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Conformational Stability of the Potato Serine Protease Inhibitor Group

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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 one stable intermediate is present. 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.

KEYWORDS: PSPI; aggregation; equilibrium

INTRODUCTION

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

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 (*3*). Serine protease inhibitors have also been reported to have inhibitory effects on tumor cell growth (*4*, *5*). In addition, when the level of cholecystokinin is increased via the inhibition of trypsin, serine protease inhibitors may also be used to reduce food intake in humans (*6*).

In potato (*Solanum tuberosum*), a wide range of protease inhibitors is expressed, of which potato serine protease inhibitor (PSPI), a Kunitz-type inhibitor (*7*), is the most abundant group. It accounts for 22% (w/w) of the soluble proteins (*8*). 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 (*7*). On the basis of 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 acid residues are involved in relatively short irregular β sheets.

In industrial processing of potato starch, potato proteins are recovered as a byproduct (*9*). 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 (*10*), it was shown that the thermal unfolding of PSPI shows an unusually sharp transition in its secondary structure from the folded to unfolded state. Therefore, the aim of the present study was to gain 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 levels of the two main PSPI isoforms at pH 7.5 were studied as a function of both the temperature and guanidinium chloride concentration.

MATERIAL AND METHODS

Materials. Potatoes of cultivar *Elkana* (AVEBE b.a., Veendam, The Netherlands) were stored at 4 °C in the dark at a relative humidity of 95−100% for a period of 6 months and used within this period. Guanidinium chloride ultrapure (GndHCl) (G-7153; Lot: 60K5423) was from Sigma Chemical Co. All other chemicals used were of analytical grade.

Preparation of PSPI Solutions. PSPI isoforms 6.1 and 6.5 were purified as described previously (*7*, *8*). After purification, PSPI 6.1 and 6.5 were dialyzed at 4 °C against 7 mM sodium phosphate buffer (pH 7.5) with an 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, 0−5 M for the CD experiments, and 0−6.5 M for the fluorescence experiments). GndHCl-induced unfolding of PSPI was in equilibrium within 2 h, as judged from CD and fluorescence spectra. All of the samples were, therefore, equilibrated for 2 h prior to measurement. All measurements were performed at 20 °C.

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

Gel Filtration. The ÄKTA explorer protein chromatography system and the columns used for the protein purification were from Amersham

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Biosciences (Uppsala, Sweden). The absorbance of the eluates was monitored at 280 and 320 nm.

A Superdex 75 HR column (30 × 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 (2000 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/h up to 85 °C and cooled 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 the 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.

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-Ultraviolet Circular Dichroism (Far-UV CD). 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 °C, using a heating rate of 30 °C/h. 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 min 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 was 0.2 nm; the bandwidth was 1.0 nm; the sensitivity was 20 mdeg; and the response time was 0.125 s. 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 the temperature (20−85 °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 the secondary structure as a function of the 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) with a pulsed Xenon source, at temperatures ranging from 20 to 85 °C with intervals of approximately 5 °C, using a heating rate of 30 °C/h. 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 min at this temperature before wavelength scans were recorded. To check the reversibility of the conformational changes, the samples were cooled to 20 °C and allowed to equilibrate for 20 min 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) 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/h, followed by using a Varian Cary Fluorimeter (Varian Cary, Inc., Palo Alto, CA).

Differential Scanning Calorimetry (DSC). DSC measurements were performed on a VP-DSC Microcalorimeter (MicroCal Incorporated, Northampton, MA). 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/h.

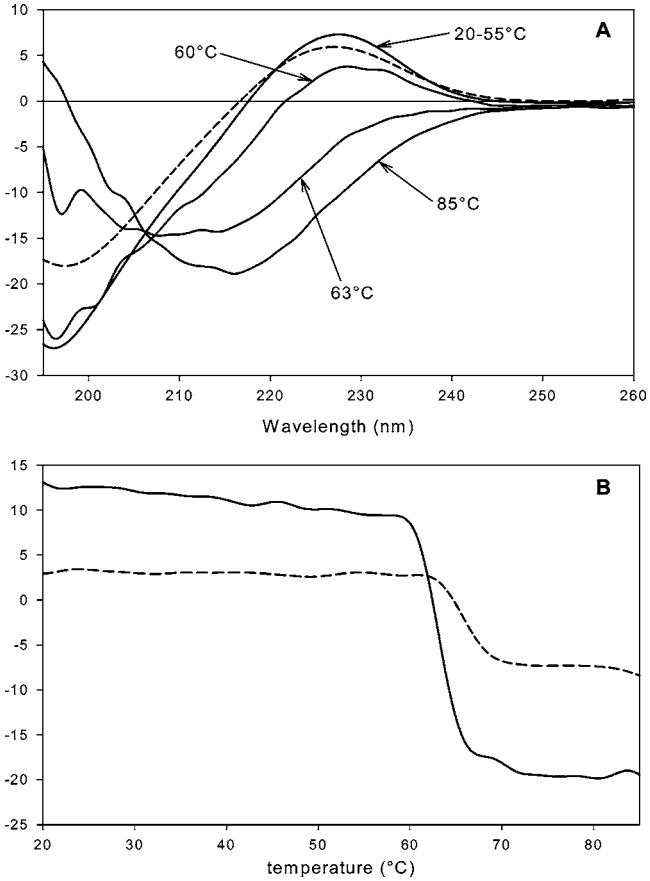

Figure 1. (A) Far UV CD spectra at pH 7.5 of PSPI 6.1 (—) at various temperatures and 6.5 (- - -) at 20 °C. (B) Thermal unfolding curves of PSPI 6.1 (—) and 6.5 (- - -) at pH 7.5, monitored by the CD signal at 222 nm.

To investigate the reversibility of unfolding, the samples were heated with a scan rate of 30 °C/h to a temperature just after the transition was complete, subsequently cooled to 20 °C, and reheated 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 and 60 °C/h. 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/h.

RESULTS

Thermal and guanidinium-induced unfolding were also performed at pH 4.0. Results were similar to those at pH 7.5 and, therefore, were not shown.

Thermal Stability. 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 6.5 at 222 nm as a function of the temperature. It can be observed that for both isoforms the shape of the thermal unfolding curves are similar, with the highest transition midpoint for PSPI 6.5. The temperature range, in which the changes in secondary structure occur, is only about 6 °C.

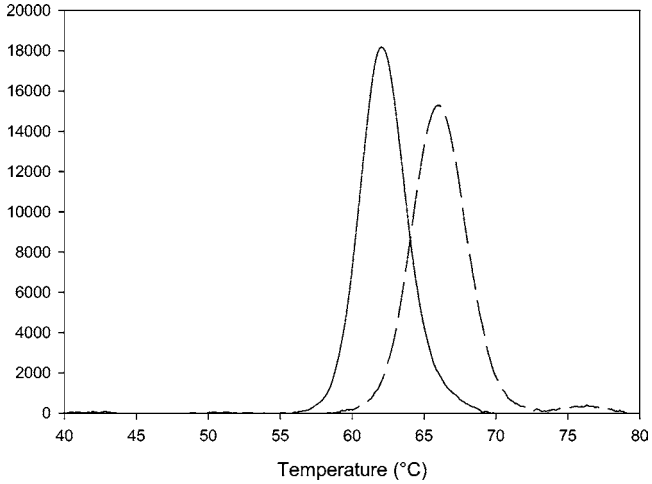

Figure 2. DSC thermograms of PSPI 6.1 (—) and 6.5 (- - -).

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
T_m (°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

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 (*11*). 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.

To obtain thermodynamic data from the thermal unfolding CD curves of PSPI, the model given by van Mierlo and coworkers (*12*) based on thermodynamic equations (*13*, *14*) 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 **Table 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 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 reheating, indicating that the transition is almost completely irreversible (data not shown).

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 an increasing protein concentration (even though the solution remains clear) (*15*).

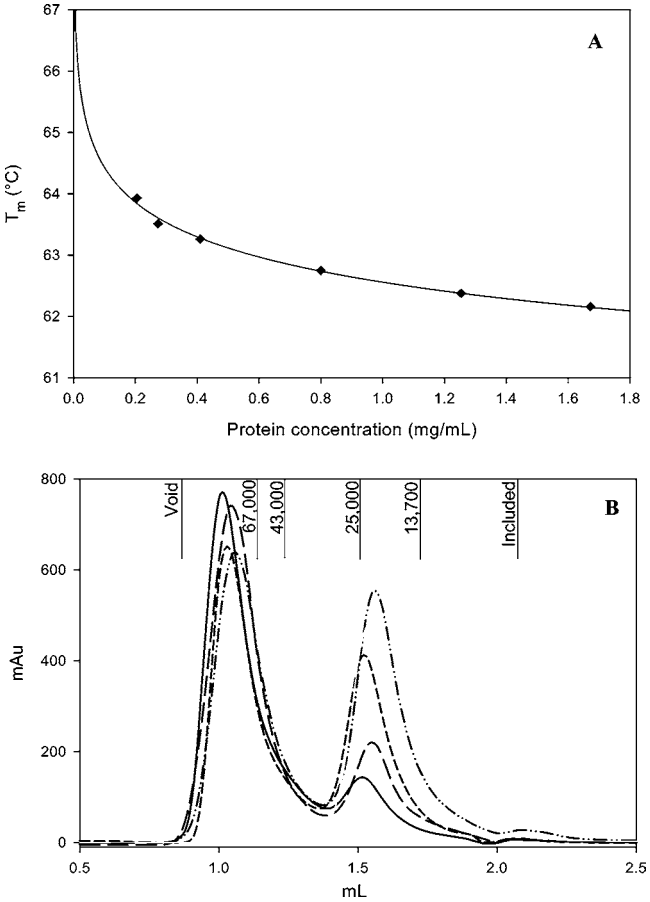

Figure 3. (A) Transition temperature of PSPI as a function of the protein concentration as determined using DSC. (B) Gel filtration of PSPI after heating (20–85 °C, at 30 °C/h) at different concentrations: 0.8 mg/mL (—), 0.6 mg/mL (– –), 0.4 mg/mL (- - -), and 0.2 mg/mL (-•-). The gray curve represents PSPI without any heat treatment (0.4 mg/mL).

To examine this aggregation behavior 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 that corresponds to nonaggregated PSPI (V_e = 1.6 mL) and one that corresponds to the aggregates formed (V_e = 1.1 mL). When the protein concentration is increased, a decrease in the proportion of nonaggregated 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.

SDS−PAGE of the aggregates under nonreducing conditions showed one single band at ±80 kDa and under reducing conditions showed two bands at 15 and 6 kDa (data not shown). These results clearly showed that the aggregate formation is linked to a rearrangement of the disulfide bridges.

Scan Rate Dependency. To establish equilibrium between the native and unfolded states at all temperatures, the scan rate should be much slower than the folding/unfolding rates (*16*). To verify if this condition is met for the thermal unfolding of PSPI, the protein was heated at various scan rates ranging from 2 to 60 °C/h.

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/h, but the shape of the peak did change. With a decreasing scan rate, the transition temperature also decreased (**Figure 4B**) and the peaks became sharper. The decrease in the transition temperature is most apparent at low

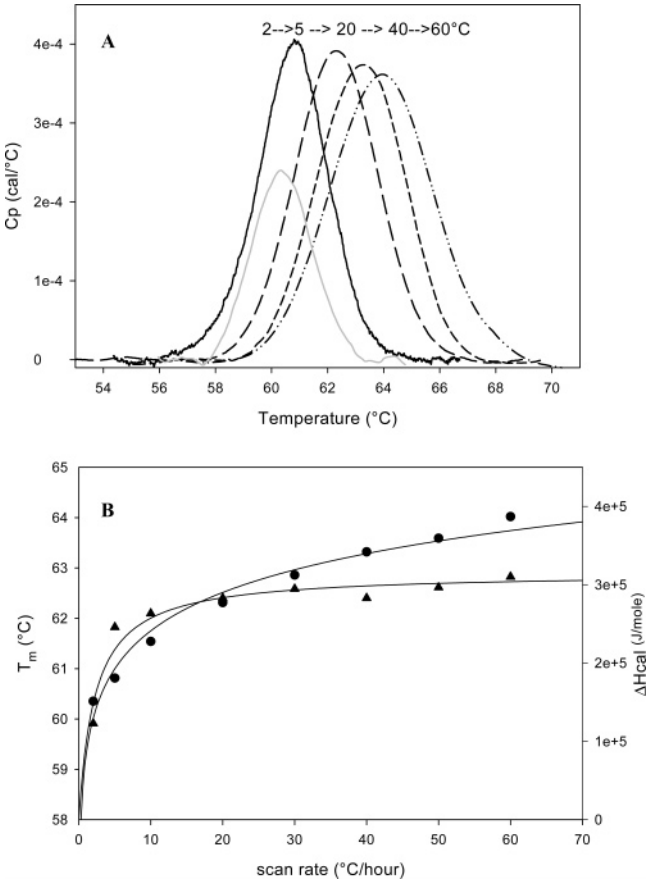

Figure 4. (A) DSC thermograms of PSPI 6.1 (pH 7.5) at various scan rates. (B) Transition temperatures of PSPI 6.1 (●) and ΔH_{cal} (▲) as a function of the scan rate.

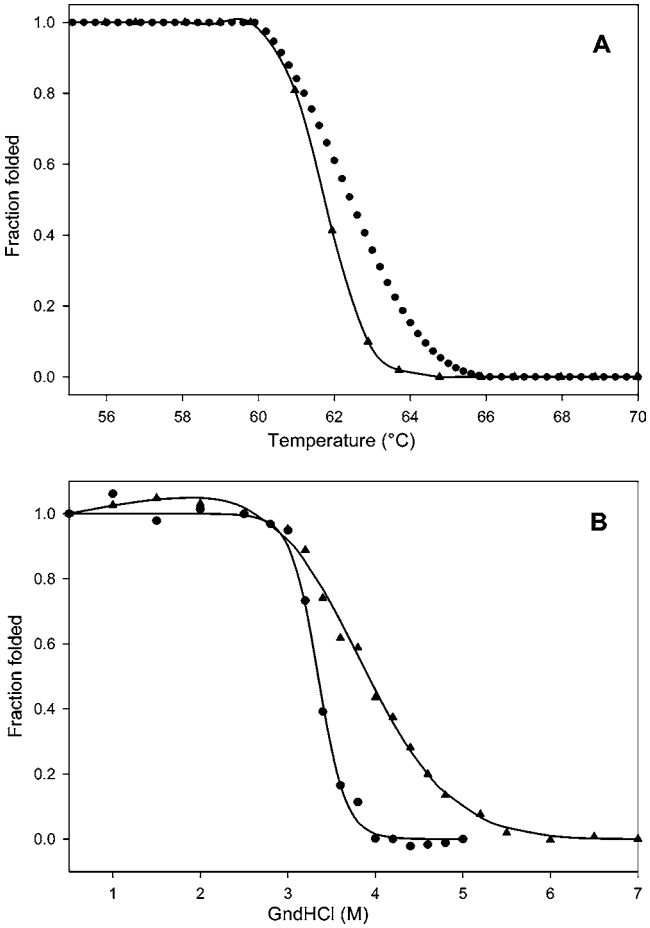

Figure 5. (A) Thermal unfolding curves as monitored by the tryptophan fluorescence at 320 nm (▲) and the far UV CD signal (222 nm) (●). (B) GndHCl-induced unfolding of PSPI 6.1 (pH 7.5) at 20 °C, as monitored by the tryptophan fluorescence at 320 nm (▲) and the far UV CD signal (222 nm) (●).

scan rates (**Figure 4B**). The calorimetric enthalpy increased from 270 to 340 kJ/mol when the scan rate was increased 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/h, 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 favored at low scan rates, as indicated by the disappearance of the peak corresponding to the nonaggregated PSPI (data not shown). The ratio $\Delta H_{vH}/\Delta H_{cal}$ increases with a decreasing scan rate and varies from 2.6 at a scan rate of 60 °C/h to 11.0 at a scan rate of 2 °C/h (no further data shown).

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 the secondary structure took place between 60 and 66 °C, whereas the changes in the tertiary structure took place between 60 and 64 °C. It thus seems that the unfolding of the secondary and tertiary structures start simultaneously, but the tertiary structure has disappeared already at temperatures where part of the secondary structure is still intact. These results are unusual but can be explained by the presence of a "molten globule" type of intermediate, by means that there is stable, partly denatured intermediate forms of the protein (*17*).

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

Effect of DTT. PSPI is known to possess two disulfide bridges (*7*). 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, with a midpoint 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 the 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.

DISCUSSION

Thermal Unfolding. From the results obtained, it can be seen that the thermal unfolding of PSPI is irreversible. Therefore,

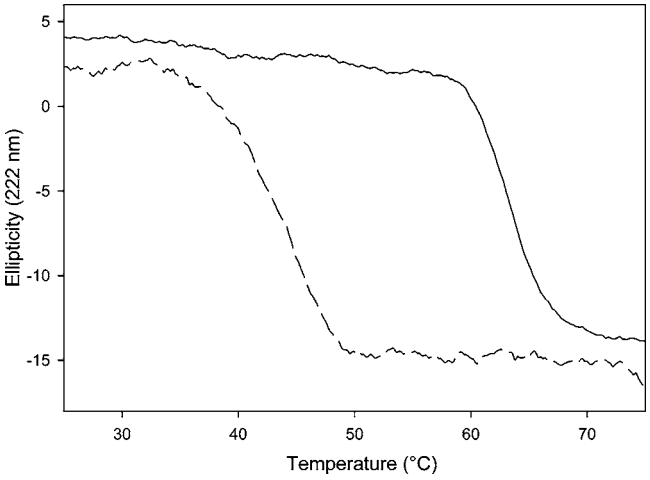

Figure 6. Thermal unfolding curves of PSPI 6.1 (pH 7.5) in the absence (—) and presence (- - -) of 1 mM DTT, monitored by the CD signal at 222 nm.

equilibrium thermodynamics cannot normally be applied in this case (*18*). In some cases, however, it is still possible to use equilibrium thermodynamics for irreversibly unfolding proteins, if several conditions are met (*19*).

First, the unfolding should be independent of the scan rate. Lowering the scan rate favors a more reliable characterization 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. Because, 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 a zero scan rate (*16*, *20*). 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. A comparison of the thermal unfolding of the secondary and tertiary structures of PSPI shows that at least one intermediate state is populated. The differences in the thermal unfolding curves can be explained by the presence of stable, partly denaturated forms of the protein (*17*, *21*). Studies have shown that these intermediates were in a stable and compact globular state but with mobile (fluctuating) side chains (*22*).

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 (*10*, *23*, *24*). 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) (*25*, *26*) 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.

The results presented indicate that the thermal as well as the GndHCl-induced unfolding of PSPI occurs via a non-two-state mechanism and that at least one stable intermediate is present (even though it is not seen in the DSC data). Unfolding of elastase showed similar results in the thermal unfolding curves. The X-ray structure of elastase shows that aromatic residues on the outside of the protein are in close contact with each other (*23*). Our proposal is that in PSPI aromatic residues are also close to each other in the 3D structure. It seems that, in the stable globular intermediate, the aromatic side-chain environment is not affected and therefore no changes in tertiary structure are apparent. 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 involves disulfide interchanges.

Aggregation of protease inhibitors in potato juice is influenced by the presence of patatin but also by other compounds such as phenolic compunds (*27*). In industrial processing of potato starch, low pH and fast heating are the two conditions used. Low protein concentrations and low ionic strengths may limit the aggregation but are difficult to apply in the industry.

ABBREVIATIONS USED

PSPI, potato serine protease inhibitor.

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