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Heat-Induced Gelation of Pea Legumin: Comparison with Soybean Glycinin

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Gel network formation of pea legumin (8.4% on a protein basis, pH 7.6) was monitored via dynamic rheological measurements. Gelation was performed in the absence and presence of the thiol-blocking reagent *N*-ethylmaleimide, at different rates of heating and cooling. Overall, it was shown that pea legumin gel formation was not effected by changes in the heating rate, and the two differently heated samples were unaffected by the addition of 20 mM NEM, which indicated that disulfide bonds were not essential within the network strands of these legumin gels. However, slowly cooling the legumin samples caused disulfide bonds to become involved within the network; this was observed by a large increase in gel strength that was then substantially reduced when repeating the sample in the presence of NEM. These experiments were repeated with soybean glycinin in order to determine whether a common model for gel formation of legumin-like proteins could be built, based upon molecular reasoning. The two proteins were affected in the same way by changes in the conditions used, but when applying a procedure of reheating and recooling the gel networks responded differently. Pea legumin gel networks were susceptible to rearrangements that caused the gels to become stronger after reheating/recooling, yet glycinin gel networks were not. It was concluded that the same physical and chemical forces drove the processes of denaturation, aggregation, and network formation. Each process can therefore be readily targeted for modification based upon molecular reasoning. Pea legumin and soybean glycinin gel networks had structurally different building blocks, however. A model of gelation aimed at texture control therefore requires additional information.

KEYWORDS: ***Pisum*; legumin; glycinin; gelation; small deformation rheology; texture control**

INTRODUCTION

Globular proteins from various sources (in the form of isolates) play important roles in many foodstuffs, both because of their nutritional value and of their contribution to food texture (*1*). These texture contributions come from the network structures created by the proteins. Since gelation is one of the most important functional properties of the globular proteins used to modify food texture (*2*), it should be important to understand which factors determine the gel network and how they are affected by processing parameters. Such an understanding would enable better control of food textures.

Protein isolates from soybean dominate the market, though presently there is a trend for alternative protein isolates having similar functional and nutritional properties as soya (*3*). A potential alternative plant protein in Europe is pea (*Pisum sativum* L.). As with soybean, it contains two major globulin proteins, namely legumin and vicilin. Pea vicilin functionality has been dealt with in a previous paper (*4*), so only legumin will be given further consideration in this paper. Legumin is a polypeptide of ~60 kDa, though this polypeptide is commonly denoted as a legumin subunit that assembles into higher molecular weight oligomers. A feature of legumin subunits is that they split into acidic (40 kDa) and basic (20 kDa) polypeptides via disulfide bond reduction. Similar subunits compose the legumin-like proteins of *Glycine max*. (*5*) and *Vicia faba* (*6*). In all cases the disulfide-bonded acidic and basic polypeptides are formed when the protein precursor is proteolytically processed in the plant (*7*). In contrast to vicilins, legumins are recognized for their cysteine content: pea and fababean legumin contain approximately 5 residues per 60 kDa subunit, and soybean glycinin approximately 8.

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Studies on the emulsification and foaming (*8*−*12*) and gelation (*12*−*14*) of pea legumin have been reported, but none of these studies compared pea legumin functionality to that of its related protein in other leguminous plants. Even though the literature exploring the functional properties of alternative leguminous protein sources is quite extensive (*15*−*21*), it is difficult to compare and contrast the functional properties as often the experimental conditions used are different. Such differences make it difficult to identify the basis needed to build a common model for gelation of legumes, and in turn hampers the introduction of alternative plant protein sources as a direct replacement for soybean.

This paper presents results on gelation of the legumin protein from peas, and demonstrates how gel formation was affected by the heating process, in both the absence and presence of the thiol-blocking reagent *N*-ethylmaleimide. The results are compared with soybean glycinin gels formed under the same conditions in order to determine whether a common model for gel formation can be built, based upon molecular reasoning.

MATERIALS AND METHODS

Preparation of Enriched Protein Fractions. Legumin was purified from peas (*Pisum sativum* L.), cv. Solara, by a non-denaturing fractionation procedure adapted from the method of Kyoro and Powers (*11*) and Bora et al. (*14*). Peas were milled in a Waring commercial blender (New Hartford, CT) 2:1 (w/w) with dry ice to avoid any heat denaturation of the proteins. Salt-soluble proteins were then extracted into a 100 mM Tris-HCl buffer, pH 8.0, with a flour-to-buffer ratio 1:10 (w/v). Extraction time was 1 h at room temperature and extract was collected by centrifugation (11900*g*, 10 °C, 25 min). Isoelectric precipitation, pH 4.8, was used to isolate the globulin proteins from the extract; the pH was adjusted with 1 M HCl. Precipitated protein was left for 2 h, 4 °C before it was collected by centrifugation (11900*g*, 10 °C, 25 min). Washing the protein pellet with water (pellet-to-water ratio 1:10 w/v) removed unwanted albumin proteins. Again the pellet was collected by centrifugation (11900*g*, 4 °C, 25 min). The crude pellet was suspended in the extraction buffer, pH 8.0 (10 mg/mL) and dialyzed at 4 °C against McIlvaine's buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid, containing 0.2 M NaCl), pH 4.8. Sample-to-buffer ratio was 1:20, and the dialysis buffer was changed three times over a 24-h period. A precipitated fraction (referred to as *legumin enriched*) was collected following centrifugation of the sample (18900*g*, 4 °C, 25 min).

Purification of Legumin. Freeze-dried *legumin-enriched* fraction was suspended in buffer A (35 mM potassium phosphate, containing 0.075 M NaCl), pH 7.6, at a concentration of 25 mg/mL (which gave a suitably low final sample viscosity for loading onto the column). Legumin-enriched isolate was centrifuged (11900*g*, 4 °C, 25 min) before further use to remove insoluble material. Clear *legumin enriched fraction* (1200 mL) was loaded onto a DEAE Sepharose Fast Flow column (5 cm diameter, 343 mL volume; Amersham Biosciences, Uppsala, Sweden), previously equilibrated with buffer A. Elution was performed with a linear salt gradient (0.075−0.5 M NaCl) in the same potassium phosphate buffer, over 6 column volumes. The eluate was monitored at 280 nm, and 15 mL fractions were collected and analyzed for purity on an SDS−PAGE gel (Bio-Rad Ready Gel Tris-HCl Gels, 10−12% linear gradient) under nonreducing conditions. Fractions containing only the band belonging to legumin (∼ 60 kDa) were pooled together. Pooled fractions were desalted by extensive dialysis against distilled water, and freeze-dried. This procedure resulted in pure *legumin*.

Purification of Glycinin From Soybean. The glycinin used in this paper was purified as previously described (*22*).

Purity of Purified Protein Preparations. Protein preparations were run on SDS−PAGE gels (Tris-HCl 12% polyacrylamide gels) and were determined as pure when, in the absence of β-mercaptoethanol, only a band at ∼60 kDa was visible, and in the presence of β-mercaptoethanol that band disappeared, revealing bands at ∼40 and ∼20 kDa. The protocol for gel electrophoresis is given below.

Nitrogen Content Determination. The percentage nitrogen content in the purified proteins was determined using the dynamic flash combustion method (NA 2100 nitrogen and protein analyzer, CE Instruments, Milan, Italy). Triplicate samples of 5, 10, and 15 mg were used for the determination, methionine was used for the calibration and the protein conversion factor used was 5.3 for pea legumin and 5.5 for soybean glycinin.

Gel Electrophoresis. Samples were prepared in the absence and presence of the reducing agent β-mercaptoethanol. For both, 10 μL of the protein (either as it eluted from the column solution, or a 1 mg/mL solution in 10 mM potassium phosphate buffer, pH 7.6) was mixed at a ratio of 1:1 with sample buffer (1.4 mL distilled water, 2.0 mL 0.5 M Tris-HCl at pH 6.8, 2.0 mL 10% SDS, 2.0 mL glycerol, and 0.4 mL 0.05% bromophenol blue) and heated for 10 min in Eppendorf tubes (1.5 mL) locked within a heating block in a boiling water bath. When used, 20 μL of β-mercaptoethanol was added to 0.78 mL of sample buffer. 10−20% linear gradient, Tris-HCl Ready Gels (Bio-Rad) were used and 3−10 μg protein was loaded into each well (according to the protein concentration of the sample). Low molecular weight protein standards, ranging from 94 to 14 kDa (Amersham Biosciences, Uppsala, Sweden) were made according to the instructions, and 10 μL was loaded for each standard lane. Gels were run at a constant 200 V. Staining was done using Coomassie Blue R-250 Biosafe stain (Bio-Rad).

Thermal Denaturation. Legumin and glycinin were dissolved in 75 mM potassium phosphate buffer pH 7.6, at 0.3% (w/v) concentration. All samples were centrifuged and degassed prior to use. Measurements were made in a VP DSC MicroCalorimeter (MicroCal Inc., Northampton, MA) using the sample buffer in the reference cell. Samples were preheated to 45 °C for 15 min, and subsequently heated to 115 °C at a rate of 1 °C/min or 0.5 °C/min. One replicate of each sample was reheated after cooling to check if any of the denaturation was reversible. One sample of each protein was also heated at 1 °C/min in the presence of 20 mM *N*-ethylmaleimide (NEM) to check if its presence affected the temperature of denaturation.

Minimum Gelling Concentration. This was determined by making 3 mL protein solutions of 8−16% (w/v) concentration, at pH 7.6, in 75 mM potassium phosphate buffer. All samples were heated, (in sealed tubes to avoid evaporation), in a boiling water bath for 30 min. Samples were cooled to room temperature for 1 h, and then stored at 4 °C overnight. The next day the tubes were inverted and the samples that did not flow were considered to have gelled, and hence the minimum gelling concentration was determined.

Small Deformation Rheology. Samples of purified proteins, 8.4% for pea legumin and 6.6% for soybean glycinin on a protein basis, were prepared in 75 mM potassium phosphate buffer, pH 7.6. Where used, the thiol-blocking agent *N*-ethylmaleimide (NEM) was added at a concentration of 20 mM. Gelling was done by heating samples in a Bohlin CVO rheometer concentric cylinder (C-14). The heating profile was 45 °C to 98 °C, holding at 98 °C for 30 min, cooling to 25 °C, and holding at 25 °C for 30 min. The sample volume was 2.8 mL, and a few drops of vegetable oil were put on the top of the sample to prevent evaporation during heating. The heating and cooling rate was 1 °C/min for control samples. In addition, one sample was heated slowly at 0.5 °C/min (yet cooled at 1.0 °C/min), and another sample was cooled slowly at 0.2 °C/min (after having been heated at 1 °C/min). Dynamic measurements were taken at 60-s intervals for all samples, and under a constant strain of 0.015 for legumin and 0.01 for glycinin (values within the linear viscoelastic strain region of the gels under the given conditions), and 0.1 Hz frequency. When analyzing the results, the temperature at which the elastic modulus (G') became greater than the viscous modulus (G'') was determined as the initiation of gelation. When performing dynamic rheological measurements this is a measure of the gel point (*2*, *23*−*25*), which is most commonly referred to in the literature as the $G'-G''$ crossover. Samples were run in triplicate, and a representative sample is presented in **Figures 1**, **2**, and **3**.

Transmission Electron Microscopy. Legumin samples were prepared at an 8.9% concentration on a protein basis, pH 7.6, $I = 0.2$, in 1.5 mL plastic vials with screw caps. The samples were heated in the water bath connected to the Bohlin rheometer. The protein concentration was higher than that used for rheological measurements in order to

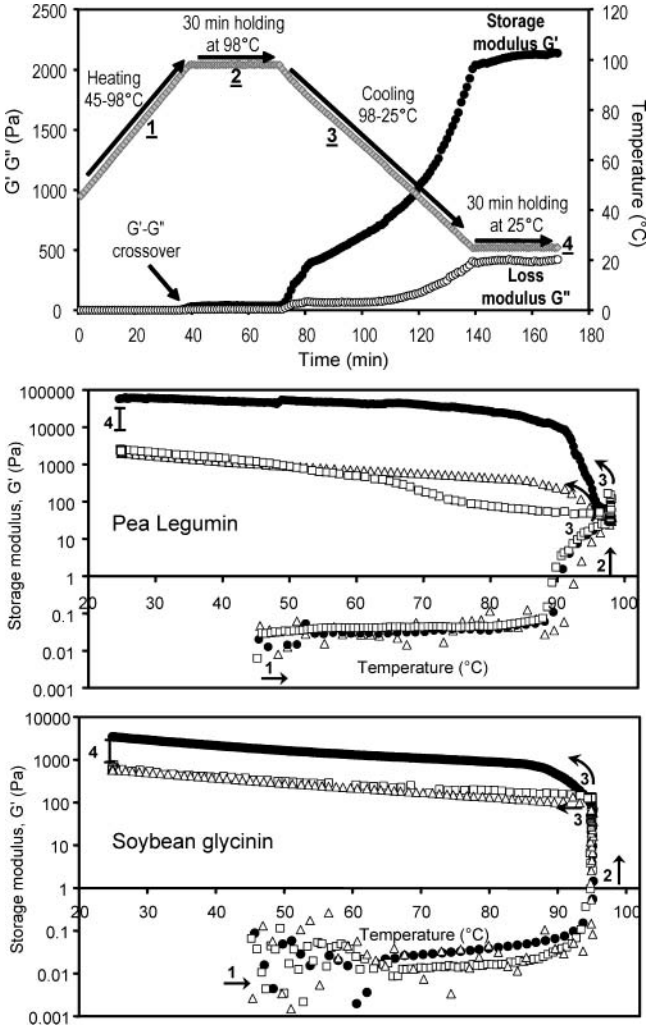

Figure 1. (**a**) Presentation of storage (*G′*) and loss (*G″*) moduli as a function of time. Heating and cooling phases are plotted on a secondary axis. Nos. 1–4 correlate with **Figures 1b/c**, **2a/b**, and **3a**–**c** where the time axis has been removed, and the storage modulus is plotted as a function of temperature. (NB: Loss modulus is not plotted in **Figures 1**–**3**. (**b**) Effect of heating/cooling rate on development of storage modulus (*G′*) during heat-induced gelation of an 8.4% protein concentration *pea legumin* solution: (△) 1 °C/min heating and cooling; (□) 0.5 °C/min heating and 1 °C/min cooling; (●) 1 °C/min heating and 0.2 °C/min. Nos. 1–4 indicate successive heating and cooling phases (as explained in the example figure). (**c**) Effect of heating/cooling on development of storage modulus (*G′*) during heat-induced gelation of a 6.6% protein concentration *soybean glycinin* solutionL (△) 1 °C/min heating and cooling; (□) 0.5 °C/min heating and 1 °C/min cooling; (●) 1 °C/min heating and 0.2 °C/min cooling. Nos. 1–4 indicate successive heating and cooling phases.

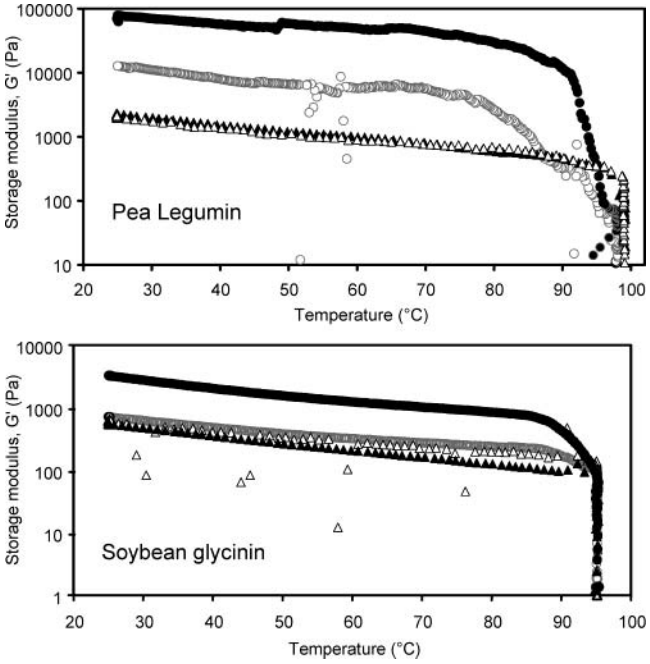

Figure 2. (**a**) Effect of the addition of 20 mM N-ethylmaleimide (NEM) on the development of storage modulus during heating and cooling an 8.4% protein concentration *pea legumin* solution: (▲) 1 °C/min heating and cooling; (△) 1 °C/min heating and cooling in the presence of 20 mM NEM; (●) 1 °C/min heating and 0.2 °C/min cooling; (gray circles) 1 °C/min heating and 0.2 °C/min cooling. (**b**) Effect of the addition of 20 mM NEM on the development of storage modulus during heating and cooling a 6.6% protein concentration *soybean glycinin* solution: (▲) 1 °C/min heating and cooling; (△) 1 °C/min heating and cooling in the presence of 20 mM NEM; (●) 1 °C/min heating and 0.2 °C/min; (gray circles) 1 °C/min heating and 0.2 °C/min cooling.

guarantee the gel was firm enough for a good sample preparation. The three heating and cooling rate combinations used were: 1.0 °C/min heating & cooling; 0.5 °C/min heating and 1.0 °C/min cooling; as well as 1.0 °C/min heating and 0.2 °C/min cooling. All samples were heated from 45 °C to 98 °C, held for 30 min at 98 °C, cooled to 25 °C, and held at 25 °C for 30 min. Gel samples were then prepared as follows. They were cut into approximately 1 mm^3 cubes and fixed using 2.5% glutaraldehyde in distilled water. After washing with distilled water the samples were dehydrated using a graded series of ethanol followed by infiltration with LX112 epoxy resin. These plastic-embedded blocks were polymerized at 60 °C for 24 h and sectioned using Leica Ultracut S. The ultrathin sections obtained (60−80 nm) were collected in 100 mesh Collodion coated grids and stained using Reynolds lead-citrate and uranyl acetate. The grids were examined in a Philips CM12 transmission electron microscope operated at 80 kV accelerating voltage.

Solubility of Gels. Samples of pea legumin (8.4% concentration on a protein basis) were prepared in the same buffer as used for the rheological experiments. 1 mL of sample was put into each test tube (5 mL with screw-cap) and heated in a water bath at 95 °C for 30 min. After heating, the samples were cooled at 4 °C for 2 h. Subsequently, 5 mL of each of the following solutions was added to one of the test tubes: 8 M urea; 8 M urea with 2% (w/v) β-mercaptoethanol; and 1.5% (w/v) sodium dodecyl sulfate (SDS). Each tube was then re-sealed and continuously rotated in a test tube rotor in order to keep the gel mobile and allow for good diffusion of the solution into the gel network. Samples were rotated for 24 h at room temperature. The amount of gel that had dissolved in each reagent was then judged visually according to the clarity/turbidity of the sample as it rotated, and the presence/ absence of sediment after leaving samples to stand for 1 h at room temperature.

RESULTS

Gel Formation Using Different Heating and Cooling Rates. Sample concentrations of 8.4 and 6.6% (on a protein basis) were used because they were determined as the minimum gelling concentrations of pea legumin and soybean glycinin, respectively. Panels **b** and **c** of **Figure 1** show legumin and glycinin gel formation at these concentrations using different heating and cooling rates, as measured by the storage modulus (*G′*). In these figures *G′* is plotted as a function of temperature, rather than against time as is more commonly done, but the direction in which to follow the data points is indicated by successive numbering from 1 to 4 (numbers 1−4 in **Figure 1b**,**c** correspond with those in **Figure 1a**, where the storage and loss

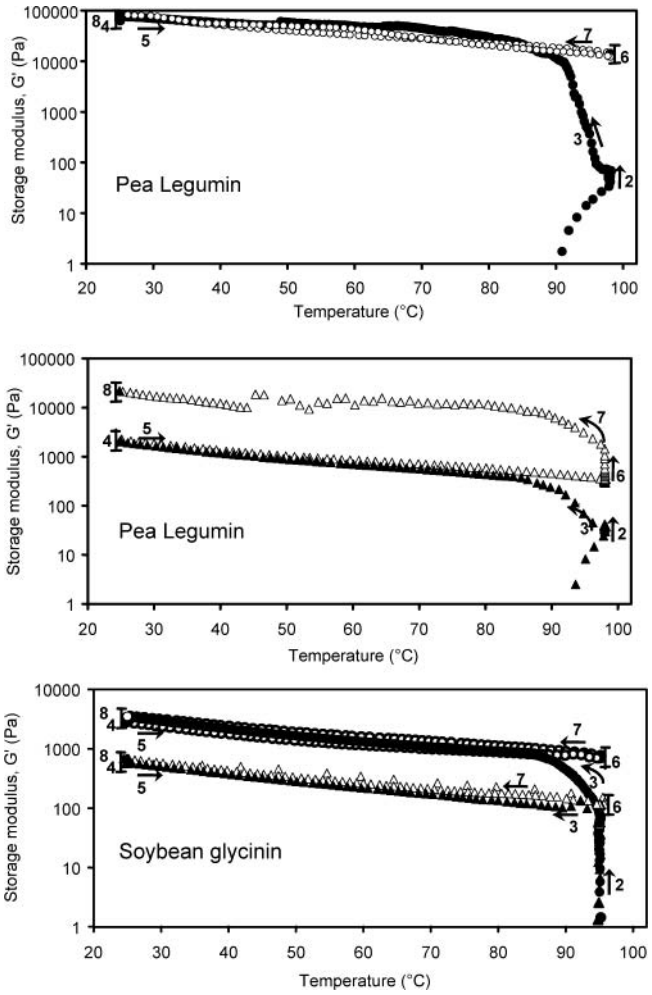

Figure 3. (**a**) 'Reheatability' of storage modulus of *pea legumin* gels originally formed with a heating rate of 1.0 °C/min, and a cooling rate of 0.2 °C/min (●). Reheating/cooling was at a rate of 1.0 °C/min (○). (**b**) 'Reheatability' of storage modulus of *pea legumin* gels originally formed with a heating rate of 1.0 °C/min (▲), and reheated/cooled at the same rate (△). (**c**) 'Reheatability' of storage modulus of two different soybean glycinin gels. A gel originally formed with a heating/cooling rate of 1.0 °C/min (▲), and reheated/cooled at the same rate (△). Another gel was originally formed with a heating rate of 1.0 °C/min and a cooling rate of 0.2 °C/min (●), and reheated/cooled at 1.0 °C/min (○).

moduli are plotted more traditionally as a function of time). A first comment on the gel formation of legumin and glycinin as a function of the heating/cooling rate is that the two proteins appeared to respond in the same way to changes in the rates. Both proteins using a slower heating rate (0.5 °C/min) instead of the control rate (1.0 °C/min) did not affect the gel formation of the legumin or the glycinin gels as detected by the rheometer. A slower cooling rate, however, caused a stronger gel to be formed, for both proteins. Looking in more detail at the plots of G' it can be seen that it was during the beginning of cooling (phase 3, ~ 98−80 °C) that the slowly cooled gel attained much of its "additional" strength. Thereafter, $\Delta G'/\Delta T$ was similar for all the gel samples, as indicated by the slopes of the plots being so similar.

Though it is not plotted, the loss modulus (G'') was monitored during all the measurements presented in **Figure 1b**,**c** because the temperature at which G' became larger than G'' (the $G'-G''$ crossover) was used as a measure of the initiation of gel formation. The temperature of the $G'-G''$ crossovers for pea legumin and soybean glycinin are presented in **Table 1**. It can

Table 1. Temperature of the $G'-G''$ Crossover Point and the Thermal Denaturation Temperature (T_d) for Pea Legumin and Soybean Glycinin Solutions Heated at 0.5 and 1.0 °C/min

heating rate (°C/min)	G'–G'' crossover (°C)		T_d (°C/min), $I = 0.2$	
	legumin	glycinin	legumin	glycinin
0.5	88	86	87	86
1.0	94	95	88	87

be seen that the samples heated slowly (0.5 °C/min) initiated gel formation at a lower temperature than the control samples (heated at 1 °C/min). Despite this, the gel networks that developed with continued heating and cooling had the same strength. **Table 1** also shows that for the slowly heated samples (0.5 °C/min), gel initiation corresponded with the peak thermal denaturation temperature (T_d), as measured by differential scanning calorimetry. However, when heating at 1 °C/min gel, initiation was not detected until the end point of the peak of denaturation (~94 °C).

Addition of *N*-Ethylmaleimide. In the control samples (heated at 1 °C/min), and those heated slowly (at 0.5 °C/min), the blocking of disulfide bond formation by the action of *N*-ethylmaleimide caused a negligible effect on the gel formation of legumin and glycinin. It was negligible in that gelation proceeded regardless, and the gel strength was unaffected, but a slight destabilization of the network during formation was apparent, as seen by a slight scattering of the data points (see the control samples in **Figure 2a**,**b**). Data for the samples heated at 0.5 °C/min are not plotted in these figures as they were the same as for the control. However, when the samples were cooled slowly (0.2 °C/min) the effect of the added NEM was very noticeable because it caused a substantial reduction of the "additional" gel strength that was previously pointed out in **Figure 1a**,**b** (between 98 and 80 °C). For pea legumin (**Figure 2a**) this reduction was such that the value of G' at ~87 °C was of the same order of magnitude at it was in the control sample (also shown in **Figure 2a**). Thereafter however, during continued cooling, an increase in G' between 85 and 75 °C caused the plot of G' to deviate from the control, and in the end the slowly cooled sample was somewhat stronger than the control. Soybean glycinin cooled slowly in the presence of NEM had no such increases during continued cooling, and the plot of G' (as seen in **Figure 2b**) was of the same order of magnitude as the control sample (cooled at 1 °C/min in the presence and absence of NEM). A final observation worth noting is that all samples heated in the presence of NEM formed transparent gels.

Reheating/Recooling of the Gel. After formation, gel samples were reheated and recooled using a constant rate of 1 °C/min. Pea legumin and soybean glycinin behaved strikingly differently to this treatment. Soybean glycinin gels were what we described as being 'reheatable'. Exactly what this means is that the part of the gel that originally formed between 85 and 25 °C of the cooling phase (in phase 3) was thermally reversible. Thus, when reheated (phase 5) and recooled (phase 7) the plot of G' between 85 and 25 °C went backward and forward along itself. This 'reheatability' of soybean glycinin can be clearly seen in **Figure 3c**. The behavior of pea legumin to the procedure of reheating/recooling differed according to the cooling rate; the slowly cooled sample (**Figure 3a**) was seen to be 'reheatable' like soybean glycinin, yet the control sample (**Figure 3b**) became stronger by one log scale after reheating/recooling. This same phenomenon of becoming stronger after reheating/recooling was also seen for the pea legumin sample heated at 0.5 °C/min.

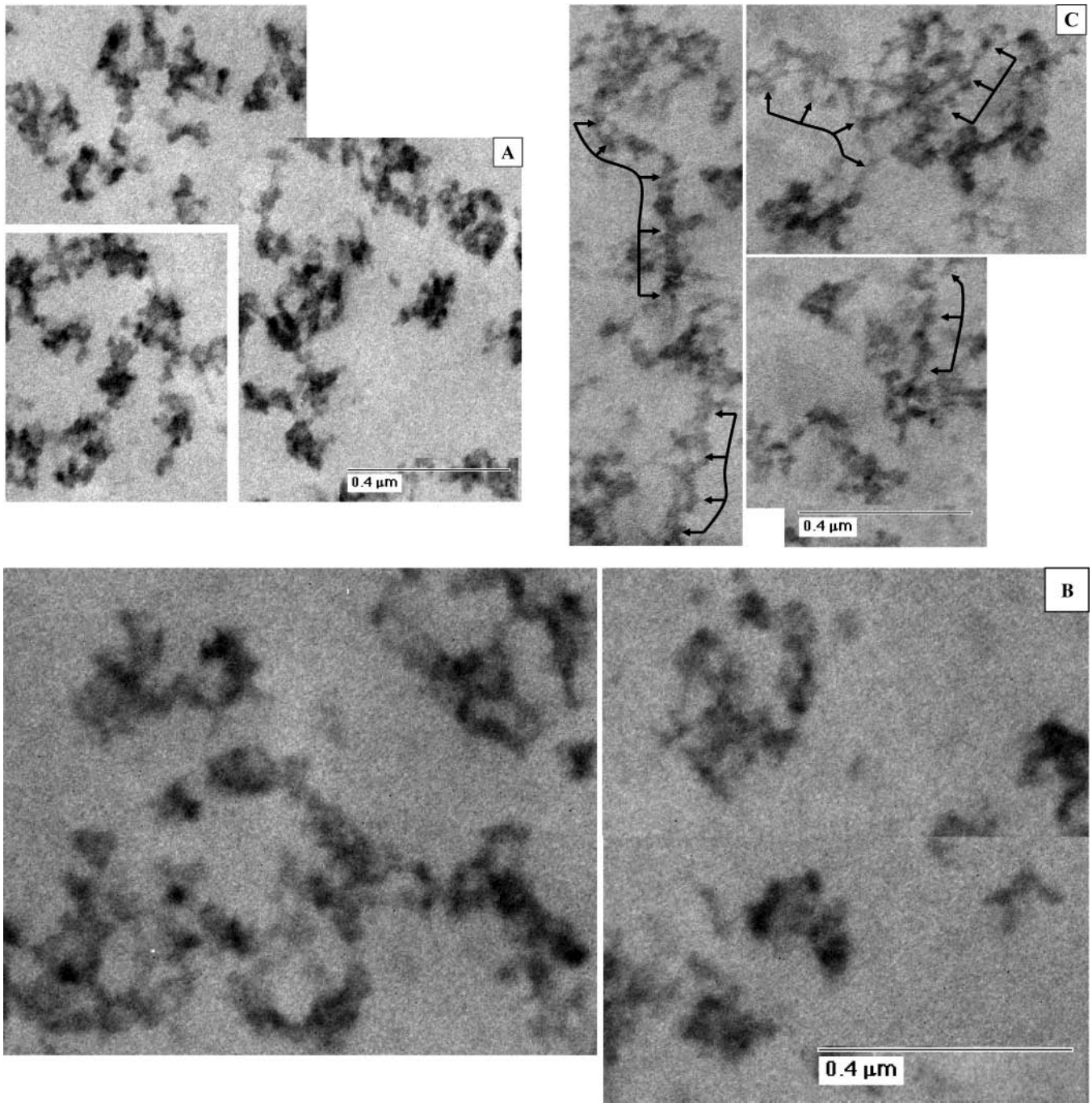

Figure 4. (**a**) TEM cross-section of a pea legumin gel heated and cooled at 1.0 °C/min. The relevant scale is indicated with the bar of 0.4 μm. (**b**) TEM cross-section of a pea legumin gel heated at 0.5 °C/min and cooled at 1.0 °C/min. The relevant scale is indicated with the bar of 0.4 μm. (**c**) TEM cross-section of a pea legumin gel heated at 1.0 °C/min and cooled at 0.2 °C/min. The relevant scale is indicated with the bar of 0.4 μm. Each series of arrows points out a 'branched area' of the gel network.

The procedure of reheating/recooling was also done with samples that were gelled in the presence of NEM. The soybean glycinin gels were again all reheatable (no results shown). The pea legumin gels however were not reheatable because the gel networks became disrupted, either during holding at 98 °C (phase 6), or during recooling (phase 7) (no results shown). This disruption was detected either by a scattering of the G' data, or by the value of G' becoming lower than that of G''.

Transmission Electron Microscopy (TEM). Panels **a**−**c** of **Figure 4** show TEM pictures of cross sections of the pea legumin gels formed with the different heating and cooling rates, in the absence of NEM. Panels **a** and **b** of **Figure 4** show two similar agglomerate gel networks. These are in fact the two legumin gels (1.0 and 0.5 °C/min heating) that had no detectable differences in their gel strengths (as measured by the Bohlin rheometer). Moreover, both of these systems had the behavior of strengthening after being reheated/recooled. The slowly cooled sample is pictured in **Figure 4c**, and appears to have a more branched and connected network than the other two samples. Also, the darkened patches of agglomerated protein seem smaller.

Solubility of Gels in Different Reagents. Tests for solubility of gels in a combination of reagents were performed for pea legumin gels as a way of determining the bond types that were structurally important for the gel network. Considering that legumin contains approximately five cysteine residues, it was

Table 2. Description of Pea Legumin Gels after Submersion in Different Reagents for a Period of 24 h

	8 M urea with 2% (w/v) β-mercaptoethanol	8 M urea	1.5% (w/v) SDS
visual appearance	clear solution within 1 h; no change after standing	turbid appearance with very small pieces of gel visible; some gel pieces settled, but turbidity remained	gel became swollen and fluffy, yet remained as one piece; SDS solution remained clear

important to determine the effect of the thiol-reducing reagent β-mercaptoethanol (β-me) on the gel structure. The results obtained are presented in **Table 2**.

DISCUSSION

As stated earlier in this paper, the purpose of comparing the gelation of pea legumin with soybean glycinin under various conditions was to determine if a common model for gel formation could be built, based upon molecular reasoning. This paper therefore also tested the assumption that is often passed around in the literature that intermolecular forces determine the structure of heat-induced gel networks, and that better understanding of these forces will enable modification and control of the resultant textural properties of the foods (*2*, *26*).

It was seen in this paper that gel formation of pea legumin and soybean glycinin, under the conditions used, was initiated at or after the peak temperature of denaturation, and their gel networks continued to develop during cooling. These observations indicated that these proteins followed the three-step process of gelation that is generally accepted for heat-induced gelation of such globular proteins. This process, which was documented in detail by Clark et al. (*27*), can be summarized as follows: (1) denaturation of the protein with subsequent exposure of hydrophobic residues, (2) intermolecular hydrophobic interaction of the unfolded proteins (aggregation), and (3) agglomeration of aggregates into a network structure. It is important to note that with cooling the network develops further and is strengthened by the formation of many short-range interactions such as hydrogen bonds. The equilibrium that exists between the native and unfolded states of globular proteins causes their process of heat-induced gelation to be under a certain extent of kinetic control (*27*). Moreover, the slower the rate of aggregation relative to denaturation, the more fine-stranded and ordered is the resultant gel network (*28*). As shown in this paper, however, a reduction in the heating rate from 1 to 0.5 °C/min caused no observable changes in the gel formation of either the legumin or the glycinin gels (**Figure 1b**,**c**), or the network structures of the pea legumin gels (see **Figure 4a**,**b**). However, when cooling the system more slowly the process of agglomeration of the aggregates into a network structure was altered, as observed by the formation of a strong branched network of the pea legumin gel (**Figures 1b** and **4c**). Slow cooling was believed to maintain the protein in its unfolded state for a longer time, slowing down the reactivity of the exposed residues, and enabling more optimal interactions to occur. Moreover, slow cooling gave the opportunity for disulfide bonds to become involved in the gel network, giving additional strength (as deduced by the observation that even in the presence of NEM the slowly cooled gel was stronger than the control (**Figure 2a**)). Thus, the more optimal gelation (seen in **Figure 4c**) was enhanced by, but not dependent on, disulfide bonds within the gel network. With soybean glycinin the results were slightly different. Though slow cooling increased gel strength due to the involvement of disulfide bonds in network branching (*29*), it had a negligible effect in the presence of NEM. This would suggest that the interaction of unfolded glycinin molecules is already close to optimal under the conditions of the control sample.

Referring for a moment to the legumin and glycinin gels heated at 0.5 and 1 °C/min, the addition of NEM caused no change in the measured gel strengths. Disulfide bonds should therefore be considered as having been nonessential in these gels. This result agrees with that of Utsumi and Kinsella (*30*), who saw that glycinin gels formed in the presence of NEM were fragile, but of equal strength to the control sample (formed at pH 8 in 30 mM Tris-HCl buffer).

Another way to determine the role of different bond types in the network structure was the solubility of the pea legumin gels in different reagents. Overall, the results showed that unless the samples were cooled very slowly disulfide bonds were involved within the individual aggregates, but it was hydrophobic and hydrogen bonds that supported network formation. The gel solubility tests were not repeated with soybean glycinin due to a lack of available protein, yet other authors have done such tests. Utsumi and Kinsella (*30*) used 8 M urea and 0.2 M 2-mercaptoethanol, and found that 90% and 30% of the gel dissolved in each reagent, respectively. This indicated a role of hydrophobic interactions, hydrogen bonds, and disulfide bonds in maintaining the gel matrix. While this may be true, rheological measurements performed in this paper indicated that disulfide bonds within the gel network of glycinin are nonessential.

To summarize, thus far it can be said that the molecular driving forces of the heat-induced gelation of pea legumin and soybean glycinin are the same, and they can be manipulated by using a slow rate of cooling. The question that then arises is why the gels of these two molecularly similar proteins have a different response to the procedure of reheating/recooling. If intermolecular forces really do determine the structure of heat-induced gel networks (as proposed by Zheng et al. (*26*) and Ikeda and Nishinari (*2*)), then legumin and glycinin, when gelled under the same conditions, would be expected to have the same network structures, and thus the same behavior of 'reheatability'. This was clearly not the case. Soybean glycinin was completely 'reheatable' under all conditions (fast or slow heating and cooling, in both the presence and absence of NEM). Results are only shown for selected conditions in **Figure 3c**. Pea legumin, on the other hand, was only 'reheatable' after having been cooled slowly in the absence of NEM (**Figure 3b**). The pea legumin gels formed in the absence of NEM became stronger after the procedure of reheating/recooling (**Figure 3a**), while those reheated in the presence of NEM were not 'reheatable' (no results shown). Since slow cooling reduces the reactivity of the exposed residues, it can be speculated that exposed residues of pea legumin react very quickly under control conditions and not all find themselves in a favorable environment. Upon reheating they therefore take the opportunity to rearrange themselves. This said however, the type of bonds involved also appears to be important for pea legumin gel 'reheatability'. In literature the phenomenon of increased gel strength after reheating was found for whey protein isolates gelled at pH 8.0 (*31*) and at neutral pH in distilled water (*32*).

Two possible explanations were offered by Rector (*31*): (i) More disulfide bonds form during reheating, reducing the flexibility of the network chains, bringing them closer together, and consequently enabling more extensive short-range cross-links to form during recooling. (ii) Aggregates unfold upon reheating, making more residues accessible for interaction. Considering that it was the gel with enhanced disulfide bonding that was 'reheatable', the first explanation offered seems most likely to apply to pea legumin gels. Thus, an optimally arranged network well stabilized with covalent bonds can be said to be important in making pea legumin gels structurally stable against rearrangements during reheating. By contrast, soybean glycinin appears to be inherently able to form structurally stable gel networks in both the absence and presence of disulfide bonds. A slow reactivity of the exposed glycinin residues seems to be the cause of this structural stability. The fact that slow cooling with NEM did not encourage a stronger glycinin gel to form certainly supports this idea.

In keeping with the idea that soybean glycinin is inherently better able to form a well-structured gel network is the fact that it has a 1.8% lower minimum gelling concentration than pea legumin. For two molecularly similar proteins that gel via the same bonding mechanisms, this difference in concentration is significant. It could reflect two possible characteristics: more organized formation of network strands, and/or inclusion of a higher amount of available protein in the network. References from the literature support the formation of more-organized strands. Hermansson (*33*) formed gels of glycinin at pH 7.0 and, using electron microscopy to visualize the structures, described the strands of the glycinin gels as "very regular". Nakamura et al. (*29*) studied the aggregation of glycinin at pH 7.6 and identified an ordered mechanism of network formation. Also, Zheng et al. (*34*) compared fababean legumin with soybean glycinin and found that under equal conditions a higher amount of legumin than glycinin was needed to form a gel of equal strength due to the more irregular network strands of fababean legumin.

Having compared the processes of heat-induced gelation of pea legumin and soybean glycinin from a molecular basis, and having compared structural elements of the two gels, it seems that the information collected is not sufficient for building a common model of gelation. Based upon molecular reasoning, changes in the gelation mechanism may well be achieved. Control over the food texture is more difficult, however, because the structural quality of the network strands appears to be determined by inherent features of the protein, rather than the molecular interactions that drive the gelation mechanism.

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