Thermal Stabilities of Lupin Seed Conglutin γ Protomers and Tetramers

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Various experimental approaches have been used in this work to assess the thermal stabilities of lupin seed conglutin γ at two pH values, 4.5 and 7.5, at which the protein exists as a protomer and a tetramer, respectively. The patterns of thermal unfolding at the two pH values differed significantly; the tetramer aggregated and became insoluble, whereas the protomer was still soluble after thermal treatment. Also, the midpoint transition temperatures were dramatically different, being 60.3 and 75.1 °C for the protomer and tetramer, respectively. The behavior of conglutin γ at neutral pH was also affected by disulfide formation/interchange, in that some unfolded protein molecules became covalently stabilized. More detailed analyses by differential scanning calorimetry and indirect fluorescence measurements, using 8-anilino-1-naphthalenesulfonic acid as a probe, confirmed the remarkable differences observed in the thermal stabilities of the two protein forms and allowed models for their unfolding patterns to be drawn.

Keywords: *Conglutin γ; Lupinus albus; oligomeric protein; thermal stability*

INTRODUCTION

In mature lupin (*Lupinus albus*) seeds, conglutin γ accounts for ∼5% of the total proteins (Duranti et al., 1981). Conglutin γ is a protomer of 46 kDa consisting of one glycosylated 29 kDa polypeptide chain disulfide-linked to one of 17 kDa, as assessed by laser mass spectrometry (Duranti et al., 1996). At neutral to slightly alkaline pH values, four protomers associate to a tetramer of relative mass ∼200 kDa. The equilibrium between protomers and tetramer is strongly pH-dependent, in that complete dissociation occurs at pH 4.5, at which only protomers are present (Duranti et al., 1996).

Conglutin γ is constitutively expressed in developing lupin seeds, where it is deposited inside the protein bodies along with the seed storage proteins (Duranti et al., 1991). Conglutin γ shares partial amino acid sequence (Kolivas and Gayler, 1993; Scarafoni et al., 1998) homology with a few other plant proteins, namely, soybean 7S basic globulin (Kagawa et al., 1989), a 57 kDa carrot glycoprotein (Satoh et al., 1992), and an antifungal cotton glycoprotein (Chung et al., 1997). Controversial results have been obtained with these proteins as far as their biological activity is concerned (Satoh et al., 1992; Duranti et al., 1994a; Komatsu et al., 1994; Chung et al., 1997). To date, no physiological role of these homologous proteins has clearly been assessed.

The interest in conglutin γ relies on its several unusual properties: it is not a storage protein, being undegraded during germination (Duranti et al., 1994a; Esnault et al., 1996); it is resistant to in vitro proteolysis when in its native conformation (Duranti et al., 1995); it has a far better essential amino acid profile than the other major lupin seed proteins (Duranti et al., 1981); and it is newly synthesized and secreted from lupin seeds incubated in water at 60 °C for 3 h (Duranti et al., 1994b). This latter finding, which might have not only physiological but also nutritional and technological implications, has prompted us to investigate the thermal stability of conglutin γ at the two pH values that affect the levels of association of the protein.

To this purpose, various experimental approaches, including size exclusion HPLC (SE-HPLC), SDS−PAGE, transverse temperature gradient gel electrophoresis (TTGGE), differential scanning calorimetry (DSC), and indirect spectrofluorometry with ANS have been used to investigate the thermal stability of conglutin γ and to compare the denaturation behaviors of the protomeric and tetrameric forms of this protein.

MATERIALS AND METHODS

Plant Material. Seeds of the sweet Multitalia variety of *L. albus* L. were kindly provided by Prof. L. Postiglione and Dr. M. Fagnano (University of Naples, Italy).

Purification of Conglutin γ from Mature Lupin Seeds. Conglutin γ was purified according to the method of Duranti et al. (1995) by using a combination of anion and cation exchange chromatography. Homogeneity of conglutin γ preparations was checked by SDS−PAGE (see below).

Protein concentration of conglutin γ solutions was determined spectrophotometrically with the Bradford method (Bradford, 1976) or by absorption at 280 nm using the absorption coefficient of 1 for 1 mg/mL concentration (M. Duranti, unpublished results).

Thermal Treatment of Conglutin γ. Conglutin γ, dissolved in 50 mM Tris-HCl, pH 7.5, or 50 mM sodium acetate, pH 4.5, both containing 0.1 M NaCl, at a protein concentration ranging from 0.3 to 1 mg/mL, was heated in a thermostated

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bath (Paratherm Julabo, Germany) at selected temperatures for the times indicated. After the treatments, the solutions were cooled in an ice bath and immediately submitted to further analyses as described below. In case of protein aggregation, the soluble fraction was recovered by centrifugation at 8000*g* for 10 min at 4 °C.

SE-HPLC. SE-HPLC of conglutin γ was carried out on a Waters HPLC model 625 with a Superose 12 HR 10/30 column equilibrated with the same buffers used for the thermal treatments. A calibration curve for M_r determination was set with the following protein markers: thyroglobulin (670 kDa); β amylase (200 kDa); glucose oxidase (180 kDa); alcohol dehydrogenase (150 kDa); transferrin (76 kDa); bovine serum albumin (67 kDa); egg albumin (45 kDa); carbonic anhydrase (29 kDa); trypsin inhibitor (20 kDa); myoglobin (18 kDa); cytochrome *c* (12 kDa); and aprotinin (6.5 kDa). The correlation coefficient of the curve was −0.99.

Electrophoretic Techniques. SDS−PAGE was carried out as described by Laemmli (1970) on 12.5% polyacrylamide gels. For runs under reducing conditions, 2% 2-mercaptoethanol was added to the sample denaturing buffer. Polypeptide bands were stained with Coomassie blue R250. The M_r of the polypeptides was determined by comparison with a standard protein solution (Pharmacia) containing phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa).

For the TTGGE experiments, a modification of the method described by Rosenbaum and Riesner (1987) was used. The main differences consisted in the use of a 3 mm thick 4.2% polyacrylamide gel at pH 4.5 and in the exposure time to the temperature gradient, which lasted only 10 min, after which the run was carried out at 12 °C. The experimental temperatures were monitored with a stick thermometer by direct immersion in the polyacrylamide gel.

Calorimetric Study. DSC measurements were made with a Microcal VP microcalorimeter, at heating rates of 1 and 1.5 °C/min. The DSC curves were corrected for an instrumental baseline obtained by heating the buffer, and the large negative peak at temperature >70 °C, due to the exothermic protein aggregation, was subtracted by hand-drawn baseline. Melting temperatures, calorimetric and van't Hoff enthalpies, and deconvolution analysis were made by using the software provided by the instrument manufacturer (Microcal). Protein concentrations varied from 0.56 to 0.21 mg/mL for the protomer and from 0.72 to 0.2 mg/mL for the tetramer, and molar enthalpies were referred to the molar concentration of protomer and tetramer, respectively.

Indirect Spectrofluorometry. The hydrophobic probe 8-anilino-1-naphthalenesulfonic acid (ANS) was used in this study to follow heat denaturation of conglutin γ. A Perkin-Elmer LS50 luminescence spectrometer equipped with a PTP-1 Peltier temperature programmer was used for these analyses. Excitation and emission wavelengths were 390 and 480 nm, respectively. Preliminary titration trials were made to optimize ANS concentration in the final assays, which was found to be 0.2 mM under the conditions used. Thermal treatments of conglutin γ in the cuvette in the presence of ANS were carried out at the respective T_m values of the protomer (60 °C) and the tetramer (75 °C) for a total time of 30 min.

RESULTS

When conglutin γ was heated at selected temperatures for a given time at the two pH values, 4.5 and 7.5, at which protomeric and tetrameric forms of this protein, respectively, do exist (Duranti et al., 1996), two remarkably different effects were seen: the protomer underwent thermal denaturation without loss of solubility, whereas the tetramer, upon losing its native conformation, aggregated and precipitated, as shown below. According to these findings, the relative extent of conglutin γ denaturation at acidic pH was quantified with SE-HPLC by comparing the area of the excluded fraction with the peak area of the native protein. An example of the elution profiles obtained by submitting conglutin γ to various temperatures is shown in Figure 1. The first eluting peak had an elution volume corresponding to the void volume of the column, that is, a relative molecular mass >300 kDa, suggesting that this peak consisted of non-native soluble protomers and/or aggregates. Conversely, because heating of conglutin γ at slightly alkaline pH above certain temperatures induced insolubilization of the protein, its thermal stability was measured by determining the amount of conglutin γ still soluble after centrifugal precipitation of the aggregated counterpart. These approaches allowed us to draw the denaturation curves of Figure 2, from which the midpoint transition temperatures (T_m) of conglutin γ heat denaturation at the two pH values tested were calculated to be 60.3 and 75.1 °C, respectively.

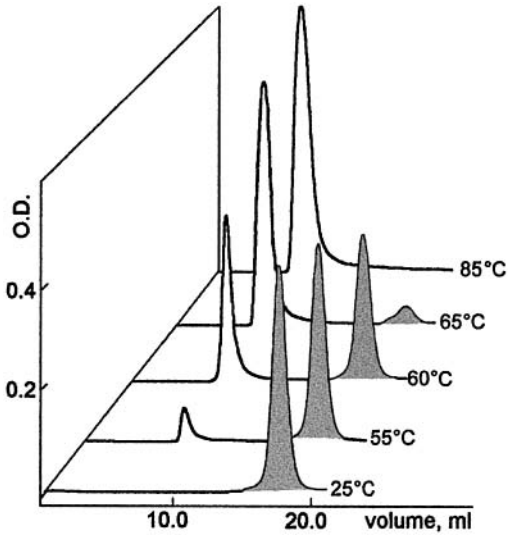

Figure 1. Elution profiles of conglutin γ at acidic pH from SE-HPLC. Conglutin γ (0.5 mg/mL) in 50 mM sodium acetate buffer, pH 4.5, was heated for 3 min at the temperatures indicated, cooled, and loaded on the column. Elution was carried out with the same buffer and monitored at 280 nm. Gray peaks represent conglutin γ protomers (M_r = 46 kDa), and empty peaks were eluted in the void volume of the column ($M_r \geq$ 300 kDa).

TTGGE was used as an alternative approach to detect structural changes of the protomer submitted to heat treatment, by virtue of its solubility upon unfolding. This technique was not suitable for the tetramer not only because of its lower solubility at the tested conditions but also because it had a too small electrophoretic mobility due to its large mass and slightly alkaline isoelectric point (Duranti et al., 1996). After Coomassie blue staining of the gel, a curved band became visible (Figure 3), showing a progressive slowing of the protein in the electric field in the region exposed to higher temperatures. This again reflected major structural changes of the protomer, resulting in greater apparent mass of the polypeptide. The phenomenon occurred at all temperatures >45 °C, but marked bending was observed from ∼63−65 °C upward (Figure 3), in very good agreement with the T_m values assessed by SE-HPLC and DSC (see below).

Because of the observed different unfolding behaviors of conglutin γ at the two pH values considered, we decided to use other techniques, allowing a direct comparison of the denaturation patterns of the protein

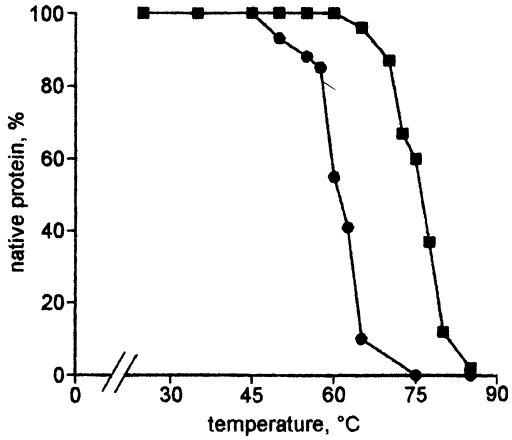

Figure 2. Denaturation curves of conglutin γ at acidic (circles) and slightly alkaline pH values (squares) as a function of the temperature. Conglutin γ (0.5−0.75 mg/mL) was heated at the temperatures indicated for 3 min at pH 4.5 and 7.5 in the proper buffers. At pH 4.5, the relative amounts of residual native protein were measured by SE-HPLC. At pH 7.5, the relative amounts of residual soluble conglutin γ were measured by direct protein concentration measurements after centrifugation (see text for details). The means of duplicate assays are given as percent of the controls at 25 °C.

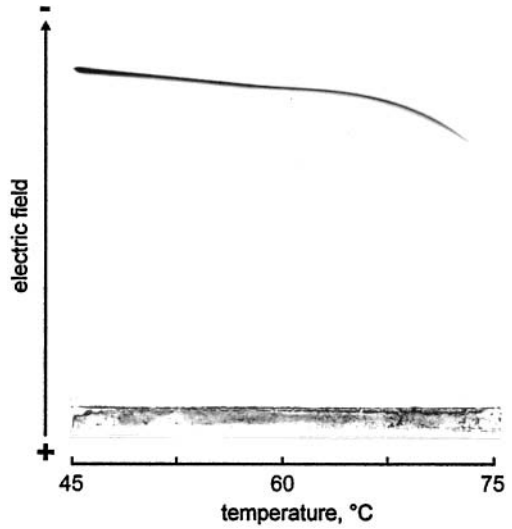

Figure 3. TTGGE of conglutin γ (0.1 mg) at pH 4.5 in 50 mM sodium acetate. The gel was stained with Coomassie brilliant blue. See Materials and Methods for experimental details.

in its different association states. One of these techniques was DSC. The thermal denaturation of conglutin γ at acidic pH is partially reversible, because a second scan showed ~30% of the enthalpy change observed during the first scan (not shown). This partial reversibility was not observed with the protein at slightly alkaline pH. With a heating rate of 1 °C/min, the scanning profile did not allow a separation between the endothermic transitions because of the protein unfolding and the large exothermic transition due to aggregation. Nevertheless, by decreasing the protein concentration to ~0.2 mg/mL and by increasing the heating rate to 1.5 °C/min, the endothermic and exothermic transitions are separated enough to allow a reasonable deconvolution of the unfolding transition. The scanning profiles obtained for conglutin γ at both acidic and alkaline pH values are shown in Figure 4. In both cases, the best curve fittings were obtained by using two transition states, which can possibly be related to the independent

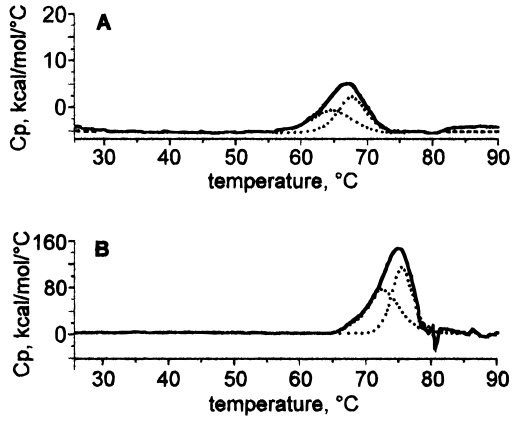

Figure 4. Melting curves of conglutin γ (0.21 mg/mL) in 50 mM sodium acetate buffer, pH 4.5 (A), and in 50 mM Tris-HCl buffer, pH 7.5 (B). DSC scans were performed at 1.5 °C/min: (thick line) experimental curve; (dotted lines) peak components obtained by deconvolution; (dashed line) sum of the two transitions. Experimental details are described under Materials and Methods.

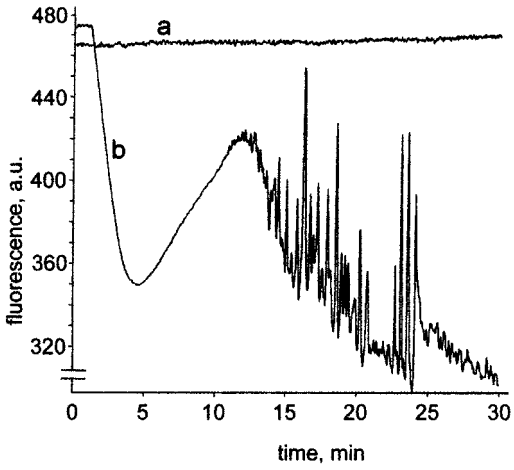

Figure 5. Fluorescence spectra of ANS binding to conglutin γ at 60 (a) and 75 °C (b). The proteins (0.5 mg/mL) were incubated with 0.2 mM ANS in 50 mM sodium acetate buffer, pH 4.5, or in 50 mM Tris-HCl buffer, pH 7.5. See text for details. Spectra were recorded with 390 and 480 nm excitation and emission wavelengths, respectively.

collapses of the two conglutin γ polypeptide chains. At pH 4.5, the two average T_m values obtained were 64.4 and 67.3 °C. Interestingly, experimental calorimetric (ΔH_{cal}) and van't Hoff (ΔH_{vH}) enthalpies of the two transitions differed significantly, being in the ratio of 0.25−0.35. This might depend, as will be detailed under Discussion, both on the observed partial reversibility of the process and on the formation of soluble aggregates consisting of three/four protomers. At neutral pH, at which massive aggregation of the protein was observed, the two average T_m values were 73.2 and 76.0 °C. In this case, the ratio between ΔH_{cal} and ΔH_{vH} was >1, suggesting that the transitions occurred independently in each protomer and that the two transitions were related to the unfolding of the two conglutin γ polypeptide chains.

Another approach to further compare conglutin γ thermal transitions was indirect fluorescence with the hydrophobic probe ANS. The spectra of the protomer and tetramer interacting with ANS during isothermal unfolding again remarkably differed (Figure 5): whereas no major changes in the binding of ANS accompanied

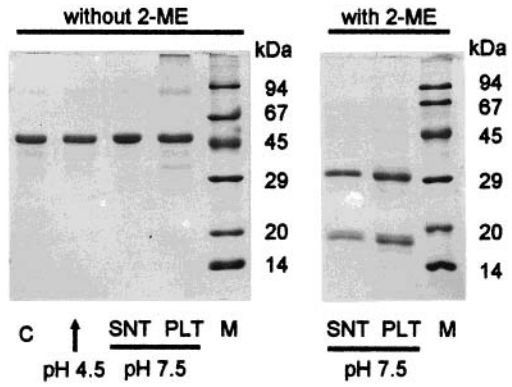

Figure 6. SDS−PAGE under reducing and nonreducing conditions of conglutin γ samples treated at pH 4.5 and 7.5 at 60 and 75 °C, respectively, for 3 min. Protein samples (0.5 mg/mL) were heated, cooled in an ice bath, and centrifuged at 10000*g* for 6 min. The pellets were resuspended in 100 μL of 1× sample denaturing buffer, and the supernatants were added to the same volume of 2× sample denaturing buffer. Mercaptoethanol (2%) was added when required. Both samples were heated at 100 °C for 5 min and aliquots submitted to SDS−PAGE analyses as described under Materials and Methods. The gel was stained with Coomassie blue. 2-ME, 2-mercaptoethanol; C, control; SNT, supernatant; PLT, pellet; M, marker proteins.

protomer unfolding, three steps characterized that of the tetramer. A first step, from 0 to 5 min exposure at the protein T_m, consisted of a remarkable decrease of ANS fluorescence; a second one, from 5 to 12 min, of partial fluorescence recovery; and a third one, above 12 min, of protein aggregation, which prevented further analysis of the spectrum. These different behaviors will be discussed below.

Finally, we intended to assess the role of disulfide bridge formation/interchange in the thermal aggregation of conglutin γ molecules. To this purpose, SDS−PAGE analyses under reducing and nonreducing conditions of heat-treated conglutin γ samples were carried out (Figure 6). Thermally aggregated insoluble polypeptides were separated from the soluble ones by centrifugation; both were denatured by SDS and then analyzed on the gel. When the reducing agent was absent, high M_r aggregates in the pellets, some of which did not enter either the separating (Figure 6) or the stacking gel (not shown), were seen. All of these bands disappeared when 2-mercaptoethanol was added, and the characteristic subunits of conglutin γ, namely 29 and 17 kDa polypeptides, predominated, indicating that those high M_r aggregates of denatured conglutin γ polypeptides were indeed stabilized by disulfide bridges. However, the amount of aggregates caused by disulfide exchange is not large, suggesting that the contribution of disulfide bonds to aggregation is relatively small. With a less dense polyacrylamide gel (7%), it has been possible to assess the M_r of some of the unreduced high M_r conglutin γ aggregates, which were found to have, respectively, M_r of 88 and 175 kDa (not shown), seemingly corresponding to the covalent dimer and tetramer of conglutin γ. When the soluble portion of conglutin γ denatured under the same conditions as above and the sample of conglutin γ denatured at pH 4.5, at which SH/S−S exchange reactions do not occur, were submitted to SDS−PAGE, no high-M_r polypeptides were found in the gel even in the absence of reducing agents (Figure 6). If the reducing agent was added already during thermal treatment of conglutin γ tetramer, aggregation at 75 °C was much more extensive (not shown), as judged by the quantitative precipitation of the protein. This suggested that the reduction of the −S−S− bridges of the native protein had facilitated its unfolding and consequently its loss of solubility. This has also been described for soybean glycinin for which, upon dissociation into acidic and basic polypeptides by heating under a reducing condition, basic polypeptides, due to their insolubility at neutral pH, precipitated (Damodaran and Kinsella, 1982).

DISCUSSION

Lupin seed conglutin γ proved to be an interesting model to study protein thermal conformational stability under different pH conditions both from a biochemical and from a technofunctional point of view. Additionally, because relatively minor pH changes strongly affected conglutin γ quaternary structure (Duranti et al., 1996), information on the respective thermal properties of the protomeric and tetrameric forms of this protein can be deduced.

Each experimental approach used in this work has highlighted particular aspects of their thermal stability and has disclosed dramatically different unfolding patterns of the protein forms at the two different pH conditions. All assays used concordantly showed a remarkably greater (∼10−15 °C) stability of conglutin γ at neutral than at acidic pH. If extrapolation on the thermal stabilities of the two forms of protein existing at the respective pH values, namely, the tetramer and the protomer, is allowed, our results compare well with the higher conformational stabilities of the oligomeric forms of various proteins (Beernink and Tolan, 1996; Yamaguchi et al., 1996; Mei et al., 1997; Pertaut et al., 1998), although we are not aware of studies specifically dealing with thermal stabilities of plant oligomeric proteins. Additionally, the molecular determinants of the increased conformational stability of the oligomeric proteins with respect to the protomeric/monomeric forms of the same protein have rarely been determined.

Among the techniques used in this study to follow the thermal unfolding of conglutin γ, far-UV circular dichroism was not applied, because the spectra of this protein were shown to be scarcely representative (Duranti et al., 1995), β-conformation being its prevalent secondary structure (Blagrove et al., 1980; Kolivas and Gayler, 1993).

Altogether the results obtained have allowed us to depict a model for the different thermal unfolding pathways of conglutin γ forms at the two pH values considered, which are described below. At acidic pH, when the protomer is prevalent, positive charges help the molecule to maintain a disperse/soluble condition upon thermal unfolding. These charges arise from the protonation of the numerous basic amino acid residues of the protein (Scarafoni et al., 1998), but those specifically involved in the transition between the monomeric and tetrameric forms of conglutin γ and in their strikingly different thermal behaviors seemingly are the 18 histidine residues, which are directly affected by the pH changes in the range considered. A significant transition of the conformational stability of barstar from pH 7.0 to 5.0 has also been attributed to protonation of one histidine residue (Khurana et al., 1995). This does not occur with the tetramer at pH 7.5, when the lack of charge repulsion allows the formation of intra- and intermolecular hydrophobic interactions. These interactions trigger the collapse of the protein molecule, as

shown by the decrease in ANS fluorescence, and subsequently cause an extensive loss of conformation, resulting in the complete exposure of previously hidden hydrophobic patches. In parallel with this leading phenomenon, a relevant role is played by disulfide interchange, which does not take place at the acidic pH values, at which denaturation of the protomer is carried out. This augments the unfolding of the protein and, at the same time, causes covalent stabilization of the aggregates, as shown by SDS−PAGE. Although various −S−S− bound high-M_r aggregates formed during heat denaturation of the conglutin γ tetramer, disulfide bond formation/interchange is not a prerequisite for conglutin γ insolubilization, because it occurred also in the presence of reducing agents (not shown). It rather appeared to be a consequence of the unfolding of conglutin γ. Indeed, conglutin γ heavy and light polypeptide chains contain nine and three cysteine residues, respectively, as deduced from nucleotide sequence analysis, at least one of which in each polypeptide chain is involved in an interchain disulfide bridge (Scarafoni et al., 1998). Although the same disulfide pattern is present in both the protomer and the tetramer, the protomer molecule appeared to be significantly more unstable. This suggests that oligomeric association enhances the overall stability of conglutin γ and that hydrophobic interactions, once positive charge repulsions are abolished, drive the process of protein aggregation upon denaturation.

As far as the sequence of events accompanying denaturation of an oligomeric protein is concerned, both dissociation of the oligomer followed by unfolding of the protomer (Gasset et al., 1997) and loss of tertiary structure before dissociation (Mei et al., 1997) have been described. This sequence can be assessed by DSC experiments by testing a wide range of protein concentrations. However, the presence of the protein aggregation prevents, in our case, this kind of approach. In a two-state reversible process, the ratio $\Delta H_{cal}/\Delta H_{vH}$ is useful to identify the number of folding units involved in the thermal denaturation of a protein, in that multistate transitions lead to a ratio >1, whereas intermolecular interactions lead to a ratio <1 (Knorle and Hubner, 1995). In the present case, the limited reversibility of the process did not allow a rigorous thermodynamic analysis because irreversible denaturation causes an overestimate of van't Hoff enthalpy (Leharne and Chowdhry, 1998). Nevertheless, whereas at acidic pH the ratio $\Delta H_{cal}/\Delta H_{vH}$ was >1, at neutral pH this ratio accounted for <1. However, if the ΔH_{cal} at neutral pH was calculated by using the protomer concentration instead of the tetramer concentration, the ratio $\Delta H_{cal}/\Delta H_{vH}$ became equal to that observed at acidic pH. This suggests that thermal denaturation involved the same folding units at both acidic and neutral pH values. On the other hand, if the low value of the ratio $\Delta H_{cal}/\Delta H_{vH}$ at acidic pH is due to an overestimate of the van't Hoff enthalpy, as previously mentioned, then the unfolding unit was conglutin γ protomer at both pH values. These data also suggest that protomer unfolding might be the first event occurring in the denaturation of conglutin γ.

When the time of exposure at 60 °C and pH 7.5 was prolonged to 3 h to mimic heat treatment of lupin seeds for conglutin γ biosynthesis and secretion (see Introduction), no changes were observed in the aggregation pattern of conglutin γ with respect to the values shown in Figure 1, after 3 min at the same temperature (not shown). This suggests that the overall stability of conglutin γ at neutral pH values below a critical temperature is not affected upon prolonged heat treatment of the protein. Whether this might have a functional relevance is still to be demonstrated.

ABBREVIATIONS USED

ANS, 8-anilino-1-naphthalenesulfonic acid; DSC, differential scanning calorimetry; SE-HPLC, size exclusion high-performance liquid chromatography; TTGGE, temperature transverse gradient gel electrophoresis.

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