# Thermal Aggregation of Patatin Studied in Situ

André M. Pots,[†] Erik ten Grotenhuis,[‡,§] Harry Gruppen,[⊥] Alphons G. J. Voragen,*[,⊥] and Kees G. de Kruif[‡,§]

Centre for Protein Technology TNO-WAU, Wageningen, The Netherlands; NIZO food research, Ede, The Netherlands; Department of Food Technology and Nutritional Sciences, Food Science Group, Wageningen Agricultural University, Wageningen, The Netherlands; and Wageningen Centre for Food Sciences, Wageningen, The Netherlands

In this work dynamic light scattering was used to study the thermal aggregation of patatin in situ, to elucidate the physical aggregation mechanism of the protein and to be able to relate the aggregation behavior to its structural properties. The dependence of the aggregation rates on the temperature and the ionic strength suggested a mechanism of slow coagulation, being both diffusion and chemically limited. The aggregation rate dependence on the protein concentration was in accordance with the mechanism proposed. The aggregation rates as obtained at temperatures ranging from 40 to 65 °C correlated well with unfolding of the protein at a secondary level. Small-angle neutron scattering and dynamic light scattering results were in good accordance; they revealed that native patatin has a cylindrical shape with a diameter and length of 5 and 9.8 nm, respectively.

**Keywords:** *Kinetic; mechanism; thermal aggregation; patatin*

## INTRODUCTION

The rationale of research concerning the aggregation of a specific globular plant protein such as patatin is, first, the fact that aggregation governs gelation and that the mechanism of gelation determines the properties of the formed particle gel (Damodaran, 1997). Second, many studies indicate that each class of proteins has its specific aggregation behavior (Hohlberg and Stanley, 1986; Kella, 1989; Roefs and de Kruif, 1994; Boye et al., 1996). Third, potato proteins are available in large amounts; the potato contains ∼2% protein (weight/fresh weight; Lisinska and Leszcynski, 1989), and potatoes are converted to starch in millions of tons annually. To be able to distinguish general pathways of aggregation mechanisms of (food) proteins, knowledge on the physicochemical properties of these proteins must be combined with a description of the aggregation mechanism. By this means the understanding of the structure−function relationships may be enhanced. This approach provides a knowledge base for the control of aggregation, possibly leading to protein products that better meet the properties required for application in a food system.

Patatin is the most abundant potato tuber protein (Racusen and Foote, 1980). It consists of 362 amino acids and shows neither extended hydrophilic nor hydrophobic sequences. It is a glycoprotein of 43 kDa (observed with SDS−PAGE), appearing in media without SDS or urea as a dimer with an apparent molar mass of ∼80 kDa (Racusen and Weller, 1984). The positive and negative charges of the side chains are randomly distributed over the sequence, and the protein contains one cysteine residue (Mignery et al., 1984; Stiekema et al., 1988). In previous work (Pots et al., 1999) the aggregation kinetics of patatin were studied by analyzing the proportions of nonaggregated and aggregated proteins after a heat treatment.

On the basis of a high or low sticking probability, two main aggregation mechanisms are distinguished: the so-called chemically limited (CLA) and diffusion limited aggregations (DLA), respectively (Lin et al., 1989). Computer models simulating these mechanisms have been developed and applied to validate the assumption concerning a specific type of coagulation (Meakin, 1988; Einarson and Berg, 1993; Elofsson et al., 1996; Kyriakidis et al., 1997).

Aggregation behavior in terms of aggregate size or form can be studied by dynamic light scattering (DLS) and small-angle neutron scattering (SANS). DLS has shown to be of great value in the study of polymerization and thermal aggregation of proteins (Ware, 1984; Schurtenberger and Augusteyn, 1991; Griffin et al., 1993). In addition, it provided information that was used to develop and validate models for the thermal denaturation and aggregation of $\beta$-lactoglobulin (Roefs and de Kruif, 1994; Elofsson et al., 1996; Hoffmann et al., 1996; Verheul et al., 1998). SANS enables the determination of the size, form, and structure of particles as well as the packing density of particles in agglomerates (Timmins and Zaccai, 1988; Verheul, 1998).

In this research the aggregation of patatin is studied in situ. One of the problems in defining protein aggregation is the difficulty in distinguishing between aggregation and precipitation, because these events tend to occur simultaneously (Boye et al., 1997). Therefore, the aggregation of patatin was studied under conditions where no precipitation occurred, that is, at sufficiently low protein concentrations. This should lead to a physical mechanism that describes the aggregation kinetics.

* Address correspondence to this author at the Food Science Group, Wageningen Agricultural University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands [telephone +31 (0)-317 482888; fax +31 (0)317 484893; e-mail Fons.Voragen@chem.FdSci.wau.nl].

[†] Centre for Protein Technology.

[‡] NIZO food research.

[§] Wageningen Centre for Food Sciences.

[⊥] Department of Food Technology and Nutrtional Sciences.

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## EXPERIMENTAL PROCEDURES

**Preparation of Patatin Solutions.** Patatin from *Solanum tuberosum* cultivar Bintje was purified as described previously (Pots et al., 1998a). After isolation, the protein solution was dialyzed (Visking V20, Carl Roth GmbH & Co., Karlsruhe, Germany) at 4 °C against a 22 mM sodium phosphate buffer (pH 7; ionic strength = 35 mM). Samples were frozen in small aliquots and stored at −20 °C until use. Protein contents were determined using the Bradford assay (Bradford, 1976) using bovine serum albumin (Sigma, A-4503) as a standard.

**DLS.** DLS measures the autocorrelation function of the fluctuating intensity resulting from the diffusional properties of particles (Berne and Pecora, 1976). For a polydisperse sample the autocorrelation function can be fitted with the method of cumulants (Koppel, 1972). The decay rate of the autocorrelation can be related to a translational diffusion constant (Koppel, 1972). From this the hydrodynamic diameter can be obtained using the Stokes−Einstein equation (Ware, 1984). Experiments were performed at NIZO food research (Ede, The Netherlands) using a self-constructed apparatus consisting of a Spinnaker 1161 laser (514.4 nm) and a detector (Thorn EMI 9863/100B, Middlesex, England) mounted on the arm of a goniometer. The output of the photomultiplier was fed into an ALV5000 autocorrelator (ALV-laser GmbH, Langen, Germany). The equipment was validated using latex standard particles with a radius of 51 ± 3.8 nm (Duke Scientific Co., Palo Alto, CA). Laser output power was set at an approximate value of 275 mW, and the intensity of the scattered light was measured at angles varying from 35 to 135°, but in this paper always at 90°. The temperature of the samples was maintained constant within 0.5 °C by immersing the sample cuvettes in a toluene bath, the temperature of which was controlled with a water bath.

Protein solutions (see below) were filtered over 0.1 $\mu$m low-protein binding filters (Millipore Millex-VV, SLVV025LS; Bedford, MA) into a cylindrical glass cuvette (radius = 9 mm). The filled cuvettes were centrifuged for 5 min at 500*g* at room temperature prior to the measurements.

Samples containing 0.3 mg/mL patatin in the above-described buffer were measured in situ at temperatures from 40 to 65 °C for times ranging from 1 to 68 h. Experiments dealing with the concentration dependence of the aggregation were performed at 55 °C with protein concentrations ranging from 0.45 to 2.0 mg/mL. The ionic strength dependence of the aggregation was investigated at 55 °C, with 0.33 mg of protein/mL. Ionic strength values >35 mM (42, 50, 57, 65, 72, and 80 mM, respectively) were obtained by the addition of sodium chloride to the buffer solutions. Experiments of 0.33 mg of patatin/mL were also performed in the presence of *N*-ethylmaleimide (NEM), by applying molar ratios of NEM/patatin of 2:1 and 1:1.

The aggregation rates were determined by taking the tangent of the initial increase in particle size.

**SANS.** SANS was performed using the D22 diffractometer at the Institut Laue-Langevin in Grenoble, France. The spectra were recorded for each sample using two different spectrophotometer configurations by changing the sample to detector distance from 3.00 to 18.00 m. The wavelength of the incident neutrons was 1 nm. In this way a range for the wave vector $q$ was covered from 0.05 to 2 nm. The data were collected by means of a two-dimensional detector, converted in intensity versus $q$, and normalized for transmission and sample path length. The data were normalized to a pure water sample.

Samples contained 0.5 mg of patatin/mL sodium phosphate buffer (pH 7) in $D_2O$ and were unheated and heated for 1 and 10 h at 55 °C prior to the measurement.

**SDS−PAGE.** SDS−PAGE was performed with a Pharmacia PhastSystem according to the instructions of the manufacturer using gradient 8−25 Phastgels. Samples at pH 7 heated for various times in the presence and absence of NEM were analyzed in the presence and absence of 20 mM $\beta$-mercaptoethanol and 6 M urea in the sample buffer and combinations thereof.

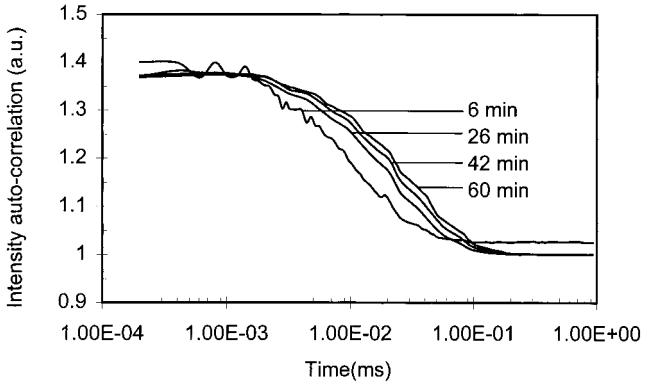


**Figure 1.** Time-dependent decay of the intensity autocorrelation function as obtained after various incubation times of 0.33 mg of patatin/mL of buffer at pH 7 at 55 °C.

## RESULTS AND DISCUSSION

The aim of this research was to elucidate the mechanism of aggregation by describing the thermal coagulation kinetics of patatin. In addition, combining the knowledge of the thermal aggregation of patatin with that of its thermal stability (Pots et al., 1998a,b) may lead to a better understanding of the structure−function relation of the protein.

**Temperature-Dependent Aggregation.** Aggregation of patatin in buffer at pH 7 was studied in situ at temperatures ranging from 40 to 65 °C using DLS. At these temperatures the intensity autocorrelation functions were measured. In Figure 1 representative autocorrelation functions are shown as obtained at 55 °C. The autocorrelation function obtained after 6 min deviated from those after longer incubation times. This is probably due to convection in the cuvette in the initial state of the measurement. This deviation was not considered significant. It can be seen that the decay became slower with longer heating times; hence, the calculated diffusion coefficient was larger as a function of heating time. From the diffusion coefficient the apparent particle size was calculated with the Stokes−Einstein equation (Berne and Pecora, 1976).

The scattering intensities measured at an angle of 90° and the corresponding apparent Stokes−Einstein radii are shown in Figure 2. It can be seen that patatin at pH 7 did not aggregate at 40 °C, whereas at 50 °C and at higher temperatures the protein coagulated with increasing rate. At 55, 60, and 65 °C a fast initial aggregation was observed. Incubations of up to 68 h at 55 °C showed that the aggregates under these conditions remained soluble and that they continued to increase in size at these long time scales (no further results shown). A relatively small difference, of ∼1 nm on a particle radius of 23 nm, was observed between the radii obtained at 60 and those obtained at 65 °C.

The aggregate radius after 1 h at 65 °C (23 nm, Figure 2) results in a maximum number of 20−25 protein molecules per aggregate, assuming a packing density of ∼2. This implies that only the beginning of the aggregation is studied and that gelation at these protein concentrations is unlikely to occur. Comparable studies on $\beta$-lactoglobulin indicate that aggregates grow to the same order of size after a few hours of incubation (Verheul et al., 1998; Hoffmann et al., 1996). Measurement of the size of the aggregates after rapid cooling to 0 °C after 1 h for each temperature revealed no changes in particle size as compared to the size prior to cooling

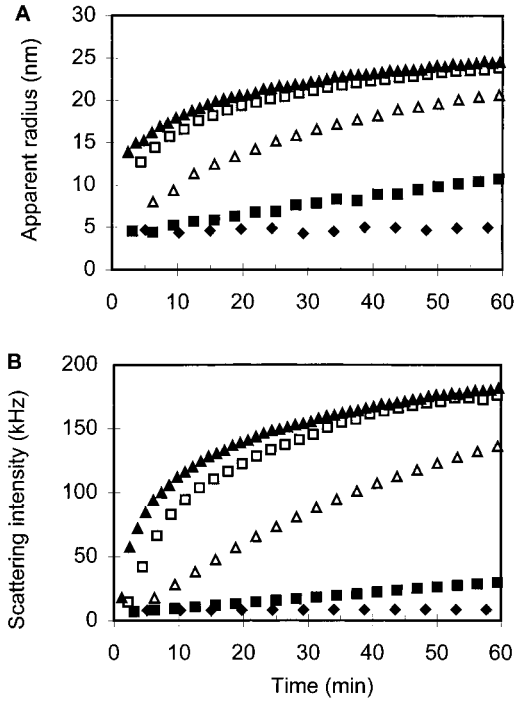


**Figure 2.** (A) Apparent Stokes−Einstein radii of 0.33 mg of patatin/mL at pH 7 at temperatures ranging from 40 to 65 °C. (B) Scattering intensity measured at an angle of 90°. Symbols: 40 °C, ◆; 50 °C, ■; 55 °C, △; 60 °C, □; 65 °C, ▲.

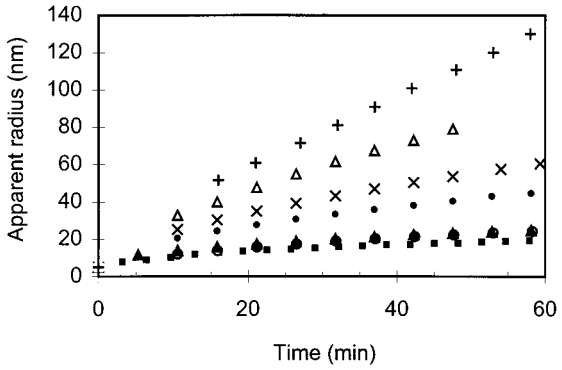


**Figure 3.** Apparent Stokes−Einstein radii of 0.33 mg of patatin/mL at 55 °C at pH 7 at ionic strengths ranging from 35 to 80 mM. Symbols: 35 mM, ■; 42 mM, ○; 50 mM, ▲; 57 mM, ●; 65 mM, ×; 72 mM, △; 80 mM, +.

(no further results shown). This observation indicates that the assumption that the particles did not aggregate further during cooling, as stated in previous work, was allowed indeed (Pots et al., 1999). The observed increase in aggregation rate coincides with the observed loss of α-helical structures of the protein, as determined with far-UV circular dichroism (Pots et al., 1998b).

**Mechanism of Aggregation.** An indication for the mechanism of flocculation can be obtained from the dependence of the aggregation rate on the ionic strength of the medium (Overbeek, 1952). The increase in particle size of patatin during incubation at 55 °C was therefore determined at various ionic strength values at pH 7. In Figure 3 it is shown that the effect of increasing ionic strength is relatively small up to 50 mM and that above 50 mM the aggregation rate increases considerably with the ionic strength.

The Smoluchowski−Fuchs mechanism for slow coagulation of particles presents a dependence of the aggregation rate on the ionic strength (Von Smoluchowski, 1917; Overbeek, 1952). This mechanism describes coagulation limited by both diffusion and reaction. It accounts by a delay or retardation factor *W* for the fact that not all collisions of particles result in permanent contact. Various experiments with colloidal particles other than proteins and model calculations have shown that a logarithmic plot of *W* as a function of the ionic strength can exhibit a linear decrease with the ionic strength and becomes constant at higher ionic strengths [e.g., Reerink and Overbeek (1954) and Amal et al. (1990)]. If the Smoluchowski−Fuchs mechanism applies, the aggregation rate is proportional to the reciprocal value of *W* (Reerink and Overbeek, 1954). Hence, a plot of the reciprocal aggregation rate as a function of the ionic strength could show the same dependence on the ionic strength as *W*. In Figure 4 it can be seen that the logarithm of the reciprocal aggregation rate remains constant up to an ionic strength of 50 mM and exhibits the expected linear decrease with increasing ionic strengths. The constant part of the reciprocal aggregation rate at high ionic strengths was not observed. It is possible that the level of ionic strength where *W* could become independent was not reached. The horizontal line in Figure 4 below 50 mM shows that the aggregation rate is independent of the ionic strength in this range. The decrease of *W* with increasing values of ionic strength, however, is typical for both diffusion and chemically limited aggregation.

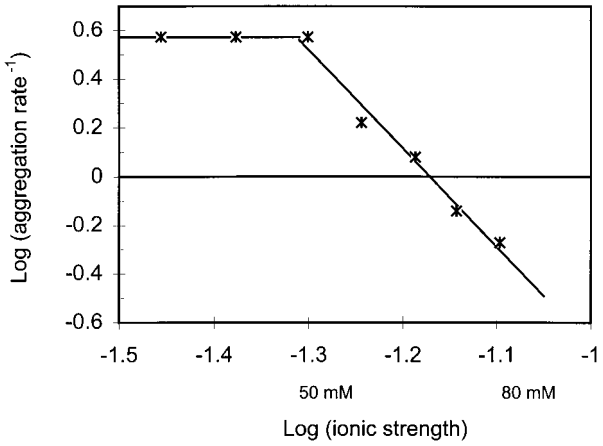


**Figure 4.** Logarithmic plot of the aggregation rate as a function of ionic strength.

If the Smoluchowski−Fuchs mechanism is valid, the kinetics of aggregation should be of second order. To test this, the aggregation rate of patatin at pH 7 at 55 °C was measured at various protein concentrations. Figure 5A shows the apparent Stokes−Einstein radii obtained at patatin concentrations ranging from 0.45 to 2.0 mg of patatin/mL (10.5−46.5 μM). It can be seen that the aggregation rate increased with increasing protein concentration. A plot of the aggregation rate should reveal a linear dependence of the squared protein concentration and it should cross the origin, if the kinetics were of second order. The solid line in Figure 5B is the best linear fit that includes the origin. It can be seen that its course is in reasonable accordance with the Smoluchowski−Fuchs model. In previous work (Pots et al., 1999) second-order kinetics were observed for the aggregation reaction, which was confirmed independently in this paper.

**Chemical Interactions during Aggregation.** To elucidate the nature of the bonds between aggregated patatin molecules, SDS−PAGE analysis was performed

**Table 1. Molar Masses of Unheated Patatin and of Particles Present after Heating under Different Circumstances, Analyzed under Several Dissociating Conditions**

| | native | heated pH 7 | heated pH 7 NEM | heated pH 3 |
|---|---|---|---|---|
| buffer[a] | dimer | aggregate | aggregate | aggregate |
| SDS[b] | monomer (43 kDa) | 43, 82, 108 kDa | 43, 82, 108 kDa | 43, 82, 108 kDa |
| SDS + $\beta$-ME[b] | monomer (43 kDa) | 43 kDa | 43 kDa | 43 kDa |

[a] Data from size exclusion chromatography. [b] In the presence of 6 M urea, data from SDS−PAGE.

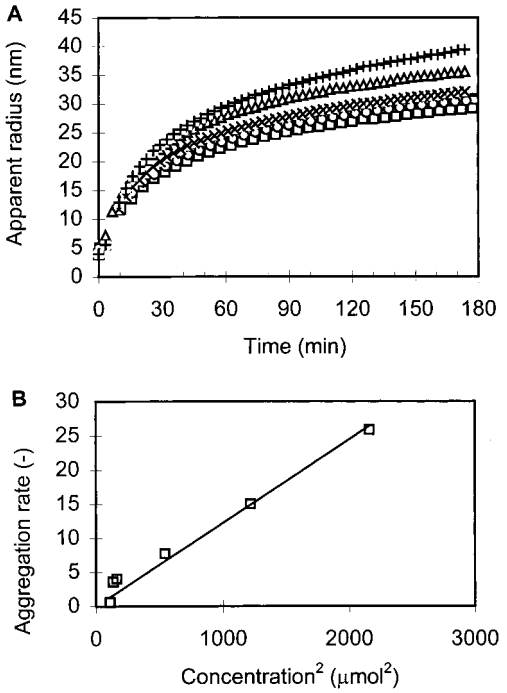


**Figure 5.** (A) Apparent Stokes−Einstein radii of patatin at 55 °C at pH 7 at protein concentrations ranging from 0.45 to 2.0 mg/mL. Symbols: 0.45 mg/mL, □; 0.5 mg/mL, −; 0.55 mg/mL, ○; 1.0 mg/mL, ×; 1.3 mg/mL, △; 2.0 mg/mL, +. (B) Plot of the initial aggregation rate as a function of the squared protein concentration. Solid line is the best linear fit of the data that passes through the origin.

under various conditions. In Table 1 the resulting molecular masses are shown of patatin that was heated under various conditions. Analysis of unheated patatin under nonreducing conditions revealed only a 43 kDa band. SDS−PAGE of heated patatin samples, however, showed under nonreducing conditions three bands, at 43, 82, and 108 kDa, respectively (Table 1). The presence of $\beta$-mercaptoethanol during SDS−PAGE analysis resulted in the dissociation of the 82 and 108 kDa bands into the patatin monomer (43 kDa, Table 1). This suggests that the proteins are connected by sulfur bridges. The bands at 82 and 43 kDa represent most probably dimer and monomer patatin, respectively. The band at 108 kDa could consist of an aggregate, constituted of three or four patatin monomers, that does not migrate in accordance with its molecular mass. Although SDS−PAGE results sometimes can exhibit considerable errors in the estimation of molar masses (Hedrich et al., 1993; Saito and Shimoda, 1997), it seems unlikely that the 108 kDa band originates from a tetramer, because 108 kDa deviates largely from the expected mass of a tetramer (172 kDa). Therefore, it is denoted a trimer from now on. According to the literature (Mignery et al., 1984; Stiekema et al., 1988) patatin contains only one cysteine residue per molecule; therefore, the formation of sulfur-bridge-linked dimers is comprehensible. The formation of the trimer, however, is more difficult to understand. It could be possible that in this trimer the third protein is entrapped by the dimer and that breakage of the dimer using $\beta$-mercaptoethanol results in the release of the third molecule.

The sensitivity to $\beta$-mercaptoethanol suggested that the dimers and trimers are disulfide linked. However, heating in the presence of NEM did not prevent the formation of the di- and trimers. In addition, patatin that was heated at pH 3 did show the 82 and 108 kDa bands on SDS−PAGE analysis and only a 43 kDa band in the presence of $\beta$-mercaptoethanol (Table 1). The intensities of the aggregate bands were similar under all conditions. Therefore, it was thought that the formation of sulfur bridges is not the determining mechanism of the aggregation. Moreover, the sulfur bridges apparently are formed when the proteins have coagulated already and are present in specific configurations. This aggregation mechanism is clearly different from that of the whey protein $\beta$-lactoglobulin where sulfur bridge formation plays a major role during the initial stage of aggregation (Roefs and de Kruif, 1994; Elofsson et al., 1996; Hoffmann and Van Mil, 1997). They both could be representative of typical classes of proteins, one in which sulfhydryl groups and sulfur bonds play an important role in the aggregation and one in which other interactions are rate determining.

**Packing Density of the Aggregates and Form of the Native Particle.** If the aggregation of patatin obeys Smoluchowski−Fuchs kinetics, it must be possible to describe its aggregation behavior with that model (Meakin, 1988; Amal et al., 1990). From the model data the apparent radius of the aggregates can be calculated if the fractal dimension or the packing density is known. Furthermore, the fractal dimension can provide information on the mechanism of the aggregation (Meakin, 1988; Lin et al., 1989). The fractal concept can be used provided the size of the aggregate and that of the monomeric unit are separated by several orders of magnitude (Rouw and de Kruif, 1989). This condition is not met here; therefore, in this study the term packing density will be used and not fractal dimension.

Using SANS it is possible to measure scattering intensities in the wave vector range of 0.05−2 nm$^{-1}$, theoretically enabling the determination of the packing density of patatin aggregates. SANS was performed because the wave vector range as covered using the DLS equipment as described above did not allow the determination of the packing density of the patatin aggregates (Rouw and de Kruif, 1989). Figure 6 shows the SANS spectra of samples containing 0.5 mg of patatin/mL at pH 7 that were unheated and heated for 1 and 10 h at 55 °C, respectively. It can be seen that the scattering data in Figure 6 are rather noisy; nevertheless, the data contain useful information. First, the scattering behavior is highly typical for an aggregating system (Renard et al., 1996; Verheul, 1998), because the sample heated for 10 h showed the highest scattering intensity and the sample heated for 1 h and native patatin induced a lower amount of scattering. The sample heated for 10 h exhibited between −0.1 and 0.1

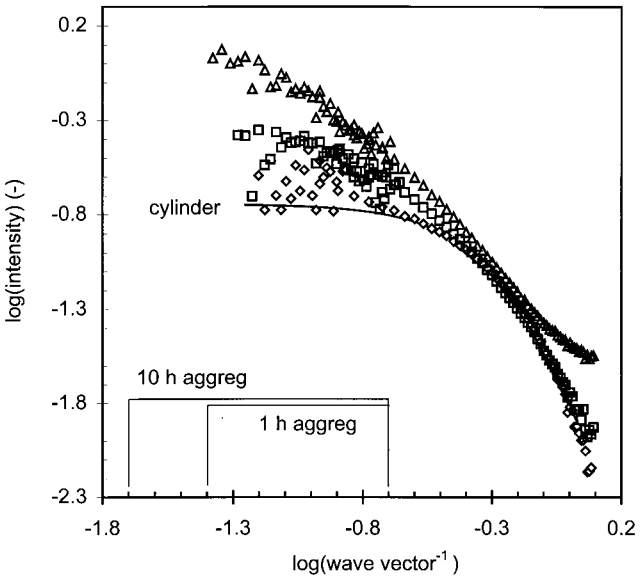


**Figure 6.** SANS spectra measured at ambient temperature of unheated patatin (◇) and patatin at pH 7 heated at 55 °C for 1 (□) and 10 h (△), respectively. Indicated are the limits of wave vector region where the packing density of the aggregates after 1 and 10 h can be obtained as well as the modeled scattering of a cylindrical particle (solid line).

(logarithm of wave vector values) a behavior deviating from that of the unheated sample and the sample heated for 1 h. In this range the scattering of nonaggregated particles is measured. Because aggregation had occurred, it was to be expected that the number of nonaggregated molecules had decreased; hence, the scattering had decreased. The expected decrease in scattering was not observed, which is not understood. In Figure 6 are indicated the regions of the wave vector range from which the packing density of the aggregates can be derived for the samples heated for 1 and 10 h. The samples heated for 1 and 10 h both showed in this area a nonlinear change in scattering intensity; therefore, no accurate measure for the packing density could be obtained.

Second, from SANS measurements information on the shape of the particles can be obtained, by fitting the scattering intensity simulated from a particle with known shape, to the experimental data (Pedersen, 1997). Pedersen presented expressions to simulate scattering for differently shaped particles. We found that the scattering of native patatin could be modeled satisfactorily ($\chi^2 = 1.3$) by assuming a cylindrical particle having a radius of 2.5 nm and a length of 9.8 nm (Figure 6). It can be seen that the model accurately describes the scattering from 0.1 to −0.7, whereas below this value the predicted intensity is too low. From DLS measurements a spherical particle with a radius of 4−5 nm was derived (Figure 2), which correlates well with the size of the cylinder as obtained from SANS (Figure 6).

In conclusion, this work showed that the aggregation of patatin could follow the mechanism of slow coagulation of the Smoluchowski−Fuchs theory. The aggregation rate dependence on the protein concentration was in accordance with that mechanism. The aggregation rates as obtained at temperatures ranging from 40 to 65 °C correlated well with unfolding of the protein at a secondary level. SDS−PAGE analysis suggested that the formation of sulfur bridges is not the determining process for the aggregation of patatin. DLS measurements, assuming a spherical shape, indicated that native patatin has a radius of 4−5 nm, which was in good accordance with the results of SANS, which suggested a cylindrical particle with a radius and length of 2.5 and 9.8 nm, respectively.

## ABBREVIATIONS USED

SANS, small-angle neutron scattering; NEM, *N*-ethylmaleimide; DLS, dynamic light scattering; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; *W*, retardation factor; DLA, diffusion-limited aggregation; CLA, chemically limited aggregation.

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