# Effect of Heat Treatment and pH on the Thermal, Surface, and Rheological Properties of *Lupinus albus* Protein

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**ABSTRACT:** Endosperm from hand-dissected and -dehulled *Lupinus albus* seeds was milled into meal, sieved through a 40-mesh screen, and suspended in phosphate buffers (pH 4, 6.8, and 8) at 20% (wt/vol). The suspensions were treated at 75, 90, or 100°C for 1 h. The heat-treated protein was characterized by SDS-PAGE, free zone capillary electrophoresis (FZCE), and DSC; and its surface hydrophobicity, surface tension, and rheological properties were examined. The presence of high M.W. aggregates was apparent from SDS-PAGE and FZCE results. Solubility was lowest at pH 4 and 100°C. DSC analysis was performed on low moisture content samples (3.1%) and 20% (wt/vol) suspensions. DSC analysis at 3.1% moisture content showed a glass transition around 85°C and an exothermic transition at 160°C, whereas the protein suspension showed a more thermally stable protein as indicated by the higher $\Delta H$ values. Lupin protein was surface active as demonstrated by its effectiveness in reducing the surface tension of the aqueous phosphate buffer. Surface hydrophobicity of the heat-treated protein decreased as the treatment temperature increased, which supports the SDS-PAGE results. The highest level of aggregation was noted at 90°C and pH 6.8 as indicated by low surface hydrophobicity values. Rheological studies showed direct relationships between the shear storage modulus ($G'$) of the lupin meal suspension and both pH and temperature treatment, although this effect is minimal at the highest temperature (100°C) and pH 6.8.

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**KEY WORDS:** DSC, interfacial tension, lupin meal, rheology, surface hydrophobicity.

Lupin, a legume, has been known for 3000 yr in the Mediterranean basin. *Lupinus albus* is also referred to as Old World lupin. Lupin endosperm contains more than 50% protein and no starch (1,2). Currently, lupin is used for feed and food. Information about isolated lupin proteins is available in the literature, but the behavior of lupin protein in whole or defatted meal is, to our knowledge, very limited. Ultracentrifugation of lupin proteins shows two or three groups of proteins based on lupin variety (3). *Lupinus albus* contains three types of globulins, conglutine α, β, and γ, with varying M.W. values. All lupin globulins have high M.W. except for the 9b fraction (4). Lupin legumins undergo association–dissociation equilibria and are arranged in trimers or hexamers. Lupin legumins contain bound sugars. The presence of bound sugars decreases trypsin proteolysis of lupin proteins (5). Privalov and Khechinashvili (6) reported lower $\Delta H$ values for oat globulins at pH 4 and 8 relative to pH 6, indicating decreased thermal stability and partial denaturation at more acidic or alkaline pH. Proteins are most stable near their isoelectric point, and increasing or decreasing the pH from this point will reduce protein stability owing to intramolecular charge repulsion (6). The surface hydrophobicity ($S_0$) property of proteins is a good indicator of their emulsifying power (7). Lupin flour was shown to have a glass transition, whereas rheological testing indicated strong viscoelastic properties (8).

Delia *et al*. (9) reported that the functional properties (surface hydrophobicity, foaming, and gelling) of soluble and insoluble (both native and thermally treated) soy protein fractions showed that heat treatment increased protein aggregation. They also reported that the concentration of soy protein extract influenced the effect of heat treatment. The objective of this work was to study the behavior of lupin protein in its native form in the presence of the remaining components of lupin meal and also the effect of heat treatment and pH on protein structure and solubility. The purpose of using different tests to study the effect of heat treatment on lupin protein was to establish whether these types of tests can be correlated with structure–function properties of the protein.

## MATERIALS AND METHODS

*Sample preparation. Lupinus albus* samples were hand-dissected and -dehulled. The endosperm was milled into meal and sieved through a 40-mesh screen. A 20% (wt/vol) suspension was prepared in phosphate buffers with pH 4, 6.8, or 8. Each suspension was treated at 75, 90, or 100°C for 1 h. The levels of pH and temperature were selected because they represent the most common levels used in the food fortification industry. The heat-treated suspension was centrifuged at $1300 \times g$. The supernatant was analyzed for protein content to determine the effect of heat treatments on protein solubility. The protein content ($N \times 6.25$) in the supernatant was determined by nitrogen

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combustion analysis using a LECO CHN-2000 instrument (St. Joseph, MI). The supernatant was also analyzed by SDS-PAGE to determine the nature of the proteins in the supernatant that would be excluded during the precipitate analysis. The precipitated pellet, which was the main product of the experiment to be tested, was freeze-dried and used for further testing of surface tension, surface hydrophobicity, free zone capillary electrophoresis (FZCE), SDS-PAGE, modulated DSC (MDSC), and rheology. Samples were defatted for the FZCE testing. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

*Surface tension*. The supernatants obtained from sample preparations at different temperatures and pH values were freeze-dried, and the resulting protein was dissolved in phosphate buffer and used in surface tension measurements. Surface tension measurements were conducted as a function of protein concentration. Surface tension of aqueous lupin solutions was measured using the axisymmetric drop shape analysis method (10) on the FTA 200 automated goniometer (First Ten Ångstroms, Portsmouth, VA 23704). In a typical procedure, the lupin solution was placed in a 10-mL disposable syringe, equipped with a 17-gauge (1.499 mm O.D.) blunt disposable needle (Becton Dickinson & Co., Franklin Lakes, NJ). The syringe was locked into place on the instrument and surface tension measurement was conducted in the automatic trigger mode. In this mode, the solution was pumped at the desired rate until a pendant drop with the specified volume was formed. This was then followed by an automatic acquisition of drop images for the specified duration, analysis of the images, and display of surface tension vs. time data (spreadsheet and plot). The data from each run were automatically saved as both a spreadsheet and a movie. The spreadsheet contained the time and surface tension for each image, and the movie contained each of the drop images as well as calibration information. In all cases, the surface tension decreased with time and leveled off to a constant equilibrium value after a long period of time. The average equilibrium surface tension values of 3–5 repeat runs were used to characterize the surface tension of the aqueous lupin solutions. The automatic trigger mode was programmed to pump a total volume of 5–18 μL of solution at 1 μL/s and to acquire images at a rate of 0.067 s/image, with a post-trigger period multiplier of 1.28× between images, which allowed for a total of 35 images to be captured during a total acquisition period of 1052.03 s.

*SDS-PAGE.* SDS-PAGE was used to examine possible aggregate formation during lupin protein manufacturing processes. SDS-PAGE was performed according to Laemmli (11) as described by Khan *et al*. (12) with 11.8% acrylamide and 0.1% bisacrylamide for the separating gel. The stacking gel was prepared with 4.5% acrylamide and 0.1% bisacrylamide. Samples (precipitate) were analyzed as native protein or else were reduced with DTT to investigate whether proteins formed aggregates involving disulfide bonds.

*FZCE*. Lupin proteins examined by FZCE were extracted from heat-treated whole and defatted precipitate. Samples (precipitate) were defatted by adding 135 mL of hexane and 1.2 g of each sample to a 250-mL Erlenmeyer flask, placing on the shaker for 3 h at 1300 rpm, and centrifuging for 10 min at 300 × *g*. Excess hexane was poured off, and the remaining hexane/solid mixture was placed overnight on a watch glass under the hood to dry. The fatted and defatted precipitates were suspended in the phosphate buffer with the proper pH, as described in the sample preparation section, or in water for the control. The suspension was centrifuged and the supernatant was analyzed.

FZCE analyses were determined on a Beckman P/ACE 2000 using a 60-cm fused-silica capillary (50 cm to the detector × 50 μm i.d.). A CElixir Solutions buffer kit and standard mesityl oxide (10 mg/mL) were supplied by MicroSolv Technology Corp. (Long Branch, NJ). Protein was extracted from heat-treated lupin meals (precipitate) by suspending 1 g in 40 mL of 0.01 M potassium phosphate at pH 8 and stirring for 3 h at 40°C. The suspension was then centrifuged at 10°C and 4500 × *g*, and the supernatant was freeze-dried. Lupin protein extract (10 mg) was resuspended in 1 mL CElixir Solution B pH 2.5 buffer. This mixture was sonicated for 5 s and filtered through a 45-μm nylon membrane (Titan filter; Sun SRI, Wilmington, NC). An aliquot of 50 μL of the lupin protein solution and the neutral marker mesityl oxide (0.5 mg/mL) was used for the FZCE analysis. The column was rinsed at 20 psi pressure for 1 and 2 min with 0.1 N NaOH and CElixir Solution A, respectively. The column was saturated for 2 min and 20 psi pressure with CElixir Solution B pH 2.5 buffer, which served as the running buffer. After a 10-s and 2.0 psi injection of the sample and water plug, the analysis of lupin proteins at 25 kV and 214 nm was carried out.

*Solubility.* The supernatant collected after each heat-treatment step was used to determine the effect of heat treatment on the solubility of lupin protein. The protein content of the supernatant was determined with the nitrogen combustion method using a LECO CHN-2000 instrument. Protein content was calculated as the total nitrogen times 6.25.

*DSC.* Samples (precipitate) were analyzed using a TA Instruments Modulated MDSC™ 2920 (TA Instruments, New Castle, DE). Sets of low moisture content (MC) (3.1%) and high MC (80%) samples (precipitate) were analyzed. For each run, a 50-mg sample was sealed in a stainless steel pan and heated to 250°C at 3°C/min, with a nitrogen flow rate of 24 $cm^3$/min. The onset and peak temperatures were determined by the tangent method used by the instrument's software, which minimizes error committed by the operator in determining the onset temperature.

*Surface hydrophobicity.* The surface hydrophobicity was determined by a method based on that of Kato and Nakai (13). Lupin precipitate (200 mg) was dispersed in 15 mL of 0.01 M phosphate buffer (pH 8.0) and stirred for 3 h at 35°C. The suspension was centrifuged at 4000 × *g* at 10°C for 20 min, and the supernatant was recentrifuged for 20 min. One milliliter of the supernatant was added to 4.0 mL of biuret reagent, mixed immediately, and left to stand for 30 min. The absorbance was then read at 540 nm (1.0 mL pH 8.0 buffer used as the blank). The standard curve dilutions were 1:2, 1:5, 1:8, and 1:10 using

0.01 M phosphate buffer, pH 8.0. The first scan was used for excitation wavelength. The initial emission wavelength was 484 nm, and the instrument was set to scan from 200 to 470 nm. The peak excitation wavelength was recorded and used to optimize the selection of emission scan parameters. The instrument was set to scan from roughly 20 nm above the excitation peak (~480 nm) to 600 nm. Results were verified by recording the peak emission wavelength with the maximum fluorescence intensity (FI), which was found to be 470 nm. Averages of two blank runs were performed (scans were repeated until agreement was <2 nm). The final FI value was obtained by subtracting the FI of the blank from the FI of the sample.

*Rheological study*. Lupin meal suspensions (30% w/w), heat-treated at different pH values and temperatures as just described, were prepared in 0.05 M phosphate buffer (pH 7) and stirred with a spatula. A total of nine suspensions were studied: a 3 × 3 matrix of three pH values (4, 6.8, and 8) and three heat treatment temperatures (25, 75, and 100°C). A TA Instruments ARES Series V controlled-strain rheometer running Orchestrator software was used to obtain the data. The geometry used was a stainless steel 25-mm diameter parallel plate fixture. The temperature throughout the test was set at 25 ± 0.1°C and controlled by a circulating water bath.

## RESULTS AND DISCUSSION

*SDS-PAGE.* As described in the Materials and Methods section, the determination of the effect of heat treatment was carried out on the freeze-dried precipitated meal and on the proteins lost in the supernatant. The SDS-PAGE profile in Figure 1 demonstrates the type of proteins found in the supernatant collected after each centrifugation following the heat treatments as compared with untreated meal. The formation of protein aggregates was obvious from the SDS-PAGE profile (data omitted for clarity) of the heat-treated meal, where pH 4 and 90 or 100°C showed the highest aggregation. Aggregate formation was indicated by the presence of high M.W. molecules at the top of the gel compared with the control. The effect of pH was more apparent on the nonreduced samples, where at pH 4 and 90°C more aggregates were formed (again, data omitted for clarity). The reduced samples confirmed this with the presence of bands of intensity closer to each other at all three pH values.

*Surface tension.* Lupin obtained by freeze-drying the supernatant was redissolved in phosphate buffer, and the surface tension of the solution was measured as a function of lupin concentration. As shown in Figure 2, lupin extracted at ambient temperature displayed significant surface activity as demonstrated by the reduction in the surface tension of the phosphate buffer as a function of lupin concentration. The surface tension of the buffer decreased from 72 to 40 dyn/cm with increasing concentration of lupin extracted at ambient temperature. Figure 2 also illustrates that the effect of lupin on surface tension is independent of the pH used to extract it at ambient temperature. Lupin extracted at 100°C showed a similar reduction of surface tension as a function of concentration.

*FZCE.* The FZCE electrophoregram of lupin proteins extracted from heat-treated meal precipitate at ambient temperature (Fig. 3) was separated into three regions relative to mesityl oxide, a neutral marker. Proteins from each region were characterized by their retention times relative to neutral mesityl oxide; positively charged proteins (region A) eluted before neutral proteins (region B), which eluted before negatively charged proteins (region C). Twelve major peaks around the positive and neutral regions were identified for both defatted and nondefatted meals and used as controls (no heat treatment). The profile also identified peaks unique to the centrifuged control meal, as noted by the SDS-PAGE analysis mentioned earlier. In addition, the profile showed three peaks unique to the nondefatted sample (region C) and four to the defatted (region C). This is an indication of the effect of centrifugation at the end of

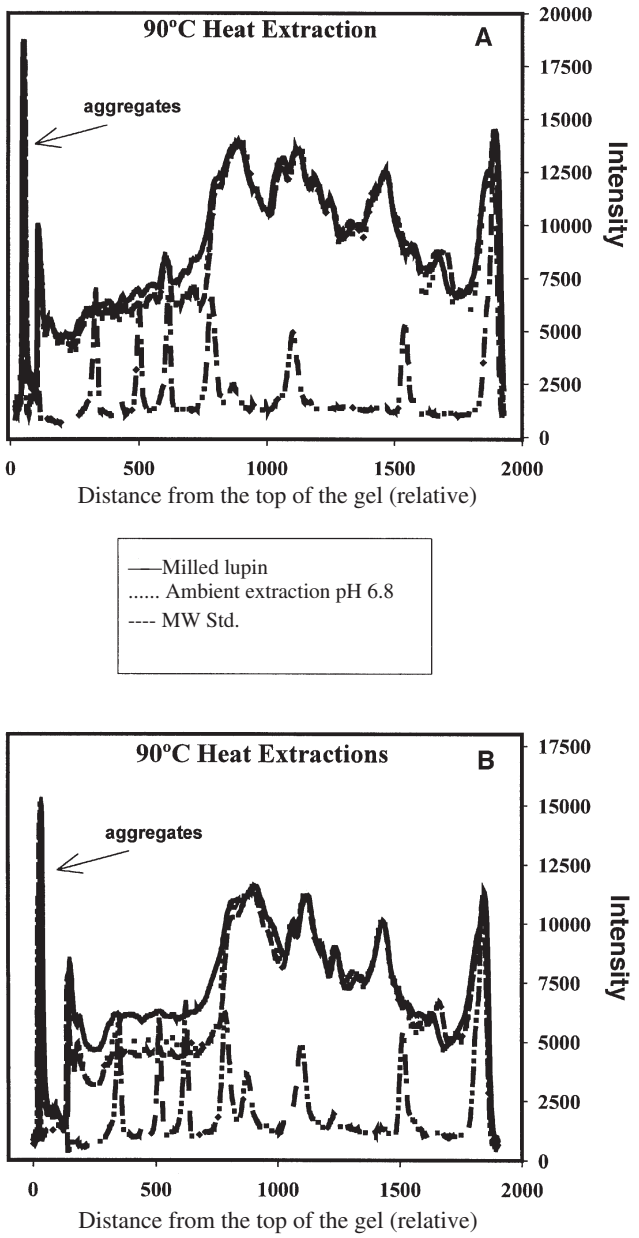


**FIG. 1.** SDS-PAGE profile of native lupin meal (A) and extracted meal (B) using phosphate buffer.

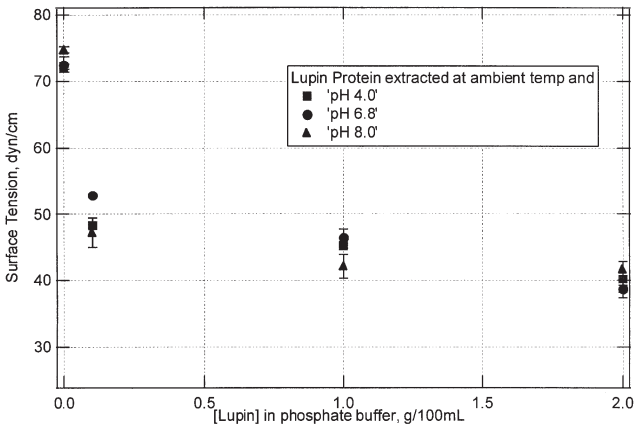


**FIG. 2.** Effect of lupin concentration on surface tension of phosphate buffer.

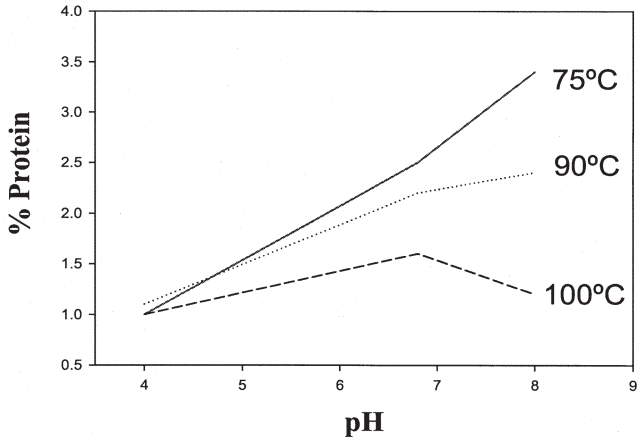


**FIG. 4.** Effect of temperature and pH on the solubility of lupin proteins.

the heat treatment and the defatting process on the protein surface properties. Centrifugation promotes separation of protein molecules as a result of the centrifugal force. A number of peaks observed in the positive and neutral regions of both defatted and nondefatted meal showed the same retention time,

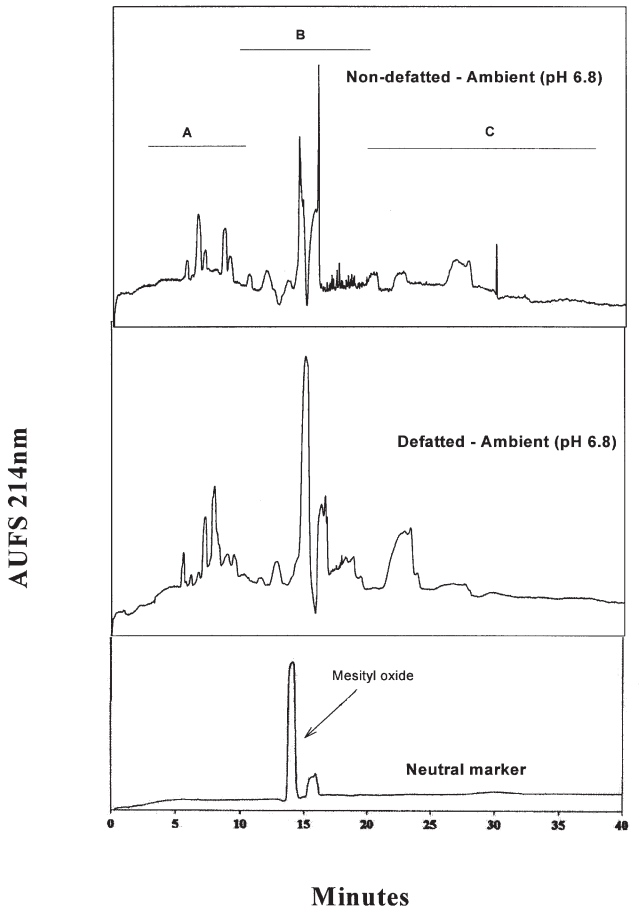


**FIG. 3.** Free zone capillary electrophoretic profile of defatted and nondefatted lupin meal extract.

which indicates a similar charge-to-size ratio. The nondefatted sample displayed a larger number of negatively charged molecules (migration time ≥ 25 min) than the defatted. These molecules may have been removed during the defatting process. This region also included unevenly shaped peaks, suggesting that clusters of proteins co-migrated through the column. This phenomenon was also observed by SDS-PAGE analysis as aggregates that did not penetrate into the stacking gel. In addition to their ability to aggregate, these molecules were characterized as being negatively charged, as shown by FZCE analysis. Both defatted and nondefatted heat-treated samples, regardless of treatment temperature, showed a prominent peak in region A (positively charged) with a mean electrophoretic mobility of $3.02 \times 10^{-4}$ cm$^2$/V·s (Fig. 3). With the exception of 75°C and pH 8.0, heat-treated lupin protein produced negatively charged molecules that were not present at the ambient temperature treatment. This suggests that negatively charged lupin proteins can be obtained by treating lupin meal at temperatures between 90 and 100°C. The pH seemed to have no influence on the type of the extracted protein species, especially when extracted at 75 and 90°C. Proteins treated at 100°C, regardless of pH, displayed neutral and positively charged molecules (regions A and B, Fig. 3) compared with the native proteins, indicating a loss of the negatively charged species of the same sample treated at 75 and 90°C. The dominant peak appearing on the positively charged region continued to appear at the 100°C heat treatment.

*Solubility.* Lupin protein solubility (protein content in the supernatant) decreased with an increase in treatment temperature (Fig. 4), possibly owing to protein aggregation as shown by the SDS-PAGE. Simultaneously, lupin protein solubility increased with pH. This agrees with the SDS-PAGE profile, where at pH 4, higher protein aggregation levels were observed, consequently reducing the solubility. Sathe *et al.* (14) reported minimal solubility of lupin protein isolate at pH 4 and a maximum at pH 10. In addition to concurring with their report, data presented here added the effect of heat treatment on the solubility.

*DSC.* The MDSC analysis profiles of the treated meal at 3.1% MC showed a glass transition around 85°C and an exothermic transition about 160°C, indicating protein aggregation.

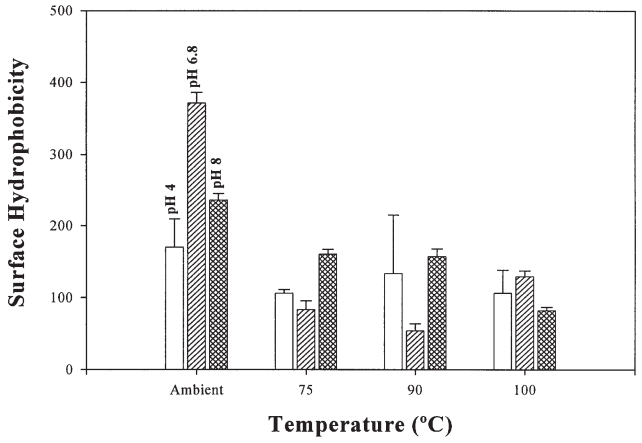


**FIG. 5.** Effect of temperature and pH on the surface hydrophobicity of lupin proteins including SD.

The presence of glass transition in lupin meal suggests the formation of a pre-gel phase. Heat treatment at 100°C produced higher glass transition temperatures compared with 75°C, 0.316 and 0.227 J/g/°C, respectively. The glass transition values were influenced by treatment temperature and pH; at pH 6.8 values were significantly higher than pH 4 and 8. The pH was more influential on the thermal properties of lupin protein than the treatment temperature. The results of the 20% (wt/vol) meal suspension were similar to the low-MC samples in having a glass transition and an exothermic transition with higher temperatures and $\Delta H$. The presence of the proteins in the 20% suspension appeared to strengthen the globular structure, thus increasing the $\Delta H$ values from an average of 9 to 20 J/g when compared with the low-moisture samples. The $\Delta H$ values of the suspension increased with temperature and decreased as the pH was reduced from 8 to 4. The low $\Delta H$ values at low pH further confirm the effect of acidic pH on the structure of lupin proteins as shown earlier by SDS-PAGE and FZCE.

*Surface hydrophobicity.* $S_0$ values changed significantly as a result of heat treatment at different pH values (Fig. 5). The $S_0$ values of the samples treated at ambient temperature were higher, indicating that at this condition, the protein is dispersed with accessible hydrophobic regions. The highest $S_0$ value for the ambient temperature extraction was at pH 6.8, demonstrating better dispersion of the protein molecules with minimal aggregation. The degree of denaturation of the protein heated at 75, 90, and 100°C is obvious from the $S_0$ values, where lower $S_0$ values indicate higher degrees of aggregation owing to the unavailability of hydrophobic regions on the surface as a result of denaturation. The effect of pH on $S_0$ is relative to temperature, except at pH 4 where the temperature has less influence (Fig. 5). At 90°C and pH 6.8, lupin protein experienced the highest degree of aggregation as indicated by the lowest $S_0$ value, whereas at 100°C and pH 6.8 the higher $S_0$ value signified protein denaturation and the exposure of hydrophobic regions otherwise buried inside globular protein.

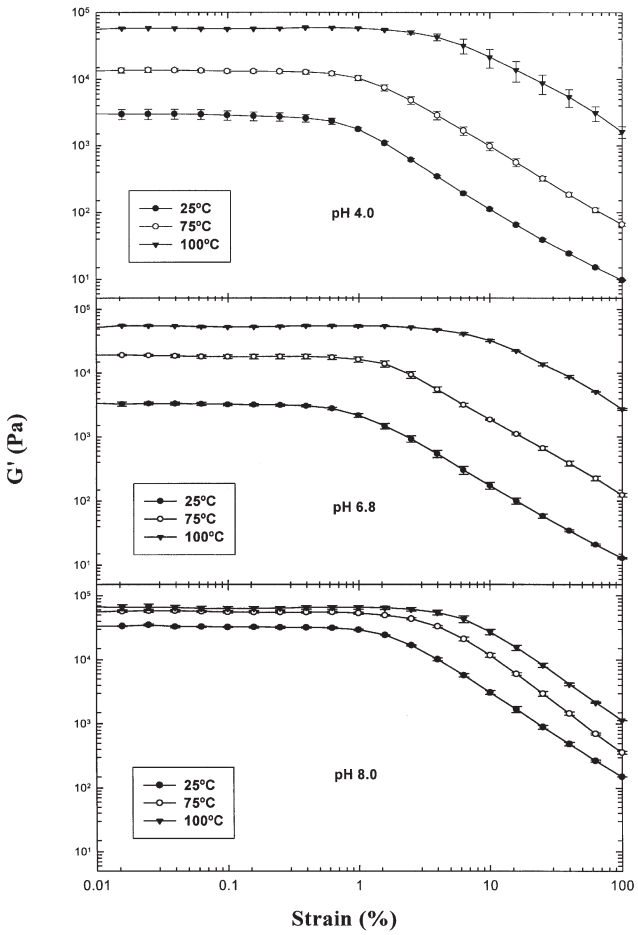


**FIG. 6.** Effect of treatment temperature on the shear storage modulus ($G'$) of lupin meal for three different pH treatments, including SD.

*Rheological properties.* Both pH and the treatment temperature of the lupin meal affected the textural properties of the suspensions. All of the suspensions were similar in that each was a semisolid material that would retain its shape and resist flow (at least on time scales up to several hours) but that could be easily mixed and deformed (i.e., a texture comparable to that of thick frosting). However, it was clear that by increasing either the pH or the treatment temperature, the resulting suspension became firmer and slightly drier. This phenomenon can be seen by quantitatively examining plots of $G'$, the shear storage modulus, vs. strain. Results shown in all plots are the averages of at least two runs for each suspension. In Figure 6, three lupin suspensions that were prepared at pH 4 and varying treatment temperatures are shown. The magnitude of $G'$ increases directly with the treatment temperature of the lupin meal. A higher $G'$ value represents a more solid-like material, which is more resistant to deformation. From this plot it can also be seen that each of these suspensions is a viscoelastic material, since each has a clear linear region where $G'$ is independent of strain followed by a nonlinear response at strains greater than roughly 1%. In Figure 7, the three suspensions prepared at 25°C are shown to illustrate $G'$ as a function of pH. From the figure it

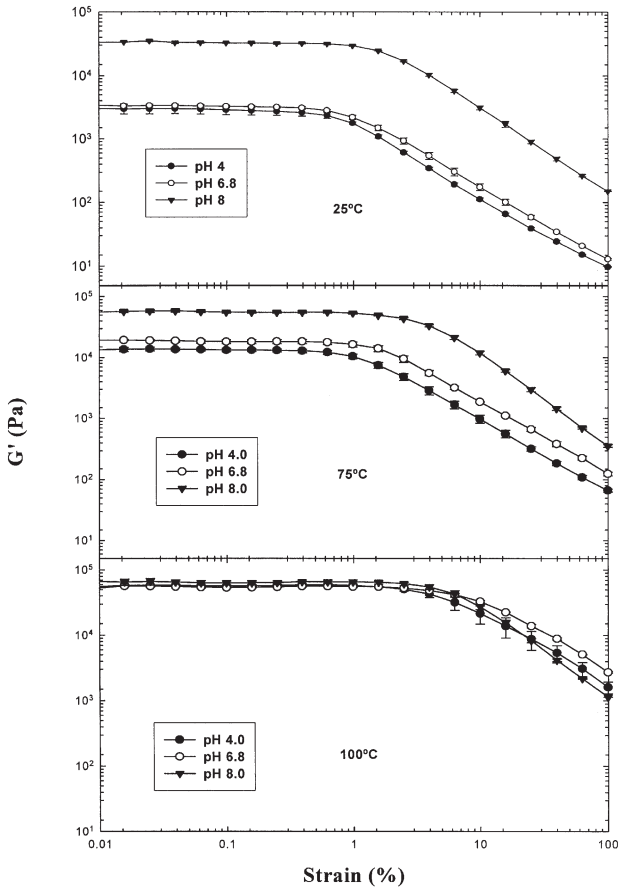


**FIG. 7.** Effect of pH on the shear storage modulus ($G'$) of lupin meal for three different treatment temperatures, including SD.

can be seen that the resulting difference in $G'$ from changing the pH from 4 to 6.8 is very small compared with the change in going from 6.8 to 8.

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