Thermal Gelation of Trypsin Hydrolysates of Sunflower Proteins: Effect of pH, Protein Concentration, and Hydrolysis Degree

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The influence of several parameters on the gelation properties of trypsin hydrolysates of sunflower proteins was studied by dynamic rheological methods. The degree of hydrolysis has very little effect on either storage modulus or gelation time. Sunflower protein gelation is strongly pH dependent. Gelation is only possible in the pH range 7−11. The storage modulus reaches its maximum value at pH 8. The gels formed at pH 7 or above pH 9 are very weak. Gelation time increases with pH and decreases with protein concentration. The storage modulus at pH 8 increases exponentially with protein concentration. The exponent of the law relating storage modulus to protein concentration changes from about 8 in the range 1.7−2.5% to 2 at concentrations >3.5%. The critical protein concentration is <1.1%.

Keywords: *Sunflower proteins; hydrolysis; gelation*

INTRODUCTION

Sunflower is a widespread protein-rich crop lacking toxicants and antinutritive factors other than phytate (Bulmaga et al., 1989a). Its protein is reputed to be only slightly deficient in lysine and threonine (Canella et al., 1982; Saviozzi et al., 1986). However, sunflower protein isolates find only very marginal use in the food industry due in part to problems associated with their chlorogenic acid content (Sabir et al., 1974). A number of processes to obtain very low phytate and chlorogenic acid protein isolates are now available (Gheyasuddin et al., 1970; Vermeersch et al., 1987; Saeed and Cheryan, 1988), but their functional properties have so far been little investigated.

Gelation is a functional property of proteins that can condition the use of protein-rich materials in foods. It implies the formation of a three-dimensional network by progressive aggregation of macromolecules or particles through chemical bonds or physical interactions.

Protein gelation takes place under conditions that allow ordered interchain, intermolecular, or interparticle interactions. Many proteins gelate during or after simple heating. Heat is thought to be required for protein unfolding and reactive group unmasking prior to aggregation (Kinsella, 1976; Clark and Lee-Tuffnell, 1986). Other proteins gelate only under moderate−high concentrations of Ca^{2+} or salt, controlled acidification, reaction conditions able to break S−S bonds, or other microenviromental manipulations that either enhance or hinder some types of physical or chemical interactions.

Gelation of sunflower proteins is not achieved by any of these treatments. However, after partial hydrolysis with trypsin, it is possible to obtain gels that show the unusual property of deeply weakening when cooled below about 80 °C (Sánchez and Burgos, 1995). Gelation requires a "critical" macromolecular concentration. Macromolecular concentration influences also gelation time and the rheological behavior of the gels (Oakenfull, 1984; Clark, 1991; Ross-Murphy, 1991; Clark, 1992). Usually protein gel properties are also strongly affected by pH (Knipe and Frye, 1990; Stading and Hermansson, 1990) and other factors.

The aim of this work is to explore the influence of protein concentration, pH, and hydrolysis degree on the rheological behavior of the thermotropic gels formed by sunflower protein hydrolysates.

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). All other chemicals used were of analytical reagent grade.

Preparation of Protein Isolates. Low phytate and chlorogenic acid isolates were prepared as described by Sánchez and Burgos (1995). For storage the isolates were freeze-dried after the pH was adjusted to 7.

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

Solubility Profiles. Solubility profiles were obtained following basically the method of Saeed and Cheryan (1988). Samples of the sunflower isolates or their hydrolysates were extracted by continual stirring (magnetic stirrer) for 1 h with 5 volumes of water at 2−4 °C and different pHs in the range 2−10. The pH was maintained stable during extraction with 0.5 N NaOH or 0.5 N HCl. Clear supernatants were obtained by centrifugation at 4000*g* for 45 min. The supernatants were filtered through Whatman No. 3 filter paper, and nitrogen was analyzed in the filtrate according to the Kjeldhal method.

SDS Electrophoresis. Electrophoretical analysis of the hydrolysates was performed on 20% polyacrylamide gels (PhastSystem, Pharmacia, Uppsala, Sweden) under reducing and dissociating conditions. Five milligram samples were dissolved in 1 mL of 0.1 M Tris-acetate buffer, pH 9.5, containing 3% mercaptoethanol and 2.5% SDS and the solutions incubated in a boiling water bath for 5 min. Samples of

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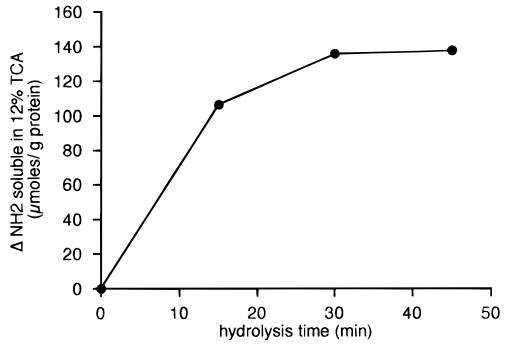

Figure 1. Increase in 12% TCA-soluble amine nitrogen of sunflower protein isolates with time of trypsin hydrolysis.

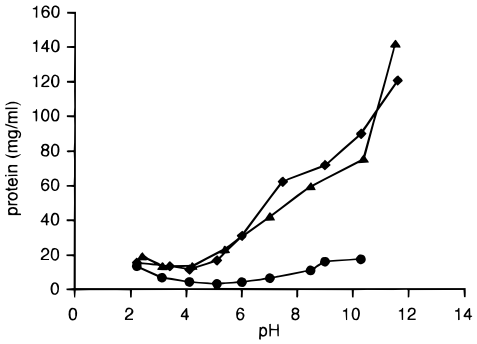

Figure 2. Solubility profile of sunflower protein isolates and their trypsin hydrolysates. Hydrolysis degree: Increase in 12% TCA-soluble amine nitrogen, 0 (●), 106 (▲) and 136 (◆) μmol/g.

0.5 μL of the incubated mixture were applied on the gels. Relative molecular mass (M_r) markers of 2 500−17 000 and 14 000−95 000 were used. Protein bands were stained with Coomassie blue as described by the instrument manufacturer (Pharmacia AB, 1986).

Rheological Measurements. Gelation was monitored by dynamic rheological measurements performed in a Bohlin CS/ETO rheometer (Bohlin, Lund, Sweden) working in the oscillatory mode. Cone and plate geometry [4° and 4 cm diameter (CP 4/40)] was used. Samples assayed consisted of 1.5 mL of a solution prepared by dissolving freeze-dried protein samples in the following buffers: 0.05 M Tris (pH 7−9), 0.05 M borate (pH 10), or 0.05 M phosphate (pH 11). To prevent water evaporation and surface drying, samples were surrounded by silicone oil without covering the cone. Test conditions were established as follows: frequency, 0.1 Hz; maximum strain, 0.02. These conditions were set after preliminary experiments by strain and frequency sweeps proved that they were in the lineal viscoelasticity range. Integration time was fixed at 10 s. Gelation time was defined as the time (seconds) after which the angle phase remained under 45°.

Gels were formed by heating the sample in the temperature range 60−98 °C at a rate of 1 °C/min followed by holding at 98 °C for 60 min to "cure" the gels. Data measured were phase angle and storage modulus (G').

RESULTS

Influence of Hydrolysis Degree. As shown in Figure 1, the proteolysis degree achieved is very limited and little affected by reaction time in the range (15−50 min) explored here. The increase of the 12% TCA-soluble NH_2 groups after 15−45 min of hydrolysis ranges over the interval 105−140 μmol/g of protein. Even such a limited hydrolysis strongly affects the protein solubility (Figure 2). There is no change in the pH of minimum solubility (4−4.5), but the solubility at the alkaline side of this pH greatly increases.

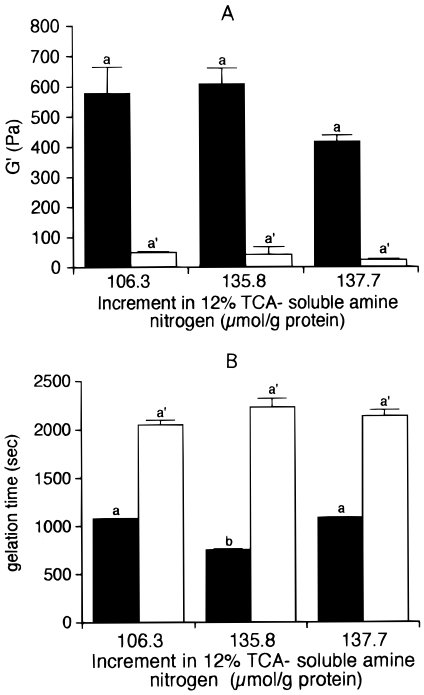

Figure 3. Influence of trypsinization degree in (A) maximum storage modulus (at 98 °C, after curing) and (B) gelation time of the gels obtained at pH 8 from trypsinized sunflower protein isolates. Protein concentrations were 4.3% (black bars) and 2% (white bars). The vertical lines represent the standard error of the mean and are not shown when masked by bars. Bars with no superscript letter in common are not significantly different ($p < 0.05$)

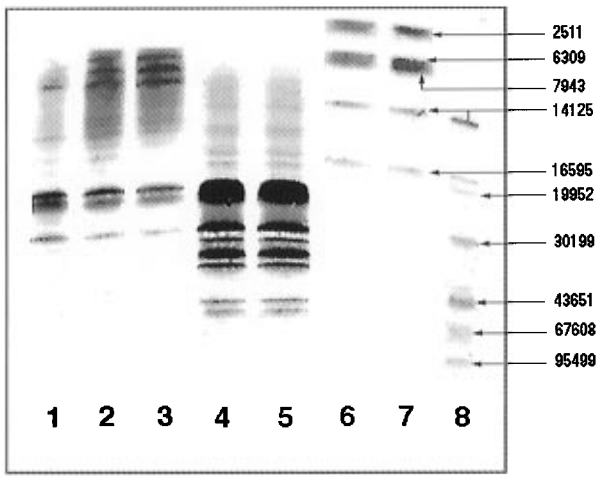

Figure 4. SDS−PAGE of sunflower proteins and their trypsin hydrolysates under reducing and dissociating conditions: (lane 1) trypsin hydrolysate (Δ 12% TCA-soluble amine nitrogen, 106 μmol/g); (lane 2) trypsin hydrolysate (Δ 12% TCA-soluble amine nitrogen; 136 μmol/g); (lane 3) trypsin hydrolysate (Δ 12% TCA-soluble amine nitrogen, 138 μmol/g); (lanes 4 and 5) nonhydrolyzed sunflower protein isolate; (lanes 6 and 7) M_r markers of 2 500−17 000; (lane 8) M_r markers of 14 000−95 000.

Hydrolysis degree affects gelation time and G'. The highest G' and the shortest gelation time were achieved with increases of 12% TCA-soluble NH_2 groups around 135 μmol/g of protein, but differences in these parameters are rather small in the range of proteolysis obtained (Figure 3).

The electrophoretic analysis of the samples (Figure 4) shows that hydrolyis affects polypeptides of M_r 30 000−50 000, which became undetectable in the elec-

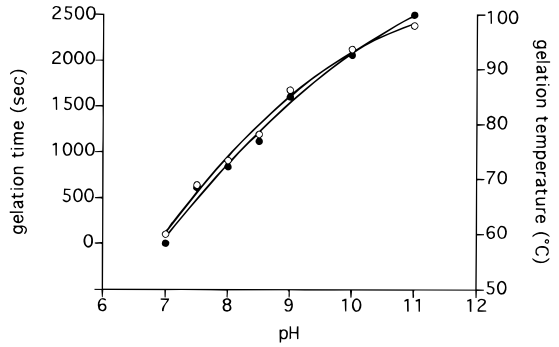

Figure 5. Influence of pH in gelation time (○) and temperature (●), under the experimental conditions (heating rate in the 60−98 °C range, 1 °C/min). Protein concentration was 5%. Hydrolysis degree was Δ 12% TCA-soluble amine nitrogen, 136 μmol/g.

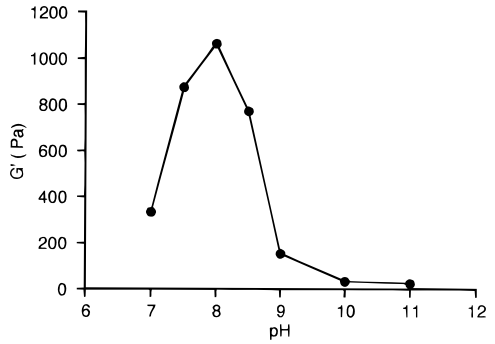

Figure 6. Influence of pH in the storage modulus (at 98 °C, after curing) of gels formed from trypsinized sunflower protein isolate (Δ 12% TCA-soluble amine nitrogen, 136 μmol/g) at 5% protein concentration.

trophoretic analysis, and chains of M_r about 20 000−28 000, which are the major components of the "native" isolates and whose contribution to total Coomassie blue stainable material diminishes markedly with the enzyme treatment and results in the appearance of three or four peptides of M_r in the range 4 000−10 000. A substantial fraction of polypeptides of M_r 20 000, 22 000, and 28 000 remain unhydrolyzed.

pH Effects. Below pH 7 the protein of the isolates precipitates, and above pH 11 there is no sol−gel transition. Gelation is only possible in the pH range 7−11.

In this pH range, G', gelation time, and gelation temperature are strongly pH dependent. Gelation time and gelation temperature increase with pH (Figure 5) and G' reaches a peak at pH 8 (Figure 6).

Protein Concentration Dependence of Storage Modulus and Gelation Time. The concentration dependence of G' and gelation time was investigated at pH 8, in the concentration range 1.7−10%. As expected (Clark, 1991, 1992; Ross-Murphy, 1991), G' increases and gelation time shortens with increasing protein concentration. As shown in Figure 7, the plot of log of gelation time versus log of concentration is a straight line of slope −1. No evidence of divergence to infinity at the lower concentrations is observed. Double-logarithmic plots of G' versus concentration (Figure 8) show increasing slopes from about 2 at 10% protein concentration to 8 at 1.7% protein concentration.

DISCUSSION

Enzymatic modification of structure and functional properties of proteins has been a current research

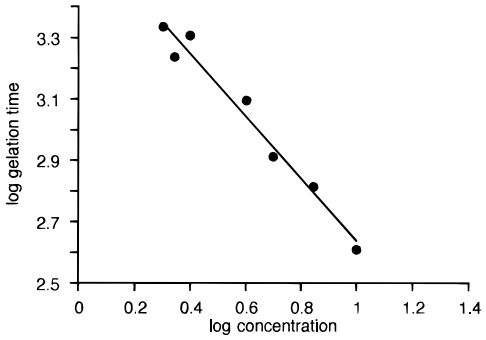

Figure 7. Protein concentration dependence of gelation time of a trypsinized sunflower protein isolate (Δ 12% TCA-soluble amine nitrogen, 136 μmol/g) at pH 8.

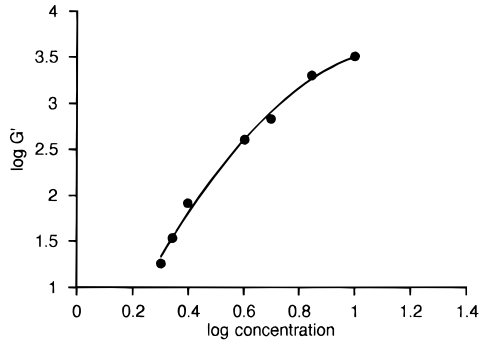

Figure 8. Protein concentration dependence of G' (at 98 °C, after standard curing) in gels of a trypsinized sunflower protein isolate (Δ 12% TCA-soluble amine nitrogen, 136 μmol/g) at pH 8.

subject during the past 20 years (Hale, 1969; Richardson, 1977; Fox et al., 1982; Dave et al., 1991). Partial hydrolysis usually increases the solubility, decreases the viscosity of concentrated solutions, and negatively affects the gelation properties of the protein isolates. Trypsin is known to have a limited proteolytic effect, specifically breaking peptide (or acylester) bonds in which the amine group of dibasic amino acids are involved. Therefore, increases of the 12% TCA-soluble NH_2 groups with reaction time must stop rather soon under optimum reaction conditions, as happens in the hydrolysis experiments here described. In seed storage proteins, a general structural disruption and a marked increase of interfacial area and charged groups exposed to the aqueous environment are to be expected from such a limited enzymatic activity. This must in turn increase solubility, as it had been previously shown for sunflower proteins by a number of authors (Kabirullah and Wills, 1981; Jones and Tung, 1983; Bulmaga et al., 1989b) and as it is confirmed in Figure 2. According to Bulmaga et al. (1989b), this partial proteolysis splits from the C-terminal sequence of the α chains of the 11S globulin short peptides rich in dibasic amino acids adjacent to a glutamic and aspartic acid rich sequence. Therefore, it has to increase the net charge of the globulins at pHs above the isoelectric point. This explains why the solubility changes take place mainly at this side of the solubility profile.

The small extent of the protein hydrolysis achieved by trypsin and the change in the net charge which this produces can also explain the observed change in the gelling properties. Gelation requires as starting material a solution of a polymer of substantial chain size. Usually proteolysis results in impaired gelling properties due to the small size of their products. As shown

in Figure 4, trypsin hydrolysis of sunflower proteins yields polypeptides big enough to be efficiently involved in network generation together with the nonhydrolyzed fractions of the chains of about 20 000, 22 000, and 28 000 M_r.

If they are not submitted to trypsin hydrolysis, sunflower protein isolates give a coarse precipitate when heated at any pH in the range 7−9, revealing an excess of protein−protein interactions. The increase of intermolecular repulsions at these pHs brought about by the splitting of the dibasic amino acid rich sequence would allow the establishment of the relatively few but ordered cross-links needed to build a network. Moreover, gel formation requires a polymer concentration above a critical value and takes place at a rate that is concentration dependent. At optimum pH, under the experimental conditions used here, gelation is difficult to observe in the trypsinized samples at concentrations lower than 1.5%, which is about the limit of solubility of the isolates. The increase in solubility brought about by trypsin is an important contribution to the change in the gelation properties.

Protein gelation is always possible only in a limited range of pH, due to the pH effect on the balance between interchain attractive and repulsive forces. This pH range usually spans several units and widens with increasing protein concentration (Shimada and Matsushita, 1980). The very narrow and alkaline pH interval at which trypsinized sunflower proteins can gelate could be expected from their high glutamic and aspartic acid richness (more than 30% of overall amino acid residues) and their low dibasic amino acid content (Saviozzi et al., 1986). The increase in gelation time and temperature with pH in this interval reflects the progressive growth of repulsive forces that hinder cross-linking.

The concentration dependence of G' has been mathematically and experimentally studied by several research workers (Hermans, 1965; Bikbov et al., 1979; Clark and Lee-Tuffnell, 1986; Clark et al., 1990; Clark, 1991, 1992) according to improvements of the basic theory of Flory−Stockmayer (Flory, 1941, Stockmayer, 1943) which imply second-order gelation kinetics. According to these theories the storage modulus is an exponential function of the polymer concentration. The theory predicts that within a concentration range spanning several times the critical one, the exponent is constant and at lower concentration values the exponent progressively increases as concentration approaches its critical value.

In most cases, the published experimental data conform to the predictions of the modified Flory−Stockmayer theory (classical model) and the constant exponent found is 2, although values higher than 2, and even close to 5, have sometimes been estimated (Walstra and van Vliet, 1991).

Data plotted in Figure 8 are also in agreement with the classical model. At relatively high concentrations, above 3.5%, G' is proportional to the square of concentration, but below 2.5%, the exponent increases, reaching values of approximately 8, to our knowledge the highest ever experimentally found for a biopolymer.

The concentration dependence of the experimentally calculated gelation times does not usually fit as well to the classical model predictions: a slope close to −1 in the double-logarithmic plots and divergence to infinity near the critical concentration.

The double-logarithmic plot of gelation time versus concentration (Figure 7) fits quite well to a straight line with slope −1 and conforms to the predictions of the classical theory for concentration values well over the critical one.

An alternative kinetic model in which no critical gel concentration is taken into account has been proposed by Oakenfull (Oakenfull, 1984, Oakenfull and Morris, 1987). According to this theory, the slope of double-logarithmic plot gives the reaction order and the number of polymer chains participating in the formation of a junction zone. Although it is not difficult to accept an apparent first order for the overall gelation reaction, assuming that the rate-limiting step is the protein denaturation, it does not make much sense for a gel to be built through junction zones involving a single polymer chain. It seems more plausible to assume that the minimal concentration used in this work (1.7%) is considerably higher than the critical one, which must be well under 1.1%, a value that can be obtained by extrapolation of double-logarithmic plots of G' versus protein concentration (Figure 8), with an arbitrarily chosen minimum value of G' of 1 Pa for the gel.

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