Gelation of Sunflower Globulin Hydrolysates: Rheological and Calorimetric Studies

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Trypsin hydrolysates of sunflower globulins gel when heated. Gelation time and gel strength are exponentially related to concentration. Critical concentration is <1%. Sunflower globulins and their tryptic hydrolysis products have a high thermal stability, compared to most plant proteins, that is slightly affected by pH in the range 6−11. Activation energies for heat denaturation are estimated as 443 ± 9 and 423 ± 22 kJ/mol·K for the globulins and their hydrolysates, respectively. The temperature dependence of the gelation rate follows the Arrhenius law. The activation energy of gelation has been estimated as 315 ± 17 kJ/mol·K. Gelation can take place at about 20 °C below T_d, but isothermal gelation experiments (at 5% hydrolysate concentration) reveal that at gelation times a fraction in the range of 15−30% of the total hydrolysate has already been denatured. This fraction becomes greater with decreasing hydrolysate concentration.

Keywords: *Sunflower globulins; gelation; temperature dependence*

INTRODUCTION

Neither sunflower protein isolates nor purified sunflower globulins form gels when submitted to heating, even after calcium enrichment or disulfide bond disruption or in the presence of added sodium chloride or propylene glycol. However, heating aqueous solutions of the trypsin hydrolysates of sunflower protein isolates causes gels that show the unusual property of deeply weakening when cooled below about 80 °C (Sánchez and Burgos, 1995, 1996, 1997). Trypsin acts mainly upon a fraction of the M_r 20 000−28 000 globulin polypeptides, yielding fragments of M_r 4 000−10 000. The gel formation ability of this hydrolysate is strongly dependent on pH. Gels are formed only in the pH range 7−11, and maximum gel strength is obtained at pH 8. Gelation time increases with pH and decreases with protein concentration. The critical concentration of hydrolyzed protein isolates has been estimated to be <1.1%. A value of 8 has been calculated for the exponent of the power law relating the storage modulus (G') to protein concentration in the concentration range 1.7−2.5% (Sánchez and Burgos, 1996).

The type of bonds and interactions involved in the gelation of hydrolyzed sunflower protein isolates has been studied by determining the influence of temperature, sodium chloride, calcium chloride, thiocyanate, acetate, and sulfate anions, mercaptoethanol, and propylene glycol on the rheological properties of the gels formed at pH 8. Hydrophobic interactions are thought to play the main role in gelation. Native disulfide bridges are believed to contribute also to the gel structure, and hydrogen bonds seem to be the cross-linkings that support the residual structure when the gels are cooled at 4 °C (Sánchez and Burgos, 1997).

Heating is generally needed for protein gelation to unfold the polypeptide chains and unmask some of the reactive groups involved in the cross-linkings. However, it has been reported (Stading and Hermansson, 1990) that thermal gelation of some proteins occurs at temperatures well under the temperature of denaturation (T_d). This has been taken as proof that under some conditions protein gelation induced by heating does not need protein denaturation.

The aim of this work was to compare the gelation behavior of the tryptic hydrolysates from purified sunflower globulins with that of the hydrolysates from sunflower protein isolates, to correlate this behavior with calorimetric properties, and to determine the extent of denaturation when the gel is formed.

MATERIALS AND METHODS

Materials. Low temperature defatted and desolvated sunflower meal, prepared from mechanically dehulled seeds, was provided by Gerdoc (Pessac, France).

Trypsin and aprotinin were obtained from Boehringer (Mannheim, Germany). All other chemicals used were of analytical reagent grade.

Preparation of Protein Isolates and Purified Sunflower Globulins. Low phytate and chlorogenic acid isolates were prepared as described by Sánchez and Burgos (1995). This procedure included repeated cold acetone treatments.

Sunflower globulins were obtained by following the method described by Raymond et al. (1981). Preparations were freeze-dried after the pH was adjusted to 7. Protein content in the preparation was estimated by determining nitrogen according to the Kjeldhal method and using a conversion factor of 5.6 as recommended by Sosulski (1979).

Protein Hydrolysis. The pH of a 10% weight/water solution of the protein preparations was adjusted to 8.1 with 2 M NaOH. Trypsin (Boehringer) was added (75 units/g of protein), and the mixture was mantained at 27−28 °C with continuous stirring. Samples were removed after 30 min, proteolysis was stopped by adding 4 units of aprotinin (Boehringer)/μg of trypsin, and the hydrolysates were freeze-dried. Protein content in the preparation was determined as in globulins. The degree of hydrolysis was estimated by the increase in 12% trichloroacetic acid (TCA)-soluble amine nitrogen, determined by the ninhydrin reaction with glycine as a standard (Clark, 1964)

Rheological Measurements. Gelation was monitored by dynamic rheological measurements performed in a Bohlin CS/ETO rheometer (Bohlin Reologi, Lund, Sweden) working in the oscillatory mode. Cone and plate geometry (4° and 4 cm

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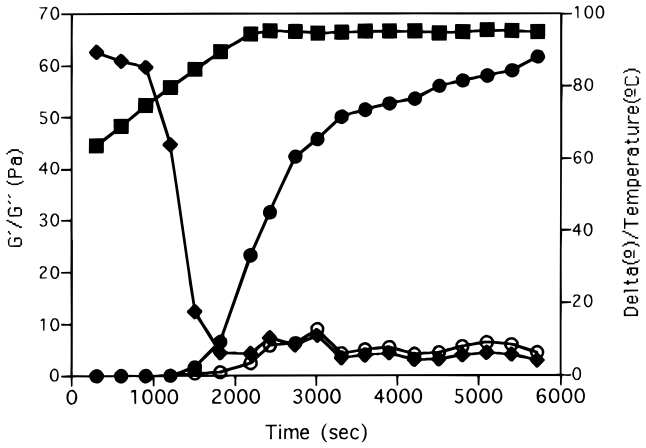

Figure 1. Changes of G' (●), G'' (○), and δ (◆), during heating of a 5% solution of sunflower globulin hydrolysates from 60 to 98 °C at a rate of 1 °C/min and holding at 98 °C (increase of 12% TCA-soluble $NH_2 = 122$ μmol/g). Temperature profile (■).

diameter, CP 4/40) was used. Samples assayed consisted of 1.5 mL of a solution prepared by dissolving freeze-dried samples in 0.05 M Tris buffer, pH 8. To prevent water evaporation and surface drying, samples were surrounded with silicone oil. Test conditions were established as follows: frequency, 0.1 Hz; maximum strain, 0.02. These conditions allowed us to work in the linear viscoelasticity range as proved by strain and frequency sweeps. Integration time was fixed at 10 s. Gelation time and gelation temperature were defined as the time and temperature after which the gelation phase gelation remained under 45°.

Gels were formed by heating the sample either in the temperature range 60−98 °C at a rate of 1 °C min^{-1} followed by holding at 98 °C for 60 min to "cure" the gels or isothermally at different temperatures. Data measured were phase angle (δ), loss modulus (G''), and storage modulus (G').

Calorimetric and Kinetic Determinations. The calorimetric studies were performed on a differential scanning calorimeter (DSC) Model DSC 10 of DuPont Instruments (Wilmington, DE) equipped with a pressure cell. For that, 3 mg of the protein preparations was weighed in aluminum pans and 10 μL of 0.05 M buffer (citric−citrate, Tris, borate, and phosphate for the pH ranges 3−6, 7−8, 9−10.6, and 11, respectively) were added. The pans were hermetically sealed. As a reference, aluminum pans containing identical samples previously heat denatured were used. Heating was performed at a rate of 10 °C/min in the temperature range 30−130 °C.

The rate constant of the thermal denaturation was calculated according to the method of Borchardt and Daniels (1957) and activation energies by means of the Arrhenius equation.

RESULTS

Linearly Increasing Temperature Experiments. The thermorheograms of the hydrolysates from globulins (Figure 1) are very similar to the ones already reported (Sánchez and Burgos, 1995) for the hydrolysates from protein isolates. However, they differ in two points: G' is much higher and gelation temperature is substantially lower at any concentration for the hydrolysates from the isolates than for those obtained from the purified globulins.

As already reported for protein isolates (Sánchez and Burgos, 1995, 1996), thermal gelation of globulin hydrolysates is only possible in a narrow pH range. Maximum gel strength is obtained at pH 8 (data not shown). Gelation rate and G' are exponentially related to protein concentration (Figures 2 and 3). The slope of the logarithmic plot of gelation time versus concentration is close to −1, without any sign of divergence to infinity in the concentration range explored (1−10%).

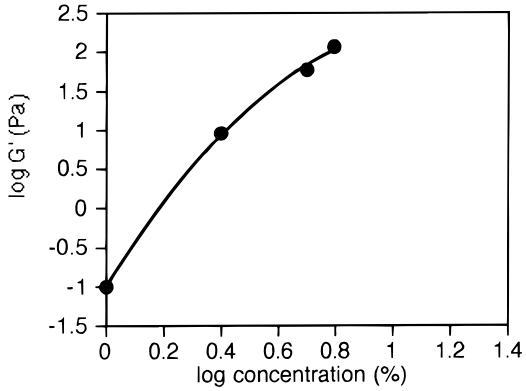

Figure 2. Log G' as function of log hydrolysate "protein" concentration. Heat treatment and hydrolysis degree are as stated in Figure 1.

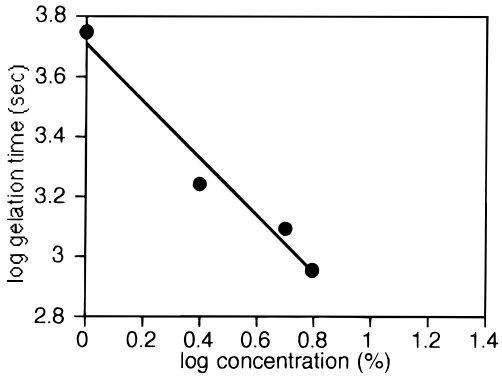

Figure 3. Log gelation time as a function of log hydrolysate "protein" concentration. Heat treatment and degree of hydrolysis are as in Figures 1 and 2.

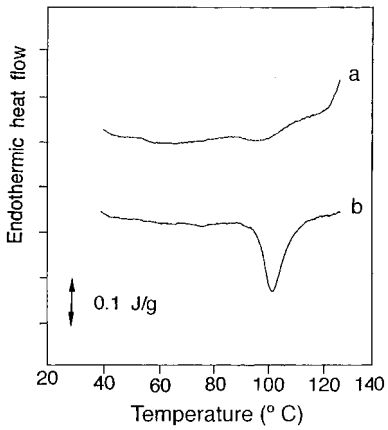

Figure 4. Thermograms of sunflower protein isolates: (a) acetone extracted; (b) non-acetone-treated. Heating rate = 10 °C/min.

The slope of the logarithmic plot of G' versus concentration is 2 in the concentration range 5−6.25% and 5.5 in the protein concentration interval 1−1.5%. The last one is a value markedly lower than that previously reported for the hydrolysates from isolates in the same concentration range.

DSC Calorimetry. Endothermic peaks are almost imperceptible in the thermograms of the protein isolates obtained according to the standard method described or their trypsinized product. However, the thermograms of protein isolates not submitted to the acetone extractions included in the standard preparation procedure show (Figure 4) an endothermic peak (enthalpy change $\Delta H = 9.2$ J/g; $T_d = 102.8$ °C). The thermograms of globulins (Figure 5) and their hydrolysis product

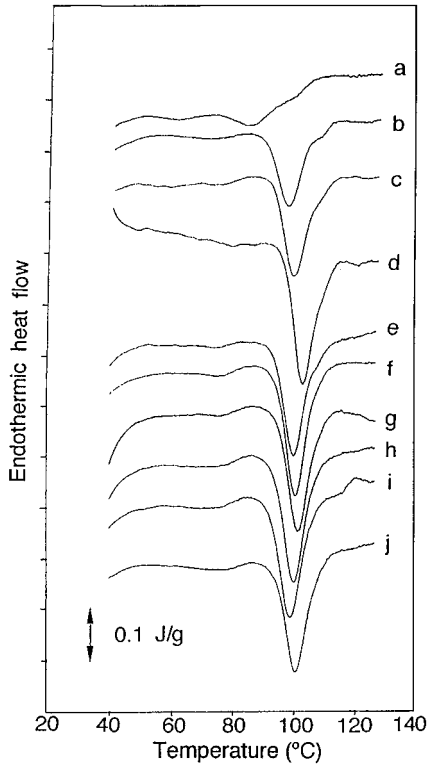

Figure 5. Thermograms of sunflower globulins at pH 3.0 (a), 4.5 (b), 5.0 (c), 6.0 (d), 7.0 (e), 8.0 (f), 9 (g), 10.0 (h), 10.6 (i), and 11 (j). Heating rate = 10 °C/min.

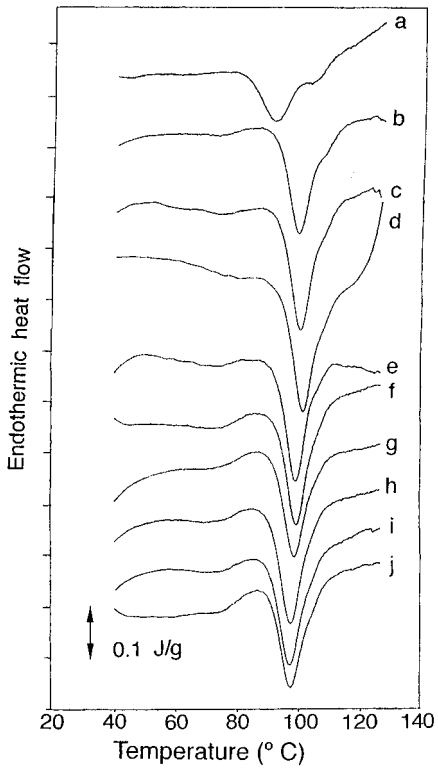

Figure 6. Thermograms of sunflower globulins hydrolysates (degree of hydrolysis as stated in Figures 1 and 2), at pH 3.0 (a), 4.5 (b), 5.0 (c), 6.0 (d), 7.0 (e), 8.0 (f), 9 (g), 10.0 (h), 10.6 (i), and 11 (j). Heating rate = 10 °C/min.

(Figure 6) show endothermic peaks, with T_d, denaturation extrapolated onset temperature, and ΔH varying with hydrolysis degree and pH as shown in Figure 7.

From Arrhenius plots, the activation energies of the denaturation process were calculated as 443 ± 9 and

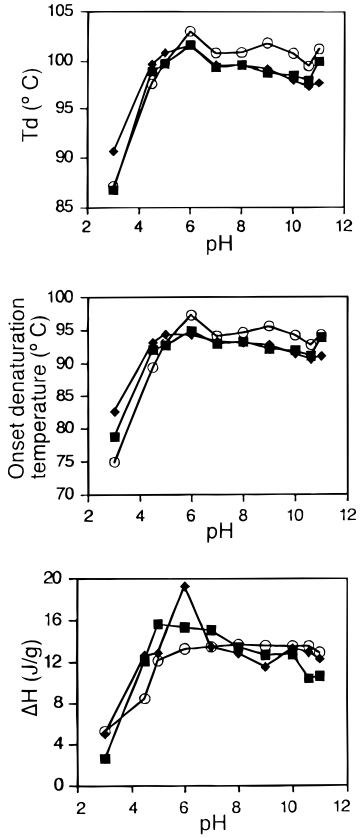

Figure 7. Changes in T_d, onset denaturation temperature, and ΔH as a function of pH in globulins (○) and their hydrolysis products at different degrees of hydrolysis: increase in 12% TCA-soluble NH_2 100 (■) and 125 (◆) μmols/g. Heating rate = 10 °C/min. Differences between hydrolyzed and nonhydrolyzed globulins are only statistically significant ($p < 0.01$) for T_d and only in the pH range 6−11.

Table 1. Gelation Temperature of Globulin Hydrolysates (Degree of Hydrolysis as in Figure 1) in Linearly Increasing Temperature Experiments (Heating Rate 1 °C/min) at Different Concentrations

protein concn (%)	gelation temp (°C)	protein concn (%)	gelation temp (°C)
1	95	5	80
2.5	88	6.25	77

423 ± 22 kJ/mol·K for globulins and their hydrolysates, respectively.

The gelation temperature observed in the thermorheograms following the standard heating procedure decreases with increasing hydrolyzed globulin concentration (Table 1) but is always lower than T_d.

Isothermal Gelation: Fraction of the Hydrolysate Already Denaturated at Gelation Time and Activation Energy of Gelation. In isothermic rheograms at constant concentration, the logarithm of gelation time decreases linearly with increasing temperature (Figure 8). At 5% hydrolyzed globulin concentration and pH 8, gels can be obtained by heating at 80 °C (about 20 °C below the T_d) for approximately 2.5 h. The heating time at constant temperature needed for gelation is also logarithmically related to protein concentration (Figure 9).

The denaturation rates for the globulin hydrolysate at the various temperatures used in isothermal gelation experiments were calculated from the activation energy of its denaturation and the denaturation rate measured at 98 °C. These data and gelation time allowed us to estimate the fraction already denatured when the gel

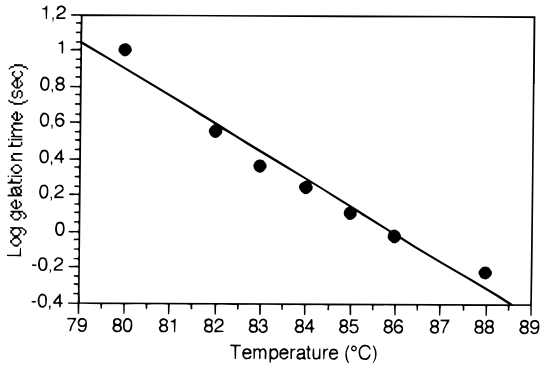

Figure 8. Semilog plot of gelation time versus temperature in a 5% solution, at pH 8, of a sunflower globulin hydrolysate. Degree of hydrolysis is as in Figure 1.

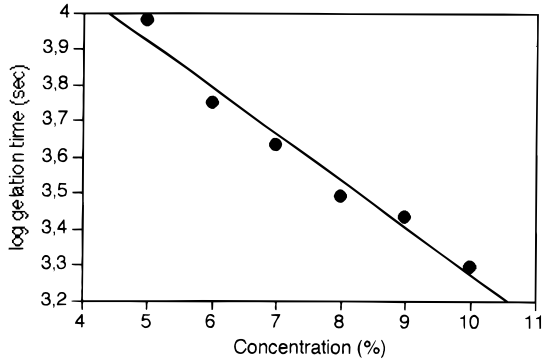

Figure 9. Semilog plot of gelation time versus concentration for sunflower globulin hydrolysate at pH 8. Isothermal heating is at 80 °C. Degree of hydrolysis is as in Figure 1.

Table 2. Gelation Time, Denaturation Rate, and Percent of Total Hydrolysate Denatured at Gelation Time in Isothermal Experiments at Different Temperatures[a]

temp (°C)	gelation time (s)	denaturation rate (s^{-1})	protein denatured at gelation time (%)
80	10100	0.00001810	18
82	3574	0.00004160	14.8
83	2311	0.00006290	14.5
84	1773	0.00009490	16.8
85	1257	0.00014200	17.8
86	943	0.00021400	20.2
88	596	0.00047950	28.6

[a] Hydrolyzed globulins concentration 5%, pH 8. Degree of hydrolysis as in Figure 1.

Table 3. Percentage of the Globulin Hydrolysate That Has Been Denatured at Gelation Time in Isothermal Heating (80 °C) Experiments, at pH 8, as a Function of Hydrolysate Concentration[a]

protein concn (%)	denatured protein (%)	protein concn (%)	denatured protein (%)
5	17.4	8	5.6
6	10.2	9	4.93
7	7.8	10	3.57

[a] Degree of hydrolysis as in Figure 1.

is established at constant hydrolysate concentration and various temperatures (Table 2) and the influence of hydrolysate concentration in this fraction at constant temperature (Table 3).

It is not experimentally possible to obtain direct measurements of gelation rate, but in gelation kinetics studies the reciprocal of gelation time at constant temperature is often taken as indicative of gelation rate. The Arrhenius plot of the rates estimated from the isothermal gelation experiments results in a straight line (Figure 10) from which activation energies of 315 ± 17 and 274 ± 28 kJ/mol·K can be calculated for the globulin hydrolysates and the hydrolysates obtained from the protein isolates, respectively.

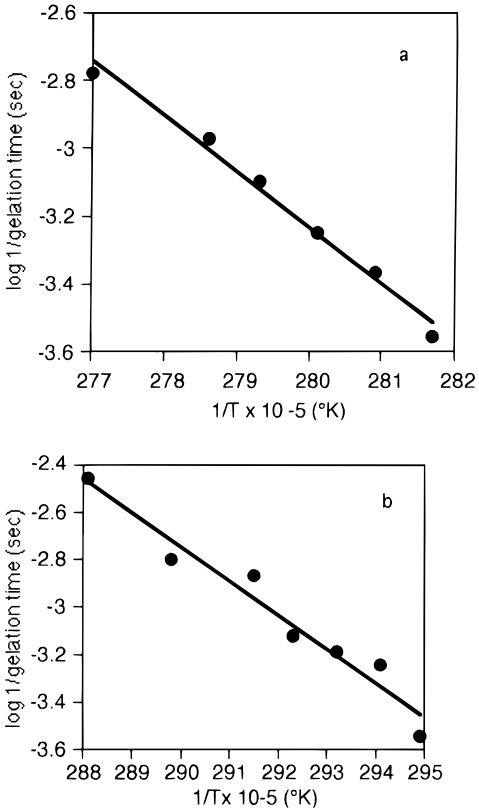

Figure 10. Arrhenius plot of gelation rate on isothermal heating of hydrolysates from globulin (a) and isolates (b). Degree of hydrolysis: globulins as in Figure 1; isolates, increase in 12% TCA-soluble NH_2 = 136 μmol/g.

DISCUSSION

Sunflower protein isolates obtained by isoelectric precipitation consist almost only of globulins (Raymond et al., 1984). This explains the great similarity of the rheological behavior of the hydrolysates from globulins and isolates. The difference in gelation temperature between the hydrolysates obtained from protein isolates and those from purified globulins can be due to the denatured state of the proteins from the isolates, revealed by the thermograms of Figure 5. In the standard protein isolate preparation procedure, denaturation is produced during acetone treatment, as can be deduced by comparing the thermograms obtained with and without acetone treatment. If denaturation is assumed to be a previous requirement for gelation, it could be argued that this initial denaturation of the protein isolates could be also the cause of the higher gel strength of their gels at the end of the standard heating.

Critical concentration is difficult to be directly determined. By extrapolation of double logarithmic plots of G' versus protein concentration to an arbitrary G' value of 1 Pa, a critical concentration of about 1.1% has been previously estimated for the hydrolysates obtained from isolates (Sanchez and Burgos, 1996). The critical concentration of globulin hydrolysates using the same criteria is very close to this value. Nevertheless, there are some signs indicating that the true critical concentration is markedly lower for the hydrolysates from

purified globulins than for the hydrolysates from protein isolates: Gelation can be experimentally measured in the hydrolysates from purified globulins at 1% concentration. The maximum slope found in the double logarithmic plot of G' versus protein concentration is lower for the purified globulin hydrolysates than for the hydrolysates from protein isolates; furthermore, since no divergence to infinity in the double logarithmic plot of gelation time versus concentration is observed with the globulin hydrolysates at 1% concentration, the critical value for them should be quite lower than 1%.

The T_d here found for sunflower native globulins is similar to those reported for the 11S globulins of other seeds (Tolstoguzov, 1988). The sharpness of the endothermic peak both in globulins (half-bandwidth = 8 °C) and in their hydrolysates (half-bandwidth = 7.5 °C) points to a high degree of cooperativity in denaturation. As has been reported for other plant proteins (Hermansson, 1978; Arntfield and Murray, 1981; Harwalkar and Ma, 1987), T_d is little affected by pH in a range close to neutrality but markedly decreases at pH <5. In fact, the T_d of sunflower globulins and their trypsin hydrolysis products remains almost constant in a wide pH interval (5−11). The limited hydrolysis produced by trypsin results in only a small reduction (about 2 °C) of T_d. This reveals that the hydrolysis products retain a high percentage of secondary structure. Extraction procedures and isoelectric precipitation of seed globulins does not usually affect their T_d but can substantially reduce ΔH (Arntfield and Murray, 1981; Arntfield et al., 1991). The ΔH of denaturation for native sunflower globulins is markedly lower than those reported by Tolstoguzov (1988) and Murray et al. (1985) for micellar protein isolates, but are in good agreement with the values calculated by Murray et al. (1985) for protein preparations submitted to isoelectric precipitation. As with T_d, ΔH was little affected by pH values in the interval 5−11 but decreased markedly at pH <5. In the pH range 5−11 the ratio $\Delta H/T_d$ remained almost constant.

The ΔH values of the hydrolysates were not significantly different at any pH from the corresponding values for the globulins, revealing that the hydrolysis procedure has not resulted in further denaturation.

The activation energy for the heat denaturation of trypsinized globulins at pH 8 was lower than values reported for some other seed globulins (Ma and Harwalkar, 1988; Hohlberg and Stanley, 1987). Tryptic hydrolysis does not seem to imply calorimetrically important structural changes.

The temperature dependence of gelation is well described by the Arrhenius equation. Activation energies for protein gelation are not frequently found in the literature. Katsuta and Kinsella (1990) and Harte et al. (1992) found for the thermal gelation of serum proteins and ovalbumin values (67−105 and 159 kJ/mol·K, respectively) that were much lower than that we have calculated for sunflower protein hydrolysates. Activation energies estimated for the gelation of polysaccharides are even lower (in the range 20−100 kJ/mol·K; Isozaki et al., 1976; Kawabata and Sawayama, 1976; Mitchell and Blanschard, 1976; Watase and Nishinari, 1981). These energies must be referred to the formation and rupture of cross-linkings in the junction zones. The low value of activation energies quoted for serum protein and their negative dependence on temperature were considered by Katsuta and Kinsella (1990) to be probably due to the fact that in the formation of their gels hydrophobic interactions are not implied. The much higher E_a value in the gelation of sunflower protein hydrolysates is consistent with the already reported involvement of hydrophobic bonds in gel formation (Sanchez and Burgos, 1996) and with the establishment at gelation time of about 15 noncovalent cross-linkings/mol.

Although denaturation is thought to be a requirement for protein gelation, there have been some reports pointing out that it does not need to be very extensive (Clark and Lee-Tuffnell, 1986; Koseki et al., 1989; Doi, 1993). Stading and Hermansson (1990) suggested that β-lactoglobulin formed heat-induced gels at pH 5.5, without previous protein denaturation. They suggestied this on the grounds of having observed a gelation temperature 20 °C below the T_d in the thermorheograms. Our experimental data with sunflower protein hydrolysates reveal similar differences between T_d and gelation temperature. However, the isothermal gelation experiments show that, at 5% protein concentration, when the gel was formed about 20% of the protein had been denatured and that the percentage of protein denatured at gelation time decreased with increasing concentration. This points to the need of a minimum concentration of denatured protein for network formation. In terms of total protein this minimum denatured protein concentration obviously means a decreasing fraction of total protein as protein concentration increases.

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