves of PCPI isoforms at pH 4.0, monitored by the far UV CD signal at 222 nm; (B) Thermal unfolding curves, monitored by the tyrosine fluorescence at 300 nm (■: PCPI 8.3; ▲: PCPI 9.4), and the far UV CD signal (222 nm).

Figure 7A shows the ellipticity of PCPI 8.3, 8.6 and 9.4 at 222 nm as a function of temperature. It can be observed that the shape of the thermal unfolding curves of PCPI 8.6 and 9.4 are similar and that the changes in secondary structure for both isoforms occur in a very narrow temperature range (approximately 4.5°C). The ellipticity at 222 nm for PCPI 8.6 (pH 4.0) showed changes between 68.5 and 73.0°C, with a midpoint at 70.3°C, whereas for PCPI 9.4, the changes occurred between 66.8 and 71.6°C, with a midpoint at 69.4°C. For PCPI 8.3, the changes occur in a broader temperature range (around 9°C) (**figure 7A**). In the secondary structure of PCPI 8.3, the changes take place between 65.9 and 74.7°C, with a midpoint at 70.0°C. In order to monitor also the changes in tertiary structure, the tyrosine fluorescence emission at 300 nm was followed as a function of temperature. **Figure 7B** shows the thermal unfolding curves as followed by the tyrosine fluorescence emission intensity at 300 nm, as well as the unfolding curves followed by the CD signal at 222 nm, for PCPI 8.3 and 9.4. In the **figure 7B**, the results are expressed as the fraction of PCPI in the folded state. The fluorescence unfolding curve for PCPI 8.6 was similar to that for PCPI 9.4 and is, therefore, not shown. It can be observed that the changes in the tertiary structure of PCPI 9.4 occur again in a very narrow temperature range (4°C), whereas the changes for PCPI 8.3 occur in a broader temperature range (5.9°C). The difference in the width of the temperature range of unfolding between the isoforms is, however, smaller than observed using far UV CD.

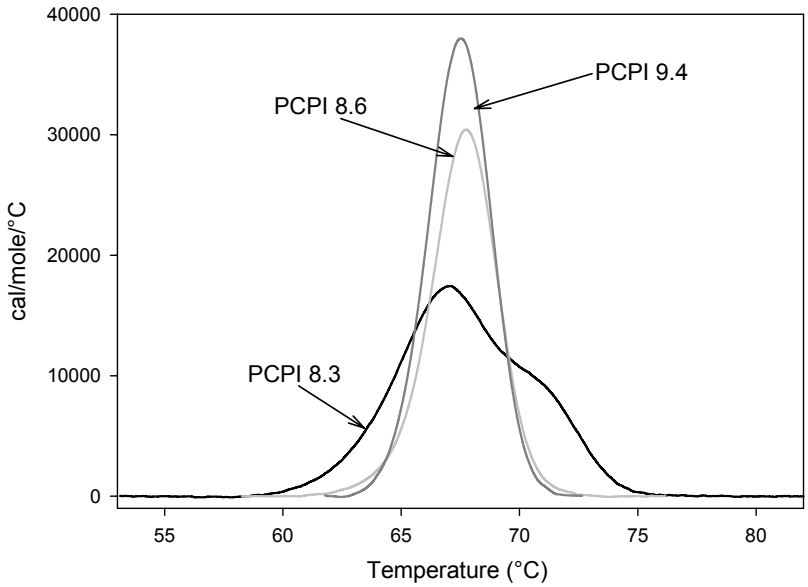

Figure 8: DSC thermograms of PCPI isoforms at pH 4.0.

DSC measurements were performed in order to determine the energy content of the heat-induced conformational changes of PCPI isoforms (Boye et al., 1997). The DSC profiles of PCPI 8.6 and 9.4 at pH 4.0 each showed a symmetric peak with transition temperatures of 67.6 and 67.5°C, respectively (**figure 8**). The calorimetric enthalpies obtained for PCPI 8.6 and 9.4 are approximately 447 and 548 kJ/mol, respectively. The DSC profile of PCPI 8.3 showed a different shape (**figure 8**). The peak is not symmetric due to a shoulder at 70°C.

These results indicate that PCPI 8.3 consists of at least two parts that unfold more or less independently with temperature. The calorimetric enthalpy for PCPI 8.3 is quite similar to that of PCPI 8.6 and 9.4 and amounts 497 kJ/mol. For all PCPI isoforms, rescanning the heated (70°C for PCPI 8.6 and 9.4 and 74°C for PCPI 8.3) samples resulted in less than 10 % of the original peak area being recovered upon reheating, indicating that the transition is almost completely irreversible (data not shown) (Makhatadze, 1998).

Table 1: Thermodynamic parameters of PCPI isoforms (cv. *Elkana*) at pH 4.0.

	Transition temperature (°C)			ΔH_{cal} (kJ/mol)	ΔH_{vH} (1) (kJ/mol)	$\Delta H_{vH}/\Delta H_{cal}$
	Far UV CD	**Tyr fluo**	**DSC**			
PCPI 8.3	70.0±0.1	69.5±0.2	67.0±0.1	498 ± 11	449 ± 25	0.90
PCPI 8.6	70.3±0.2	69.6±0.1	67.6±0.1	448 ± 6	885 ± 44	1.97
PCPI 9.4	69.4±0.2	69.1±0.2	67.5±0.2	548 ± 8	909 ± 56	1.66

(1) ΔH_{vH} was determined from the CD thermal unfolding curves.

Transition temperatures and calorimetric and Van't Hoff enthalpies as obtained from CD, fluorescence and DSC measurements are shown in **Table 1**. The ratios of the Van't Hoff enthalpy and the calorimetric enthalpy are also shown in this table. This ratio differs from 1 for all three isoforms, and ranges from 0.9 for PCPI 8.3 to approximately 2 for PCPI 8.6. (Makhatadze, 1998) These results indicate that PCPI does not unfold via a simple two state mechanism, but that at least one intermediate is present.

3.4 Concentration and scan rate dependency

Figure 9A shows the transition temperature as well as the calorimetric enthalpy as a function of the protein concentration for the three PCPI isoforms, as obtained by DSC. It can be clearly seen that the transition temperature decreases when the protein concentration increases, whereas the ΔH_{cal} remains constant. These results indicate that increasing protein concentration leads to more extensive (invisible) aggregation (Makhatadze, 1998). The changes in transition temperature with protein concentration followed the same trend for all three isoforms.

In order to examine the aggregation behaviour of PCPI 9.4, samples with concentrations between 0.2 to 1 mg/mL were studied by gel filtration, after heating at 85°C (**figure 9B**). The results show that heating leads to the formation of aggregates at all concentrations tested. Even at the lowest concentration (0.2 mg/mL) aggregation occurred and no non-aggregated PCPI was observed (Ve=15 mL). However, also a peak, eluting at 17.5 mL, appeared, indicating a molecular mass of approximately 7 kDa. Similar results were obtained for PCPI 8.3 and 8.6, including the appearance of the 7 kDa peak (data not shown).

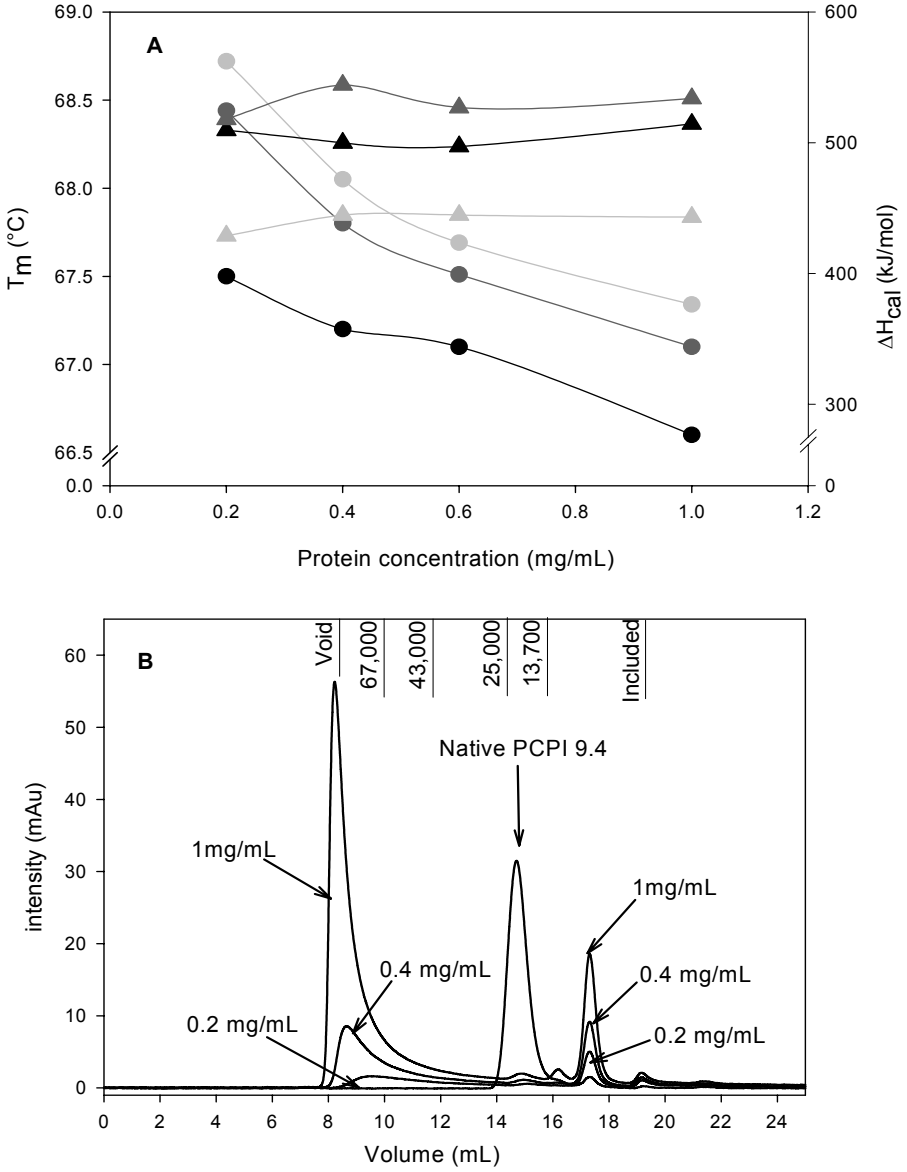

Figure 9: (A) Effect of protein concentration on the transition temperature (•) and the ΔH_{cal} (▲) for PCPI 8.3 (black), PCPI 8.6 (clear grey) and PCPI 9.4 (dark grey); (B) Gel filtration of PCPI 9.4 after heating at 85°C, at different concentrations.

In order to establish equilibrium between the native and the unfolded state at all temperatures, the heating rate should be much lower than the folding/unfolding rates (Yu et al., 1994). Therefore, PCPI isoforms were heated at scan rates between 2 and 60°C/hour.

Figure 10 shows thermograms of PCPI 8.3 and 9.4 at various scan rates. For PCPI 9.4 the peak remained symmetric, but became less sharp and shifted to a higher temperature, with increasing scan rate in the range of 2 to 60°C/hour (**Figure 10A**). A similar, but even larger, effect of scan rate was observed for PCPI 8.3 (**Figure 10B**). The transition temperatures for all three isoforms decreased with decreasing scan rate, whereas the calorimetric enthalpies increased from 280 to 460kJ/mol, from 210 to 400 kJ/mol and from 385 to 530 kJ/mol for

PCPI 8.3, 8.6 and 9.4, respectively. These results indicate that no equilibrium is reached, even at the lowest scan rate.

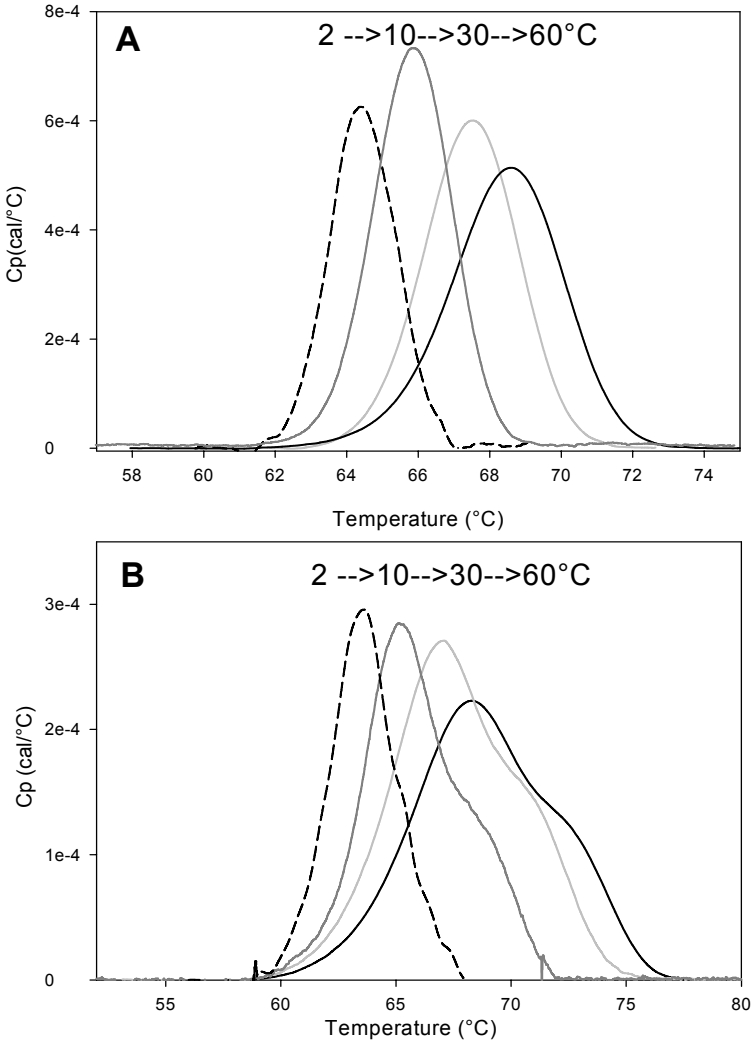

Figure 10: DSC thermograms of PCPI 9.4 (A) and PCPI 8.3 (B) at various scan rates.

4 DISCUSSION

4.1 PCPI: its similarity to PSPI

In chapter 4, PSPI was classified as a β-II protein. The far UV CD spectra of all investigated PCPI isoforms show extremes at approximately 200 and 228 nm, similar to the spectra obtained for PSPI (chapter4). Together with the sharp peaks in the near UV CD spectra and the narrow temperature range in which thermal unfolding occurs, this indicates that also PCPI can be classified as a β-II protein. Moreover, the large similarity of both the far UV CD and the infrared spectra of PCPI to those of PSPI and to those of another β-II protein, the Kunitz-type inhibitor STI, confirm this classification.

The maximum in the far UV CD spectrum at 228 nm has been observed for various protease inhibitors such as STI (Wu et al., 1992)and clitocypin, a cysteine protease inhibitor from mushroom (Kidric et al., 2002). This positive maximum seems to be a common characteristic of many protease inhibitors belonging to the β-II protein class. Therefore, the common structure (fold), consisting of distorded β-sheets, of the protease inhibitors from the β-II protein subclass maybe responsible for this positive maximum.

4.2 Comparison between PCPI isoforms

The results presented indicate that the thermal unfolding of PCPI occurs via a non two state mechanism in which at least one intermediate is formed, thus preventing the use of equilibrium thermodynamics. Interpretation of the thermal unfolding data is further complicated by the occurrence of aggregation, which, especially at low scan rates, increases the apparent cooperativity of the unfolding ($\Delta H_{vH}/\Delta H_{cal}$) and makes the system kinetically rather than thermodynamically controlled.

In this study, several differences between the three isoforms of PCPI were observed. PCPI 8.6 and 9.4 gave in all experiments very similar results, while in some cases PCPI 8.3 showed a different behaviour. PCPI isoforms showed similar structural properties, although the zero crossing in the far UV CD spectrum of PCPI 8.3 indicates that PCPI 8.3 may have a slightly different secondary structure. No differences between the 3 isoforms could be observed in the ATR-IR spectra.

The differences between these isoforms became more apparent upon thermal unfolding. The changes in secondary and tertiary structure occur in a broader temperature range for PCPI 8.3 than for the other two isoforms. The absence of complete overlap between the temperature ranges in which the secondary and tertiary structure of all three PCPI isoforms unfold, indicates the presence of at least two more or less independently unfolding parts. The existence of these two parts is, however, much more clear for PCPI 8.3 (figure 8).

LITERATURE CITED

Boye, J. I.; Ma, C.-Y.; Harwalkar, V. R. Thermal denaturation and coagulation of proteins. In *Food proteins and their applications*; Damodaran S and Parat A, eds; Marcel Dekker Inc.: New-York, **1997**;

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, 72, 248-254.

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.

Chang, C. T.; Wu, C.-S. C.; Yang, J. T. Circular dichroic analysis of protein conformation: Inclusion of the b-turns. *Anal. Biochem.* **1978**, 91, 13-31.

de Jongh, H. H. J.; Goormachtigh, E.; Killian, A. Analysis of circular dichroism spectra of oriented protein-lipid complexes: toward a general application. *Biochemistry* **1994**, 33, 14521-14528.

De Meester, P.; Brick, P.; Lloyd, L. F.; Blow, D. M.; Onesti, S. Structure of the Kunitz-type soybean trypsin inhibitor (STI): implication for the interactions between members of the STI family and tissue-plasminogen activator. *Acta Crystallogr. D. Biol. Crystallogr.* **1998**, 54, 589-597.

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.

Goormaghtigh, E.; Cabiaux, V.; Ruysschaert, J.-M. Determination of soluble and membrane protein structure by Fourier transform infrared spectroscopy. III. Secondary structures. *Subcell. Biochem.* **1994**, 23, 405-450.

Greenfield, N. J.; Fasman, G. D. Computed circular dichroism spectra for evaluation of protein conformation. *Biochemistry* **1969**, 8, 4108-4116.

Gruden, K.; Strukelj, B.; Ravnikar, M.; Poljsak-Prijatelj, M.; Mavric, I.; Brzin, J.; Pungercar, J.; Kregar, I. Potato cysteine proteinase inhibitor gene family: molecular cloning, characterisation and immunocytochemical localisation studies. *Plant Mol. Biol.* **1997**, 34, 317-323.

Haris, P. V.; Severcanb, F. FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media. *J. Mol. Catal.* **1999**, 7, 207-221.

Kelly, S. M.; Price, N. C. The application of circular dichroism to studies of protein folding and unfolding. *Biochim. Biophys. Acta* **1997**, 1338, 161-185.

Kidric, M.; Fabian, H.; Brzin, J.; Popovic, T.; Pain, R. H. Folding, stability, and secondary structure of a new cysteine dimeric proteinase inhibitor. *Biochem. Biophys. Res. Commun.* **2002**, 297, 962-967.

Knorr, D.; Kohler, G. O.; Betschart, A. A. Potato protein concentrates: the influence of various methods of recovery upon yield, compositional and functional characteristics. *J. Food Process. Preserv.* **1977**, 1, 235-247.

Kos, J.; Stabuc, B.; Schweiger, a.; Krasovec, M.; Cimerman, N.; Kopitar-Jerala, N.; Vrhovec, I. Cathepsins B, H, and L and their inhbitors stefin A and cystatin C in sea of melanoma patients. *Clin. Cancer Res.* **1997**, 3, 1815-1822.

Krizaj, I.; Drobnic-Kosorok, M.; Brzin, J.; Jerala, R.; Turk, V. The primary stucture of inhibitor of cysteine proteinases from potato. *FEBS Lett.* **1993**, 333, 15-20.

Lisinska, G.; Leszczynski, W. *Potato science and technology*, Elsevier Applied Science, London, **1989**.

Makhatadze, G. I. Measuring protein thermostability by differential scanning calorimetry. In *Current protocols in protein science*; Coligan JE, Dunn BM, Ploegh HL, Speicher DW and Wingfield PT, eds; John Wiley and sons: New York, **1998**; 7.9.1-7.9.14.

Pace, C., N.; Shirley, B. A.; Thomson, J. A. Measuring the conformational stability of a protein. In *Protein structure; a practical approach*; Creighton, T. E. eds; IRL Press: Oxford: **1989**;

Ryan, C. A. Proteolytic enzymes and their inhibitors in plant. *Ann. Rev. Plant Physiol.* **1977**, 24, 173-196.

Song, H. K.; Suh, S. W. Kunitz-type soybean trypsin inhibitor revisited: refined structure of its complex with porcine trypsin reveals an insight into the interaction between a homologous inhibitor from *Erythrina caffra* and tissue-type plasminogen activator. *J. Mol. Biol.* **1998**, 275, 347-363.

Tetenbaum, J.;Miller, L. M. A new spectroscopic approach to examining the role of disulfide bonds in the structure and unfolding of soybean trypsin inhibitor. *Biochemistry* **2001**, 40, 12215-12219.

Turk, V.; Turk, B.; Guncar, G.; Turk, D.; Kos, J. Lysosomal cathepsins: structure, role in antigen processing and presentation, and cancer. *Adv. Enzyme Regul.* **2002**, 42, 285-303.

Wu, J.; Yang, J. T.;Wu, C.-S. C. β-II conformation of all-β proteins can be distinguished from unordered form by circular dichroism. *Anal. Biochem.* **1992**, 200, 359-364.

Yu, Y.; Makhatadze, C.; Pace, N.; Privalov, P. L. Energetics of ribonclease T1 structure. *Biochemistry* **1994**, 33, 3312-3319.

Chapter 7

Structural characterisation of potato inhibitor I (cv. *Bintje*) after expression in *Pichia pastoris*

ABSTRACT

In the present study the structural properties of potato protease inhibitor 1 (PI-1) were studied as a function of temperature in order to elucidate its precipitation mechanism upon heating. A cDNA coding for PI-1 from cv. *Bintje* was cloned and expressed in *Pichia pastoris*. Using the recombinant PI-1 it was suggested that PI-1 behaves as a hexameric protein rather than as a pentamer, as previously proposed in literature. The recombinant protein seems to have either a predominantly unordered structure or belongs to the β-II proteins. Differential scanning calorimetry analysis of PI-1 revealed that its thermal unfolding occurs via one endothermic transition in which the hexameric PI-1 probably unfolds having a dimer instead of a monomer as cooperative unit. The transition temperature for the recombinant PI-1 was 88^{o}C. Similar results were obtained for a partially purified pool of native PI-1 from cv. *Bintje*.

Based on:

L. A. M. van den Broek, L. Pouvreau, G. Lommerse, B. Schipper, G. A. van Koningsveld, and H. Gruppen. Structural characterisation of potato inhibitor I (cv. *Bintje*) after expression in *Pichia pastoris. Accepted for publication in Journal of Agricultural and Food Chemistry*

1 INTRODUCTION

Potato juice, which is a by-product of the potato starch production, contains a relatively high amount of protein (Knorr et al., 1977). Patatin and protease inhibitors are the most abundant proteins. The interest in protease inhibitors, as a food ingredient, is derived from their positive nutritional effects (Hill et al., 1990), carcinogenesis prevention (Kennedy, 1998), foam-forming and stabilising properties (van Koningsveld et al., 2002), and good emulsifying properties over a large pH range (Ralet and Gueguen, 2000). According to chapter 2, the potato protease inhibitors can be classified into seven different groups. Potato protease inhibitor I (PI-1) is one of these seven groups. PI-1 inhibits serine proteases and is highly active towards chymotrypsin. It represents 2% of the soluble potato tuber proteins of the apical cortical tissues from cv. *Russet Burbank*, but its content may vary with variety (Ryan et al., 1976). In potato juice of cv. *Elkana* e.g. PI-1 represents 4.5 % of the total protein (chapter 2).

PI-1, which was first described by Ryan and Balls (1962), is composed of protomers (Melville and Ryan, 1970). Four protomer types (A-D) have been identified (Melville and Ryan, 1972), which consist of 70-71 amino acid residues (Richardson and Cossins, 1975) and show a high degree of homology among all four types. For cv. *Ulster Prince* type A and B have Glu as their N-termini, whereas type C and D both have an additional Lys at their N-termini (Richardson, 1974; Richardson and Cossins, 1974). The molecular weight of the protomers has been reported to vary between 7.9-8.1 kDa (Richardson and Cossins, 1975). The molecular weight of the oligomer in solution has been estimated as 39 kDa (Melville and Ryan, 1972), which implies that the complex is pentameric. Oligomers that differ in their protomer composition are usually called isoinhibitors (Melville and Ryan, 1972). In cv. *Elkana* (chapter 2) eight different isoforms of PI-1 with pI's between pH 5.1 and pH 7.8 have been isolated.

The inhibitor PI-1 is known to be highly stable against heating (Ryan and Balls, 1962; Melville and Ryan, 1972; Huang et al., 1981) and proteolytic digestion at neutral pH (Ryan, 1966). PI-1 from cv. *Russet Burbank* was still heat stable after 5 minutes incubation at 97^{o}C at either pH 3 or pH 8 as determined by quantitative immunological assays (Plunkett and Ryan, 1980).

The present study was undertaken to investigate the structural stability of PI-1 at different temperatures, which is important in elucidating the mechanism of irreversible precipitation of PI-1 during the industrial recovery of potato protein from the potato juice. Due to the heterogeneous character of the isoinhibitors a more homologues PI-1 form would make a structural characterisation much easier to interpret. Therefore, one PI-1 protomer was cloned and expressed in *Pichia pastoris*, because heterologous protein expression in this methylotrophic yeast can be advantageous due to (i) a high expression level and (ii) extracellular secretion, which can reduce the amount of necessary purification steps (Cereghino, 2000).

Here, we report the expression and characterisation of the structural properties, both at ambient temperature as well as at elevated temperatures, of recombinant PI-1 from cv. *Bintje*.

2 MATERIALS AND METHODS

2.1 Materials.

Plasmid pPIC9 and *P. pastoris* strain KM71 and GS115 were part of a *P. pastoris* expression kit that was purchased from Invitrogen (San Diego, CA, USA). BMG medium (100 mM potassium phosphate buffer, pH 6.0, 1.34 % (w/v) yeast nitrogen base with ammonium sulfate without amino acids, 4 10^{-5} % (w/v) biotin, and 1.0 % (v/v) glycerol) and BMM medium (BMG medium with 0.5 % (v/v) methanol instead of 1.0 % (v/v) glycerol) were prepared according to the instructions in the *Pichia* Expression Kit manual. Bovine pancreas trypsin (T-8642, lot 114H7100), bovine chymotrypsin (C-4129, lot 58H7001), and N-succinyl-L-Phe-*p*-nitroanilide were obtained from Sigma Chemical Co. Benzoyl-DL-Arg-*p*-nitroanilide (DL-BAPA) was from Merck and a commercial PI-1 preparation was from Calbiochem.

2.2 Cloning of PI-1 in *Pichia pastoris*.

A PI-1 cDNA clone (kindly provided by M. Jongsma, Plant Research International BV, The Netherlands; accession number AY496262) was obtained from a tuber-specific potato cDNA library from cv. *Bintje*. The part coding for the mature protein was amplified from the cDNA by PCR. Primer P5 contained an extra *Xho*I restriction site and a signal peptide sequence (5'-CCCCCCCTCGAGAAAAGAGAGGCTGAAGCTAAGGAATTTCAATGCAATGG-3') for extracellular targeting of recombinant PI-1. Primer P3 contained an extra *Not*I restriction site (5'-CCCCCCGCGGCCGCTTAACCAACCACAGGAATTTG-3'). The amplified fragment and the pPIC9 vector were digested with *XhoI* and *Not*I and hereafter ligated. For plasmid propagation the ligated vector was transformed into *Escherichia coli* XL1 Blue MRF´. Cells were grown overnight at 37^{o}C on solidified (15 g/L agar) Luria-Bertani (LB) broth plates supplemented with 50 μg/mL ampicillin. A PCR with plasmids from the recombinant *E. coli* colonies as template and the primers P5 and P3 was performed to estimate if the PI-1 gene was present. One of the positive clones was used for further cloning. The plasmid was isolated and digested with *Sal*I. Transformation into and growth of *P. pastoris* KM71 and GS115 was performed according to the instructions of the supplier (Invitrogen, San Diego, CA, USA). The nucleotide sequence was determined as described previously (chapter 3)

Twelve colonies were randomly picked from each transformed *P. pastoris* strain and grown overnight in 10 ml BMG-medium at 30^{o}C and 275 rpm. Cells were subsequently centrifuged (2000*g*, 10 min, RT) and suspended in 10 ml BMM-medium to an OD_{600} of 1. The cell cultures were grown overnight. After 24 h an aliquot (1 mL) of cell culture was taken for analysis and replaced by 1 mL BMM in 5% (v/v) methanol. This was repeated 4 times. To estimate the expression of PI-1 into the cell supernatant a radial diffusion assay with chymotrypsin as protease was used (Jongsma et al., 1993).

2.3 Expression of recombinant PI-1.

The colony of *P. pastoris* KM71 with the highest expression level of recombinant PI-1 was chosen for large-scale production using a BioFlo3000 fermentor (New Brunswick Scientific BV, Nijmegen, The Netherlands). *P. pastoris* was grown for 24 hours in 190 ml BMG-medium. Hereafter the cells were transferred into the fermentor, which contained 1.8 litre BMG-medium and 8 ml PTM_1 trace salt (Invitrogen, San Diego, CA, USA). Glycerol was added as carbon source. When the cell culture reached an OD_{600} of 198, the glycerol was replaced by methanol to induce the expression of PI-1. The fermentation was stopped after adding methanol for 52 h. Cells were centrifuged (10,000*g*, 15 min, 4°C) and the supernatant was filtered through a 0.2 μm filter and frozen till further use.

2.4 Recombinant P1-1 purification.

The cell supernatant was dialyzed overnight against water (MWCO: 2,000) and the pH was adjusted to pH 8 with NaOH. Subsequently the sample was filtered through a 0.2 μm filter. Purification was performed using an Äkta Explorer (Amersham Biosciences, Uppsala, Sweden) equipped with a Source 15 Q anion exchange column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 10 mM Tris-HCl buffer (pH 8). After loading the sample onto the column, elution took place with a linear gradient of 0-0.5 M NaCl in 10 mM Tris-HCl buffer (pH 8), at a flow rate of 62.9 cm h^{-1} and detection was done at 280 nm. Fractions (25 mL) were analyzed for trypsin inhibiting activity and fractions with a high inhibiting activity were pooled. Parts of the pools obtained were dialyzed overnight against water (MWCO: 2,000) and subsequently freeze-dried. The freeze-dried material was dissolved in 7 mM sodium phosphate buffer (pH 7.5) (ionic strength 15 mM).

Protein concentration was determined by the method of Bradford (Bradford, 1976) using BSA and a commercial PI-1 as standards, or the concentration was calculated from the absorbance measured at 278 nm. In this case the sample was diluted in 0.02 M sodium phosphate buffer containing 6 M Gnd HCl, pH 6.5, and the molar extinction coefficient used was 5,600 M^{-1} cm^{-1}.

2.5 Partial purification of native P1-1 from cv. *Elkana* and *Bintje*.

PI-1 pools were isolated from cv. *Bintje* and *Elkana* according to Melville and Ryan (Melville and Ryan, 1972). Potato juice was adjusted to pH 3.0 with 5 M HCl and the suspension was subsequently centrifuged (14,000*g*, 20 min, 4°C). The supernatant was adjusted to pH 7 with NaOH and heated for 5 min at 75°C. After centrifugation the supernatant obtained was filtered through a 0.2 μm filter. Purification was performed using a Äkta Explorer (Amersham Biosciences, Uppsala, Sweden) equipped with a Superdex 75 prep grade size exclusion column (Amersham Biosciences, Uppsala, Sweden) for purification of PI-1. Equilibration and elution took place with 25 mM TRIS/HCl buffer (pH 7.0), with a flow rate of 30.6 cm.h^{-1} and detection was performed at 280 nm. Fractions (25 mL) corresponding to PI-1, as judged with SDS-PAGE, were pooled.

2.6 Protein characterization.

The apparent native molecular weight was determined by size exclusion chromatography using a Superdex 75 column (Amersham Biosciences, Uppsala, Sweden) on an Äkta Purifier (Amersham Biosciences, Uppsala, Sweden). The column was calibrated with BSA (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), chymotrypsinogen A (25,000 Da), ribonuclease A (13,700 Da), cytochrome C (12,400 Da), and aprotinin (6,500 Da). Elution was performed with 10 mM TRIS-HCl buffer (pH 8), containing 0.15 M NaCl at a flow rate of 37.3 cm h^{-1}, while detection was performed at 280 nm.

MALDI-TOF MS analysis in the linear mode was performed using a Voyager DE RP instrument (Perseptive Biosystems, Framingham, MA) as described previously (chapter 2).

SDS-PAGE in presence of β-mercaptoethanol was carried out on the Pharmacia Phastsystem according to the instructions of the supplier. Coomassie Brilliant Blue R-250 staining was used for detection of proteins on PhastGel 8-25% gradient gels (Amersham Biosciences, Uppsala, Sweden).

2.7 Inhibitor assays.

Protease activity in the presence or absence of inhibitor was measured by the hydrolysis of DL-BAPA for trypsin and N-succinyl-L-Phe-*p*-nitroanilide for chymotrypsin with aid of a microtiterplate reader (μQuant, Bio-Tek instruments Inc.) or a Shimadzu spectrophotometer (Shimadzu Corporation). Trypsin inhibition and chymotrypsin inhibition was estimated as described previously (chapter 2). From these results the trypsin inhibitory activity (TIA) and chymotrypsin inhibitory activity (CIA) were calculated for the different recombinant PI-1 pools. The TIA and CIA values were expressed in mg (active) enzyme/g inhibitor.

2.8 Spectroscopic measurements.

Samples were measured in 7 mM sodium phosphate buffer, pH 7.5, and filtered through a 0.22 μm filter before measurement.

Far-ultra violet CD

Far-ultra violet circular dichroism (far UV CD) spectra of 0.2 mg/mL samples, at temperatures varying from 20 to 95°C, were recorded on a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) as described previously (Pots et al., 1998). The samples were heated till the desired temperature and equilibrated for 6 minutes at this temperature before spectra were recorded. Spectra were corrected by subtracting the spectrum of a protein free sample obtained under identical conditions. Noise reduction was applied using the Jasco software and analysis was performed using a non-linear regression procedure (De Jongh et al., 1994).

Changes in secondary structure of recombinant PI-1 during heating (20-95°C; 30°C.h^{-1}) were monitored by measuring the ellipticity at 222 nm.

Near-ultra violet CD

Near-ultra violet circular dichroism (near UV CD) spectra at 20°C of 1 mg/mL samples of PI-1 were also recorded on a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan). The scan interval was 250-350 nm, the scan speed 50 nm min^{-1}, the data interval 0.2 nm, the bandwidth 1.0 nm, the sensitivity 20 mdeg, and the response time was 0.125 s. Spectra were also corrected by subtracting the spectrum of a protein free sample obtained under identical conditions.

Tryptophan fluorescence

Tryptophan fluorescence spectra of 0.2 mg/mL samples were recorded on a Perkin Elmer Luminescence Spectrophotometer LS 50 B (Perkin Elmer Corp. Boston, MA, USA) equipped with a pulsed Xenon source at 20°C. Spectra of the samples were obtained by excitation at 295 nm and three emission spectra 300 to 400 nm were recorded and averaged using a scan speed of 100 nm.min^{-1}. Both the excitation and emission slit was set at 5 nm. Spectra were corrected by subtracting the spectrum of a protein free sample obtained under identical conditions. Changes in tertiary structure of PI-1 during heating (20 till 95°C, heating rate 30°C h^{-1}) were monitored by the changes in fluorescence at 330 nm, using a Varian Cary fluorimeter (Varian Cary Inc., Palo Alto, CA, USA).

2.9 Differential Scanning Calorimetry.

DSC measurements were performed on a VP-DSC Microcalorimeter (Microcal Incorporated, Northampton MA, USA). Solutions containing 1 mg/mL PI-1 in 7 mM sodium phosphate buffer, pH 7.5, were heated from 20 to 130°C with a scan rate of 30°C h^{-1}. Data were analyzed using DSC-Microcal Origin software.

3 RESULTS AND DISCUSSION

3.1 Expression and purification of recombinant PI-1.

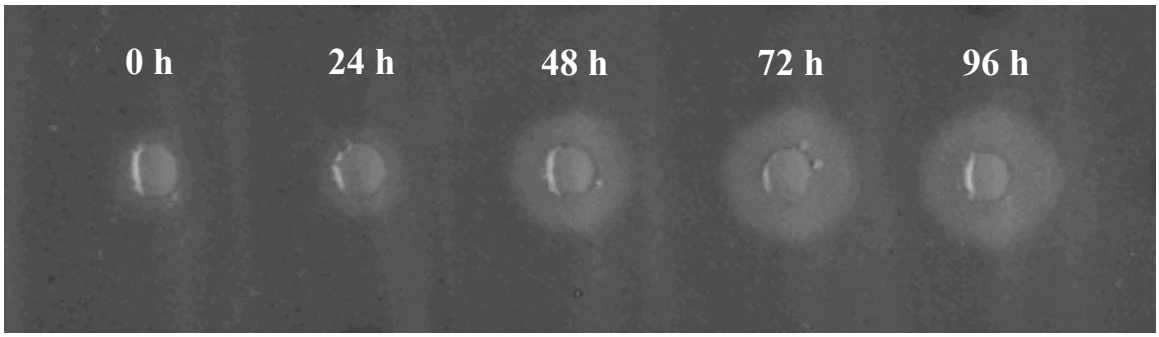

Figure 1: Progression profile of recombinant PI-1 from potato (cv. *Bintje)* accumulation in cell supernatant of *Pichia pastoris*.

To obtain high levels of recombinant PI-1 from potato cv. *Bintje* the corresponding cDNA (AY496262) was cloned into *P. pastoris* using a commercial kit. The deduced amino acid sequence of the cDNA showed the highest identity (91%) with PI-1 from cv. *Russet Burbank* (Q00783; S26717 and CAA78259). The pPIC9 vector was used for extracellular production of the protein to reduce the amount of contaminating proteins. After transformation 92% and 75% of the total clones from *P. pastoris* strain GS115 and KM71, respectively, showed inhibiting activity towards α-chymotrypsin. The inhibiting activity of PI-1 in methanol-induced recombinant *P. pastoris* was almost maximal at 48 h as estimated with a radial diffusion assay (**figure 1**). For a high production of recombinant PI-1 fermentation was performed with one of the clones of strain KM71 that showed the highest expression level. Purification of recombinant PI-1 in the cell supernatant was performed using a Source 15 Q anion exchange column (**figure 2**). Based on the protein elution profile and the inhibiting activity towards trypsin six pools were collected (**figure 2**). With SDS-PAGE under reducing conditions all pools showed a single band at approximately 8 kDa (data not shown).

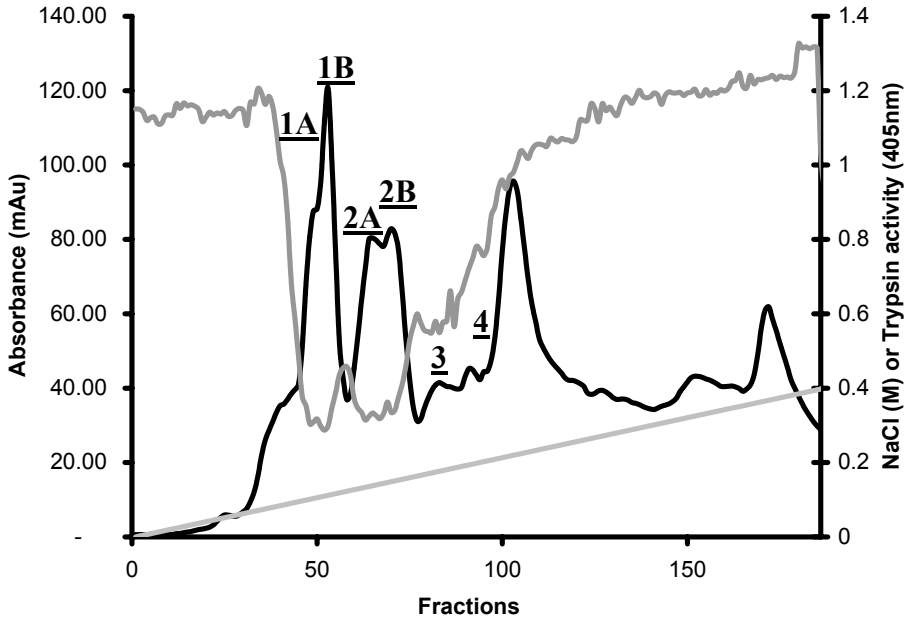

Figure 2: Source 15 Q anion exchange chromatography of recombinant PI-1 from potato (cv. *Bintje)* in cell supernatant of *Pichia pastoris*. Black line, 280 nm; dark gray line, trypsin activity; clear gray line, salt gradient. Pooled fractions are indicated as 1A, 1B, 2A, 2B, 3, and 4.

Only pool 3 showed some other faint bands. The inhibition data towards trypsin and α-chymotrypsin are given in **Table 1**. Pools 3 and 4 showed lower TIA and CIA values than the other pools. This is probably due to the presence of some contaminating proteins in these pools. Pools 1A and 1B have slightly higher TIA and CIA values than pools 2A and 2B.

Table 1. Inhibition activity towards trypsin (TIA) and α-chymotrypsin (CIA) of recombinant potato PI-1 from potato (cv. *Bintje).*

Pool	TIA (mg/g)	CIA (mg/g)
1A	580 ± 8	4939 ± 105
1B	582 ± 17	5381 ± 282
2A	552 ± 3	4331 ± 75
2B	525 ± 9	3812 ± 165
3	101 ± 1	650 ± 29
4	130 ± 1	511 ± 30

Based on pools 1A, 1B, 2A, and 2B approximately 105 mg of recombinant PI-1 per litre cell supernatant was obtained. Pools 3 and 4 were excluded because they had low TIA and CIA values. In comparison with other recombinant plant proteins produced by *P. pastoris* a high yield was obtained for the recombinant PI-1 (Cereghino, 2000).

3.2 Molecular mass and oligomer size determination of PI-1.

The gene inserted in the pPIC9 vector was sequenced and it appeared that one nucleotide was changed during PCR. This change resulted in Arg at position 50 instead of His and the deduced molecular mass thus became 7,857 Da. The molecular mass of the proteins in the different pools was determined with MALDI-TOF MS (**figure 3**). Three major peaks were observed for all pools (7,728±1, 7,857±1, and 8,057±1 Da). A mass of 7,857 Da corresponds to the deduced molecular mass of the cloned PI-1. The peak of 7,729 Da corresponds to the mass of a protomer without the first amino acid (Lys) present. Richardson and Cossins (1974) reported the amino acid sequence of four protomers from cv. *Ulster Prince*. Two protomers contained Glu at their N-terminus whereas the other two had an additional Lys at their N-terminus. But in this case these protomers were expressed from different genes because they showed slight differences in their amino acid sequence. In our study only one gene was used but still two forms of recombinant PI-1 were obtained, with or without a Lys at the N-terminus. Another mass that was clearly observed is 8,057±1 Da, which corresponds to a form that has an additional Glu and Ala from the α-factor prepro leader of the pPIC9 vector, used for extracellular production of recombinant PI-1, which was not cleaved off during processing. Variability in the amino acid sequence of the N-terminus is commonly seen with heterologously expressed proteins by *P. pastoris* and is due to inefficient cleavage of the α-factor mating signal sequence (Cereghino, 2000). The obtained masses of the smaller peaks in some pools were 7,928±2 Da, (which corresponds to an additional Ala), 8,128±2 Da (with an

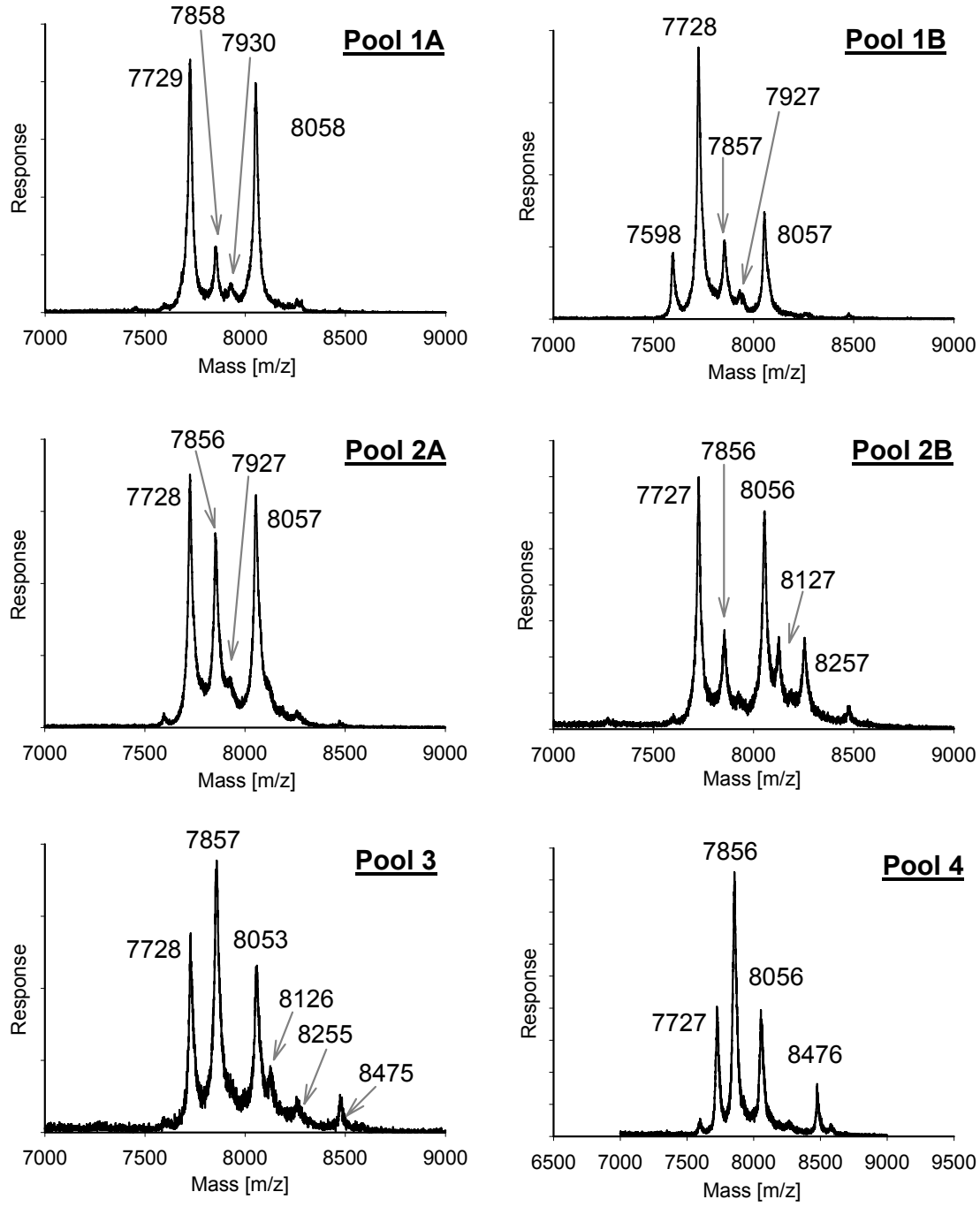

Figure 3: MALDI-TOF spectra of the different pools of recombinant PI-1 from potato (cv. *Bintje)* obtained after anion exchange chromatography. Masses are given in Dalton.

extra Ala-Glu-Ala), and 8,258±3 Da (with an additional Glu-Ala-Glu-Ala at the N-terminus, all originating from the α-factor prepro leader sequence). A mass of 7,598 Da in pool 1B corresponds to a form missing both Lys and Glu at the N-terminus. Further investigations are needed to determine the exact composition of the peak with a mass of 8,475 Da in pools 3 and 4. From the above it is clear that the recombinant PI-1 consists of different protomers varying in molecular mass. The pool of natural PI-1 showed a broad peak from 7,597 Da till 7,974 Da with the highest intensity at 7,731 Da, indicating that several different forms were present.

For all pools the apparent native molecular weight was estimated by size exclusion chromatography. The native molecular weight varied from 48 to 53 kDa (pool 1A and 1B 48 kDa; pool 2A 50 kDa; pool 2B and 3 52 kDa; pool 4 53 kDa). It was observed that oligomers with a higher apparent molecular weight eluted later from the anion exchange column. When combining the MALDI-TOF MS analysis and size exclusion data it can be concluded that the mature recombinant PI-1 is presumable a hexameric protein. In the literature available PI-1 is described as a pentamer (Melville and Ryan, 1972; Richardson et al., 1976) as estimated from ultracentrifugation and size exclusion chromatography experiments. To investigate if the recombinant PI-1 behaves differently from the native form, PI-1 was partially purified from potato cultivars *Bintje* and *Elkana* according to Melville and Ryan (1972). Both PI-1's showed very similar elution volumes with size exclusion chromatography and also similar to those obtained for the recombinant PI-1, indicating that the native form of PI-1 also seems to be a hexamer, taking into account the MALDI-TOF data of these PI-1's.

3.3 Structural properties of recombinant PI-1 at 20°C.

Pools 1A, 1B, 2A, and 2B were used for investigating the structural properties of recombinant PI-1. Far-UV CD spectra were recorded to determine whether any differences exist in the secondary structure of the recombinant PI-1's in these pools (Fig. 4). All spectra showed a clear minimum at approximately 204 nm, which could imply that the protein has a mostly unordered structure (Venyaminov and Yang, 1996). All pools showed a maximum at 228 nm, which can be due to a combination of contributions from secondary structures like β-turns and loops (Perczel and Hollosi, 1996), the presence of disulfide bridges, and the interaction between aromatic side chains (Woody and Dunker, 1996). The presence of a

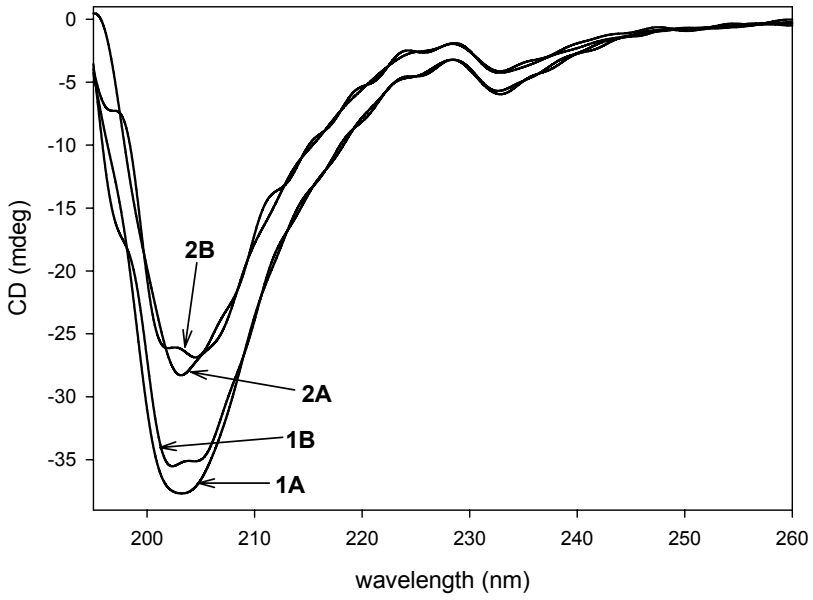

Figure 4: Far-UV CD spectra at 20°C of different pools of purified recombinant PI-1 from potato (cv. *Bintje)*.

disulfide bridge was already found by Plunkett (Plunkett and Ryan, 1980) for PI-1 from cv. *Russet Burbank.* From the deduced amino acid sequence of PI-1 from cv. *Bintje* the presence of four aromatic amino acids (three Phe and one Trp) was established. The observed minimum at 204 nm and the maximum at 228 nm may also indicate that PI-1 belongs to the β-II proteins (Wu et al., 1992). Since all spectra were highly similar, it can be concluded that there is no real difference in secondary structure between the different recombinant PI-1's . In addition, a similar spectrum was also obtained for the partially purified native PI-1 from cv. *Bintje*. Near-UV CD spectra were recorded for pools 1A and 2A. No clear difference was observed between the two isoinhibitors (data not shown). The spectra were also similar to those obtained for native PI-1 from cv. *Russet Burbank* (Plunkett and Ryan, 1980).

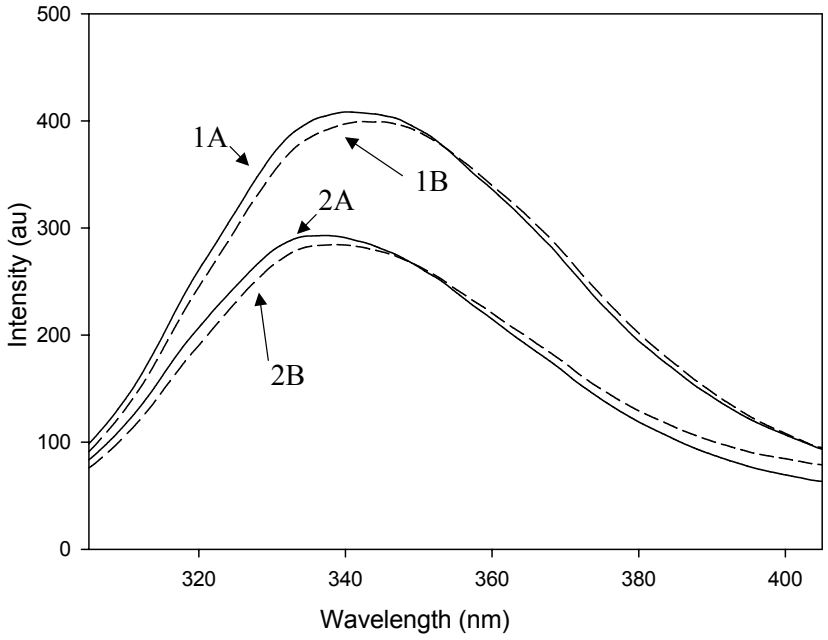

Figure 5: Tryptophan fluorescence spectra at 20°C of different pools of purified recombinant PI-1 from potato (cv. *Bintje)*.

Fluorescence spectra can give information about the polarity of the environment of phenylalanine, tyrosine, and tryptophan residues (Lakowicz, 1999). Tryptophan fluorescence spectra of the different pools were recorded and are shown in **Figure 5**. Pools 1A and 1B showed higher intensities than pools 2A and 2B. The intensity maximum of the different pools was at 340, 343, 335, and 338 nm for pools 1A, 1B, 2A, and 2B, respectively. A maximum at a higher wavelength indicates that the environment of the tryptophan residues is more polar, which indicates a less compact structure. Pools 1A and 1B thus seem to have a less compact structure than pool 2A and 2B, however, the differences are small.

3.4 Thermal stability.

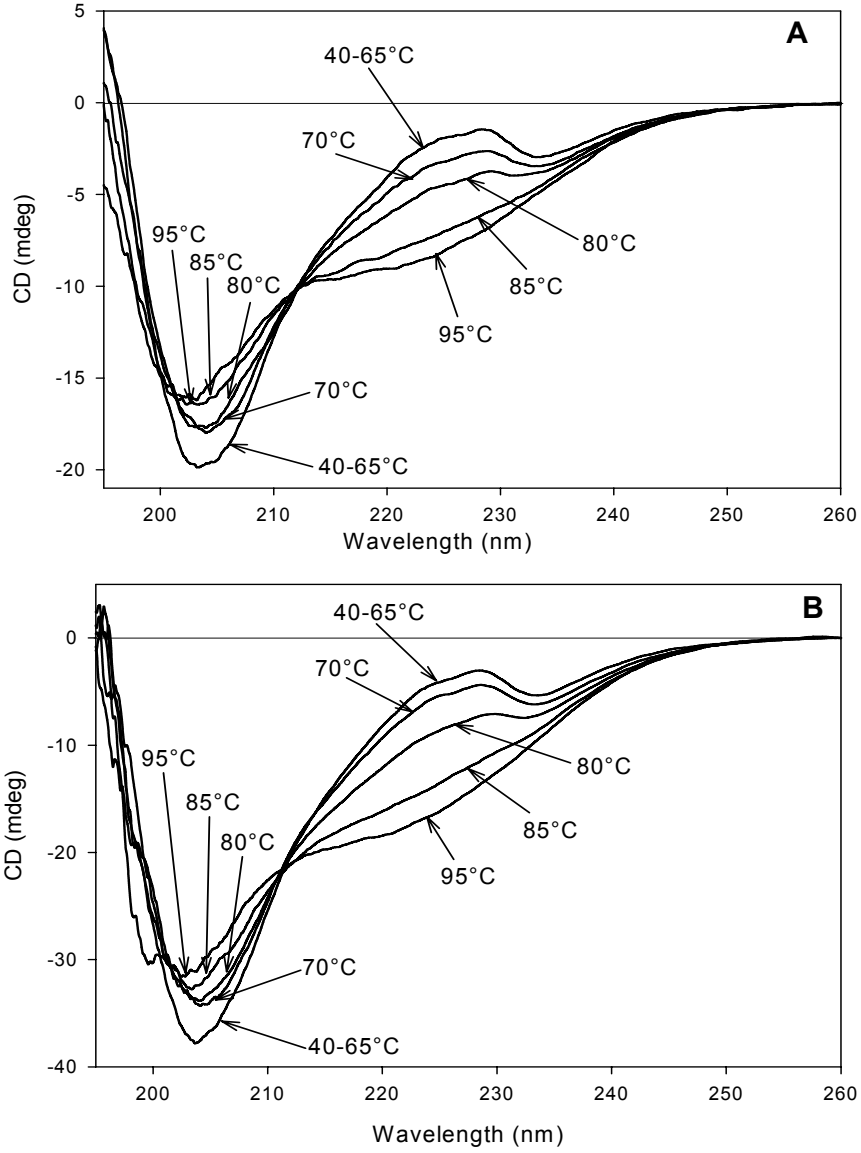

Figure 6: Far-UV CD spectra at different temperatures of (A) Pool 1B and (B) Pool 2A of purified recombinant PI-1 from potato (cv. *Bintje)*.

The recombinant PI-1 was studied in more detail with regard to its thermal stability. Far-UV CD spectra were recorded at various temperatures for pools 1B and 2A (**figure 6**). A similar course as a function of temperature was observed for both recombinant PI-1 isoinhibitors. No changes appeared between 40°C and 65°C as the temperature increased. The absolute intensity of the minimum (204 nm) decreased and the maximum (228 nm) disappeared with increasing temperature above 70°C. The spectral changes occurred with a clear isodichroic point at 212 nm. The presence of an isodichroic point is an indication that the transition precedes essentially via a two-state mechanism (Tamura et al., 1991). The

ellipticity of the recombinant PI-1 pools at 222 nm as a function of temperature was also recorded. All isoinhibitors showed similar unfolding curves (data not shown) and the calculated transition temperature obtained for each isoinhibitor is given in **Table 2**. Fluorescence emission spectra as a function of temperature were also recorded. From the unfolding curves obtained from these spectra, which were similar for all isoinhibitors, the transition temperature was calculated for each isoinhibitor (**Table 2**).

Table 2: Thermodynamic parameters of recombinant potato from potato cv. *Bintje*.

Pool	Transition temperature (°C)			ΔHcal (kJ/mol) [a]	ΔHvH (kJ/mol) [a]	ΔHvH/ΔHcal
	Far UV CD	Trp fluo	DSC			
1A	86.5	87.4	88.7±0.1	325.2±2.4	657.3±9.5	2.02
1B	86.2	87.1	88.7±0.1	335.2±4.3	635.9±19.3	1.90
2A	86.7	87.0	87.8±0.2	344.7±5.2	665.2±5.3	1.93
2B	85.8	87.3	88.1±0.2	339.0±3.7	652.7±8.5	1.93
Native PI-1 pool	nd[b]	nd[b]	84.9	360.7	736.7	2.04

[a]Protein concentration was based on a molecular weight of the protomer (8,000 Da).

[b]Not determined.

Also, DSC was performed to analyze the thermal unfolding behavior of the recombinant PI-1 isoinhibitors. From the DSC thermograms the enthalpy involved in unfolding and the transition temperature can be estimated. For all isoinhibitors the thermal event was endothermic and there was no indication that aggregation took place. The thermodynamic data calculated from the DSC profiles are given in **Table 2**. No clear differences were observed in the calorimetric enthalpies (ΔH_{cal}), the van't Hoff enthalpies (ΔH_{vH}), and transition temperatures between the different isoinhibitors. The transition temperatures obtained by DSC were slightly higher than those estimated by far UV CD and fluorescence spectroscopy, which may be due to a higher protein concentration used in the DSC measurements. The ratio of ΔH_{vH} and ΔH_{cal} is approximately 2 for all pools (**Table 2**) when using 8 kDa as the molecular mass of the cooperative unit. This indicates that the PI-1 unfolds with a dimer (16 kDa) instead of a monomer as cooperative unit (Privalov, 1979; Makhatadze, 1998). As can be seen in **Figure 7** the endotermic peak for PI-1 is slightly asymmetric (sharper on the high temperature side), which can be regarded as indication that dissociation occurs during the unfolding process (Sturtevant, 1987). Indeed, fitting of the DSC results with Microcal software using a dissociation model gave good fits, especially for a model assuming PI-1 built from 3 subunits (results not shown). It thus seems likely that the PI-1 hexamer is built up from 3 tightly associated dimers, which dissociate upon thermal

unfolding. However, more research would be necessary to confirm this model. Similar DSC results were also obtained with the natural PI-1 pool from cv. *Bintje* (**Table 2**) and this implies that the recombinant PI-1 resembles the native PI-1 form. Pool 1A was used to measure the reversibility of the thermal unfolding of recombinant PI-1. No endotherm was detected upon rescanning a heated (95°C) sample indicating that the unfolding was irreversible.

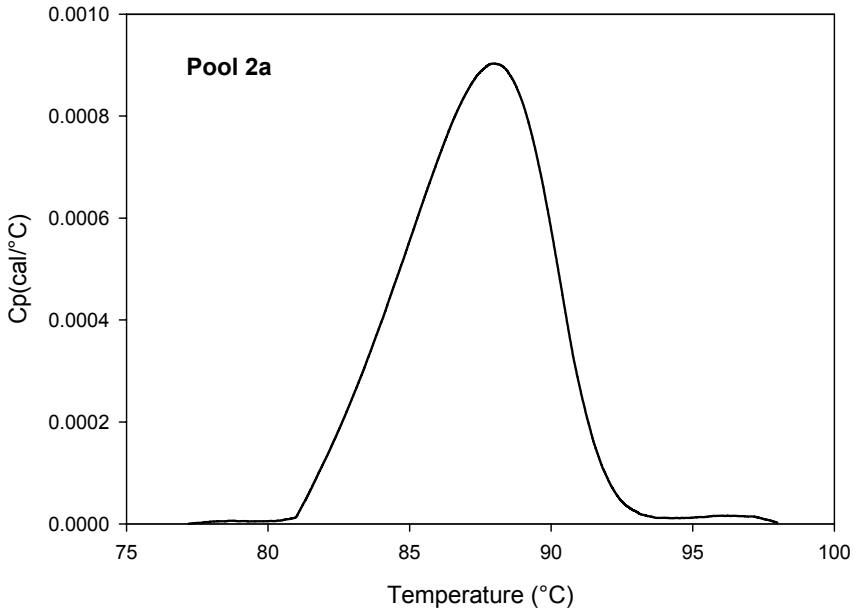

Figure 7: DSC thermograms of pool 2a.

The high transition temperature (88°C) is in agreement with the high thermostability of native PI-1 (Ryan and Balls, 1962; Melville and Ryan, 1972; Huang et al., 1981). In addition, Van Koningsveld et al. (van Koningsveld et al., 2001) showed that in a protease inhibitor pool, obtained from potato fruit juice cv. *Elkana*, most of the protease inhibitors could be inactivated by heat treatment. However, the chymotrypsin inhibiting activity was still present for about 30% at 80°C. In potato juice from cv. *Elkana* PI-1 is responsible for 19% of the chymotrypsin inhibiting activity (chapter 2). Thus the measured inhibiting activity of chymotrypsin at 80°C is probably due to PI-1.

4 CONCLUSION

Although only one gene (PI-1) form potato cv. *Bintje* was cloned in *P. pastoris* different protomers were expressed. They differed in their N-terminal amino acid composition due to variations induced by the post-translational processing in *P. pastoris*. Nevertheless, the recombinant protomers formed hexamers, showed similar structural characteristics, and were highly thermostable. Furthermore, the data obtained were similar to those obtained for a

partially purified pool of native PI-1 from cv. *Bintje*, which also suggest that also the native PI-1 is present as a hexamer rather than a pentamer, as previously proposed in literature. Therefore, the recombinant PI-1 is a good model to study the structural properties and the heat induced unfolding mechanism of PI-1.

LITERATURE CITED

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, 72, 248-254.

Cereghino, J. L. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol. Rev.* **2000**, 24, 45-66.

De Jongh, H. H. J.; Goormaghtigh, E.; Killian, A. Analysis of circular dichroism spectra of oriented protein-lipid complexes: Toward a general application. *Biochemistry* **1994**, 33, 14521-14528.

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.

Huang, D. Y.; Swanson, B. G.; Ryan, C. A. Stability of proteinase inhibitors in potato tubers during cooking. *J. Food Sci.* **1981**, 46, 287-290.

Jongsma, M. A.; Bakker, P. L.; Stiekema, W. J. Quantitative determination of serine proteinase inhibitor activity using a radial diffusion assay. *Anal. Biochem.* **1993**, 212, 79-84.

Kennedy, A. R. Chemopreventive agents: protease inhibitors. *Pharmacol. Ther.* **1998**, 78, 167-209.

Knorr, D.; Kohler, G. O.; Betschart, A. A. Potato protein concentrates: the influence of various methods of recovery upon yield, compositional and functional characteristics. *J. Food Process. Preserv.* **1977**, 1, 235-247.

Lakowicz, J. R. *Principles of fluorescence spectroscopy*, Kluwer Academic/Plenum Publishers, New York, **1999**.

Makhatadze, G. I. Measuring protein thermostability by differential scanning calorimetry. In *Current protocols in protein science*; Coligan JE, Dunn BM, Ploegh HL, Speicher DW and Wingfield PT, eds; John Wiley and sons: New York, **1998**; 7.9.1-7.9.14.

Melville, J. C.; Ryan, C. A. Chymotrypsin inhibitor I from potatoes: A multisite inhibitor composed of subunits. *Arch. Biochem. Biophys.* **1970**, 138, 700-702.

Melville, J. C.; Ryan, C. A. Chymotrypsin inhibitor I from potatoes: Large scale preparation and characterization of its subunit components. *J. Biol. Chem.* **1972**, 247, 3443-3453.

Perczel, A.; Hollosi, M. Turns. In *Circular dichroism and the conformational analysis of biomolecules*; Fasman GD, eds; Plenum press: New York, **1996**; 285-380.

Plunkett, G.; Ryan, C. A. Reduction and carboxyamidomethylation of the single disulfide bond of proteinase inhibitor I from potato tubers. *J. Biol. Chem.* **1980**, 255, 2752-2755.

Pots, A. M.; de Jongh, H. H. J.; Gruppen, H.; Hamer, R. J.; Voragen, A. G. J. Heat-induced conformational changes of patatin, the major potato tuber protein. *Eur. J. Biochem.* **1998**, 252, 66-72.

Privalov, P. L. Stability of proteins. Small globular proteins. *Adv. Protein Chem.* **1979**, 33, 167-241.

Ralet, M.-C.; Gueguen, J. Fractionation of potato proteins: solubility, thermal coagulation and emulsifying properties. *Lebensm. Wiss. Technol.* **2000**, 33, 380-387.

Richardson, M. Chymotrypsin inhibitor I from potatoes: The amino acid sequence of subunit A. *Biochem. J.* **1974**, 137, 101-112.

Richardson, M.; Baker, R. D. J.; McMillan, R. T. Re-examinationn of the molecular weight of chymotryptic inhibitor I from potatoes. *Biochem. Soc. Trans.* **1976**, 4, 1077-1079.

Richardson, M.; Cossins, L. Chymotrypsic inhibitor I from potatoes: The amino acid sequences of subunits B, C and D. *FEBS Lett.* **1974**, 45, 11-13.

Richardson, M.; Cossins, L. Corrigendum. Chymotrypsin inhibitor I from potatoes: The amino acid sequences of subunits B, C and D. *FEBS Lett.* **1975**, 52, 161.

Ryan, C. A. Chymotrypsin inhibitor I from potatoes: Reactivity with mammalian, plant, bacterial, and fungal proteinases. *Biochemistry* **1966**, 5, 1592-1596.

Ryan, C. A.; Balls, A. K. An inhibitor of chymotrypsin from *Solanum tuberosum* and its behavior toward trypsin. *Proc. Natl. Acad. Sci. U. S. A.* **1962**, 48, 1839-1844.

Ryan, C. A.; Kuo, T.; Pearce, G.;Kunkel, R. Variability in the concentration of three heat stable proteinase inhibitor proteins in potato tubers. *Am. Potatp J.* **1976**, 53, 443-455.

Sturtevant, J. M. Biochemical applications of differential scanning calorimetry. *Annu. Rev. Phys. Chem.* **1987**, 38, 463-488.

Tamura, A.; Kimura, K.; Takahara, H.; Akasaka, K. Cold denaturation and heat denaturation of Streptomyces subtilisin inhibitor. 1. CD and DSC studies. *Biochemistry* **1991**, 30, 11307-11313.

van Koningsveld, G. A.; Gruppen, H.; de Jongh, H. H. J.; Wijngaards, G.; van Boekel, M. A. J. S.; Walstra, P.; Voragen, A. G. J. The effects of pH and heat treatment on the structure and solubility of potato proteins in different preparations. *J. Agric. Food Chem.* **2001**, 49, 4889-4897.

van Koningsveld, G. A.; Walstra, P.; Gruppen, H.; Wijngaards, G.; van Boekel, M. A. J. S.; Voragen, A. G. J. Formation and stability of foam made with various potato protein preparations. *J. Agric. Food Chem.* **2002**, 50, 7651-7659.

Venyaminov, S. Y.; Yang, J. T. Determination of protein secondary structure. In *Circular dichroism and the conformational analysis of biomolecules*; Fasman GD, eds; Plenum press: New York, **1996**; 69-109.

Woody, R. W.; Dunker, K. A. Aromatic and cystine side-chain circualr dichroism in proteins. In *Circular dichroism and the conformational analysis of biomolecules*; Fasman GD, eds; Plenum press: New York, **1996**; 109-158.

Wu, J.; Yang, J. T.; Wu, C.-S. C. β-II conformation of all-β proteins can be distinguished from unordered form by circular dichroism. *Anal. Biochem.* **1992**, 200, 359-364.

Chapter 8

General discussion

1 INTRODUCTION

Potato proteins are a promising source of plant protein ingredients for human consumption. Therefore, a considerable amount of work has been performed to obtain undenaturated proteins than can be applied for human consumption (Knorr et al., 1977; Lindner et al., 1980; Gonzalez et al., 1991; van Koningsveld et al., 2002). Pots and coworkers (Pots et al., 1999) studied in detail the mechanism of unfolding and aggregation/precipitation of patatin, the major potato tuber protein. Detailed knowledge considering unfolding and aggregation of the PIs in potato juice was, however, missing in literature.

Therefore, the aim of this study was to determine in which proportion each family of PIs was present in potato juice. Subsequently, our aim was to study the structural properties of the main PIs and the thermal unfolding mechanism of these proteins upon heating. This information, combined with the available information for patatin should be of help to elucidate the mechanism of irreversible precipitation as occurring in the industrial process, and provide tools to enable efficient potato protein recovery containing or devoid of protease inhibitory activity.

2 PROTEASE INHIBITORS IN POTATO TUBER

Protease inhibitors are ubiquitously present in tubers and plant seeds (Ryan et al., 1974). In higher plants, several gene families have been characterised, particularly those constituting the serine protease inhibitors from Leguminosae, Solanaceae and Graminae (Garcia-Olmeda et al., 1987). As shown in Table 1 of chapter 1, serine protease inhibitors are more abundant than to cysteine, aspartate and metallo- protease inhibitors.

Potato tubers contain approximately 1.5% protein on a fresh weight basis (Lisinska and Leszczynski, 1989). Until now, PIs were reported to represent up to 30% of the total amount of protein (Melville and Ryan, 1972). However, in chapter 2, we showed that, in cv. *Elkana,* PIs represent up to 50% of the total amount of protein (chapter 2). These PIs were classified in 7 different groups according to their inhibitory specificity, their molecular mass and their protein architecture.

Although many plant species are reported to contain protease inhibitors, only limited data are present concerning the content and relative proportions of PIs and specific PI families in various products. In sweet potato roots, 60% of the total protein content consists of a 33 kDa protein (Lin and Chen, 1980), which shows inhibitory activity towards trypsin as well as antioxidant activities (Hou et al., 2001). (Roozen and De Groot, 1989)For soybeans, it can be calculated that PI consitute about 5% of all proteins present in (ungerminated) soybeans (Roozen and De Groot, 1989; Liu, 2000). These PIs can be classified in two families: the Bowman-Birk inhibitors and the Kunitz-type of inhibitors (Werner and Wemmer, 1991).

When expressed on fresh weight basis, potatoes (cv. *Elkana*) contain 0.75 % PIs, sweet potato 0.7% and soybeans 2%. Therefore, although potatoes and sweet potatoes contain

relative to their protein content more PIs than soybean, on a fresh weight basis soybeans contain 3x more protease inhibitor. (Werner and Wemmer, 1991)

3 INHIBITION MECHANISM

All protease inhibitors prevent access of substrates to the catalytic site of their associated enzyme through steric hindrance (Bode and Huber, 1992). PIs can be divided in two groups according to the interactions formed in the complex with enzyme. For the first group, the PIs bind the enzyme through interactions directly to the catalytic site of the enzyme, in a 'substrate-like binding mechanism'. The second group of PIs do not bind to the catalytic site but show interactions in the surrounding of the catalytic site and thereby closing it for entrance of substrate ('non substrate-like binding'). **Figure 1** shows examples of the two different types of interactions within the complex enzyme/protease inhibitors.

The first group is by far the largest group. This group consists of relatively small proteins (or domains of multiheaded inhibitors) of 30 to 200 amino acids and consists mainly of serine protease inhibitors. They all possess an exposed binding loop of a characteristic and specific conformation, in which the residue P1 is exposed on the outside (Jackson and Russell, 2001). P1 refers to the nomenclature of Schechter and Berger (1967) in which P stands for Peptide and the number refers to the position of the amino acid with respect to the scissile bond in the N- (e.g. P1) or C- (e.g. P1') terminal direction. The PIs (I) appear to interact with enzymes (E) according to the following scheme:

$$E + I \rightleftharpoons EI \rightleftharpoons E + I^*$$

The stable complex (EI) is rapidly formed and usually dissociates very slowly into free enzyme and cleaved or uncleaved inhibitor (I*). The cleaved inhibitor is specifically hydrolysed at the scissile peptide bond between P1 and P1'. However, the conformation of the inhibitor remains unchanged due the presence of a disulfide bridge in the binding loop. The side chain of residue P1 (e. g. arginine residue for trypsin inhibitor) shows Van der Waals interactions with the serine residue of the enzyme catalytic site and the residues from P4 to P4' form an antiparrallel β-pleated sheet structure by combination with amino acids of the enzyme.

The Kunitz-type of inhibitors, such as e.g. Potato Serine Protease Inhibitor and Potato Cysteine Protease Inhibitor, are typical examples of this group.

The second group consists mainly of PIs belonging to the cysteine protease inhibitors (except the ones belonging to the Kunitz-type) and the hirudin families. Hirudin is an extremely tight binding and selective inhibitor of thrombin. Its amino acid segment 1HI-3HI forms a parallel β-pleated sheet structure with thrombin segment Ser214-Gly219. The catalytic residue Ser195 of thrombin is not blocked, nor its specificity pocket. The extended C-terminal tail of hirudin also forms several hydrophobic contacts and salt bridges with the "fibrinogen secondary binding site" of thrombin.

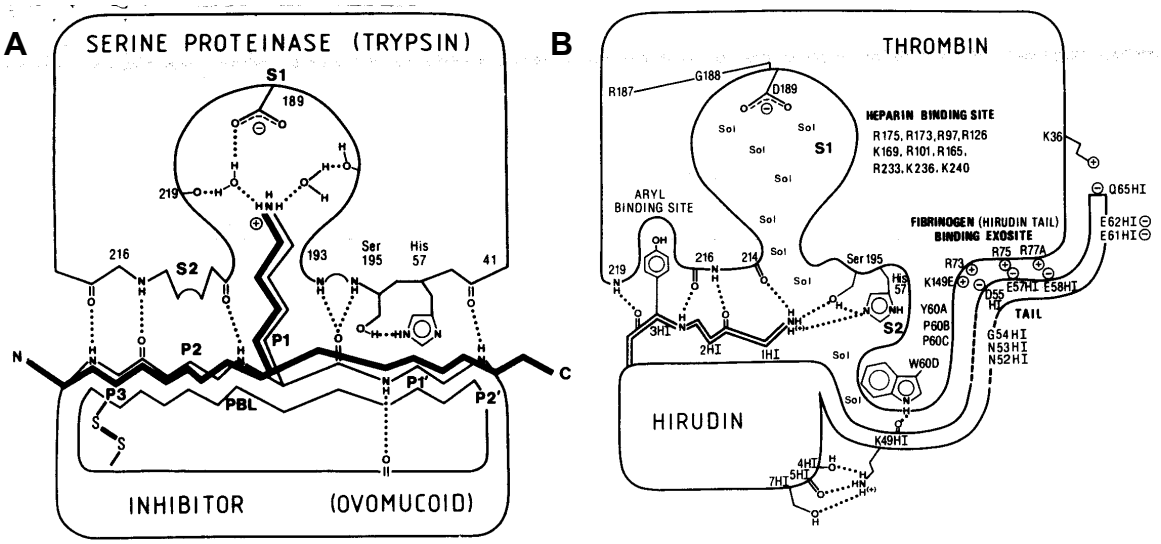

Figure 1: Comparison of the 'substrate-like' binding inhibition mode (**A**) with the 'non substrate-like binding' (**B**), exemplified by ovomucoid and hirudn, respectively.

The mechanism of binding is, therefore, very specific and involves several interactions between the extended binding loop and the enzyme. A specific rigid conformation is required to facilitate such specific binding.

4 MANY PROTEASE INHIBITORS ARE *ALL β-PROTEINS*

The X-ray structures of several proteinaceous protease inhibitors have been solved (Bode and Huber, 1992). Most of them are serine protease inhibitors, but also X-ray structures of cysteine protease and metallo-protease inhibitors have been solved (Clore et al., 1987; Stubbs et al., 1990). Knowledge on the structure of aspartate protease inhibitors is, however, still lacking. The 3D structures of various PIs show that many PIs share a common large binding loop (Jackson and Russell, 2001). The residual structure outside the binding loop may possess quite different motifs (Bode and Huber, 1992). In all cases, however, the inhibitors have a compact structure and, in some cases, contain intramolecular disulfide bridges. Protease inhibitors have representatives in the all-α, all-β and α+ β classes, but it appears that many of them are members of the all-β protein class, indicating that most of their amino acid residues are involved in β-sheets and β-turns. Protease inhibitors, which do not show any homology in sequence, mostly have a secondary structure containing mainly β-sheets, such as e.g. the Bowman-Birk inhibitor (BBI), Bovine Pancreatic Trypsin Inhibitor (BPTI), Thaumatin and soybean trypsin inhibitor (STI) (a Kunitz-type of inhibitor) (Sweet et al., 1974; Li de la Sierra et al., 1999; Charron et al., 2004). **Figure 2** shows the structure of typical examples of PIs, which are members of all-β protein class.

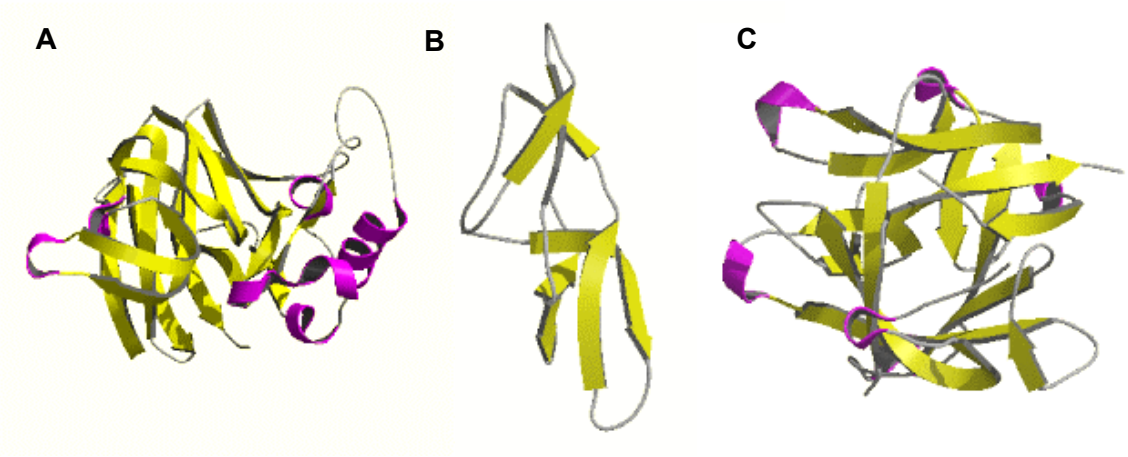

Figure 2: 3D structures of PIs from various families: (A) Thaumatin, (B) Bowman-Birk Inhibitor, and (C) Soybean trypsin inhibitor (STI).

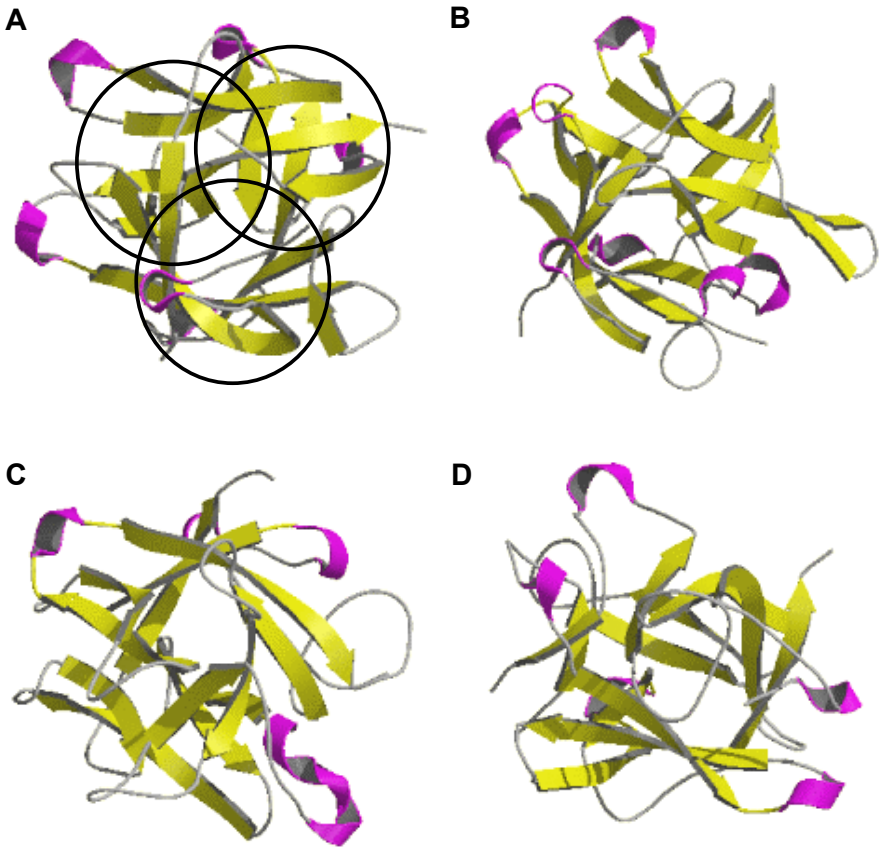

Figure 3: 3D structures of four Kunitz-type inhibitors, showing the β-trefoil structure. (A) STI (with the β-trefoil shape indicated), (B) Winged bean inhibitor (WBI), (C) α-amyalase/subtilisin inhibitor and (D) Erythrina trypsin inhibitor (ETI).

Therefore, it seems that the presence of β-sheet (parallel of antiparallel) is of importance for the inhibitory activity of PIs. PIs have the particularity to be susceptible for cleavage only at the scissile bond, whereas most of the "substrate" proteins have more splitting sites. Amino acids in β-sheets are less accessible for hydrolysis compared to those involved in e. g. α-helices in which the side chains are at the outside of the helix and, therefore, more easily accessible for the enzyme.

5 MANY KUNITZ-TYPE INHIBITORS ARE *β-II PROTEINS*

As shown in chapters 2 and 3, the most abundant PIs in potato (PSPI, PCPI and most of the Potato Aspartate Protease Inhibitors (PAPI)), are Kunitz-type inhibitors. As explained in chapter 1, being a Kunitz-type inhibitor means that the proteins show a high degree of sequence homology (at least 20%) with the soybean trypsin inhibitor (STI). All members also possess in their primary structure several specific conserved amino acids.

The SCOP (Structural classification of Proteins) database provides a detailed and comprehensive description of the structural and evolutionary relationships of the proteins of known structure (Murzin et al., 1995). This classification does not follow the same principle as the classification described in chapter 1 for protease inhibitors. It is based on the presence of common folds in protein structures. Proteins in this database are classified in classes, superfamilies and families. As explained in chapter 1, proteins can be divided in four different *classes* according to the proportion and alignment of α-helix and β-sheet. The four classes are: all-α, all-β, α+β and α/β classes. *Superfamilies* consist of proteins which may have a low sequence homology but whose structures and, in many cases, biological activities (e.g. specific binding affinity) suggest that a common evolutionary origin is probable. Proteins have a common fold if they have some major elements of secondary structure in the same arrangement with the same topological connections. Proteins within the superfamilies are subsequently clustered in *families* according to their sequence homology, further implying their common evolutionary origin, such as e.g. the Kunitz type inhibitors.

Using far and near UV CD, fluorescence and FTIR spectroscopy, it has been shown that PSPI and PCPI possess highly similar characteristics on a secondary and tertiary level as STI, a well-known β-II protein (Wu et al., 1992). Therefore, PSPI and PCPI have been classified as β-II proteins. The β-II proteins are a subclass of all-β proteins, in which most of the amino acid residues are involved in relatively short irregular β-sheets. β-Sheets are considered irregular when the extended plane structure is absent and the β-sheets show several bends.

It is interesting to notice that all the Kunitz-type inhibitors of which the 3D structure is known present a common structure (fold): ***a β-trefoil fold***. For example, STI, Erythrina trypsin inhibitor (ETI), the α-amylase/subtilisin inhibitor and winged bean inhibitor (WBI) (Sweet et al., 1974; Onesti et al., 1991; Vallée et al., 1998; Dattagupta et al., 1999) are all Kunitz-type inhibitors that share highly similar structural characteristics at the secondary and tertiary level among them, and with PSPI and PCPI (**Figure 3**). The structural architecture of these proteins is characterised by the presence of three repeating units, in which each unit is

made up of four β-strands with an intervening loop, which forms a characteristic ββLβ (L stands for loop) (Heidary and Jennings, 2002). Only the small helical part differs in these proteins.

Based on the SCOP classification, all Kunitz-type inhibitor belong to the β-trefoil (lectin) superfamily. It has to be noticed that proteins such as acidic fibroblast growth factor, interleukin 1 β, hisactophilin (actin binding protein) and agglutinin (a lectin), belong to the same superfamily as the Kunitz-type inhibitors (McCoy and Kortt, 1997; Transue et al., 1997; East and Isacke, 2002; Heidary and Jennings, 2002; Kim et al., 2002; Dunn et al., 2003). Although, these proteins are involved completely in different processes, they are all proteins which should have a high binding affinity for specific other proteins or carbohydrates. These proteins bind their target using a substrate-like binding mechanism, similar to that described before. These results indicate that the β-trefoil fold is common for proteins with a biological activity that involves thight binding to other molecules. The β-trefoil fold probably forms a kind of skeleton for the binding loops thereby fixing their conformation.

From the discussion above, it can be concluded that classification based on sequence homology alone is not sufficient, as proteins with low sequence homology may share a highly similar structure. A good example of this is Potato Inhibitor I (PI-1). PI-1 appears as a hexameric protein, which upon heating unfolds having a dimer as its cooperative unit (chapter 7). PI-1 does not show a high degree of sequence homology to Kunitz-type inhibitors, such as STI, PCPI and PSPI, but it does show a far UV CD spectrum similar to those of STI, PSPI and PCPI. As a consequence, despite its low sequence homology to Kunitz-type inhibitors, PI-1 can be classified as a β-II protein. Therefore, the primary step for classifying PIs should not be based on their sequence homology but e.g. on their target proteases. Also, from an application point of view, it is of primary interest to know which classes of protease will be inhibited by a certain inhibitor family.

6 CLASSIFICATION OF PROTEASE INHIBITORS

6.1 Scheme of classification

Figure 4 represents a simple procedure, which may be of help to classify protease inhibitors into families. The scheme was made based on the known classes and families of PIs and their characteristics.

The first criterion is, of course, the type of enzyme the protein inhibits. There are 4 different classes: the serine, the cysteine, the aspartate and the metallo- proteases. In some cases, the protein will inhibit two classes of proteolytic enzymes such as e.g. trypsin and papain. In this case, the most distinctive inhibitory activity is used for classification. Some other families of inhibitors will inhibit, besides a proteolytic enzyme, also a non-proteolytic enzyme such as e.g. α-amylase.

The second criterion is the molecular mass of the protein. This characteristic can be easily determined using SDS-PAGE. This molecular mass is, however, only an estimation since migration of a protein can be influenced by parameters, such as the presence of disulfide

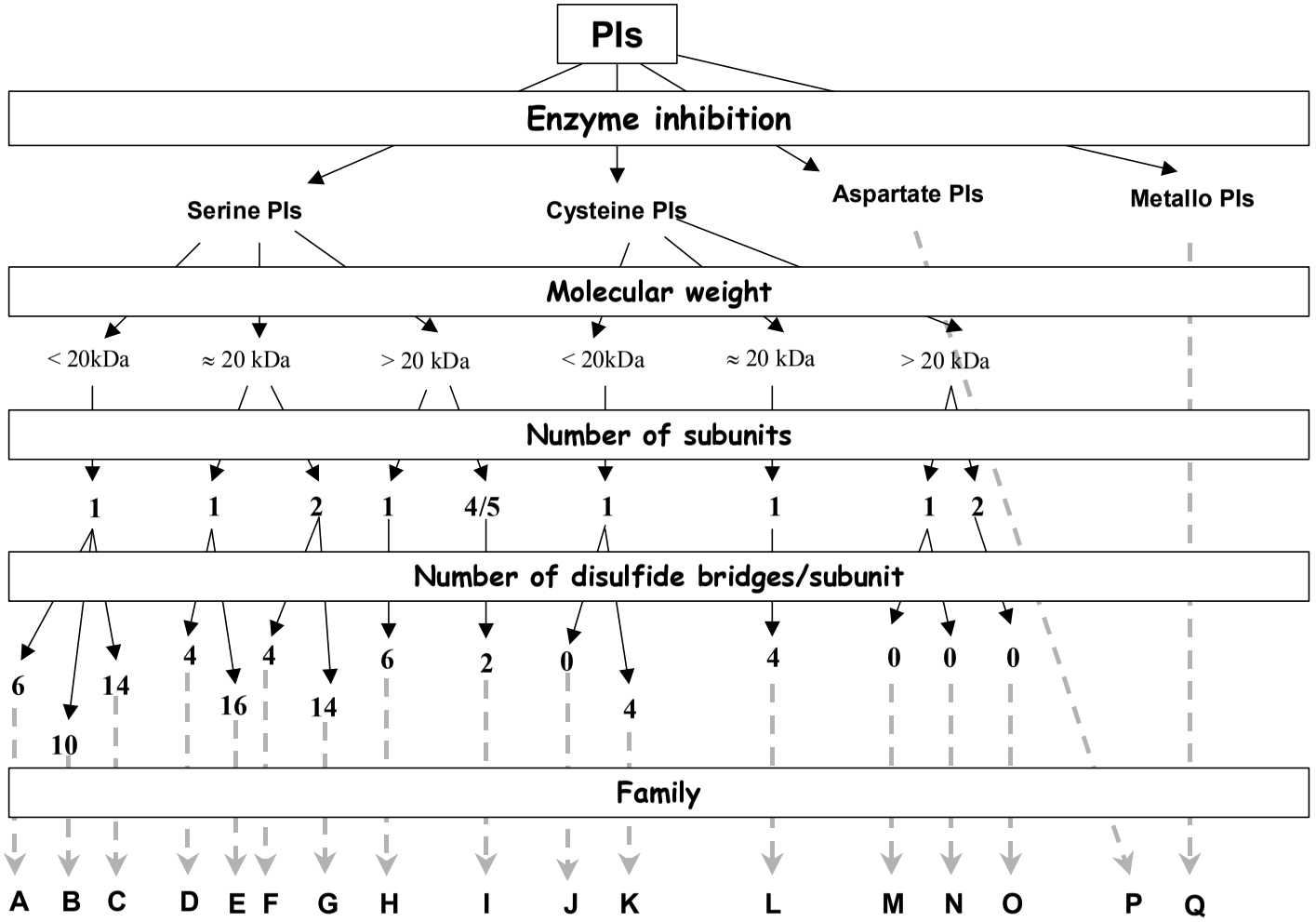

Figure 4: Schematic procedure to classify a protease inhibitor.

A: Hirudin, BPTI, Kazal Cucurbit; **B**: Chelonianin, Cereal superfamily; **C**: Chelonianin, BBI; **D**: Kunitz-type serine protease inhibitor (e.g. PSPI group, PKPI group, and OSPI group); **E**: Thaumatin; **F**: SSI; **G**:PI-2; Serpins; **I**:PI-1; **J**: Stefin; **K**: Cystatin; **L**: Kunitz-type cysteine protease inhibitor (e.g. PCPI group); **M**: Kininogen; **N**: Multicystatin; **O**: Clytocypin; **P**: Kunitz-type aspartate protease inhibitor (e.g. PAPI); **Q**: PCI.

bridges and glycosylation (Lane, 1978). MALDI-TOF MS is an alternative method and has the advantage that it is much more precise.

The third criterion is the number of subunits. This criterion can be easily determined using SDS-PAGE and/or MALDI-TOF MS. Three cases are possible:

- Monomeric protein: the protein will show the same electrophoretic or mass pattern under reducing as under non-reducing conditions. Example: PCPI.
- Multimeric protein, in which the subunits are not disulfide linked: the protein will show the same electrophoretic or mass pattern under reducing as under non-reducing conditions. Example: Potato Inhibitor I.
- Multimeric protein, in which the subunits are disulfide linked: The protein will show a single band under non-reducing conditions, whereas under reducing conditions several bands of lower molecular masses will appear. Example: PSPI.

The fourth criterion is the number of cysteine residues and the number of disulfide bridges present per subunit. The number of cysteine residues can be easily determined using dithiothreitol (DTT) in combination wit an alkylating agent. In the presence of SDS and DTT, the protein will unfold and the disulfide bridges will be broken. In order to prevent the reformation of the disulfidebridges, an alkylating agent (e.g. iodoacetamide) is added to block the free cysteine residues. Using MALDI-TOF MS, the increase in molecular mass after treatment of the protein is proportional to the number of cysteine residues.

The fifth criterion is the origin. In **figure 4**, the different families of PIs are denoted with a letter. However, within one family, PIs can be further subdivided in groups according to their origin. As an example, PSPI is a group of PIs belonging to the Kunitz-type serine protease inhibitor family isolated only within from potato tuber. PSPI group members share common characteristics such as e.g. forming dimers.

As an example, we can take a protein that inhibits trypsin, and which consists of one polypeptide chain of approximately 20 kDa containing 2 S-S bridges. This protease inhibitor will probably be a Kunitz-type inhibitor. If the protein would have the same characteristics, except that it contained 16 cysteine residues, the protein would presumably be a member of the Thaumatin family.

These criteria may be of help to classify PIs. However, in order to confirm the classification the N-terminal sequence (approximately 20 amino acids) needs to be known to make a search for sequence homology possible.

6.2 Use of antibodies

An alternative for N-terminal sequence determination may be the use of antibodies against a specific PI family.

In this study, as an example, polyclonal antibodies have been raised against PSPI 6.1 and 6.5, and against PCPI 8.6 and 8.3. The antibodies against PSPI showed the same affinity for both isoforms of PSPI (**Figure 5**). In order to study the structure homology between the different families of protease inhibitors, the cross-reactivity of these antibodies was tested for various purified representatives of the different families in potato cv. *Elkana* (**figure 5**). PSPI antibodies showed medium affinity for members of the PAPI family (NID and PDI), whereas

they showed only a very low affinity for the three main isoforms of PCPI and for PCI and PI-1. PCPI 8.6 antibodies showed a high affinity for PCPI isoforms, but only a low affinity for PSPI isoforms and, similar to PSPI antibodies, medium affinity for PAPI members.

Therefore, it can be concluded that, although PSPI and PCPI seem to have a highly similar structure, antibodies against PSPI and PCPI do not bind to the same epitope. Therefore, polyclonal antibodies may be a tool to help classifying PIs.In this perspective antibodies against representatives of all different PI families have to be raised. Elaborating on this approach could be the use of monoclonal antibodies which have a higher specificity and, therefore, may give more precise results.

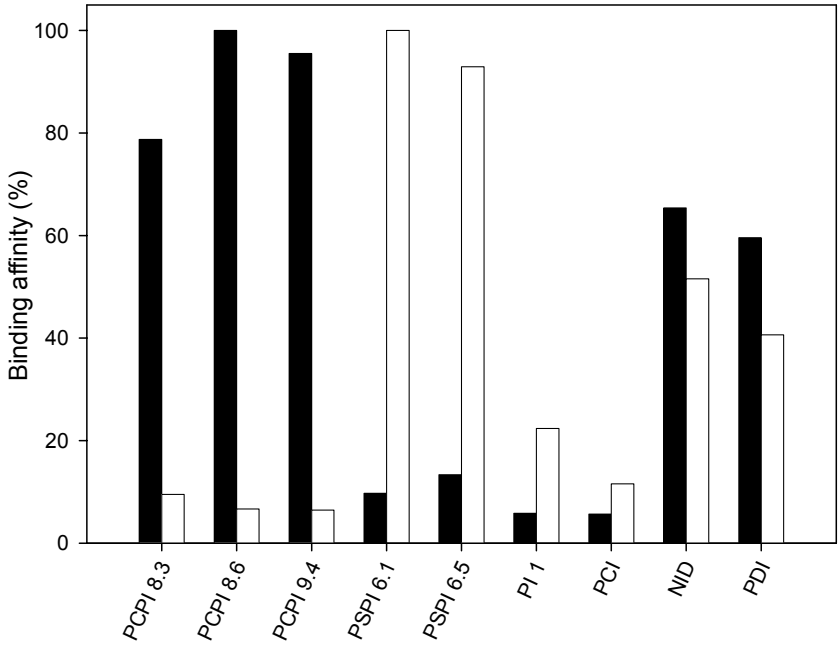

Figure 5: Binding of PSPI 6.1 (□), PCPI 8.3 (■) antibodies against PSPI and PCPI isoforms and potato protease inhibitors from different families. NID (Novel Inhibitor of cathepsin D) and PDI (Potato cathepsin D inhibitor) are members of the Potato Aspartate Protease Inhibitor (PAPI) family.

7 THERMAL AND PH STABILITY

7.1 pH Stability

Melville and Ryan (Melville and Ryan, 1972) have shown that potato protease inhibitors can be easily isolated from potato juice just by lowering the pH to 3.0. At this pH value, almost all patatin was precipitated and a mixture of active PIs was obtained. These results indicate that potato PIs are stable at low pH. Our experiments show (data not shown) that PIs in potato juice showed similar inhibition constants after low pH treatment. In chapter 4, it was shown that PSPI, the most abundant PI in potato juice, showed no changes in secondary and tertiary structure in the pH range 3 to 7.5. Also, PCPI, the second most abundant family of PIs in potato, showed not to be affected by pH. It was also shown that

exposure to low pH does not change the inhibitory activity of these proteins, confirming that no significant changes had occurred in their structure.

This high pH stability is of interest for biofunctional applications in which active PIs are needed. In chapter 1, it was described that the interest in PIs is renewed due to their anticarcinogenic potency and their possible use in skin disease. In the case of skin disease, pools of PIs showing a broad range of inhibiting activity are needed. As shown in chapter 2, PIs from potato are a good candidate for such applications since they show inhibition against serine, cysteine, aspartate and metallo-proteases. Lowering the pH would be in this case the best method to obtain PIs in large amounts and devoid of other proteins from potato juice. Subsequently, using techniques such as ultrafiltration, the PIs present in the acid treated potato juice may be concentrated and purified from low molecular weight impurities.

7.2 Thermal stability

Irreversible precipitation is a recurrent problem in starch manufacture for the recovery of potato proteins. Although, PIs are known to be stable in a broad pH range, precipitation in industry is generally done by a combinationof acidic pH (3.5-5.2) and high temperatures (80 to 120°C) (Knorr et al., 1977). In order to be able to relate the precipitation of proteins in potato juice to their heat-induced structural changes, the thermal (structural) stability of purified PIs was studied.

As already mentioned, most potato PIs are not susceptible to conformational changes at low pH at ambient temperature, whereas patatin, the main protein in potato, undergoes significant structural changes at low pH (Pots et al., 1998b) resulting in irreversible aggregation and precipitation in the presence of salts (Melville and Ryan, 1972). Patatin was also shown to form soluble and insoluble aggregates upon heating, formed by non-covalent interactions (Pots et al., 1998a).

Thermal unfolding of PSPI and PCPI, which together represent 40% of the total protein content in potato juice, is almost completely irreversible and leads to the formation of small soluble aggregates. The aggregation mechanism of PSPI seems to be specific and leads to the formation of a tetrameric form of PSPI, as shown by gel filtration (chapter 5), probably via disulfide bridge interchanges. Even though, PSPI and PCPI have similar structure and a similar molecular weight, the aggregates formed upon heating are of different sizes. Soluble PCPI aggregates have a molecular weight higher than 100 kDa, as shown by gel filtration. Precipitation occurs only around the isoelectric point of these proteins (pH 5.0-6.0). In potato juice, precipitation of PIs also occurs at lower pH. Therefore, it seemed that, in potato juice, precipitation of PIs is influenced by the aggregation/precipitation of patatin, and also by the presence of other compounds, for example phenolic compounds, which may form covalent and non covalent interactions with proteins.

Aggregation of PIs in potato juice maybe limited using specific conditions such as a low pH, low ionic strength, fast heating as well as a low protein concentration. Low pH and fast heating are conditions, which are already used in industry. Low protein concentrations and low ionic strengths seem, however, difficult to apply on a large scale as they imply dilution of the potato juice.

7.3 Thermal treatment and inhibitory activity

As explained in chapter 1, PIs may be used as a biofunctional ingredient due to their indirect effect on the level of CCK. In most food applications the inhibitory effect of PIs, which may lead to a low protein digestibility in the diet and loss of endogenous proteins, is not desired. It is, therefore, of interest to know the range of conditions at which PIs remain active, or at which conditions their inhibitory activities disappear.

Table 1: Inhibition constant of PSPI 6.1 after pre-incubation (10 min) at various temperatures at pH 7.0.

	Inhibition constant (x 10^{-8} M)
Preincubation temperature (°C)	**PSPI 6.1**
30	4.7±0.2
40	7.6±0.1
50	32.3±0.4
60	47.2±0.1
70	nd

nd: not detectable.

The inhibition constant of PSPI at pH 7.0, after pre-incubation at various temperatures, was determined (table 1) using a Lineweaver-Burk representation. In chapter 4, the transition temperature of PSPI 6.1 at pH 7.0 was observed to be approximately 64°C. From Table 1, it can be clearly seen that the inhibition constant increases with increasing preincubation temperature. After preincubation at 60°C, the inhibition constant is already 10-fold higher than that at 30°C, indicating that unfolding has already taken place. After incubation at 70°C, no inhibition could even be measured anymore.

Similar experiments have been done for potato carboxypeptidase inhibitor (PCI), but the inhibition constant could not be determined since no enzymatic assay for carboxypetidase A is available that allows monitoring hydrolysis in time. Therefore, the inhibition, after preincubation (10 min) at various temperatures (25 to 150°C), is expressed as proportion of the initial inhibiting activity. PCI is the smallest protease inhibitor present in potato, with a molecular weight of 4.3 kDa. PCI is known to be extremely stable upon heating (Huang et al., 1981). Inhibition experiments have been performed at two different pHs: one close to its pI, pH 7.0, and one far away from it, at pH 3.0. The results (**figure 6**) clearly show that PCI is more stable upon heating at pH 7.0 than at pH 3.0, or that the refolding of the protein upon cooling is more effective at pH 7.0. PCI at pH 3.0 shows almost 100% of its initial inhibiting activity after heat treatment up to 110°C. With heat treatment above 110°C, PCI shows a decrease in inhibiting activity with increasing preincubation temperature, whereas at pH 7.0 a clear decrease in inhibition occurs only after treatment at 150°C.

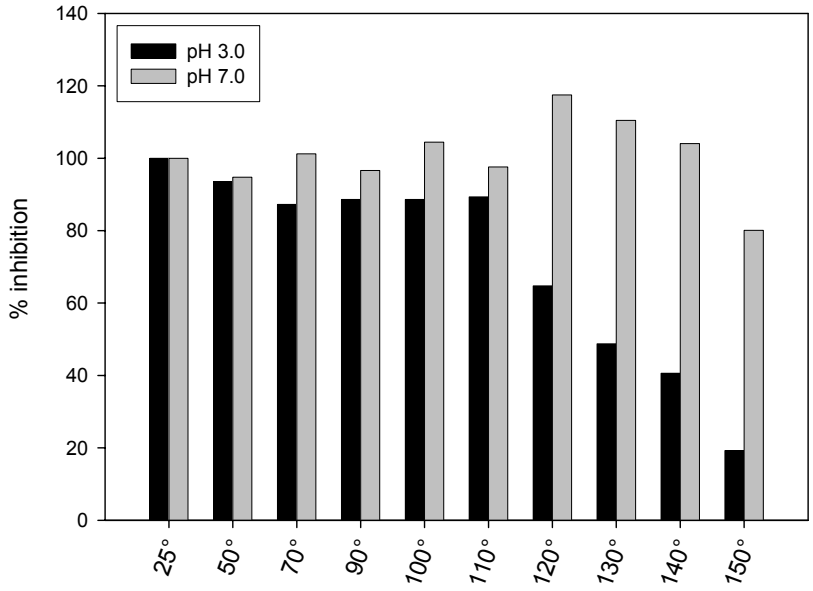

Figure 6: Percentage of PCI inhibition towards carboxypeptidase A after heating at various temperatures, at pH 3.0 and 7.0.

From these results, and from the knowledge acquired during this study, it follows that inactivation of PIs can be easily performed by heating the PIs at temperatures slightly higher than their transition temperatures.

These results are in accordance with those of Van Koningsveld and coworkers (van Koningsveld et al., 2002) with pools of PIs. A 50% decrease in trypsin and papain inhibiting activity was observed after heating at 60°C, whereas a similar decrease was only observed after treatment at temperatures above 70°C for cathepsin D. A more surprising result was the decrease of almost 40% in inhibition of carboxypeptidase after heating at 70°C. This may be explained by a co-aggregation and co-precipitation of active PCI with other proteins present. These results, in combination with our results, seem to indicate that the unfolding of one group of proteins may influence the aggregation and the inhibitory activity of other groups of proteins. This problem of co-aggregation/precipitation may be limited by the use longer heating at low temperature or shorter heating at a high temperature.

LITERATURE CITED

Bode, W.; Huber, R. Natural protein proteinase inhibitors and their interaction with proteinases. *Eur. J. Biochem.* **1992**, 204, 433-451.

Charron, C.; Giegé, R.; Lorber, B. Structure of thaumatin in a hexagonal space group: comparison of packing contacts in four crystal lattices. *Acta Crystallogr. D. Biol. Crystallogr.* **2004**, 60, 83-89.

Clore, G. M.; Gronenborn, A. M.; Nilges, M.; Ryan, C. A. Three-dimensional structure of potato carboxypeptidase inhibitor in solution. A study using nuclear magnetic resonance, distance geometry, and restrained molecular dynamics. *Biochemistry* **1987**, 26, 8012-8023.

Dattagupta, J. K.; Podder, A.; Chakrabarti, C.; Sen, U.; Mukhopadhyay, D.; Dutta, S. K.; Singh, M. Refined crystal structure (2.3 A) of a double-headed winged bean alpha-chymotrypsin inhibitor and location of its second reactive site. *Proteins* **1999**, 35, 321-331.

Dunn, E. F.; Gay, N. J.; Bristow, A. F.; Gearing, D. P.; O'Neill, L. A.; Pei, X. Y. High-resolution structure of murine interleukin 1 homologue IL-1F5 reveals unique loop conformations for receptor binding specificity. *Biochemistry* **2003**, 42, 10938-10944.

East, L.; Isacke, C. M. The mannose receptor family. *Biochim. Biophys. Acta* **2002**, 1572, 364-386.

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.

Gonzalez, J. M.; Lindamood, J. B.; Desai, N. Recovery of protein from potato plant waste effluents by complexation with carboxymethylcellulose. *Food Hydrocolloids* **1991**, 4, 355-363.

Heidary, D. K.; Jennings, P. A. Three topologically equivalent core residues affect the transition state ensemble in a protein folding reaction. *J. Mol. Biol.* **2002**, 316, 789-798.

Hou, W.-C.; Chen, Y.-C.; Chen, H.-J.; Lin, Y.-H.; Yang, L.-L.; Lee, M.-H. Antioxydant activities of trypsin inhibitor, a 33 KDa root storage protein of sweet potato (*Ipomoea batatas* (L.) Lam cv. Tainong 57). **2001**,

Huang, D. Y.; Swanson, B. G.; Ryan, C. A. Stability of proteinase inhibitors in potato tubers during cooking. *J. Food Sci.* **1981**, 46, 287-290.

Jackson, R. M.; Russell, R. B. Predicting function from structure: examples of the serine protease inhbitor canonical loop conformation found in extracellular proteins. *Comput. Chem.* **2001**, 26, 31-39.

Kim, J.; Blaber, S. I.; Blaber, M. Alternative type I and I' turn conformations in the beta8/beta9 beta-hairpin of human acidic fibroblast growth factor. *Protein Sci.* **2002**, 11, 459-466.

Knorr, D.; Kohler, G. O.; Betschart, A. A. Potato protein concentrates: the influence of various methods of recovery upon yield, compositional and functional characteristics. *J. Food Process. Preserv.* **1977**, 1, 235-247.

Lane, L. C. A simple method for stabilizing protein-sulfhydryl groups during SDS-gel electrophoresis. *Anal. Biochem.* **1978**, 86, 655-664.

Li de la Sierra, L.; Quillien, L.; Flecker, P.; Gueguen, J.; Brunie, S. Dimeric crystal structure of a bowman-birk protease inhibitor from pea seeds. *J. Mol. Biol.* **1999**, 285, 1195-1207.

Lin, Y. H.; Chen, H. L. Level and heat stability of trypsin inhibitor activity among sweet potato (Ipomoea batatas LAm.) varieties. *Bot. Bull. Acad. Sin.* **1980**, 21, 1-13.

Lindner, P.; Kaplan, B.; Weiler, E.; Ben-Gara, I. Fractionation of potato juice proteins into acid-soluble and acid-coagulable fractions. *Food Chem.* **1980**, 6, 323-335.

Lisinska, G.; Leszczynski, W. *Potato science and technology*, Elsevier Applied Science, London, **1989**.

Liu, K. Expanding soybean food utilisation. *Food technology* **2000**, 54, 46-48.

McCoy, A. J.; Kortt, A. A. The 1.8 A crystal structure of winged bean albumin 1, the major albumin from *Psophocarpus tetragonolobus* (L.) DC. *J Mol Biol* **1997**, 269, 881-891.

Melville, J. C.; Ryan, C. A. Chymotrypsin inhibitor I from potatoes: Large scale preparation and characterization of its subunit components. *J. Biol. Chem.* **1972**, 247, 3443-3453.

Murzin, A. G.; Brenner, S. E.; Hubbard, T.; Chothia, C. SCOP: A Structural Classification of Proteins Database for the investigation of sequences and structure. *J. Mol. Biol.* **1995**, 247, 536-540.

Onesti, S.; Brick, P.; Blow, D. M. Crystal structure of a Kunitz-type trypsin inhibitor from Erythrina caffra seeds. *J. Mol. Biol.* **1991**, 217, 153-176.

Pots, A. M.; de Jongh, H. H. J.; Gruppen, H.; Hamer, R. J.; Voragen, A. G. J. Heat-induced conformational changes of patatin, the major potato tuber protein. *Eur. J. Biochem.* **1998a**, 252, 66-72.

Pots, A. M.; de Jongh, H. H. J.; Gruppen, H.; Hessing, M.; Voragen, A. G. J. The pH dependence of the structural stability of patatin. *J. Agric. Food Chem.* **1998b**, 46, 2546-2553.

Pots, A. M.; ten Grotenhuis, E.; Gruppen, H.; Voragen, A. G. J.; de Kruif, K. G. Thermal aggregation of patatin *in situ*. *J. Agric. Food Chem.* **1999**, 47, 4600-4605.

Roozen, R. J.; de Groot, J. Analysis of residual trypsin inhibitor activity in feed flour. In *Recent advances of research in antinutritional factors in legume seeds.*; Pudoc - The Netherlands: Wageningen, **1989**; 114-117.

Ryan, C. A.; Hass, G. M.; Kuhn, R. W. Purification and properties of a carboxypeptidase inhibitor from potatoes. *J. Biol. Chem.* **1974**, 249, 5495-5499.

Schechter, I.; Berger, A. On the size of the active site in proteases. I papain. *Biochem. Biophys. Res. Commun.* **1967**, 27, 157-162.

Stubbs, M. T.; Laber, B.; Bode, W.; Huber, R.; Jerala, R.; Lenarcic, B.; Turk, V. The refined 2.4 A X-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: a novel type of proteinase inhibitor interaction. *EMBO J.* **1990**, 9, 1939-1947.

Sweet, R. M.; Wright, H. T.; Janin, J.; Chothia, C. H.; Blow, D. M. Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6-A resolution. *Biochemistry* **1974**, 13, 4212-4228.

Transue, T. R.; Smith, A. K.; Mo, H.; Goldstein, I. J.; Saper, M. A. Structure of benzyl T-antigen disaccharide bound to Amaranthus caudatus agglutinin. *Nat. Struct. Biol.* **1997**, 4, 779-783.

Vallée, F.; Kadziola, A.; Bourne, Y.; Juy, M.; Rodenburg, K. W.; Svensson, B.; Haser, R. Barley alpha-amylase bound to its endogenous protein inhibitor BASI: crystal structure of the complex at 1.9 A resolution. *Structure* **1998**, 6, 649-659.

van Koningsveld, G. A.; Gruppen, H.; de Jongh, H. H.; Wijngaards, G.; van Boekel, M. A.; Walstra, P.; Voragen, A. G. J. Effects of ethanol on structure and solubility of potato proteins and the effects of its presence during the preparation of a protein isolate. *J. Agric. Food Chem.* **2002**, 50, 2947-2956.

Werner, M. H.; Wemmer, D. E. ^{1}H assignments and secondary structure determination of the soybean trypsin/chymotrypsin Bowman-Birk inhibitor. *Biochemistry* **1991**, 30, 3356-3364.

Wu, J.; Yang, J. T.; Wu, C.-S. C. b-II conformation of all-b proteins can be distinguished from unordered form by circular dichroism. *Anal. Biochem.* **1992**, 200, 359-364.

SUMMARY

Potato proteins are present in a by-product of the potato starch industry, the so-called potato juice. They are recovered by an acidic heat-treatment of the potato juice. This results in a completely irreversible precipitation of the proteins, with a complete loss of functionality for food applications. This explains that so far potato proteins are only used in low-value applications such as feed. Potato proteins have a relatively high nutritional value and therefore have a high potential for application in food.

The aim of this research was to investigate and understand the thermal unfolding behaviour of potato protease inhibitors. The first step was to make an inventory of the relative abundance and inhibitory activity of the protease inhibitors present in potato tuber. The second step was to investigate the stability of the most abundant and, therefore, representative protease inhibitors in potato juice, as a function of temperature and pH. This information should be of help to understand the mechanism of the irreversible precipitation occurring in industrial processes, thereby creating possibilities to obtain soluble and/or biologically active potato proteins that can be used in food and pharmaceutical applications.

In contrast to patatin, the most abundant protein in potato juice, the protease inhibitors are a more heterogeneous group of proteins. In chapter 2, protease inhibitors from potato juice (cv. *Elkana)* were purified and quantified. The protease inhibitors represent approximately 50 % of the total soluble proteins in potato juice. They were classified in seven different groups: Potato Inhibitor I (PI-1), Potato Serine Protease Inhibitor (PSPI formerly called 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' (OSPI). The most abundant groups were the PSPI and PCPI, representing 22 and 12 % of the total protein in potato juice, respectively. In chapter 3, the gene of the most abundant protease inhibitor in potato (cv. *Elkana*) was isolated and sequenced. The amino acid sequence deduced from this gene showed 98 % identity with Potato Serine Protease Inhibitor (PSPI), a member of the Kunitz-type inhibitor, and not, as was assumed in literature, with PI-2. It can be concluded that, in cv. *Elkana*, not PI-2 but PSPI is the most abundant group of proteases inhibitors. Potato protease inhibitors inhibit an extraordinary broad spectrum of enzymes. All the groups (except PCI) inhibited also trypsin and/or chymotrypsin. PSPI isoforms exhibit 82 and 50 % of the total trypsin and chymotrypsin inhibiting activity, respectively. A strong variation within the activities was observed within one group as well as between the protease inhibitor groups. 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 showed that presumably approximately 70% of the protease inhibitors present in potato juice belongs to the Kunitz-type inhibitor.

In chapter 4, PSPI isoforms were shown to have a highly similar structure at both the secondary and tertiary level. From the results described, PSPI is classified as a β-II protein based on: (1) the presence of sharp peaks in the near UV spectra, indicating a rigid and compact protein, (2) the sharp transition from the native to the unfolded state upon heating (only 6°C) and (3) the similarity in secondary structure to soybean trypsin inhibitor, a known β-II protein, as indicated by a similar far UV CD spectrum and a similar amide I band in the IR spectrum. The conformation of PSPI was shown also to be stable at ambient temperature in the pH range 4 to 7.5. Upon lowering the pH to 3.0, only minor changes in the protein core occur, as observed from the increase of the intensity of the phenylalanine peak in the near UV CD spectrum.

In chapter 5, the unfolding behaviour of PSPI was studied in detail using far UV CD spectroscopy, fluorescence spectroscopy and DSC. The results indicate that the thermal as well as the guanidinium-induced unfolding of PSPI occurs via a non-two state mechanism in which at least two parts of the protein unfold more or less independently. Additionally, the occurrence of aggregation, especially at low scan rates, increases the apparent cooperativity of the unfolding and makes the system kinetically rather than thermodynamically controlled. Aggregate formation seems to occur via a specific mechanism of which PSPI in a tetrameric form is the end product, and which may involve disulfide interchanges.

In chapter 6, the conformational stability of Potato Cysteine Protease Inhibitor (PCPI), the second most abundant protease inhibitor group in potato tuber, was investigated, at ambient temperature and upon heating, using far and near UV CD spectroscopy, fluorescence spectroscopy and DSC. The PCPI isoforms investigated were shown to have a highly similar structure at both the secondary and tertiary level. PCPI isoforms show structural properties similar to those of Potato Serine Protease Inhibitor and the Kunitz-type soybean trypsin inhibitor. Therefore, PCPI isoforms are also classified as members of the β-II protein subclass. Results show that the thermal unfolding of PCPI isoforms also does not follow a two-state mechanism, and that at least one intermediate is present. The occurrence of this intermediate is most apparent in the thermal unfolding of PCPI 8.3, as indicated by the presence of two peaks in the DSC thermogram. Additionally, the formation of large aggregates (>100 kDa), especially at low scan rates, increases the apparent cooperativity of the unfolding and makes the system again kinetically rather than thermodynamically controlled.

In chapter 7, the structural properties of potato protease inhibitor 1 (PI-1) were studied as a function of temperature, in order to elucidate its precipitation mechanism upon heating. A cDNA coding for PI-1 from cv. *Bintje* was cloned and expressed in *Pichia*

pastoris. Using the recombinant PI-1 it was suggested that PI-1 behaves as a hexameric protein rather than as a pentamer, as previously proposed in literature. The recombinant protein seems to have either a predominantly unordered structure or also belongs to the β-II proteins. DSC analysis of PI-1 revealed that its thermal unfolding occurs via one endothermic transition in which the hexameric PI-1 probably unfolds having a dimer instead of a monomer as cooperative unit. The transition temperature for the recombinant PI-1 was 88^{o}C. Similar results were obtained for a partially purified pool of native PI-1 from cv. *Bintje*.

In chapter 8, the common structural characteristics of potato protease inhibitors from different groups are discussed and compared to those of soybean trypsin inhibitor, a Kunitz-type inhibitor. This leads to the conclusion that all these proteins belong to the β-II protein sub-class and have a more or less common β-trefoil fold. A scheme is introduced, defining the main characteristics, which should be of help to classify any unknown protease inhibitor in the correct family. Finally, the pH and the thermal stability of the protease inhibitors are discussed in relation with the aggregation and precipitation processes occurring in industrial potato juice.

SAMENVATTING

Samenstelling en fysisch-chemische eigenschappen van protease remmers uit aardappel (*Solanum tuberosum*)

Aardappeleiwitten zijn aanwezig in een bijproduct van de aardappelzetmeelindustrie, het zogenoemde aardappelsap. Zij worden gewonnen door een zure hittebehandeling van het aardappelsap. Dit resulteert in een complete irreversibele precipitatie van de eiwitten, met een volledig verlies van de functionaliteit voor levensmiddeltoepassingen. Dit verklaart dat ,tot dusver, aardappeleiwitten alleen gebruikt worden in goedkope toepassingen als veevoeder. Aardappeleiwitten hebben een relatief hoge voedingswaarde en hebben daarom een groot potentieel voor levensmiddeltoepassingen.

Het doel van dit onderzoek was om het door hitte geïnduceerde ontvouwinggedrag van aardappelproteaseremmers te onderzoeken en begrijpen. De eerste stap was het inventariseren van de hoeveelheid en de remmende activiteit van de proteaseremmers aanwezig in de aardappelknol. De tweede stap was het onderzoeken van de stabiliteit van de meest voorkomende, en dus meest representatieve, proteaseremmers in aardappelsap, als functie van temperatuur en pH. Deze informatie zou moeten helpen om het mechanisme van de irreversibele precipitatie die optreedt in industriële processen te begrijpen, waardoor de mogelijkheid wordt gecreëerd om oplosbare en/of biologisch actieve aardappeleiwitten in handen te krijgen, die gebruikt kunnen worden in levensmiddelen en in farmaceutische toepassingen.

In tegenstelling tot patatine, dat het meest voorkomende eiwit in aardappelsap is, bestaan de proteaseremmers uit een meer heterogene groep van eiwitten. In hoofdstuk 2 wordt de zuivering en kwantificering van proteaseremmers uit aardappelsap (cv. *Elkana*) beschreven. De proteaseremmers vertegenwoordigen ongeveer 50% van de totaal hoeveelheid op gelost eiwit in aardappelsap. Ze worden in zeven verschillende groepen ingedeeld: Potato Inhibitor I (PI-1), Potato Serine Protease Inhibitor (PSPI, voorheen PI-2 genoemd), Potato Cysteine Protease Inhibitor (PCPI), Potato Aspartate Protease Inhibitor (PAPI), Potato Kunitz-type Protease Inhibitor (PKPI), Potato Carboxypeptidase Inhibitor (PCI) en 'other serine protease inhibitors' (OSPI). De meest voorkomende groepen zijn PSPI en PCPI, die respectievelijk 22 en 12 % van het totale eiwit in uitmaken. In hoofdstuk 3 wordt beschreven hoe het gen van de meest voorkomende protease remmer in aardappel (cv. *Elkana*) werd geïsoleerd en gesequenced. De aminozuurvolgorde verkregen van dit gen vertoonde een homologie van 98 % met Potato Serine Protease Inhibitor (PSPI), een Kunitz-type inhibitor, en niet, zoals in de literatuur gesuggereerd, met PI-2. De conclusie kan worden getrokken dat, in cv. *Elkana*, niet PI-2, maar PSPI de meest voorkomende groep van proteaseremmers is. Aardappel proteaseremmers remmen een ongewoon breed spectrum van enzymen. Alle groepen (m.u.v. PCI) remmen, naast hun eventuele specifieke activiteit,

trypsine en/of chymotrypsine. Alle PSPI isovormen zijn verantwoordelijk voor, respectievelijk, 82 en 50 % van de totale trypsine en chymotrypsine remmende activiteit. Een sterke variatie in de activiteiten werd waargenomen zowel binnen een groep, als tussen de verschillende proteaseremmergroepen. Antilichamen werden gemaakt tegen de twee meest voorkomende isovormen van PSPI. De binding van deze antilichamen aan PSPI isovormen en proteaseremmers uit verschillende groepen toonde aan dat ongeveer 70 % van de proteaseremmers aanwezig in aardappelsap waarschijnlijk tot de Kunitz-type remmers behoren.

In hoofdstuk 4 wordt aangetoond dat PSPI isovormen een grote gelijkenis in structuur vertonen, op zowel secundair als tertiair niveau. Uit de beschreven resultaten kan PSPI als een β-II eiwit worden geclassificeerd op basis van: (1) de aanwezigheid van scherpe pieken in het nabij-UV CD spectrum, wat duidt op een stijf en compact eiwit, (2) de scherpe overgang van de natieve naar de ontvouwen toestand bij verhitting (slechts 6°C) en (3) de overeenkomst in secundaire structuur met de Kunitz trypsineremmer uit sojaboon, een bekend β-II eiwit. Deze overeenkomst blijkt uit een soortgelijk ver-UV CD spectrum en een vergelijkbare amide I band in het IR spectrum. De structuur van PSPI bleek stabiel te zijn bij kamertemperatuur in het pH-gebied van 4 tot 7,5. Wanneer de pH werd verlaagd tot pH 3,0 traden slechts kleine veranderingen in de eiwitkern op, zoals werd waargenomen uit de toename van de intensiteit van de fenylalaninepiek in het nabij-UV UV CD spectrum.

In hoofdstuk 5 werd het ontvouwingsgedrag van PSPI in detail bestudeerd met behulp van ver-UV CD spectroscopie, fluorescentie spectroscopie en DSC. De resultaten tonen aan dat zowel de hitte- als de denaturant geïnduceerde ontvouwing van PSPI plaatsvindt via een niet-twee toestanden mechanisme, waarin tenminste twee delen van het eiwit min of meer onafhankelijk van Elkana ontvouwen. Bovendien, zorgt het plaatsvinden van aggregatie, vooral bij lage scansnelheden, voor een verhoogt de schijnbare coöperativiteit van de ontvouwing, wat het systeem meer kinetisch gedomineerd maakt dan thermodynamisch. Aggregaatvorming lijkt op te treden via een specifiek mechanisme, waarvan PSPI in een tetramere vorm het eindproduct is, waarbij ook het verbreken en her vormen van zwavel brugggen uitwisselingen betrokken kunnen zijn.

In hoofdstuk 6 werd de structuurstabiliteit bij kamertemperatuur en bij verhitting van Potato Cysteine Protease Inhibitor (PCPI), de op een na meest voorkomende groep van proteaseremmers in de aardappelknol, onderzocht met behulp van ver-UV en nabij-UV CD spectroscopie, fluorescentie spectroscopie en DSC. De onderzochte PCPI isovormen blijken een sterk gelijkende structuur op zowel secundair als tertiair niveau te hebben. PCPI isovormen vertonen structuur eigenschappen vergelijkbaan met die van Potato Serine Protease Inhibitor en de Kunitz-type sojaboon trypsineremmer. Daarom worden PCPI isovormen ook geclassificeerd als leden van de β-II eiwit subklasse. Resultaten toonden verder aan dat de hitte geïnduceerde ontvouwing van PCPI isovormen ook geen tweetoestanden mechanisme volgt en dat minstens één intermediair aanwezig is. De aanwezigheid van dit intermediair valt het meest op in de hitte geïnduceerde ontvouwing van PCPI 8.3, zoals wordt aangetoond door de aanwezigheid van twee pieken in het DSC thermogram. Bovendien wordt de schijnbare coöperativiteit van de ontvouwing verhoogd

door de vorming van grote aggregaten (>100 kDa), met name bij lage scansnelheden. Dit maakt het systeem wederom meer kinetisch dan thermodynamisch gedomineerd.

In hoofdstuk 7 werden de structuureigenschappen van potato protease inhibitor (PI-1) bestudeerd als functie van temperatuur, om het precipitatie mechanisme bij verhitting op te helderen. Een cDNA sequentie van PI-1 uit cv. *Bintje* werd gekloond en tot expressie gebracht in *Pichia pastoris*. Met behulp van recombinant PI-1 werd aannemelijk gemaakt dat PI-1 zich als een hexameer eiwit gedraagt in plaats van een pentameer, zoals eerder in de literatuur werd voorgesteld. Het recombinante eiwit lijkt een overwegend ongeordende structuur te hebben of behoort ook tot de β-II eiwitten. DSC analyse van PI-1 toonde aan dat zijn hitte geïnduceerde ontvouwing via één endotherme overgang plaatsvindt, waarin het hexamere PI-1 ontvouwt met waarschijnlijk een dimeer in plaats van een monomeer als coöperatieve eenheid. De overgangstemperatuur voor gerecombineerd PI-1 was 88°C. Soortgelijke resultaten werden gevonden voor een gedeeltelijk gezuiverde fractie van natief PI-1 uit cv. *Bintje*.

In hoofdstuk 8 worden de algemene structuureigenschappen van aardappel proteaseremmers uit verschillende groepen besproken en vergeleken met de eigenschappen van sojaboon trypsineremmer, een Kunitz-type remmer. Hieruit volgt de conclusie dat al deze eiwitten tot de β-II eiwit sub-klasse behoren, en dus een min of meer algemene β-trefoil vouwing hebben. Ook wordt er een schema geïntroduceerd, waarin de belangrijkste eigenschappen worden gedefinieerd en wat kan helpen bij het classificeren van onbekende proteaseremmers in de correcte familie. Tot slot worden de pH- en hittestabiliteit van proteaseremmers bediscussieerd in relatie tot de aggregatie- en precipitatieprocessen die plaatsvinden in industrieel aardappelsap.

Composition et propriétés physico-chimiques des inhibiteurs de protéase de pomme de terre (*Solanum tuberosum*)

Les protéines de pomme de terre sont présentes dans un sous-produit de l'industrie féculière, appelé jus de pomme de terre. Elles sont issues d'un traitement du jus de pomme de terre où l'application d'une température élevée et d'un bas pH est combinée. Le résultat est une précipitation irréversible des protéines, accompagnée d'une perte totale de fonctionnalité. Ceci explique pourquoi les protéines de pomme de terre ont été jusqu'ici seulement utilisées comme complément dans l'alimentation animale. Les protéines de pomme de terre ont une haute valeur nutritionnelle et ont donc un fort potentiel pour une utilisation dans l'alimentation.

Le but de cette recherche était d'analyser et de comprendre le dépliement, en fonction de la température, des inhibiteurs de protéase de pomme de terre. La première étape était de déterminer l'abondance relative et l'activité inhibitrice des inhibiteurs de protéase présents dans le tubercule de pomme de terre. La seconde étape était d'analyser la stabilité des inhibiteurs de protéase les plus abondants, et donc les plus caractéristiques dans le jus de pomme de terre, en fonction de la température et du pH. Ces informations pourraient aider à comprendre le mécanisme de la précipitation irréversible des protéines dans les féculeries, permettant ainsi d'obtenir des protéines de pomme de terre solubles et/ou actives biologiquement afin de pouvoir les utiliser dans l'industrie alimentaire et/ou pharmaceutique.

A la différence de la patatine, la protéine la plus abondante dans le jus de pomme de terre, les inhibiteurs de protéase sont un groupe hétérogène de protéines. Le chapitre 2 décrit la purification et la quantification des inhibiteurs de protéase présents dans le jus de pomme de terre (cv. *Elkana*). Les inhibiteurs de protéase représentent approximativement 50% de la totalité des protéines solubles dans le jus de pomme de terre. Ils ont été classifiés dans sept groupes différents: Inhibiteur de protéase de type I (PI-1), Inhibiteur de protéase à sérine (PSPI) (précédemment appelé Inhibiteur de protéase de type II: PI-2), Inhibiteur de protéase à cystéine (PCPI), Inhibiteur de protéase à acide aspartique (PAPI), Inhibiteur de type Kunitz (PKPI), Inhibiteur de la carboxypeptidase (PCI) et «Autres inhibiteurs de protéase à sérine» (OSPI). Les groupes les plus abondants sont PSPI et PCPI, représentant, respectivement, 22 et 12% de la totalité des protéines présentes dans le jus de pomme de terre. Dans le chapitre 3, le gène de l'inhibiteur de protéase le plus abondant a été isolé et séquencé. La séquence en acides aminés déduite de ce gène est identique à 98% à celle d'un inhibiteur de protéase à sérine (PSPI), un inhibiteur de type Kunitz, et non pas avec PI-2 (décrit comme le plus abondant dans la littérature). La conclusion est que, dans le cultivar *Elkana,* l'inhibiteur de protéase le plus abondant est PSPI et non pas PI-2. Les inhibiteurs de protéase de pomme de terre inhibent un très large spectre d'enzymes. Tous les groupes (excepté PCI) inhibent la trypsine et/ou la chymotrypsine. Les isoformes de PSPI montrent une inhibition le l'activité trypsique et chymotryspique de 82 et 50%, respectivement. Une grande variation des activités inhibitrices était observée aussi bien à l'intérieur d'un même groupe qu'entre les différents

groupes d'inhibiteurs de protéase. Des anticorps dirigés contre les deux plus abondants isoformes de PSPI ont été produits. La liaison de ces anticorps aux isoformes de PSPI et aux inhibiteurs de protéase de groupes différents montrent que (approximativement) 70% des inhibiteurs de protéase de pomme de terre sont probablement des inhibiteurs de type Kunitz.

Dans le chapitre 4, il a été montré que les isoformes de PSPI ont une structure secondaire et tertiaire très similaire. A partir des résultats décrits, PSPI est classé comme une protéine de type β-II basé sur: (1) la présence de pics étroits dans le spectre de proche UV DC (Dichroïsme Circulaire), indiquant une protéine rigide et compacte; (2) la transition « rapide » due à la chaleur, entre l'état natif et l'état déplié (6°C seulement); (3) la similitude avec la structure secondaire de l'inhibiteur trypsique de soja, une protéine de type β-II connue, comme l'indique le spectre de loin UV DC et une bande similaire «amide I» dans le spectre IR. Il a été observé que la conformation de PSPI était stable à température ambiante ainsi qu'entre pH 4.0 et pH 7.5. A pH 3.0, seuls des changements mineurs apparaissent dans le cœur de la protéine, comme l'indique l'augmentation de l'intensité du pic de phenylalanine dans le spectre de proche UV DC.

Dans le chapitre 5, le dépliement de PSPI a été étudié en détail, utilisant la spectroscopie de DC (loin UV), la spectroscopie de fluorescence et le DSC. Les résultats indiquent que le dépliement de PSPI induit par la chaleur et par le chlorure de guanidinium ne se produit pas via un mécanisme a deux états (natif et déplié), et qu'au moins deux parties de la protéine se déplient plus ou moins indépendamment. De plus, la présence d'agrégation, particulièrement à vitesse lente de chauffage, augmente l'apparente coopérativité du dépliement et rend le système contrôlé de manière cinétique plutôt que thermodynamique. La formation d'agrégats se produit via un mécanisme spécifique pouvant impliquer des échanges entre les ponts S-S et formant un produit final qui est un tétramère de PSPI.

Dans le chapitre 6, la stabilité structurale de PCPI, le second group d'inhibiteur de protéase le plus abondant, a été analysée à 20°C et en fonction de la température, utilisant la spectroscopie de DC (proche et loin UV), la spectroscopie de fluorescence et le DSC. Les isoformes de PCPI étudiés ont une structure secondaire et tertiaire très similaire. Les isoformes de PCPI ont des propriétés structurales similaires à celles de PSPI et de l'inhibiteur trypsique de soja de type Kunitz. Donc les isoformes de PCPI ont aussi été classifiés comme des protéines de type β-II. Les résultats montrent que le dépliement dû a la chaleur ne suit pas un mécanisme à deux états, et qu'au moins un intermédiaire est présent. La présence de cet intermédiaire est particulièrement apparente dans le dépliement dû à la chaleur de PCPI 8.3, comme l'indique la présence de deux pics dans le thermogramme (DSC). De plus, la formation de larges agrégats (>100 kDa), particulièrement à vitesse lente de chauffage, augmente l'apparente coopérativité du dépliement et rend le système, de nouveau, contrôlé de manière cinétique plutôt que thermodynamique.

Dans le chapitre 7, les propriétés structurales de PI-1 ont été étudiées en fonction de la température afin d'élucider le mécanisme de précipitation. Un ADNc de PI-1 (cv. *Bintje*) a été cloné et exprimé dans *Pichia pastoris*. En utilisant le PI-1 recombinant, il a été suggéré que PI-1 se comporte comme une protéine hexamérique plutôt que comme une protéine pentamérique jusqu'ici décrite dans la littérature. La protéine recombinante semble avoir une structure peu ordonnée (pas d'éléments de structure secondaire) ou être une protéine de type

β-II. L'analyse des thermogrammes (DSC) de PI-1 révèle que le dépliement dû la chaleur se produit via une transition endothermique dans laquelle PI-1 se déplie probablement en ayant un dimère plutôt qu'un monomère comme unité coopérative. La température de transition du PI-1 recombinant était de 88°C. Des résultats similaires ont été obtenus pour un pool partiellement purifié de PI-1 natif (cv. *Bintje*).

Dans le chapitre 8, les caractéristiques structurales communes des inhibiteurs de protéase de pomme de terre sont discutées et comparées à celles de l'inhibiteur trypsique de soja (un inhibiteur de type Kunitz). Ceci amène à la conclusion que toutes ces protéines sont des protéines de type β-II et ont plus ou moins un état de pliement commun: β-trefoil. Un schéma est proposé définissant les principales caractéristiques pouvant aider à classifier les inhibiteurs de protéase inconnus dans la famille correcte d'inhibiteurs. Finalement, la stabilité des inhibiteurs de protéase de pomme de terre en fonction de la température et du pH est discutée en relation avec le processus d'agrégation et de précipitation présent dans le jus de pomme de terre industriel.

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Laurice

CURRICULUM VITAE

Laurice Pouvreau was born on 25 November 1972 in Nantes, France. In 1996, she graduated obtaining her ”maitrise” in cell biology (specialisation Biochemistry) at the Sciences University of Nantes. In 1996-1997, she obtained her “Diplome d’Etudes Approfondies” (DEA) at the university of Nantes. During this last year, she spent 7 months at the INRA (Institut National de la Recherche Agronomique) of Nantes in the LEIMA (Laboratoire d’Etudes des Interactions des Molecules Alimentaires) for a stage. She studied the effect of two different serine protease inhibitors on modified and wild-type trypsin, as well as effect of bivalent cations. In 1999, she started her PhD thesis at the Laboratory of Food Chemistry of Wageningen University, and the work is presented in this thesis.

LIST OF PUBLICATIONS

- Effect of pea and bovine trypsin inhibitors on wild-type and modified trypsins – Pouvreau, L., Chobert, J.-M., Briand, L., Quillien, L. and Haertle, T. – *FEBS Letters* **1998**, 423, 167-172.
- Relative abundance and inhibitory distribution of protease inhibitors in potato juice from cv. *Elkana* - Pouvreau, L., Gruppen, H., Piersma, S. R., van den Broek, L. A. M., van Koningsveld, G. A., Voragen, A. G. J. – *Journal of Agricultural and Food Chemistry* **2001**, 49, 2864-2874.
- Functionele eigenschappen van aardappeleiwitten zijn veelbelovend – van Koningsveld, G., Pouvreau, L. and Gruppen, H. – *V.M.T.* **2001**, 34, 45-48.
- Promising properties of potato proteins – van Koningsveld, G., Pouvreau, L. and Gruppen, H. – *Industrial proteins IOP* **2002**, 10, 20-23.
- The most abundant protease inhibitor in potato tuber (cv. *Elkana*) is a serine protease inhibitor from the Kunitz family - Pouvreau, L., Gruppen, H., van Koningsveld, G. A., van den Broek, L. A. M., Voragen, A. G. J. – *Journal of Agricultural and Food Chemistry* **2003**, 51, 5001-5005.
- *In vivo* uniform ^{15}N-isotope labelling of plants: Using the greenhouse for structural proteomics - Ippel, J. H., Pouvreau, L., Kroef, T., Gruppen, H., Versteeg, G., van den Putten, P., Struik, P. C. and van Mierlo, C. P. M. – *Proteomics* **2004**, 4, 226-234.
- Structural characterisation of potato inhibitor I (cv. *Bintje*) after expression in *Pichia pastoris* - L. A. M. van den Broek, L. Pouvreau, G. Lommerse, B. Schipper, G. A. van Koningsveld, and H. Gruppen – *Accepted for publication in Journal of Agricultural and Food Chemistry*.
- Tentative assignment of the Potato Serine Protease Inhibitor group as β-II proteins based on their spectroscopic characteristics - Pouvreau, L., Gruppen, H., van Koningsveld, G. A., van den Broek, L. A. M., Voragen, A. G. J. - *Submitted for publication.*

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