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# Structural Characterization of Potato Protease Inhibitor I (Cv. Bintje) after Expression in *Pichia pastoris*

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In the present study the structural properties of potato protease inhibitor 1 (PI-1) were studied as a function of temperature 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 the literature. The recombinant protein seems either to have a predominantly unordered structure or to belong to the $\beta$-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 °C. Similar results were obtained for a partially purified pool of native PI-1 from cv. Bintje.

**KEYWORDS: Potato; *Solanum tuberosum*; protease inhibitor; *Pichia pastoris*; recombinant PI-1**

## INTRODUCTION

Potato juice, which is a byproduct of potato starch production, contains a relatively high amount of protein (*1*). 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 (*2*), carcinogenesis prevention (*3*), foam-forming and stabilizing properties (*4*), and good emulsifying properties over a large pH range (*5*). According to Pouvreau and co-workers (*6*) the potato protease inhibitors can be classified into seven different families. Potato protease inhibitor I (PI-1) is one of these seven families. PI-1 inhibits serine proteases and is highly active toward 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 (*7*). In potato juice of cv. Elkana, for example, PI-1 represents 4.5% of the total protein (*6*).

PI-1, which was first described by Ryan and Balls (*8*), is composed of protomers (*9*). Four protomer types (A−D) have been identified (*10*), which consist of 70−71 amino acid residues (*11*) and show a high degree of homology among all four types. For cv. Ulster Prince types A and B have Glu as their N termini, whereas types C and D both have an additional Lys at their N termini (*12*, *13*). The molecular mass of the protomers has been reported to vary between 7.9 and 8.1 kDa (*11*). The molecular mass of the oligomer in solution has been estimated as 39 kDa (*10*), which implies that the complex is pentameric. Oligomers that differ in their protomer composition are usually called isoinhibitors (*10*). In cv. Elkana (*6*) eight different isoforms of PI-1 with p*I* values between pH 5.1 and pH 7.8 have been isolated.

The inhibitor PI-1 is known to be highly stable against heating (*8*, *10*, *14*) and proteolytic digestion at neutral pH (*15*). PI-1 from cv. Russet Burbank was still heat stable after 5 min of incubation at 97 °C at either pH 3 or pH 8 as determined by quantitative immunological assays (*16*).

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 homologous PI-1 form would make a structural characterization 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 number of necessary purification steps (*17*).

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

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## MATERIALS AND METHODS

**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). BMG medium [100 mM potassium phosphate buffer, pH 6.0, 1.34% (w/v) yeast nitrogen base with ammonium sulfate without amino acids, $4 \times 10^{-5}$% (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.

**Cloning of PI-1 in *P. pastoris*.** A PI-1 cDNA clone (kindly provided by M. Jongsma, Plant Research International BV, The Netherlands; accession no. 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′-CCCCCCCTC-GAGAAAAGAGAGGCTGAAGCTAAGGAATTTCAATGCAATGG-3′), for extracellular targeting of recombinant PI-1. Primer P3 contained an extra *Not*I restriction site, (5′-CCCCCCGCGGCCGCTTAAC-CAACCACAGGAATTTG-3′). The amplified fragment and the pPIC9 vector were digested with *Xho*I and *Not*I and thereafter ligated. For plasmid propagation the ligated vector was transformed into *Escherichia coli* XL1 Blue MRF′. Cells were grown overnight at 37 °C on solidified (15 g/L agar) Luria−Bertani (LB) broth plates supplemented with 50 $\mu$g/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 were performed according to the instructions of the supplier (Invitrogen, San Diego, CA). The nucleotide sequence was determined as described by Pouvreau et al. (*18*).

Twelve colonies were randomly picked from each transformed *P. pastoris* strain and grown overnight in 10 mL of BMG medium at 30 °C and 275 rpm. Cells were subsequently centrifuged (2000*g*, 10 min, room temperature) and suspended in 10 mL of 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 of BMM in 5% (v/v) methanol. This was repeated four times. To estimate the expression of PI-1 into the cell supernatant a radial diffusion assay with chymotrypsin as protease was used (*19*).

**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 h in 190 mL of BMG medium. Thereafter the cells were transferred into the fermentor, which contained 1.8 L of BMG medium and 8 mL of $PTM_1$ trace salt (Invitrogen). 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 the addition of methanol for 52 h. Cells were centrifuged (10000*g*, 15 min, 4 °C), and the supernatant was filtered through a 0.2 $\mu$m filter and frozen until further use.

**Recombinant P1-1 Purification.** The cell supernatant was dialyzed overnight against water (MWCO = 2000), and the pH was adjusted to pH 8 with NaOH. Subsequently, the sample was filtered through a 0.2 $\mu$m filter. Purification was performed using an Äkta Explorer (Amersham Biosciences, Uppsala, Sweden) equipped with a Source 15 Q anion exchange column (Amersham Biosciences) equilibrated with 10 mM Tris-HCl buffer, pH 8. After the sample had been loaded 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 = 2000) 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 according to the method of Bradford (*20*) 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 5600 $M^{-1}$ $cm^{-1}$.

**Partial Purification of Native PI-1 from Cv. Elkana and Bintje.** PI-1 pools were isolated from cv. Bintje and Elkana according to the method of Melville and Ryan (*10*). Potato juice was adjusted to pH 3.0 with 5 M HCl, and the suspension was subsequently centrifuged (14000*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 $\mu$m filter. Purification was performed using a Äkta Explorer (Amersham Biosciences) equipped with a Superdex 75 preparatory grade size exclusion column (Amersham Biosciences) 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.

**Protein Characterization.** The apparent native molecular mass was determined by size exclusion chromatography using a Superdex 75 column (Amersham Biosciences) on an Äkta Purifier (Amersham Biosciences). The column was calibrated with BSA (67000 Da), ovalbumin (43000 Da), carbonic anhydrase (29000 Da), chymotrypsinogen A (25000 Da), ribonuclease A (13700 Da), cytochrome *c* (12400 Da), and aprotinin (6500 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 $h^{-1}$, whereas 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 (*6*).

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

**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 the aid of a microtiter plate reader ($\mu$Quant, Bio-Tek Instruments Inc.) or a Shimadzu spectrophotometer (Shimadzu Corp.). Trypsin inhibition and chymotrypsin inhibition were estimated as described previously (*6*). From these results the trypsin inhibitory activity (TIA) and chymotrypsin inhibitory activity (CIA) were calculated for the different recombinant PI-1 pools (*6*). The TIA and CIA values were expressed in milligrams of (active) enzyme per gram of inhibitor.

**Spectroscopic Measurements.** Samples were measured in 7 mM sodium phosphate buffer, pH 7.5, and filtered through a 0.22 $\mu$m filter before measurement.

Far-ultraviolet 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.) as described previously (*21*). The samples were heated to the desired temperature and equilibrated for 6 min 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 nonlinear regression procedure (*22*). Changes in the 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-ultraviolet circular dichroism spectra (near-UV CD) at 20 °C of 1 mg/mL samples of PI-1 were also recorded. 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 0.125 s. Spectra were also corrected by subtracting the spectrum of a protein-free sample obtained under identical conditions.

Tryptophan fluorescence spectra of 0.2 mg/mL samples were recorded on a Perkin-Elmer LS 50 B luminescence spectrophotometer

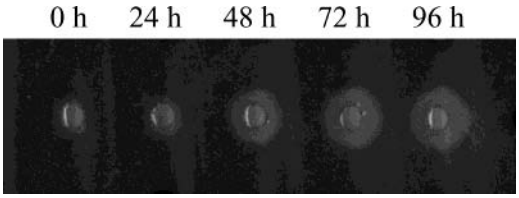


**Figure 1.** Progression profile of recombinant PI-1 from potato cv. Bintje accumulation in cell supernatant of *P. pastoris*.

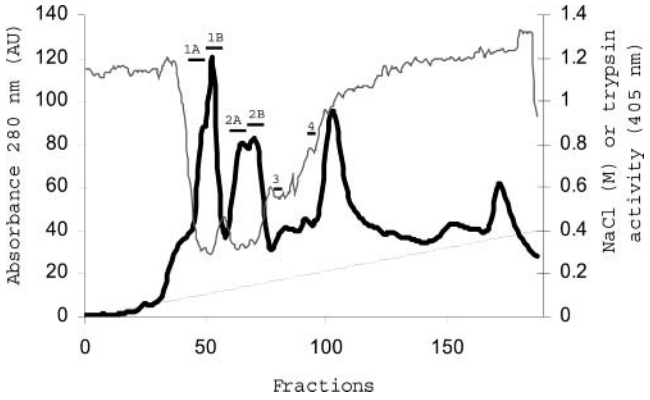


**Figure 2.** Source 15 Q anion exchange chromatography of recombinant PI-1 from potato cv. Bintje in cell supernatant of *P. pastoris*: (thick black line) 280 nm; (thick gray line) trypsin activity; (thin gray line) salt gradient. Pooled fractions are indicated as 1A, 1B, 2A, 2B, 3, and 4.

equipped with a pulsed xenon source at 20 °C. Spectra of the samples were obtained by excitation at 295 nm, and three emission spectra from 300 to 400 nm were recorded and averaged using a scan speed of 100 nm $min^{-1}$. Both the excitation and emission slits were set at 5 nm. Spectra were corrected by subtracting the spectrum of a protein-free sample obtained under identical conditions. Changes in the tertiary structure of PI-1 during heating (20−95 °C, heating rate = 30 °C $h^{-1}$) were monitored by the changes in fluorescence at 330 nm, using a Varian Cary fluorometer.

**Differential Scanning Calorimetry (DSC).** DSC measurements were performed on a VP-DSC microcalorimeter (Microcal Inc., Northampton, MA). 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.

## RESULTS AND DISCUSSION

**Expression and Purification of Recombinant PI-1.** 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* strains GS115 and KM71, respectively, showed inhibiting activity toward 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**). On the basis of the protein elution profile and the inhibiting activity toward trypsin, six pools were collected (**Figure 2**). With SDS-PAGE under reducing conditions all pools showed a single band at ∼8 kDa (data not shown). Only pool 3 showed some other faint bands. The inhibition data toward 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 toward 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 |

On the basis of pools 1A, 1B, 2A, and 2B, ∼105 mg of recombinant PI-1/L of 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 (*17*).

**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 7857 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 (7728 ± 1, 7857 ± 1, and 8057 ± 1 Da). A mass of 7857 Da corresponds to the deduced molecular mass of the cloned PI-1. The peak of 7729 Da corresponds to the mass of a protomer without the first amino acid (Lys) present. Richardson and Cossins (*12, 13*) reported the amino acid sequence of four protomers from cv. Ulster Prince. Two protomers contained Glu at their N termini, whereas the other two had an additional Lys at their N termini. However, 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 8057 ± 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 (*17*). The obtained masses of the smaller peaks in some pools were 7928 ± 2 Da (which corresponds to an additional Ala), 8128 ± 2 Da (with an extra Ala-Glu-Ala), and 8258 ± 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 7598 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 8475 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 7597 to 7974 Da, with the highest intensity at 7731 Da, indicating that several different forms were present (**Figure 3**).

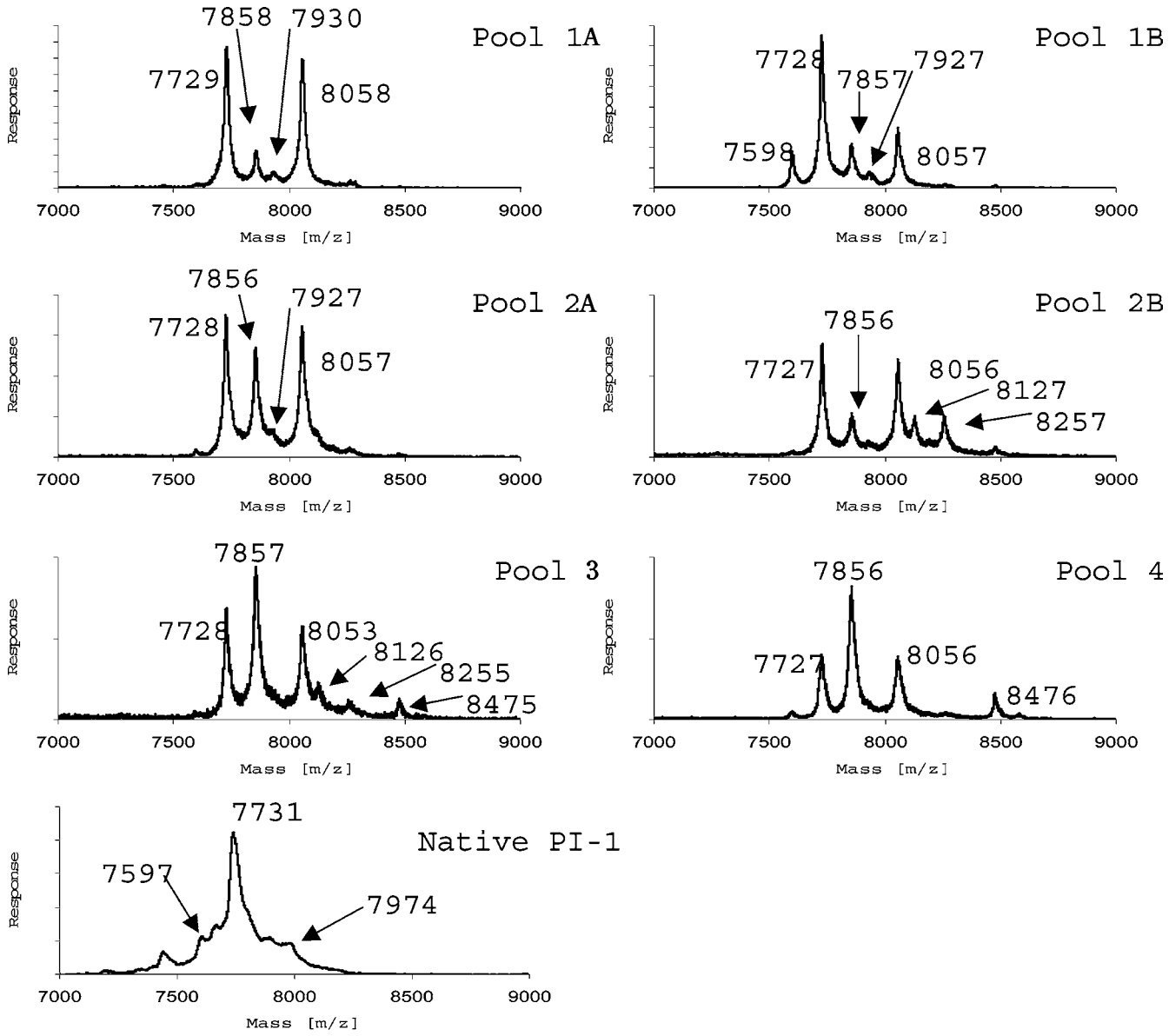


**Figure 3.** MALDI-TOF spectra of the different pools of recombinant PI-1 obtained after anion exchange chromatography and from the partial purified native PI-1 from potato cv. Bintje. Masses are given in daltons.

For all pools the apparent native molecular mass was estimated by size exclusion chromatography. The native molecular mass varied from 48 to 53 kDa (pools 1A and 1B, 48 kDa; pool 2A, 50 kDa; pools 2B and 3, 52 kDa; pool 4, 53 kDa). It was observed that oligomers with a higher apparent molecular mass eluted later from the anion exchange column. When the MALDI-TOF MS analysis and size exclusion data are combined, it can be concluded that the mature recombinant PI-1 is presumably a hexameric protein. In the literature available PI-1 is described as a pentamer (*10*, *23*) 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 cv. Bintje and Elkana according to the method of Melville and Ryan (*10*). Both PI-1s showed elution volumes very similar 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-1s.

**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-1s in these pools (**Figure 4**). All spectra showed a clear minimum at ∼204 nm, which could imply that the protein has a mostly unordered structure (*24*). All pools showed a maximum at 228 nm, which can be due to a combination of contributions from secondary structures such as $\beta$-turns and loops (*25*), the presence of disulfide bridges, and the interaction between aromatic side chains (*26*). The presence of a disulfide bridge was already found by Plunkett (*16*) 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 $\beta$-II proteins (*27*). Because all spectra were highly similar, it can be concluded that there is no real difference in secondary structure between the different recombinant PI-1s. 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

**Table 2.** Thermodynamic Parameters of Recombinant Potato PI-1 from Potato Cv. Bintje

| | transition temperature (°C) | | | $\Delta H_{cal}$[a] (kJ/mol) | $\Delta H_{vH}$[a] (kJ/mol) | $\Delta H_{vH}/\Delta H_{cal}$ |
|---|---|---|---|---|---|---|
| pool | far-UV CD | Trp fluorescence | DSC | DSC | DSC | 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 ± 0.1 | 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 P1-1 | ND[b] | ND[b] | 84.9 | 360.7 | 736.7 | 2.04 |

[a] Concentration of protein was based on the molecular weight of the protomer (8000 Da). [b] Not determined.

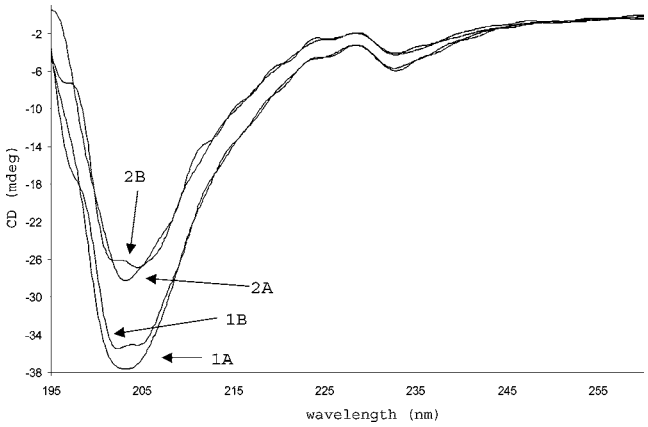


**Figure 4.** Far-UV CD spectra at 20 °C of different pools of purified recombinant PI-1 from potato cv. Bintje.

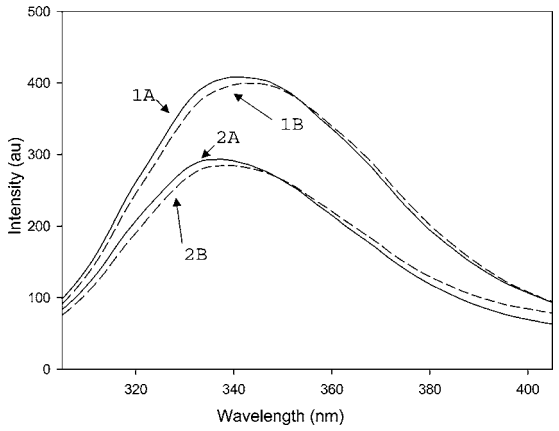


**Figure 5.** Tryptophan fluorescence spectra at 20 °C, obtained by excitation at 295 nm, of different pools of purified recombinant PI-1 from potato cv. Bintje.

the two isoinhibitors (data not shown). The spectra were also similar to those obtained for native PI-1 from cv. Russet Burbank (*16*).

Fluorescence spectra can give information about the polarity of the environment of phenylalanine, tyrosine, and tryptophan residues (*28*). 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 maxima of the different pools were 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.

**Thermal Stability.** The recombinant PI-1 was studied in more detail with regard to its thermal stability. Far-UV CD

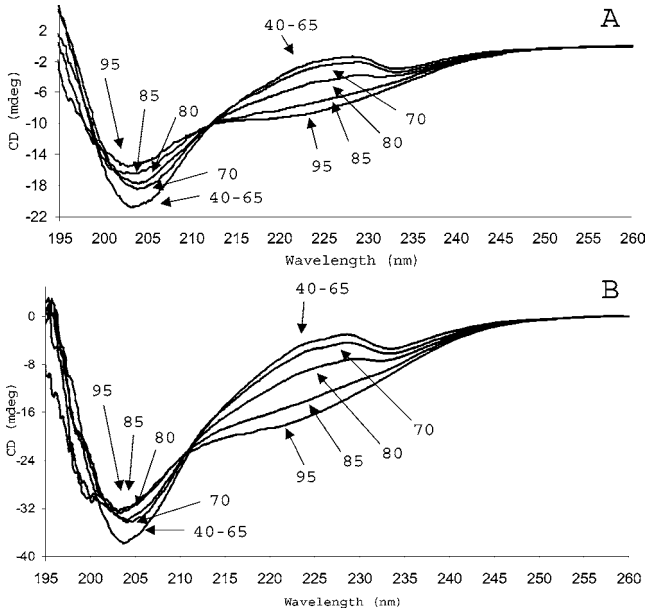


**Figure 6.** Far-UV CD spectra at different temperatures of (**A**) pool 1B and (**B**) pool 2A of purified recombinant PI-1 from potato cv. Bintje.

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 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 (*29*). 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**). To obtain these thermodynamic data from the thermal unfolding curves (CD and fluorescence), the model given by van Mierlo et al. (30), based on thermodynamic equations (*31*, *32*), was used.

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 ($\Delta H_{cal}$), the van't

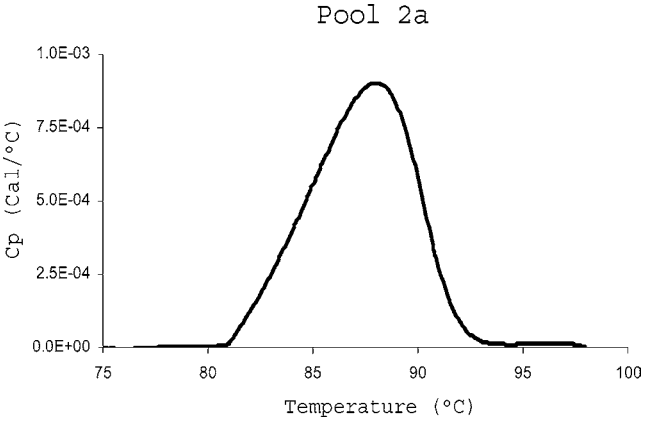


**Figure 7.** DSC thermogram of pool 2A of purified recombinant PI-1 from potato cv. Bintje.

Hoff enthalpies ($\Delta H_{vH}$), 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 $\Delta H_{vH}$ and $\Delta H_{cal}$ 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 (*33*, *34*). As can be seen in **Figure 7** the endothermic peak for PI-1 is slightly asymmetric (sharper on the high-temperature side), which can be regarded as an indication that dissociation occurs during the unfolding process (*35*). Indeed, fitting of the DSC results with Microcal software using a dissociation model gave good fits, especially for a model assuming PI-1 was built from three subunits (results not shown). It thus seems likely that the PI-1 hexamer is built from three 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.

The high transition temperature (88 °C) is in agreement with the high thermostability of native PI-1 (*8*, *10*, *14*). In addition, Van Koningsveld et al. (*36*) 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 ~30% at 80 °C. In potato juice from cv. Elkana PI-1 is responsible for 19% of the chymotrypsin-inhibiting activity (*6*). Thus, the measured inhibiting activity of chymotrypsin at 80 °C is probably due to PI-1.

**Conclusion.** Although only one gene (PI-1) from 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 suggest also that the native PI-1 is present as a hexamer rather than a pentamer, as previously proposed in the literature. Therefore, the recombinant PI-1 is a good model to study the structural properties and the heat-induced unfolding mechanism of PI-1.

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