Kinetic Modeling of the Thermal Aggregation of Patatin

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A kinetic model of the thermal aggregation of patatin is presented based on chromatographic analysis of the proportions of nonaggregated and aggregated patatin. It was observed that the decrease of the amount of nonaggregated patatin proceeded initially quickly and was followed by slower aggregation at longer incubation times. It was shown that this behavior was not due to heterogeneity of the starting material. It was noted that overestimation of the amount of native molecules after a heat treatment, caused by refolding of the unfolded protein during the cooling step prior to the analysis, was significant and could not be neglected. Hence, corrections based on information on the structural properties of patatin were applied. Taking this into account, a model was proposed consisting of a first-order formation of reactive particles, followed by a second-order aggregation reaction. This model described the thermal aggregation of patatin rather accurately and was confirmed by experiments at various protein concentrations.

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

INTRODUCTION

Potato proteins are of interest as ingredients for fabricated foods because they exhibit promising functional properties in the undenatured state, such as foam and emulsion forming and stabilizing capacity (Holm and Eriksen, 1980; Wojnowska et al., 1981; Jackman and Yada, 1988) and they have a high nutritional value (Kapoor et al., 1975; Liedl et al., 1987). The potato contains 1.5−2.0% (weight/fresh weight) protein. Its protein is a byproduct of the potato starch industry, in industrial processes recovered by a combined acid and heat treatment of the so-called potato fruit juice resulting in irreversibly precipitated proteins. These precipitates have lost functional properties and, consequently, they can be applied only as a low-value feed. Despite work performed to obtain undenatured potato proteins, an industrial method has not been developed so far (Lindner et al., 1980; Ahldén and Trägårdh, 1992).

The rationale of studying the thermal behavior of a protein such as patatin, the major potato protein, is that many studies indicate that each class of proteins has its specific aggregation behavior (Hohlberg and Stanley, 1986; Kella, 1989; Bacon et al., 1989; Roefs and de Kruif, 1994; 1996; Boyer et al., 1996). Patatin, as a plant storage protein that is low in cysteine, could be a typical example of such a class of proteins. In addition, knowledge of the temperature-dependent behavior of the protein might help to establish the link between the observed irreversible precipitation in the potato fruit juice and the applied heat treatment. This could lead to the design of alternative procedures of isolation of potato proteins, which allow the protein to resolubilize and thus enable novel food applications comparable to, for example, those of storage proteins of oats and legumes. These proteins are used in the food industry for their functional properties, such as their capacity to form and stabilize foams and emulsions and to form gels (Damodaran, 1997; Oakenfull et al., 1997). A gel, that is, a continuous protein network, can be formed upon prolonged aggregation under suitable conditions (Damodaran, 1997). Aggregation occurs usually upon (thermal) denaturation of proteins (Damodaran, 1997; Catsimpoolas and Meyer, 1970), and the mechanism of aggregation determines the type, hence, the properties, of the gel. To establish the aggregation mechanisms for various classes of (food) proteins, fundamental knowledge of the physicochemical properties of these proteins must be combined with an understanding of the molecular aggregation mechanism. This would provide means to optimize the aggregation and subsequent gelation, leading to gels that better meet the desired functionality in food systems.

Patatin is the most abundant potato tuber protein, accounting for ∼40% of the soluble tuber proteins (Racusen and Foote, 1980). The apparent molar mass of patatin determined by SDS−PAGE is 43 kDa, whereas in media without SDS or urea it appears as a dimer with an apparent molar mass of ∼80 kDa (Racusen and Weller, 1984). Patatin, in fact, consists of glycoproteins encoded by two multigene families, one of which is expressed only in the tuber in relatively large quantities (Park et al., 1983; Pikaard et al., 1987; Sonnewald et al., 1989). Recently, it was shown that these isoforms behave uniformly under various conditions; hence, they can be studied as a single protein species (Pots et al., 1999a). Patatin does not contain extended clusters of hydrophobic or charged amino acids, and only one

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cysteine residue is present (Mignery et al., 1984; Stiekema et al., 1988). The molecular properties of patatin during heat treatment under various conditions have been described (Pots et al., 1998a,b).

In the present work chromatographic analysis of the amounts of nonaggregated and aggregated patatin as a function of incubation time and temperature is presented. This enabled kinetic modeling of the aggregation rate of the protein.

EXPERIMENTAL PROCEDURES

Preparation of Patatin Solutions. Patatin was purified from *Solanum tuberosum* cultivar Bintje as described elsewhere (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 by the Bradford assay (Bradford, 1976) using bovine serum albumin (Sigma, A-4511; St. Louis, MO) as standard.

Heating Experiments. In closed glass tubes (13 × 100 mm Kimax culture tube, Kimble Glass Inc.) 0.2 mL solutions containing 0.1−1.5 mg of patatin/mL of buffer (pH 7) were incubated in a water bath at temperatures ranging from 40 to 65 °C (±0.5 °C; temperature is reached within 30 s). The samples were heated for times ranging from 2 min to 24 h and stored on ice prior to and immediately after heating (temperature of the sample below 5 °C within 30 s). After heat treatment, the samples were analyzed at 20 °C to determine the proportions of nonaggregated and aggregated protein as a function of incubation time using gel filtration chromatography. No corrections were made for the times required to reach the incubation temperature and to cool the samples.

Analytical Gel Filtration Chromatography. Chromatographic analysis of heat-treated samples, loaded via a 50 μL loop, was performed using a Smart System (Pharmacia Biotech, Uppsala, Sweden). Columns used were Superose 6 and Superdex 75, but generally a Superdex 200 column (all 3.2 × 300 mm; Pharmacia Biotech), equilibrated and run at 20 °C and 80 μL/min in the above-described pH 7 sodium phosphate buffer. The Superdex 200 and Superose 6 columns were calibrated using blue dextran (MW = 2×10^3 kDa), thyroglobulin (667 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (BSA; 67 kDa), and sodium ascorbate (176 Da). The Superdex 75 column was characterized using blue dextran, BSA, and sodium ascorbate. Detection of protein was at 280 nm. Changes in the amounts of aggregated and nonaggregated protein after a heat treatment were negligible for at least 24 h of storage. Therefore, analysis of the heated samples was performed on the day of the heat treatment.

Preparative Gel Filtration Chromatography. A patatin sample (0.5 mg/mL) that had been incubated for 30 min at 55 °C in a 22 mM sodium phosphate buffer (pH 7) was subsequently injected at 20 °C via a 2 mL loop and fractionated using a Superose 6 column (1 × 30 cm) attached to an FPLC system (Pharmacia Biotech). The column was equilibrated and run at 20 °C with a flow rate of 0.5 mL/min using a 22 mM sodium phosphate buffer (pH 7). Detection of protein was at 280 nm, and fractions of 1 mL were collected. Fractions containing nonaggregated and aggregated proteins were pooled and analyzed by capillary electrophoresis (CE) as described before (Pots et al., 1999b). The heated, nonaggregated protein fraction was subjected to a second heating experiment at 55 °C.

Kinetics of Heat-Induced Aggregation. The amount of protein present in the aggregated and nonaggregated fraction was determined by integrating the corresponding peak area after gel filtration. The peak areas of nonaggregated and aggregated patatin were expressed relative to the peak area of unheated patatin [the latter denoted as $N(0)$]. To determine the rates of the changes in nonaggregated and aggregated proteins, kinetic models were fitted to the data by applying the mathematical program Matlab for Windows (version 4.2c1; The Mathworks, Inc., Natick, MA), making use of a Levenberg−Marquardt procedure (Press et al., 1986). A Gear algorithm was applied to numerically solve differential equations describing the kinetic processes (Stoer and Bulirsch, 1980). Monte Carlo simulations were performed by assuming an experimental error of on average 10%. To estimate the necessary number of simulations 100, 200, and 400 repetitions were performed and the sizes of the resulting 90% confidence intervals were compared (Alper and Gelb, 1990).

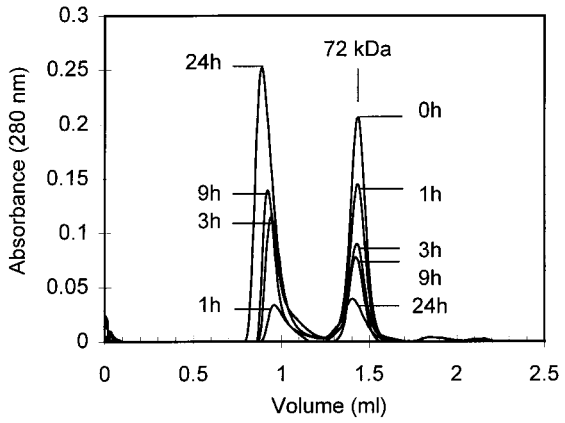

Figure 1. Gel filtration (Superdex 200) chromatograms as measured at 20 °C after incubation for various times at 50 °C of 0.33 mg of patatin/mL of 22 mM sodium phosphate buffer (pH 7). Indicated are the apparent molar mass of the nonaggregated protein and the heating times. The included and excluded volumes of the column were 0.9 and 2.1 mL, respectively.

RESULTS AND DISCUSSION

The aim of this work was to describe the thermal aggregation of patatin in terms of a kinetic model. Although numerous papers describe the thermal denaturation and subsequent aggregation and gelation of proteins of various origins, a description of the underlying mechanism is usually not presented (Hohlberg and Stanley, 1986; Kella, 1988; Bacon et al., 1989; Boyer et al., 1996; Elofsson et al., 1996; Sathe and Sze, 1997). Only a few papers propose a kinetic model based on the occurring reactions [e.g., Roefs and De Kruif (1994)]. To establish general rules for the thermal denaturation and aggregation behavior of various classes of (food) proteins, a description of the physical and chemical mechanism of unfolding and aggregation combined with knowledge on the physicochemical properties of the proteins is essential. In that respect, patatin could, as a supposed multidomain glycoprotein (Pots et al., 1998b) with only one cysteine residue (Mignery et al., 1984), provide an example of a class of proteins exhibiting a typical denaturation and aggregation behavior.

Temperature Dependence of the Aggregation. Representative gel filtration elution profiles of patatin that had been heated for various times at 50 °C are shown in Figure 1. Nonaggregated protein eluted as a dimer of ∼72 kDa, as expected (Racusen and Weller, 1984). Upon incubation for a defined period of time at 50 °C or higher, a second peak appeared at shorter retention times, as an aggregate with an apparent molar mass of at least 1300 kDa. In addition, the area of the nonaggregated patatin peak decreased concomitantly. Interestingly, no distinct peaks were observed between 1.0 and 1.4 mL elution volumes, indicating that hardly any particles with apparent masses between about 80 and 1300 kDa were present. It may be that during the

incubation relatively small aggregates were formed but that these particles are highly reactive and aggregated rapidly to larger and more stable particles, which were detected after cooling.

After 24 h at 50 °C (Figure 1) the peak area of the aggregated protein was 1.5 times that of the corresponding amount of unheated native patatin. A similar effect could be observed after 3 h at 55 °C (no further results shown), for which the peak area of aggregated protein was 1.1 times that of the native protein. This may be due to an increase in the absorbance coefficient of the protein. Patatin contains two tryptophan residues that are stacked in the native state. This configuration is lost after unfolding (Pots et al., 1998a,b), which may result in an increased absorbance coefficient (Schmid, 1989). This increase in optical density could also be caused by an increased level of scattering of the incident light. At 55 °C, however, the peak area of the aggregated protein decreased and it virtually disappeared after longer incubation times (6−24 h). Because visually no precipitation was observed, this may have been caused by adsorption of particles to the column material or by particles that were too large to elute from the column. Dynamic light scattering measurements of patatin under comparable conditions indicated that the maximum radius of the particles present (measured both at 65 °C and after cooling) after 1 h was ∼24 nm (no further results shown), which should allow elution in the excluded volume of the Superdex 200 column. However, these opposite effects were negligible for incubation times up to 1 h. Similar aggregation experiments were performed at temperatures from 40 to 65 °C. The resulting amounts of aggregated and nonaggregated patatin are depicted in Figure 2, in which the quantities of protein are expressed as proportion of the peak area of the unheated patatin.

It can be seen in Figure 2 that patatin at 40 °C aggregated at a negligible rate at the indicated time scales and conditions, whereas at temperatures exceeding 50 °C the protein coagulated at increasing rates. The initial fast decrease of nonaggregated patatin was followed by a slower decrease at longer incubation times. Samples heated at 50 and 55 °C (Figure 2B) were measured for 24 h but for clarity are shown only up to 10 h. When heated at 50 °C the residual amount of nonaggregated protein decreased to ∼10% after 24 h, whereas the sample heated at 55 °C contained virtually only aggregated protein after 10 h.

Homogeneous Behavior of the Patatin Sample. The fast initial decrease and the slower subsequent decrease suggest either heterogeneity of the starting material or multiple rate-limiting steps in the unfolding and subsequent reactions (Creighton, 1990). The former seemed the most obvious option to be examined, because it is known that patatin consists of various isoforms (Park et al., 1983; Pikaard et al., 1987). To test for heterogeneity in the patatin sample, nonaggregated protein was separated from the aggregated protein after the sample had been heated for 30 min at 55 °C. Next, the nonaggregated protein was heated again, and the aggregation rate of this heated, nonaggregated patatin fraction was determined and compared to the behavior of nonpreheated protein. The aggregation kinetics of the heated, nonaggregated protein were similar to those observed at 55 °C for the non-preheated protein, showing again an initial fast phase followed by a slower one (no further results shown). Furthermore, comparison of

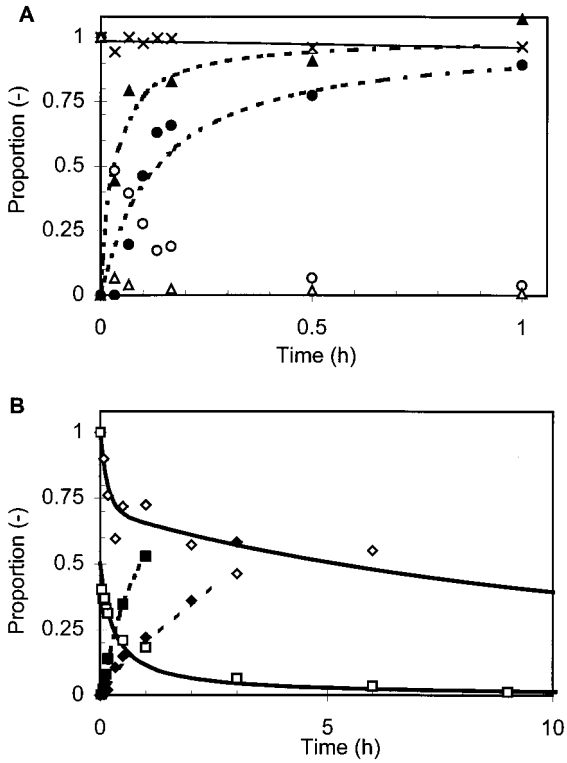

Figure 2. Proportion of nonaggregated and aggregated patatin at temperatures ranging from 40 to 65 °C as a function of time: (A) temperatures 40, 60, and 65 °C, depicted up to 1 h; (B) temperatures 50 and 55 °C, depicted up to 10 h. Nonaggregated and aggregated proteins are represented by open and solid symbols, respectively: 40 °C, ×, aggregate not present; 50 °C, ◇, ◆; 55 °C, □, ■; 60 °C, ○, ●; 65 °C, △, ▲. Fits of these points are depicted by full lines (decrease of nonaggregated patatin) and dashed lines (increase of aggregated protein).

electropherograms (CE) of heated, nonaggregated protein and nonheated protein revealed that all isoforms (Pots et al., 1999a) were present in the same peak ratios in both samples (results not shown). All of these results indicate that the patatins behave as a homogeneous system and that the behavior as observed in Figure 2 is due to a more complex reaction mechanism rather than to heterogeneity of the starting material.

Modeling of the Aggregation. Assuming a single-step reaction and first-, second-, or *n*th-order kinetics (Van Boekel, 1996), fitting of the decrease of the amount of nonaggregated patatin gave poor (unacceptable) fits for the data at 50 and 55 °C. In addition, a model consisting of a single-step first-order formation of reactive particles and a second-order aggregation reaction did not fit the data: In the models tested, the distribution of the residuals was not random (results not shown). Therefore, more complex reaction models were examined (Lumry and Eyring, 1954; Pyun, 1971; Sadana, 1991; Schokker and Van Boekel, 1997). The simplest plausible model, consisting of two consecutive reactions, is presented in Scheme 1, in which, besides the reactions, also the corresponding differential equations are given. This model describes the formation of reactive particles R (1), followed by an aggregation reaction of reactive particles (2).

The formation of these reactive particles itself is most likely also a process that consists of two steps: first, a rapid equilibrium unfolding, which may be followed by a slower conformational change that results in the formation of the reactive particle R. This is a general

Scheme 1. Proposed Model of the Aggregation of Patatin with Its Corresponding Mathematical Equations[a]

$$N \underset{\leftarrow}{\overset{K}{\rightleftharpoons}} U \xrightarrow{k_1} R \qquad (1)$$

$$R_p + R_q \xrightarrow{k_2} R_{(p+q)} \qquad (2)$$

$p,q \in \{1,2,3...\}$

$$\frac{d([N]+[U])}{dt} = -\frac{k_1}{1+1/K}([N]+[U])$$

$$\frac{d[R_{(p+q)}]}{dt} = k_2([R_p]+[R_q])^2$$

[a] K represents the equilibrium constant between the native and the unfolded molecule, k_1 is the reaction constant of the formation of the reactive particle R from the unfolded state U. The aggregation of the reactive particle is summed in eq 2.

model describing the heat denaturation of proteins (Lumry and Eyring, 1954). It was proposed because modeling of the data assuming a reversible reaction 1 resulted in reaction rates that were too slow to account for unfolding only (reaction half-times up to 396 s; no further results shown). In addition, from previous work (Pots et al., 1998a,b) it is known that the thermal unfolding of patatin is a rapid process. Therefore, it is proposed that the observed reaction rate, k_1 in Scheme 1, is the overall resulting reaction rate for the formation of reactive particles that takes the proportion of unfolded (U) and native patatin (N) into account by the equilibrium constant K (Scheme 1), the ratio of unfolded-to-native protein. The reactive state R may consist of a mixture of unfolded conformations, because generally the increased conformational freedom of unfolded proteins results in a number of unfolded molecules with differing structures (Creighton, 1990; Pots et al., 1998a,b). In previous work, however, it was observed with differential scanning calorimetry that patatin has a relatively small enthalpy of unfolding (~20 kJ/mol) and that a large part of the protein remains structured upon a thermal treatment (Pots et al., 1998a). Therefore, the presence of strongly differing unfolded species of the unfolded patatin is considered unikely.

The second reaction (eq 2 in Sheme 1), as an aggregation reaction typically of second order, presents an averaged ensemble of simultaneously occurring reactions. The second-order aggregation reaction could be of a more complex nature. Nevertheless, a single-step second-order reaction seems to describe the behavior of the protein adequately.

The correctness of a kinetic model can be tested by fitting it to data describing both the decrease of reactants and the increase of products as a function of time. Moreover, the temperature and the concentration dependence must be consistent with theory and the prerequisites of the model. Furthermore, a model implying the lowest possible number of parameters is preferred and the residuals must be randomly distributed over the whole time range (Van Boekel, 1996).

Using a least-squares fit procedure the data were fitted to the differential equations. It should be noted, however, that the proportion of nonaggregated protein (N) at any time will be an overestimate because it was measured as the sum of the fraction of native protein plus the fraction of unfolded protein (U) that had refolded to N upon cooling. Similarly, the amount of aggregated protein ($R_{(p+q)}$; Scheme 1) is overestimated because it is based on the sum of aggregated protein plus the fraction of R that aggregates to $R_{(p+q)}$ during cooling. Most of the studies in the literature neglect or merely note the overestimation of the amount of native protein due to the reversible character of the unfolding [e.g., Manji and Kakuda (1987), Levieux et al. (1995), and Verheul et al. (1998)]. When we applied the model without the assumptions correcting for the overestimation of native protein, an adequate fit of the data and a consistent dependence on temperature and concentration were obtained (χ^2 values ranging from 0.01 to 0.015; further results not shown). Nevertheless, this is known to be incorrect because the native protein content determined at room temperature is not equal to the native protein content at the reaction temperature. Therefore, the overestimation was corrected by using our knowledge of the thermal stability of patatin (Pots et al., 1998a,b). The following assumptions were made: The amount of U at 50 °C was estimated as 5% of the nonaggregated protein present, because previous work indicated that ~5% of the protein was unfolded at that temperature (Pots et al., 1998b). This results in a K value of 0.05 (Scheme 1). The midpoint of unfolding of patatin at pH 7 is at 55 °C; therefore, at this temperature the amount of U must equal that of N, and, hence, K should equal 1. To obtain the correct measure for the proportion of N during the heat treatment at 55 °C, the measured amount of N was multiplied by 0.5. These corrected data are presented in Figure 2. Finally, it was assumed that at 60 and 65 °C virtually all protein was in the R form (Pots et al., 1998b). This would imply that the increase of $R_{(p+q)}$ at these temperatures could be fitted accurately with a single-step second-order reaction (reaction 2 in Scheme 1). At all temperatures the amount of R aggregating to $R_{(p+q)}$ during cooling is neglected. This is allowed because it was shown that virtually no aggregation occurred at 40 °C or below (Figure 2A) and because the samples were cooled to below 5 °C within 30 s. As illustrated by the full and dashed lines in Figure 2B, this model describes sufficiently (χ^2 values ranging from 0.01 to 0.035) the temperature-dependent decrease of the primary particle and the growth of the aggregates at 50 and 55 °C. Due to the assumption that all protein would be in the reactive state at 60 and 65 °C, only the growth of the aggregates had to be modeled at these temperatures (Figure 2A). Therefore, only k_2 could be obtained at 60 and 65 °C. The fit of the aggregation at 60 °C seemed not as good as the fit at 65 °C. The confidence intervals as obtained with this procedure are greatly overestimated, due to a large standard deviation and a small number of degrees of freedom (Arabshahi and Lund, 1985; Cohen and Saguy, 1985). A better estimate of the confidence intervals can be obtained by a Monte Carlo simulation procedure (Alper and Gelb, 1990). Assuming an experimental error of 10%, 200 and 400 repetitions gave essentially the same confidence intervals; therefore, 200 simulations were performed. In Table 1 the so-obtained reaction rates, with their 90% confidence intervals, are given. The obtained correlations between the parameters at 50 and 55 °C were 0.977 between k_1 and k_2. This value indicates that the problem of parameters correlating too strongly does not occur here. Correlating parameters is a problem that often occurs

Table 1. Reaction Constants with Their 90% Confidence Intervals as a Function of the Temperature

temp (°C)	k_1 (s^{-1}) [90% ci]	k_2 ($L \cdot mol^{-1} \cdot s^{-1}$) [90% ci]
50	1.75×10^{-3} [1.6×10^{-3}–3.3×10^{-3}]	149.3 [76.6–253.2]
55	3.5×10^{-2} [2.1×10^{-2}–5.0×10^{-2}]	144.5 [133.8–195.9]
60	nd[a]	274.7 [249.6–296.2]
65	nd	1205.2 [1016.5–1379.6]

[a] Not determined.

in kinetic analysis because the experimental range of temperatures is small as compared to the absolute temperature range (Bates and Watts, 1988; Van Boekel, 1996).

It was observed that a conformational change of the protein is essential to allow aggregation (Pots et al., 1998b; Figure 2). As a result of unfolding, hydrophobic or otherwise reactive parts of the protein molecule will become available to interact with other molecules. It is known from previous work (Pots et al., 1998a) that patatin unfolds partly but rapidly during an increase in temperature and that the secondary structure content remains constant when the temperature is kept constant. The model presented in Scheme 1 implies, however, a continuing decrease of the amount of native particles N, by reaction of R to aggregates at a constant temperature. This suggests that the reactivity in the unfolded state R would not primarily depend on the change in the amount of secondary structure but on other structural changes of the protein. Although the model can be used to describe the thermal behavior of patatin and physicochemical information from previous work was used to explain the observed behavior, the model does not provide detailed insight into the physical mechanism of the aggregation.

In Figure 2 it was shown that the model fits the results mathematically reasonably well. Because only at 50 and 55 °C could a value for the temperature dependence of k_1 be obtained, it is difficult to interpret the values for the reaction constants. According to Table 1 k_1 would strongly increase with increasing temperature. The confidence intervals of k_1 and k_2 as shown in Table 1 are fairly large, and it is possible that k_1 and k_2 are underestimated and overestimated, respectively. The reaction constant k_2 exhibits no significant differences up to 60 °C to show a strong increase at 65 °C. From step 2 in Scheme 1, one would expect a gradual increase of k_2 with rising temperatures. The fact that this behavior is not observed may suggest that the proposed model does not represent the entire mechanism. It may be possible, for example, that the polydispersity of the system increases with temperature, resulting in an increased aggregation rate (Overbeek, 1952). Another possible reason could be that the sticking probability, that is, the probability that two colliding particles become and remain aggregated, of the aggregation reaction changes with temperature. Step 2 in Scheme 1 neglects differences between aggregation rates of, for example, primary particles with multiple ones and the multiple ones among themselves. The theory of Smoluchowski–Fuchs does take these reactions into account (Overbeek, 1952) but did not provide a better fit of the measured data than our model (no results shown). Therefore, we did not use the Smoluchowski–Fuchs approach and applied the reaction as presented in step 2 of Scheme 1. Nevertheless, it should be noted that the aggregation mechanism is of a more complex and not fully understood nature.

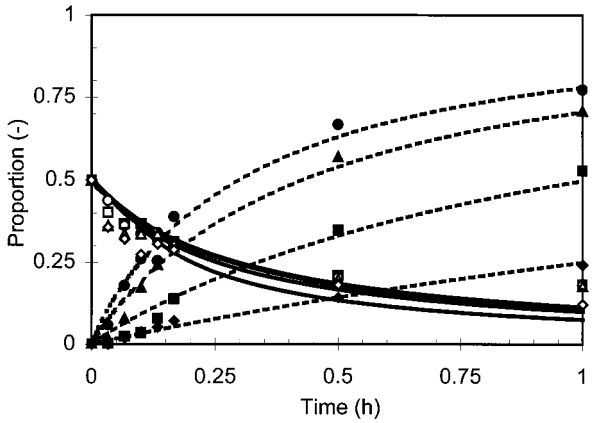

Figure 3. Concentration dependence of the decrease of nonaggregated and the increase of aggregated patatin, determined at 55 °C. Protein concentrations (nonaggregated and aggregated proteins are represented by open and solid symbols, respectively): 0.1 mg/mL (2.3 μM), ◇; 0.33 mg/mL (7.7 μM), □; 1.0 mg/mL (23.2 μM), △; 1.5 mg/mL (34.9 μM), ○. Fits are represented by lines (aggregated protein, dashed lines; nonaggregated protein, solid lines).

Table 2. Reaction Constants with Their 90% Confidence Intervals as a Function of Protein Concentration

concn (μM)	k_2 ($L \cdot mol^{-1} \cdot s^{-1}$) [90% ci]
2.3	155.3 [143.8–167.2]
7.7	159.0 [147.3–175.1]
23.2	115.9 [105.1–126.6]
34.9	113.8 [103.8–125.0]

Concentration Dependence of the Aggregation. The reaction rate of the aggregation (step 2, Scheme 1) should exhibit a quadratic dependence of the reactant concentration as implied by its second-order kinetics, whereas the reaction constant of aggregation should, by definition, be independent of the protein concentration (Sadana, 1991). To test this, the concentration dependence of the aggregation was examined at 55 °C. In Figure 3 the decrease of nonaggregated and the increase of aggregated patatin are shown at protein concentrations ranging from 0.1 to 1.5 mg/mL (2.3–34.9 μM). Peak areas of both fractions were normalized for each concentration using the peak area of the amount of unheated native protein. The peak area of nonaggregated protein was corrected by a factor 0.5, as discussed above. The values of the reaction constants k_1 and k_2, as obtained at 55 °C in the experiments dealing with the temperature dependence of the reactions, were used here, and only k_2 was allowed to vary. It can be seen that the model fits the results and that the curves of the decrease of native patatin coincide (Figure 3). Therefore, it cannot be concluded that the relative decrease of native particles was dependent on the protein concentration; it is thus unlikely that it is a first-order reaction. It was observed that the aggregation constant was not strongly dependent on the protein concentration (Table 2), whereas the aggregation rate, as determined from the initial tangent, clearly increased with increasing protein concentration.

Summarizing, a model was proposed for the thermal aggregation of patatin at pH 7 that consisted of two steps: first, the formation of reactive particles, which is followed by a second-order aggregation reaction. It was observed that the overestimation of the amount of native molecules after a heat treatment, due to refolding of the unfolded protein during the cooling step prior to the analysis, was significant and could not be neglected.

This overestimation was corrected by using the information on the structural information on patatin, as reported previously (Pots et al., 1998a,b). The resulting model described the thermal aggregation of patatin reasonably well.

ABBREVIATIONS USED

ci, confidence interval; CE, capillary electrophoresis; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MW molecular weight.

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