JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Influence of pH and Ionic Strength on Heat-Induced Formation and Rheological Properties of Soy Protein Gels in Relation to Denaturation and Their Protein Compositions

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The influence of pH and ionic strength on gel formation and gel properties of soy protein isolate (SPI) in relation to denaturation and protein aggregation/precipitation was studied. Denaturation proved to be a prerequisite for gel formation under all conditions of pH and ionic strength studied. Gels exhibited a low stiffness at pH >6 and a high stiffness at pH <6. This might be caused by variations in the association/dissociation behavior of the soy proteins on heating as a function of pH, as indicated by the different protein compositions of the dissolved protein after heating. At pH 3−5 all protein seems to participate in the network, whereas at pH >5 less protein and especially fewer acidic polypeptides take part in the network, coinciding with less stiff gels. At pH 7.6, extensive rearrangements in the network structure took place during prolonged heating, whereas at pH 3.8 rearrangements did not occur.

KEYWORDS: Soy protein isolate; gelation; differential scanning calorimetry; protein solubility; rheology

INTRODUCTION

The ability of soy proteins to form a gel on heating makes them well suited to improve the texture of food products. Nevertheless, soy proteins are not frequently used as texture enhancers. Partly, this is due to the difficulties of predicting gel properties and in controlling food texture for all the different conditions (e.g. pH, ionic strength, and heating temperature) during the manufacturing of food products.

The main soy proteins are glycinin and β-conglycinin. The quaternary structure of these proteins depends on pH and ionic strength. Glycinin is composed of acidic (ca. 38 kDa) and basic polypeptides (ca. 20 kDa) linked by a single disulfide bridge, except for the acidic polypeptide A_4 (*1*). At ambient temperatures and pH 7.6 glycinin forms hexameric complexes (11S), while at pH 3.8 it is mainly present as trimeric complexes (7S) (*2*,*3*). At pH 2−10 and ionic strength higher than 0.1, β-conglycinin is a trimeric glycoprotein (a 7S globulin) (*4*) consisting of three different subunits (α', α, and β with molecular masses of ca. 65, 62, and 47 kDa, respectively) in at least six different combinations (*5*). At an ionic strength less than 0.1, β-conglycinin exists as a hexamer (9S) at pH 5 and higher, whereas at pH 2−5 β-conglycinin dissociates into a 2-3S and 5-6S fraction (*4*).

Heat denaturation is often a prerequisite for gel formation of globular proteins. Denaturation temperatures depend strongly on pH and ionic strength (*6*, *7*). The onset denaturation temperature of glycinin is around 80−90 °C for the 11S form and 60−70 °C for the 7S form (*8*, *9*). β-Conglycinin starts to denature at 60−75 °C (*10*−*13*). The relation between denaturation and the onset of gelation has not been extensively studied for soy proteins as a function of pH and ionic strength.

Ionic strength and pH also affect the characteristics of soy protein gels (*13*−*22*). The influence of pH on gel formation by soy proteins has been studied by rheology (*17*, *20*), microscopy (*23*), gel swelling or dissolving experiments (*17*, *21*), and FTIR spectroscopy (*20*). Beside different methods, different materials (glycinin, β-conglycinin, and soy protein isolate (SPI)) were used for each of these studies, which makes comparison and an integration of the results into an overall picture of gel formation difficult.

This study is focused on the influence of pH on gel formation by SPI in relation to denaturation at three salt concentrations. Furthermore, gel properties are determined by rheological measurements at small deformation and related to the effect of heating on protein aggregation/precipitation and to the extent of participation of polypeptides/subunits in the network formation. The relations between network structure and rheological properties of SPI gels as a function of pH will be studied in future work.

MATERIAL AND METHODS

Sample Preparation. For the experiments, a soy protein isolate (SPI) was used with a calculated protein content of 97% using $N \times 6.25$. The SPI (pH 4.8) was prepared from mildly treated, defatted PDI 80 soy flakes (Cargill, Amsterdam, The Netherlands) according to a method

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10.1021/jf020061b CCC: $22.00
Published on Web 09/07/2002

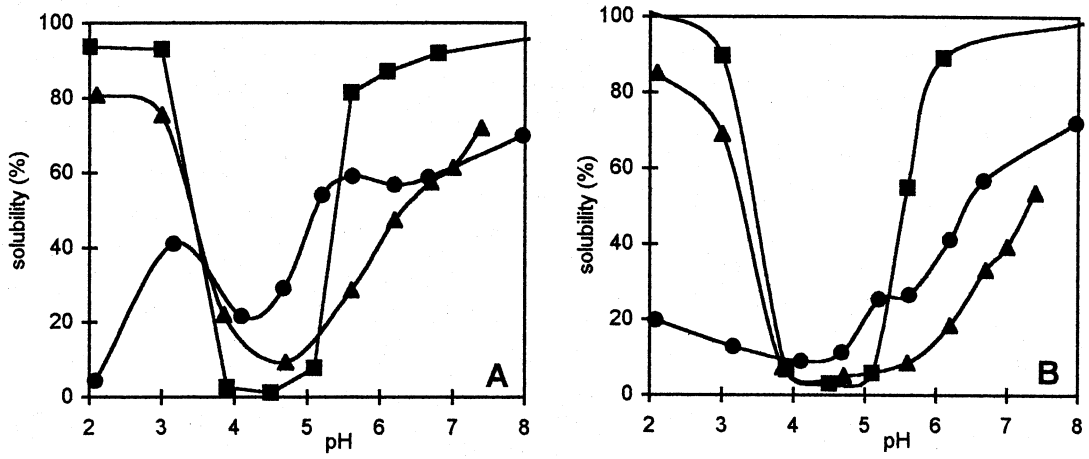

Figure 1. Solubility of unheated (A) and heated (B) 1% soy protein dispersions as a function of pH at added salt concentrations of 0 (■), 0.2 (▲), and 0.5 M NaCl (●).

described previously (*22*). Protein dispersions (120 mg/g) for gel formation and DSC experiments were prepared by suspending 6 g of SPI in 35 g of double-distilled water and 0.2 and 0.5 M NaCl solution. After it was stirred for 1 h at 4 °C, the suspension was brought to pH 7.6 with 0.5 M NaOH. After 2 h the pH was adjusted with 0.5−1 M HCl to the desired value. If necessary, the mass of the dispersion was adjusted to 50 g by adding water or salt solution to obtain the desired protein concentration. The protein dispersions were stirred overnight to enhance protein dissolution. Protein dispersions were prepared at 4 °C to prevent proteolysis by endogenous enzymes.

As a reference for identification of endotherms in DSC experiments, protein dispersions (0.1 g/g) of purified glycinin and a β-conglycinin-rich fraction in double-distilled water were used. Isolation of these proteins was described in a previous paper (*13*). Purified glycinin is ∼95% pure, whereas the β-conglycinin-rich fraction consists of β-conglycinin (∼60%), glycinin (∼15−20%), and other proteins (∼20−25%) (*13*).

Determination of Protein Solubility and Protein Composition. SPI dispersions (10 mg/mL) were prepared by stirring 1 g of SPI in 90 mL of double-distilled water or salt solution for 1 h at 4 °C. In all cases, the protein dispersions were first brought to pH 7.6 with a defined amount of 0.5 M NaOH. After 1 h, the pH was adjusted to the desired pH with 0.5 M HCl or NaOH. After pH adjustment, the volume of the dispersion was adjusted to 100 mL. The dispersions were stirred for 16 h at 4 °C. Preparation of the dispersions was performed at 4 °C to prevent proteolysis by endogenous enzymes. Part of the protein dispersion was heated from 20 to 95 °C at a rate of 1 K/min, kept at 95 °C for 1 h, and cooled at a rate of 1 K/min to 20 °C. The unheated and heated dispersions were centrifuged at 32 000 *g* for 30 min at 4 °C. The protein content of the dispersions (total protein) and of the supernatants (dissolved protein) was determined in duplicate by the micro-Kjeldahl method (*24*) using a Kjeldahl factor of 6.25. Solubility was defined as (dissolved protein/total protein) × 100%.

The protein composition of the supernatants of the heated and unheated dispersions was determined by SDS-PAGE under both reducing (with 2.5% β-mercaptoethanol) and nonreducing conditions on a Phast System (Pharmacia of Sweden) according to the instructions of the manufacturer. Equal volumes of each supernatant were diluted 1:1 in sample buffer, which resulted in sample protein concentrations ranging from 0 to 0.5 mg/mL. The samples were left overnight at 20 °C. Phastgel Gradient 10−15 gels (Pharmacia) were used, which were stained with Coomassie Brilliant Blue. The gel was calibrated with low-molecular-mass markers ranging from 14 to 94 kDa (Pharmacia).

The protein composition of the supernatants was quantified by visual density evaluation of the SDS-PAGE profiles. The conditions of pH 8.1 and 0 M NaCl were regarded as a reference, because under these conditions the protein solubility was highest.

Differential Scanning Calorimetry (DSC). Denaturation temperatures of SPI dispersions (120 mg/g) were determined by differential scanning calorimetry at a scanning rate of 1 K/min. The measurements were performed in a micro-DSC (Setaram of France) equipped with 0.9 mL stainless steel sample vessels. The temperature at which denaturation starts, the onset denaturation temperature (T_o), was estimated by taking the intercept of the baseline and the extrapolated slope of the peak. For the peak denaturation temperature (T_{max}), the temperature at maximum heat flow was taken.

Gelation. Gel formation was followed in duplicate by dynamic measurements in a Bohlin CVO rheometer using a serrated concentric cylinder geometry (C25). The storage (G') and loss (G'') moduli were measured in the linear region at a constant maximum strain of 0.01 and an angular frequency of 0.63 rad/s. To induce gel formation, protein dispersions were heated from 20 to 95 °C at a rate of 1 K/min, kept for 1 h at 95 °C, and cooled to 20 °C at a rate of 1 K/min. To prevent solvent evaporation, a thin layer of soy oil was put on top of the samples. Selected gels were reheated from 20 to 95 °C at a rate of 1 K/min. At 95 °C, the time-dependent behavior of the gels was studied by a frequency sweep up and down from 0.063 to 63 rad/s in 16 logarithmic steps, followed by a frequency sweep down and up from 0.063 to 0.0063 rad/s in 6 logarithmic steps. The temperature at which G' started to increase over 0.5 Pa/K was defined as the gelation temperature. At most conditions, a slow increase in G' (total about 1 Pa) was observed before the steep increase. We chose to neglect this slow increase.

RESULTS

Solubility. Figure 1 shows the protein fraction that remains dissolved after centrifuging of unheated and heated SPI dispersions (10 mg/mL) in water and 0.2 and 0.5 M NaCl. This protein fraction consists of proteins, polypeptides, subunits, and aggregates smaller than approximately 0.2 μm, as calculated by using the Stokes equation, and is further denoted as solubility.

In water and 0.2 M NaCl, SPI had a low solubility between pH 4 and 5 (**Figure 1A**). In 0.5 M NaCl, SPI also had a low solubility in this pH range, but the actual minimum was observed at pH values below 3. An increase of salt concentration caused an increase in the amount of dissolved protein between pH 4 and 5 and a decrease at pH values lower than 3 and higher than 7. This agrees with results by others *(25, 26)*. Heating did not affect the solubility in the absence of NaCl (**Figure 1B**). At 0.2 M NaCl the solubility decreased with 20−30% at pH values higher than 5, and at 0.5 M NaCl it decreased with 10−30% at pH 3−7.

Protein Composition of Dissolved Fractions. Protein compositions of the dissolved fractions of the unheated and heated dispersions (10 mg/mL) were determined by SDS-PAGE analysis under reduced and nonreduced conditions and are presented in **Table 1**.

Table 1. Protein Composition of Supernatant of Heated 1% Soy Protein Isolate Dispersions As Determined by SDS-PAGE[a]

		unheated reduced conditions				heated reduced conditions				heated nonreduced conditions					
NaCl concn, M	pH	A	B	α, α′	β	A	B	α, α′	β	A	B	AB	α, α′	β	agg
0	3	+++	+++	+++	+++	++	++	+	+++	?	?	++	+	+	++
	3.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	4.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	5.1	–	–	+	–	–	–	–	–	–	–	–	–	–	–
	5.6	+++	+++	+++	+++	+++	+++	+++	+++	+	–	–	+	+	+++
	6.1	+++	+++	+++	+++	+++	+++	+++	+++	+	–	–	+	+	+++
	6.8	+++	+++	+++	+++	+++	+++	+++	+++	+	–	–	+	+	++
	8.1	+++	+++	+++	+++	+++	+++	+++	+++	++	–	–	++	++	++
0.2	2.1	+++	+++	+++	+++	++	++	–	++	–	–	+	–	++	-
	3	+++	+++	+++	+++	++	++	–	++	–	–	+	–	++	+
	3.9	+	+	++	++	–	–	–	–	–	–	–	–	–	–
	4.7	-	-	+	–	–	–	–	–	–	–	–	–	–	–
	5.7	+	+	++	++	+	–	–	–	–	–	–	–	–	–
	6.2	++	++	+++	+++	++	–	+	-	+	–	–	–	–	++
	6.7	+++	+++	+++	+++	+++	+	++	++	+	–	–	+	+	++
	7	+++	+++	+++	+++	+++	+	++	++	+	–	–	+	+	+++
	7.4	+++	+++	+++	+++	+++	+	++	++	+	–	–	+	+	+++
0.5	2.1	–	–	+++	+++	–	–	–	–	–	–	–	–	–	–
	3.2	+++	+++	++	++	–	–	–	–	–	–	–	–	–	–
	4.1	+	+	++	++	–	–	–	–	–	–	–	–	–	–
	4.7	++	++	+++	+++	–	–	–	–	–	–	–	–	–	–
	5.2	++	++	+++	+++	+	+	–	–	–	–	–	–	–	–
	5.6	+++	+++	+++	+++	++	+	–	–	–	–	+	–	–	–
	6.2	+++	+++	+++	+++	++	+	+	–	+	–	+	–	–	+
	6.7	+++	+++	+++	+++	+++	++	++	++	++	–	++	++	+	++
	8	+++	+++	+++	+++	+++	++	+++	++	++	–	++	++	+	++

[a] Legend: A and B, acidic and basic polypeptides of glycinin; α, α′, and β, subunits of β-conglycinin; agg, aggregates; –, no polypeptides/subunits present in the dissolved fraction; +++, all polypeptides/subunits present in the dissolved fraction; ++ and +, part of the polypeptides/subunits present in the dissolved fraction; ?, no clear profile. Quantification is based on visual density evaluation of the SDS-PAGE profiles and with the condition at pH 8.1 and 0 M NaCl as a reference.

Unheated. The SDS-PAGE profiles of the supernatants of the unheated dispersions at pH 2−3 and pH 5.6 and higher showed a normal pattern of glycinin polypeptides and β-conglycinin subunits, except for pH 2−0.5 M. Under these conditions, only β-conglycinin was present in the supernatant. At the intermediate pH values, dissolved protein was only observed at 0.2 and 0.5 M NaCl and consisted of more β-conglycinin than glycinin.

Heated. At pH >5 (further denoted as *high pH*) and 0 M NaCl, as much acidic (A) as basic (B) polypeptides of glycinin were found under reduced conditions, whereas in the presence of salt more acidic than basic polypeptides remained dissolved. Nonreducing SDS-PAGE revealed that the original AB subunit was no longer present at 0 and 0.2 M NaCl, in contrast to the case for 0.5 M NaCl. At all salt concentrations, free acidic polypeptides and aggregates larger than 100 kDa were observed in the nonreduced SDS-PAGE gels, whereas free basic polypeptides were not noticed. The basic and the remaining acidic polypeptides must be present as (part of the) dissolved aggregates and, at 0.5 M NaCl, also as part of the AB subunits. Reduced SDS-PAGE showed that, on heating, the amount of dissolved β-conglycinin subunits decreased with increasing salt concentration, whereas the composition of β-conglycinin remained largely unchanged. Under nonreduced conditions, however, fewer β than α and α′ subunits were observed at 0.5 M NaCl. This means that also part of the β subunits is present as (part of the) dissolved aggregates.

At pH 3−5, no protein could be detected on the SDS-PAGE gels because of the low amount of dissolved protein.

At pH 2 and 3 (further denoted as *low pH*), the A and B polypeptides of glycinin were present in the same amounts at 0 and 0.2 M NaCl when analyzed under reduced conditions. At 0.5 M NaCl, no dissolved protein was detected. Under nonreduced conditions, the original AB subunit and aggregates larger than 100 kDa were observed and no free polypeptides were present at 0 and 0.2 M NaCl. This means that the acidic and basic polypeptides were only present in the AB subunit. At 0 M NaCl, the composition of the β-conglycinin subunits differed between reduced and nonreduced conditions. This indicates that part of the β subunits was present as (part of the) soluble aggregates.

The aggregates larger than 100 kDa formed at low and high pH could only be seen on SDS-PAGE gels under nonreduced conditions. The results indicate that at least part of every polypeptide and subunit is present in an aggregated form. Whether aggregates consist of only acidic or basic polypeptides of glycinin or subunits of β-conglycinin or of complexes formed by specific polypeptides or subunits has not been studied.

Denaturation. The influence of pH and ionic strength on denaturation was studied by differential scanning calorimetry (DSC). **Figure 2** shows typical examples of DSC thermograms of soy protein isolate (a−d) at several pH values. All thermograms showed two endothermic transitions, except for pH 3. Separate analysis of purified glycinin (e) and β-conglycinin (f) at this pH showed that heat denaturation took place for both proteins. The enthalpy, ΔH, however, was very low at pH 3. β-Conglycinin had an onset and peak denaturation temperature of 55 and 61 °C, respectively. The thermogram of glycinin showed a broad endothermic transition with an onset denaturation temperature of 45 °C and two peak denaturation temperatures at 55 and 63 °C.

In **Figure 3**, the onset and peak denaturation temperatures of SPI were maximal close to the isoelectric point and minimal at pH values smaller than 4. Similar results were obtained for

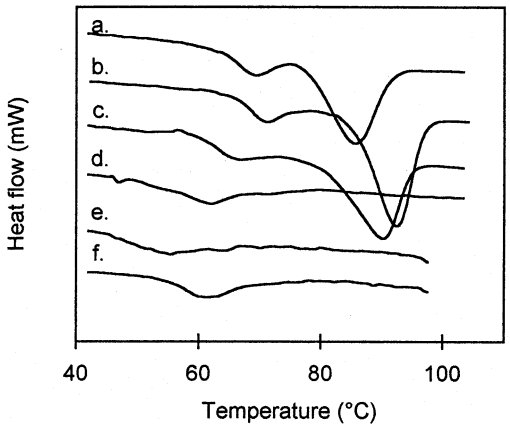

Figure 2. Examples of DSC thermograms of 12% soy protein isolate dispersions (a–d) and 10% dispersions of purified glycinin (e) and β-conglycinin (f) at different pH values and 0 M NaCl: (a) pH 7.8; (b) pH 5.2; (c) pH 3.8; (d–f) pH 3. The scanning rate was 1 K/min.

SPI (*27*), β-conglycinin (*13*, *20*), and glycinin (*9*, *28*). Figure 3 also shows that heat denaturation temperatures were higher at higher salt concentrations than at low salt concentrations. Furthermore, with increasing salt concentration the pH dependence became less pronounced at pH 5−8.

Gelation. Figure 4 shows typical gelation curves of SPI gels in 0.2 M added NaCl at pH 3.8, 5.2, and 7.6, as observed by dynamic rheological measurements. At a certain temperature, the gelation temperature, G', starts to increase. During subsequent heating at 95 °C G' kept increasing, but a much stronger increase was observed on cooling.

Gelation temperatures are plotted in **Figure 3** together with the denaturation temperatures. At a pH lower than 6, gelation temperatures were lowest and coincided with the first denaturation peak for SPI. At pH 6 and higher, gelation temperatures were higher and coincided with the second denaturation peak.

Figure 5 shows the storage or elastic modulus, G', of soy protein isolate gels after completion of the heating and cooling cycle as a function of pH at the three different salt concentrations. G' is a measure of the stiffness of gels or, in other words, of the resistance to deformation. At every salt concentration studied, gels were stiffer at low pH than at high pH. In the absence of NaCl, a maximum in G' was found at pH 6.5, but an even higher stiffness of the gels was measured at pH values lower than 6. At pH values 4.5−5.5 and in the absence of NaCl, no measurements could be performed owing to protein precipitation before heating. At 0.2 M NaCl, only three data points are given. However, another series of measurements, performed with SPI from a different batch, showing a gradual increase in G' with decreasing pH (not shown) allowed drawing of the dotted line. Also, at 0.5 M NaCl, G' increased gradually with decreasing pH. Similar results were obtained by van Kleef (*17*).

Thermoreversibility and Frequency Dependence. Thermoreversibility and the occurrence of rearrangements at high temperatures were investigated by reheating gels to 95 °C, where the frequency dependence was tested, and consecutively cooling gels down to 20 °C. **Figure 6** shows the increase in storage modulus, G', of a 12% SPI dispersion in 0.2 M NaCl of pH 3.8, 5.2, and 7.6 during a normal heating and cooling cycle (line with one arrow). At pH 7.6, G' is thermoreversible on reheating to 40 °C. At higher temperatures, the reheating curve started to deviate from the first cooling curve. The second cooling curve started at a lower stiffness and differed completely from the previous curves. Immediate cooling after reheating of the gel did not result in such a large difference at 20 °C (data not shown). At pH 3.8, the reheating curve follows the first cooling curve completely, but G' of the second cooling curve is about 1 kPa higher at each temperature. At pH 5.2, the reheating and second cooling curves were only slightly deviating, which means that this gel was almost completely thermoreversible. Keeping the gels for a long time at 95 °C apparently caused changes in (the properties of) the gels at pH 7.6 and, to a small extent, at pH 3.8 and pH 5.2.

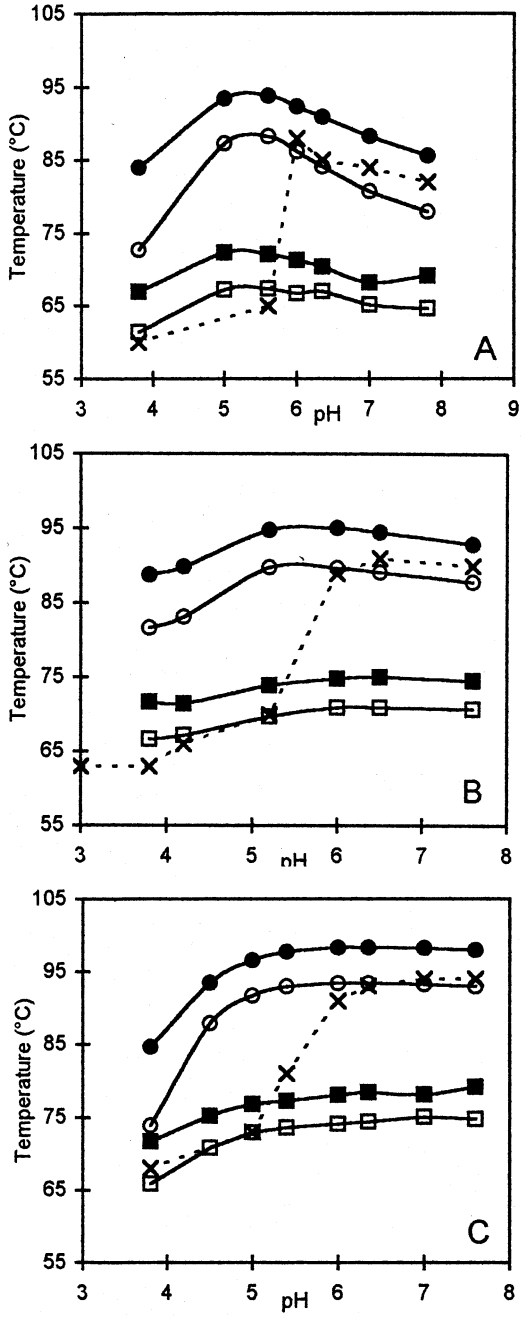

Figure 3. Onset (□, ○) and peak (■, ●) denaturation and gelation (×) temperatures of 12% soy protein isolate dispersions as a function of pH at 0 (A), 0.2 (B), and 0.5 M (C) added NaCl: (■, □) denaturation temperatures of the first endothermic transition; (●, ○) denaturation temperatures of the second endothermic transition.

Figure 7 shows the frequency dependence of the storage modulus G' (A) and the loss tangent tan δ (B) at 95 °C of SPI gels at 0.2 M NaCl and pH 3.8, 5.2, and 7.6. At pH 3.8, G' values were slightly higher for a higher angular frequency, ω, whereas at pH 5.2 and, especially, pH 7.6, G' was more

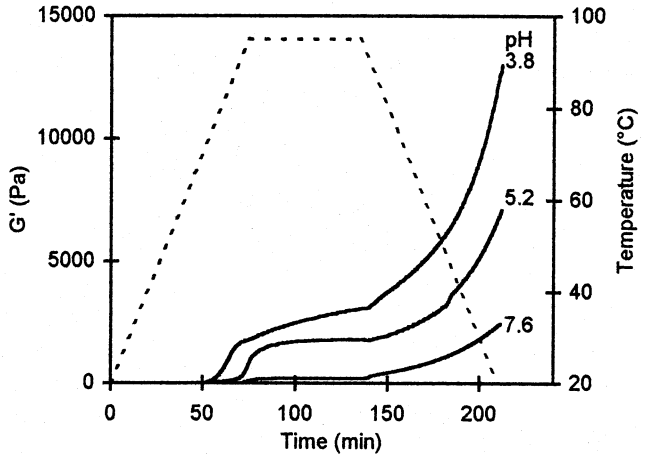

Figure 4. Typical gelation curves of 12% soy protein isolate at 0.2 M NaCl at three different pH values: (—) storage modulus G'; (- - -) temperature.

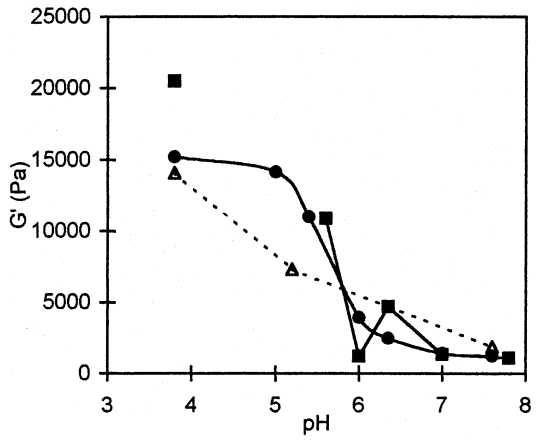

Figure 5. Storage modulus G' after a complete temperature cycle of 12% soy protein dispersions as a function of pH at added salt concentrations of 0 (■), 0.2 (▲), and 0.5 M NaCl (●).

frequency dependent. At low frequencies lower G' values were measured than at high frequencies, because at larger experimental time scales ($=1/\omega$) more protein−protein bonds have the opportunity to become stress-free during the periodic deformation. **Figure 7B** shows that tan δ is also more frequency dependent at pH 5.2 and 7.6 than at pH 3.8. At pH 7.6 the curves of the frequency sweeps down and up from 0.063 to 0.0063 rad/s were not the same, which showed that long incubation times at 95 °C induced large changes in the pH 7.6 gel, leading to a decrease in tan δ. A control experiment in a Bohlin VOR rheometer, in which the dynamic moduli of a pH 7.6 gel were recorded during heating at 90 °C for 10 h at a constant strain of 0.01 and an angular frequency of 0.0063 rad/s, confirmed that tan δ decreased on prolonged heating (data not shown).

DISCUSSION

Heat Denaturation as a Prerequisite for Gelation. Heat denaturation temperatures vary as a function of pH and ionic strength (**Figure 3**). Highest denaturation temperatures were found close to the isoelectric point (pH ∼5.5) and at higher salt concentrations. This is common for most globular proteins (*29*), for proteins tend to be most stable against denaturation when they have no net charge or when their charge is screened.

Gel formation started at the onset of denaturation, which shows that heat denaturation is a prerequisite for gel formation

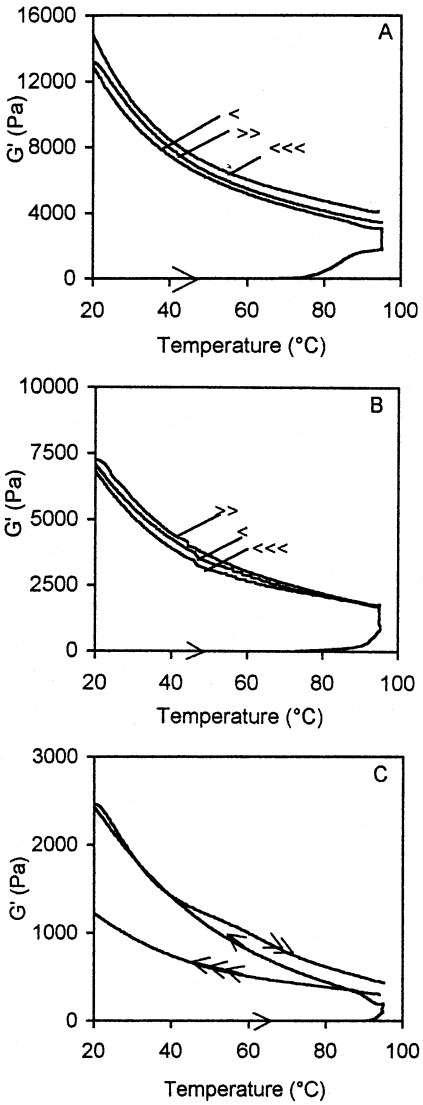

Figure 6. Thermoreversibility of the storage modulus G' of a 12% soy protein isolate gel at pH 3.8 (A), 5.2 (B), and 7.6 (C) and 0.2 M NaCl. The line with one arrow represents the first heating and cooling curve, the line with two arrows the second heating curve, and line with three arrows the second cooling curve (after performance of the frequency sweeps at 95 °C (duration ∼10 h)).

by soy proteins. This is valid for all the studied conditions of pH and ionic strength. Unexpectedly, gelation of SPI coincided with the first denaturation peak at pH <6, whereas at pH >6 gelation started at the second denaturation peak (**Figure 3**). At the studied pH range, the endothermic transition observed at the lowest temperature is caused by heat denaturation of β-conglycinin and the one at the highest temperature by glycinin (*27, 30*). At pH 3.8, the endothermic transition at the lowest temperature might be partly caused by heat denaturation of the 7S form of glycinin which is present predominantly at this pH (*3*) and has a lower denaturation temperature than the 11S form (*8, 9, 31*). Although this might suggest that at pH <6 gelation is also caused by denaturation of 7S glycinin, results from our laboratory showed that, for the present conditions, gel formation by glycinin did only start at heat denaturation of the 11S form (*22*).

The results suggest that β-conglycinin cannot form a gel at pH >6, but other studies (*13, 20*) showed that gel formation does take place at pH >6. However, at the SPI concentration (120 mg/g) in this study, the initial increase in G' as a result of

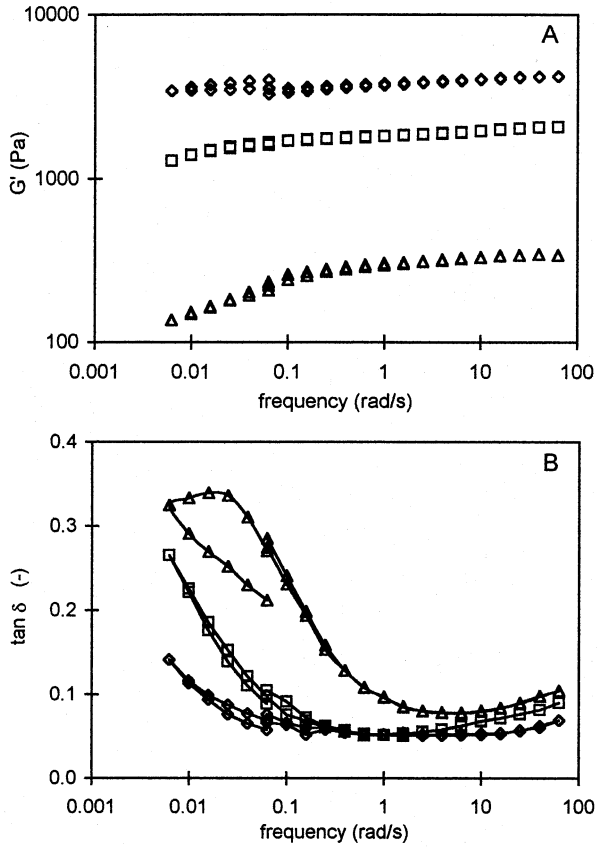

Figure 7. Frequency dependence of the storage modulus (A) and loss tangent (B) at 95 °C of 12% soy protein isolate gels at pH 3.8 (◇), 5.2 (□), and 7.6 (△) and 0.2 M NaCl.

denaturation of β-conglycinin was too low ($\ll$0.5 Pa/K) to be considered as gel formation. This is due to the lower efficiency of β-conglycinin to form a gel with a certain G' at pH 7.6 compared to pH 3.8 (*13*). The critical protein concentration of β-conglycinin required for gel formation did not differ much at low or high pH values (*13*).

At pH 3, only one broad endothermic transition is observed (**Figure 2**). Further analysis showed that this is a result of denaturation of both glycinin and β-conglycinin. Exceptionally, the denaturation temperatures of glycinin were lower than those of β-conglycinin. According to Wolf and co-workers (*2*) most of the glycinin is in a 3S (1AB) and part of the glycinin in a 7S form at pH 3 and low ionic strength. The 3S form does not give a cooperative transition (*8*), which implies that at this condition 7S glycinin is not very heat stable.

Rheological Properties of Soy Protein Gels. The stiffness of soy protein gels varies as a function of pH and ionic strength (**Figure 5**). In general, higher values for G' were obtained at pH <6 than at pH >6. Irrespective of pH, the stiffness of the gels decreased when the temperature of the gels was increased. At pH 3.8 and 5.2, reheating of the gels completely undid the stiffening of the gels that was induced by cooling, in contrast to the case at pH 7.6 (**Figure 6**). The fact that the gels were not thermoreversible on reheating at pH 7.6 indicates that at high temperatures changes in the network occur. We believe that these changes are most likely induced by rearrangements. Indications for the occurrence of these rearrangements are obtained from the frequency dependence of G' and tan δ at 95 °C. Higher tan δ values at lower frequencies (**Figure 7**), as found at pH 7.6 and to some extent pH 5.2, mean that these gels had a stronger viscous-like behavior than at pH 3.8. It implies that, at 95 °C, bonds between protein molecules can be broken and re-formed more easily at pH 7.6 and 5.2 (*32*).

Low G' values, as found at higher pH values, correlate with a high amount of dissolved protein in heated 1% dispersions (**Figures 1B** and **5**). It is expected that the protein that remained dissolved after heating is not incorporated in the network in contrast to the precipitated polypeptides and subunits. These lower concentrations of aggregated protein result in a lower stiffness of the gels (*33*). However, despite the higher amounts of dissolved protein, gels at pH 5.2−0.5 M NaCl have higher G' values than at 0.2 M NaCl. This means that the amount of protein incorporated in the network cannot fully explain the differences in G'. Indeed from other work, we know that variations in network structure of the soy protein gels also contribute to variations in gel stiffness (*34*).

The dip in the G' curve at pH 6 and 0 M NaCl is not an experimental artifact (**Figure 5**). Measurements of G' at pH 6 as a function of NaCl concentration (data not shown) also showed that G' is very low at NaCl concentrations of 0−0.03 M. Up to 0.1−0.2 M NaCl, G' increased, owing to a salting-in effect, because solubility measurements showed a decreasing solubility in this region (data not shown). At salt concentrations higher than 0.2 M, a decrease in G' was observed corresponding with an increase in solubility (data not shown). The dip in the G'−pH curve has also been observed by van Kleef (*17*) for soy protein isolate (at pH 4.5) and glycinin (at pH 6) under conditions without salt. It might indicate the presence of a (small) pH zone in which the network had a different structure than at both sides of the zone, as was observed for heat-induced ovalbumin gels (*35*).

Association/Dissociation Behavior on Heating. The increase in solubility of the unheated SPI at pH 4−5 with increasing ionic strength was in the first place due to an increased solubility of β-conglycinin, but also more glycinin became dissolved (**Table 1**). At other pH values no difference in solubility between both proteins was observed, except at pH 2 and 0.5 M NaCl, where all glycinin had precipitated. This latter observation agrees with results for glycinin (*3*). At pH 4−5, salting-in effects can explain the increase in the amount of dissolved proteins at higher salt concentrations. The solubility decrease at pH <3 and pH >7 with increasing ionic strength seems to be a salting-out process. However, salting out of globular proteins normally happens at NaCl concentrations much higher than 0.5 M (*36*). At pH <3 and pH >7, the proteins have a strong net charge, which promotes dissolution at low ionic strength. At higher ionic strength, the charge is screened, resulting in a lower electrostatic repulsion and lower solubility.

After heating, a different protein composition in the supernatant was found for samples at pH 2 and 3 (further denoted as low pH) than for samples at pH >5 (further denoted as high pH). At high pH the absence of the AB subunit and the presence of free acidic polypeptides in the supernatants of the heated dispersions imply rupture of the disulfide bond and the noncovalent interactions between the acidic and the basic polypeptide of glycinin. Reshuffling of S−S/SH groups probably broke the disulfide bond. This is in agreement with results by others (*9, 37*). At 0.5 M NaCl, part of the AB subunit stayed intact on heating. This might be explained by the dissociation/association processes being slower compared to those at 0.1 M NaCl as observed by Wolf and Tamura (*38*). At low pH, the disulfide bonds seem to remain intact, because intact AB subunits were observed under nonreduced conditions and as much acidic as basic polypeptides under reduced conditions. This suggests a difference in denaturation mechanism at low

and high pH, which might be due to the activity of the S−S/SH interchange reaction being much higher around pH 7 than around pH 3.

In SPI, part of the basic polypeptides remained dissolved as (part of the) soluble aggregates on heating a 1% dispersion at high pH. In the case of purified glycinin all the basic polypeptides would have been precipitated (*9*, *37*, *39*). The presence of β-conglycinin in SPI prevented complete precipitation of the basic polypeptides, as was observed earlier by other researchers (*40*−*42*). They concluded that a heat-induced complex was formed between basic polypeptides of glycinin and β subunits of β-conglycinin. The smaller amount of dissolved β subunits compared to α and α′ at 0.5 M in our results might be explained by this complex formation. At low pH, there are no indications of complex formation between polypeptides of glycinin and subunits of β-conglycinin. The lower solubility of α and α′ subunits of β-conglycinin at low pH is not understood but might be due to their pI being lower than that of β subunits.

Heat-induced aggregates larger than 100 kDa could only be seen on SDS-PAGE gels under nonreduced conditions. As has been discussed before, it is very likely that disulfide bridges play a role in aggregate formation at high pH. The absence of aggregates at low pH under reduced conditions might indicate that disulfide bridges are also important for aggregation at low pH. However, it is more likely that the reduction of the S−S bridge between the acidic and basic polypeptides during the analysis has facilitated the breakup of the aggregates.

Variations in the association/dissociation behavior of the soy proteins on heating as a function of pH might be a reason for differences in the rheological properties of the soy protein gels. At pH 3−5 all protein seems to participate in the network, whereas at pH >5 less protein and especially fewer acidic polypeptides take part in the network, resulting in less stiff gels. However, a cautioning remark has to be made. The association/dissociation behavior of soy protein during heat denaturation is sensitive to protein concentration (*43*, *44*). Therefore, the difference in protein concentration between the solubility and the gelation experiments limits the comparison of both experiments. In this study, the effect of pH on gel formation and gel properties was larger than that of salt concentration. Denaturation proved to be a prerequisite for gel formation under all conditions studied.

ABBREVIATIONS USED

SPI, soy protein isolate; PDI, protein dispersability index; DSC, differential scanning calorimetry; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; A, acidic polypeptide of glycinin; B, basic polypeptide of glycinin.

ACKNOWLEDGMENT

We thank Erik van der Linden for critical reading of the manuscript and Catriona Lakemond and Harmen de Jongh for valuable discussions.

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Received for review January 18, 2002. Revised manuscript received July 19, 2002. Accepted July 19, 2002. This research was financially supported by the Dutch Ministry of Economic Affairs, through the program IOP-Industrial Proteins, and Cargill BV (The Netherlands).

JF020061B