pH-Induced Modifications in the Thermal Stability of Soybean Protein Isolates

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pH-induced modifications in the thermal stability of soybean protein isolates were studied by differential scanning calorimetry. The thermal stability of the 11S globulin was higher than that of the 7S globulin and was very sensitive to pH changes. The glycinin denaturation temperature decreased by 10 °C when the pH was increased from 6 to 11, while the 7S globulin denaturation temperature did not change. At pH 11, soybean isolate gave only one endotherm representing both globulins. 11S globulin underwent conformational changes as the pH increased, which were reflected by lower cooperativity in the denaturation process. 11S globulin also had a higher activation energy for the denaturation process than did 7S globulin. The activation energy of 11S globulin was higher than that of 7S globulin (in the pH 6−10 range) and shows a maximum kinetic stability at pH 8. The 7S and 11S globulin half-lives at different treatment temperatures were calculated to pHs between 6 and 11, and thermal treatments causing different degrees of denaturation were carried out to produce a maximum increase of the surface hydrophobicity (Ho). For thermally untreated isolates, the increase in pH led to an increase in exposed hydrophobicity. The combination of pH 10−11 and thermal treatments at temperatures of about 65 °C led to higher exposure of hydrophobic groups, conditions for which would be most suitable for obtaining isolates with a higher emulsifying capacity. Denaturing thermal treatments at this pH value induced aggregation along with thus a fall in Ho.

Keywords: *Thermal stability; soybean; denaturation; globulin*

INTRODUCTION

At pH 7.6 and an ionic strength (μ) of 0.5, the structure of soybean 7S and 11S globulins is very compact, showing trimeric and hexameric forms, respectively (Badley et al., 1975; Nielsen, 1985). The modification of these conditions brings about association and/or dissociation reactions. For instance, at an ionic strength above 0.5, the β-conglycinin possesses a trimeric forms of 7S, while at an ionic strength below 0.2, it shows a hexameric 9−10S form. At an ionic strength in the range of 0.2−0.5, both forms coexist in equilibrium, which is maintained for pHs between 4.8 and 11. At very low ionic strengths (μ < 0.01) and acidic pH, the α subunit dissociates from the trimer, leading to 2S and 5S forms (Thanh and Shibasaki, 1979). In a similar fashion, at an ionic strength higher than 0.5, the glycinin presents a hexameric form [11S $(AB)_6$] which converts to a dodecameric form when the ionic strength falls below 0.1 (Kinsella, 1979). Moreover, when exposed to very low ionic strengths (μ = 0.001), extreme pH values, or treatments with detergents, glycinin dissociates into 7S [$(AB)_3$] and 3S (AB) forms. In turn, when reducing treatments are applied, glycinin acquires a 2S form with free A and B polypeptides (Wolf and Briggs, 1956; Wolf, 1978). This association/dissociation behavior may modify the thermal stability.

When a protein is initially heated, it becomes denatured. Depending on the medium and treatment conditions, this protein may be reversibly, totally, or partially denatured, or else it undergoes aggregation (Damodaran, 1988). Exposure to extreme pH values also produces conformational changes, which are often more intense than those caused by thermal treatments at controlled conditions (Das and Kinsella, 1990). Since the interaction of the protein in the oil/water interface is mainly hydrophobic where the hydrophobic groups are unexposed in the native structure, the exposure of such groups through thermal treatments, or modifications in the medium, can improve the emulsifying properties (Kato and Nakai, 1980; Kato et al., 1981, 1983, 1986; Townsend and Nakai, 1983; Voutsinas et al., 1983a, b; Matsudomi et al., 1985; Shimizu et al., 1986).

The objective of this work was to analyze the modifications in the thermal stability of 7S and 11S globulins from soy protein isolates produced by changes in the pH of the medium. Once the kinetic parameters of the denaturation process were determined, treatments were designed to induce denaturation with as little aggregation as possible. These treatments were then carried out at each pH, and the resulting surface hydrophobicity changes were analyzed.

MATERIALS AND METHODS

Preparation of Isolates. Protein isolates were obtained from a defatted flour (Sanbra, S. A., Brazil) using conditions described in a previous work (Petruccelli and Añón, 1995a). The pellet obtained after the isoelectric precipitation was suspended in water (15−20% w/v) and was fractionated in six aliquots, each of them being adjusted to a specific pH in the range of 6.20−11. The pH was adjusted by slow addition of 2 N NaOH. The samples were mantained under agitation to assure a good homogeneity.

Preparation Fractions 7S and 11S. Proteins were obtained from defatted soybean flour (Sanbra, S. A., Brazil), which was dispersed in water containing 10 mM β-mercaptoethanol at pH 8 (the pH was adjusted with 2 N NaOH). The suspension so obtained was filtered through gauze, and the filtrate material was centrifuged at 10000*g* for 30 min at 4 °C. The pH of the supernatant was adjusted to 6.4 and the supernatant was left at 4 °C for 18 h. After that, it was

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centrifuged at 10000*g* for 30 min at 4 °C. The pellet thus obtained was mainly composed of 11S globulin. The supernatant was adjusted to pH 4.5 and was centrifuged at 10000*g* for 30 min at 4 °C. The resulting pellet was mainly composed of 7S globulin. It was washed three times with water to eliminate whey proteins. The pellets were each dissolved in water containing 10 mM β-mercaptoethanol, and the pH of each was adjusted to 8. Ammonium sulfate fractional precipitation was performed in the range between 75 and 100%. The fractions obtained were dialyzed and then freeze-dried.

The 7S and 11S freeze-dried fractions were suspended in water (15−20% w/v) and were fractionated in aliquots. Each aliquot was adjusted to a specific pH (between 6 and 11) by slow addition of 2 N NaOH, under continuous agitation.

Thermal Treatments. Soy protein isolates were treated at different conditions of temperature and pH. For these experiments, the isolate protein suspensions (2.5 mg/mL) adjusted to a specific pH as described previously were fractionated in 1 mL aliquots and placed in 1.5 mL Eppendorf tubes, which were inmersed in a constant temperature bath at 100 °C. The heating rate was measured by a thin Cu-Constantan thermocouple placed in the protein suspension and connected to a recorder. Once the Eppendorf tubes reached a preselected final temperature, they were removed from the thermostatic bath and were immediately cooled in an ice/water bath. Six thermal treatments were performed where final temperatures were 67.8, 70, 76.5, 80.5, 84.5, and 90.2 °C. In all of them, the protein concentration was 2.5 mg/mL.

Differential Scanning Calorimetry. A Rheometric Scientific Calorimeter was used for these studies.The samples analyzed were soy protein isolates and 7S and 11S protein fractions adjusted at different pHs as described previously. Hermetically sealed aluminum pans were prepared to contain 1.7−3.4 mg of isolate suspended in water (10−20% w/w). The addition of water did not change the pH of the samples. These capsules were heated from 30 to 130 °C using various rates, 5−20 °C/min. A preheated pan was used as a reference. At least duplicate runs were done for each set of conditions.

Determination of Surface Hydrophobicity. Isolate heated according to the previously described conditions was diluted in 0.1 M phosphate buffer (pH 7) at different protein concentrations. Surface hydrophobicity was then measured with a ANS probe (Hayakawa and Nakai, 1985a,b). Duplicate determinations were performed. The protein concentration was determined by the technique of Lowry et al. (1951) using bovine albumin as the standard.

RESULTS AND DISCUSSION

Since the association/dissociation state of soybean globulins depends on the environment conditions, the pH was varied from 6.2 to 11, and the influence of such changes on the thermal stability was analyzed by differential scanning calorimetry. To eliminate the effects of the heating rate on the denaturation temperature, determinations were carried out at different rates, and the denaturation temperature was extrapolated to a zero rate. Results so obtained are shown in Figure 1. As the pH was increased from 6.2 to 10.2, the denaturation temperature of 7S globulin did not change. On the other hand, the denaturation temperature of 11S globulin decreased by about 10 °C when evaluated over the same pH range. This indicates a thermal destabilization of glycinin. At pH 11, the isolates showed only one endotherm for temperatures below 70 °C, which would correspond to both globulins.

To more extensively assess the analysis of the pH-induced modifications in the thermal stability, we determined the effect of pH on purified 7S and 11S fractions. Results are shown in Figure 2. The denaturation temperatures of the 7S and 11S purified fractions were slightly lower than those in the isolates. This behavior may be caused both by differences in the

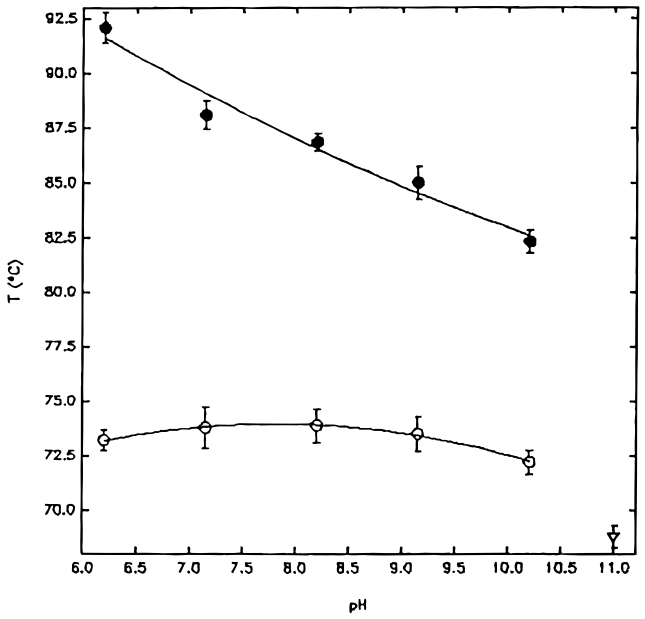

Figure 1. pH effect on the denaturation temperature, extrapolated at a zero heating rate, of the 11S globulin (●) and 7S globulin (○). (▽) The value at pH 11 corresponds to both globulins.

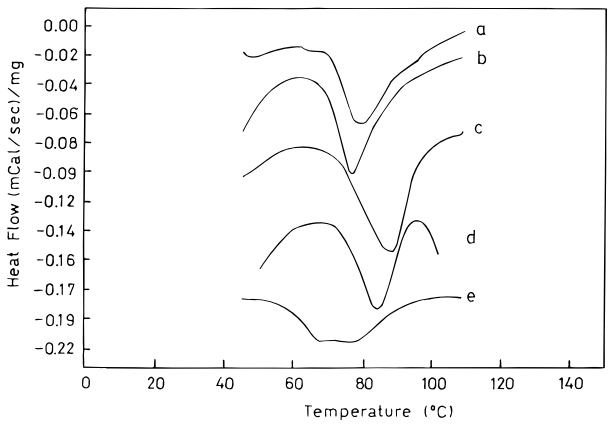

Figure 2. DSC thermograms of thermal curves of 7S globulin at pH 7.0 (a) and 11.0 (b) and 11S globulin at pH 6.5 (c), 8.0 (d), and 11.0 (e).

ionic environment and/or by interactions between the two globulins. Other authors (Hohlberg and Stanley, 1987) have also observed thermal stability changes in purified proteins versus protein mixtures. The denaturation temperature (T_d) of 7S globulin was not modified by the increase in pH. In addition, we observed no change in the peak width, which indicates no loss of cooperativity in the denaturation process. Also, since we observed no change in the ΔH of denaturation, there was no change in conformation. However, the denaturation temperature of 11S globulin decreased by 2 °C as the pH increased from 6.2 to 8. At pH 11, we observed an increase in the width of the endotherm. This indicates a loss of cooperativity in the process. Also, we observed two endothermic event that would correspond to species of distinct thermal stability. In addition, we observed a 50% decrease in the ΔH which demonstrates that denaturation occurred when enthalpies at pH 11 are compared with those at pH 6.5. This behavior of the glycinin may be due to a dissociation of the hexameric form to trimeric and/or monomeric forms. The loss of cooperativity in the denaturation process has also been observed in oats and fababean (Arntfield and

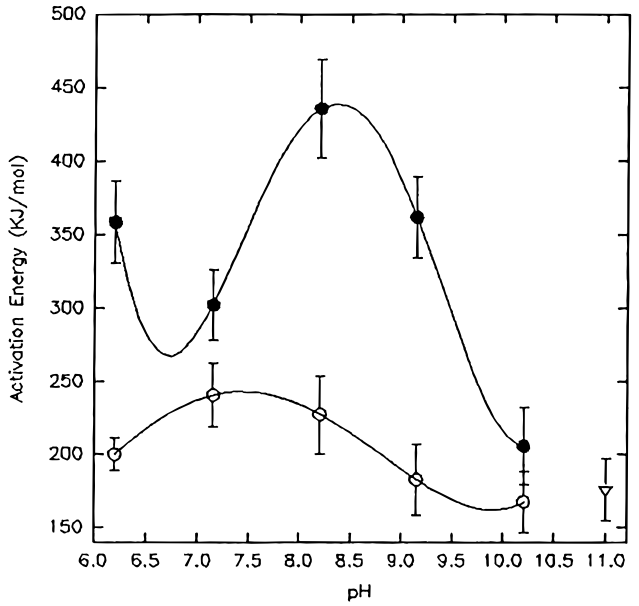

Figure 3. pH effect on the activation energy for the denaturation process of 7S globulin (○) and 11S globulin (●). The (▽) value at pH 11 corresponds to both globulins.

Murray, 1981; Harwalkar and Ma, 1987); these proteins present maximum thermal stability at pHs close to the isoelectric point.

According to our results, the thermal stability of 11S globulin was higher than that of 7S globulin and its structure more sensitive to pH changes in the range studied. These results also agree with those described by several other authors who evaluated modifications in the association/dissociation state as induced by changes in pH and ionic strength (Thanh and Shibasaki, 1979; Wolf and Briggs, 1956; Wolf, 1978). In these treatment conditions, the 7S globulin would be in an equilibrium between 7S and 9S forms, whereas the 11S globulin would dissociate into trimeric forms and free subunits. The greater ionization of glycinin free sulphydryl groups caused by the pH increase would contribute to a loss of cooperativity in the denaturation process, since they would promote denaturation through interchange reactions.

To determine the kinetic parameters in the denaturation process of 7S and 11S globulins in isolates, we used the dynamic method of Ozawa (1970). For both 7S and 11S globulins, we analyzed the effect of pH changes on the activation energies of the denaturation process. The results obtained are shown in Figure 3. The activation energy of 11S globulin was higher than that of 7S globulin (in the pH range under study) and showed a maximum kinetic stability at pH 8. From the thermodynamical point of view, the maximum thermal stability of 11S globulin occurred at pH values close to its isoelectric point (higher denaturation temperature). 7S globulin showed a slight decrease in its activation energy at pHs greater than 8. The activation energies of 7S and 11S globulins calculated by other authors by spectroscopic methods (Iwabuchi et al., 1991a,b; Watanabe, 1988) coincide with those obtained in our work by differential scanning calorimetry.

If the denaturation process is assumed to follow first-order kinetics, then the half-life of the protein fractions can be calculated. The results of such a procedure are shown in Figure 4. For pHs from 6.2 to 10.2, the 7S globulin required temperatures above 60 °C to start denaturation, and more than 75 °C to complete it.

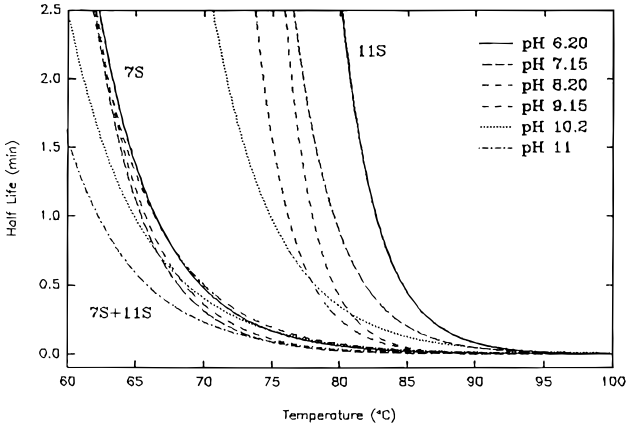

Figure 4. Heating temperature effect on the half-life of 7S and 11S globulins.

Therefore, if protein isolates with partially denatured 7S globulin are required in such a pH range, the treatments should be done in the range of 65−75 °C. At these temperatures, the pH changes did not significantly modify the half-life of β-conglycinin.

At pH 11, the half-lives corresponded to both globulins, since, at this pH, the isolates showed only one endotherm. Treatments at 65 °C caused considerable denaturation; therefore, temperatures above 70 °C can be assumed to totally denature at pH 11.

In the range of pH 6.2−10.2, the half-life of 11S globulin decreased with an increase in pH. Treatments at 75 °C and pH 10 caused substantial denaturation, while at pH 6−7, temperatures higher than 80 °C were required to reach a similar degree of denaturation. The treatments at temperatures above 90 °C are totally denaturing at all pH values.

For pH values between 6.2 and 11, different thermal treatments were applied to soy protein isolates with final temperatures varying from 65 to 90 °C, as described in Materials and Methods. The heating rate was high, and the samples were cooled immediately after they reached the target final temperature. Therefore, the effect of both heating and cooling rates on the denaturation process was minimized. At all pH values of the range studied (6.2−10.2), the β-conglycinin was partially denatured by temperatures ranging from 65 to 75 °C and totally denatured for values between 80 and 90 °C. In the same pH range, the 11S globulin was completely denatured only for thermal treatments performed at temperatures above 90 °C. Treatments done at pH 6−7 and temperatures of 80−85 °C and those carried out at pH 6−10 and temperatures below 75 °C caused partial denaturation of the glycinin. Treatments performed at pH 11 produced a complete denaturation in both globulin fractions (7S and 11S) for all temperatures, except at 65 °C, where denaturation was partial. These experimental results corroborated the behavior predicted by the calculated half-lives. It was observed that the thermal treatments caused a slight increase of the denaturation temperatures and a decrease in $\Delta T_{1/2}$ which suggests a protein with more compact and orderly structure. This behavior could be due to denaturation of the less stable fractions with an enrichment in the more stable fractions, or else due to formation of aggregates. In some cases, the denaturation temperature of the preheated isolates was beyond 100 °C. Similar behavior was also observed for other preheated proteins (Ma et al., 1990). In previous work done in our laboratory (Petruccelli and Añón, 1995b), we had ob-

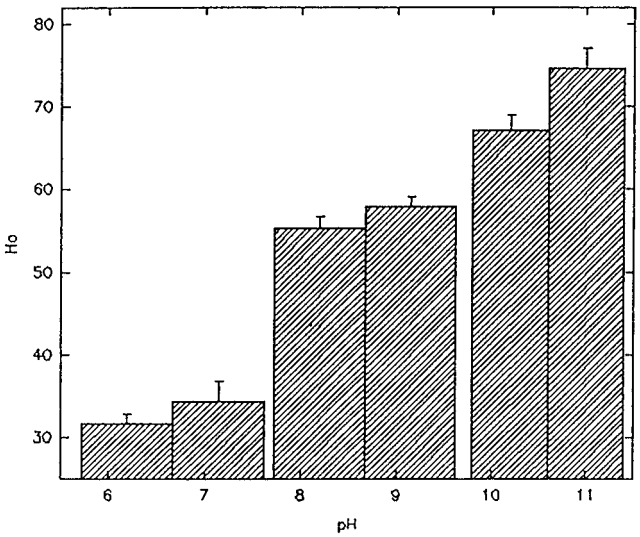

Figure 5. pH effect on the surface hydrophobicity (Ho) of soybean protein isolates without thermal treatments.

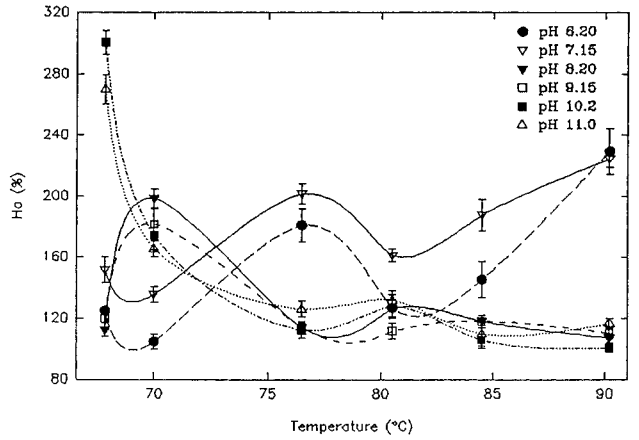

Figure 6. Temperature treatment effect on the surface hydrophobicity (Ho) where Ho (percent) represents the ratio of values for heat versus no heat treatment × 100.

served formation of aggregates of the AB subunit in treatments carried out at pH 7 for temperatures above 80 °C. In that work, no formation of such aggregate was observed at pH 6; moreover, the temperature required to produce aggregation of the AB subunit became lower as the pH increased. Besides, such a pH increase also produced dissociation of the subunit and formation of β/B aggregates (β-β-conglycinin/B-glycinin) (Petruccelli and Añón, 1995b).

The existence of the above-mentioned aggregates can explain the behavior of the preheated aggregates of the present work, which indicates that the behavior of these proteins in isolates is different from that in purified fractions. Figure 5 shows the hydrophobicity (Ho) of samples that were not exposed to thermal treatment, as a function of pH. It is observed that a pH increase from 6−7 to 8−9 causes a 1.7-fold increase of hydrophobicity. The increase of hydrophobicity was even higher when the pH reaches 10−11. These values of Ho can be attributed to the fact that the protein structure unfolds and/or the protein subunits dissociate.

Over the range of pHs evaluated, Figure 6 shows the modifications of exposed hydrophobicity of protein isolates when treated at temperatures between 65 and 90 °C and expressed as percentages of values with no thermal treatment. At pH values of 6.2 and 7.15, isolates showed a surface hydrophobicity maximum at 77 °C, which is in agreement with the denaturation of fraction 7S previously shown. Besides, an increase of Ho was observed at 90 °C, which coincides to the total denaturation of glycinin. Likewise, at pH 8.2 and 9.15, and at pH 10.15 and 11, the maxima of Ho showed by the isolates were 70 and 65 °C, respectively, and a further decrease that may be attributed to aggregation phenomena. The absolute values of exposed hydrophobicity at pH 10.15 and 11.0 were higher than those in an equivalent thermal treatment applied at other pH values.

The surface hydrophobicity depends not only on the combination of denaturation processes, which tend to increase it, but also on the aggregation phenomena, which tend to decrease it. A pH increase from 6.2 to 11 leads, on the one hand, to an increase in the protein charge, which tends to decrease its tendency to aggregate, but on the other hand, it also produces dissociation and partial denaturation which leads to more exposed hydrophobic groups that would favor aggregation. Besides, we observed a synergism between modifications produced by the pH change as well as those caused by temperature. At pH 10 and 11, modifications were higher than those normally attributed to thermally induced denaturation alone or to the effect of pH.

At pH values that did not lead to complete denaturation, the thermal treatments at 65 °C did lead to a noticeable change in Ho. This phenomenon could be attributed to little aggregation. At such pH values, aggregation is caused by treatments at higher temperatures, which justify the observed fall of Ho. We had observed in a previous work (Petrucelli and Añón, 1994) that conditions where the pH was increased from 7 to 9 in combination with treatments which led to partial denaturation of 7S globulin produced isolates with better emulsifying properties. The results shown in the present work indicate that the best treatment conditions to obtain soluble unfolded proteins are pH 10−11 and temperatures of 65 °C. Higher temperatures induce aggregation, whereas less alkaline pH values produce a less important exposure of hydrophobic groups.

CONCLUSIONS

As the pH is increased, the thermal stability of soy globulin 11S undergoes important modifications. For instance, its denaturation temperature decreased by 10 °C when the pH changes from 7 to 11. On the contrary, there was no practical change in the thermal stability of globulin 7S when evaluated over the same pH range.

11S globulin undergoes conformational changes as the pH increases, which are reflected by lower cooperativity in the denaturation process. When the pH reaches 11, a 50% denaturation was observed.

For unheated isolates, the increase of pH leads to increased exposed hydrophobicity. The combination of pH 10−11 and thermal treatments at temperatures of about 65 °C leads to higher exposure of hydrophobic groups, which would be the most suitable condition to obtain isolates with higher emulsifying capacity. Denaturing thermal treatments at this pH value induce aggregation with a resultant fall in Ho.

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