Physicochemical Studies of Caroubin: A Gluten-like Protein

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It has been reported that caroubin, a protein mixture obtained from carob seeds, has rheological properties similar to those of gluten. Comparative studies of the effects of hydration and temperature on caroubin and gluten were carried out with the aid of NMR, FTIR, scanning electron microscopy, and differential scanning calorimetry techniques. The results show that caroubin has a more ordered structure than gluten and that hydration has little effect on its secondary structure when compared to gluten. Caroubin is more easily accessible to water than gluten, suggesting that caroubin is more hydrophilic in nature. On hydration, caroubin, like gluten, forms fibrillar structures and sheets.

Keywords: *NMR; FTIR; electron microscopy; DSC; caroubin; gluten*

INTRODUCTION

Caroubin is a water-insoluble mixed-protein system isolated from carob bean embryo. The carob tree (*Ceratonia siliqua* L.) is a Mediterranean plant, also known as St. John's bread, locust bean, and Egyptian fig. It is a legume and evergreen and takes 20 years to reach maturity. Trees start to bear fruit at the age of 5−7 years, yielding ∼10 kg/tree. At maturity they will yield 250−500 kg/tree and will continue to produce for up to 200 years. The fruit consists of long pods of which 90% is pulp and 10% seed. The pulp is sweet and can be either milled for use in confectionery or extracted to make syrup (*1*). The unique characteristics of the syrup make it ideal food flavoring. The seeds can be milled to extract a gum from the cell wall, which is used as a food thickener. The embryos are removed during milling and, as a byproduct, they are processed for animal feedstuffs (*2*, *3*). Almost 50% of the weight of the embryo is a protein (*4*) known as caroubin (*5*). Caroubin is a mixture of a large number of proteins that are different in size and degree of polymerization, covering a molecular weight range from more than one million to several thousands (*5*). Seventy-eight percent of the protein has a molecular weight range of 65000 < MW (Da) < 1000000 (*5*). In comparison, gluten has 54% of its contents in the molecular weight range 20000 < MW (Da) < 65000. When hydrated, caroubin can form a dough, which has some similarities to wheat gluten in it rheological properties (*5*, 6). The amino acid composition of the caroubin differs from that of the wheat gluten in that it contains more arginine, aspartic acid, and lysine and less cystine, phenylalanine, and proline (*3*). A slightly lower level of glutamic acid content was found in caroubin (32%) compared to gluten (38%), although the glutamic acid levels are very high in both of the proteins (*5*).

Although caroubin possesses properties similar to those of gluten and might have potential as a gluten replacement, little attention has been paid to it. The principal reason for replacing gluten is for the relief of celiac disease, which is a sensitivity of the lining of the small intestine to gluten, causing failure to digest food unless gluten is strictly excluded from the diet. In this paper, we report an investigation of caroubin using nuclear magnetic resonance spectrometry (NMR), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and microscopy. ^{2}H NMR was used to examine the effects of temperature on water intake of caroubin. FTIR measurements allowed an insight into the effects of hydration and temperature on the secondary structure of protein. Complementary studies of the effects of temperature and hydration on caroubin were carried out using DSC and microscopy. The results obtained are compared with those of wheat gluten.

MATERIALS AND EXPERIMENTAL METHODS

Materials. Caroubin was isolated from carob germ flour by gently stirring 20 g of germ flour with 200 mL of deionized water for 3 h at room temperature and centrifuging for 15 min at 5500*g*. The supernatant was discarded. Caroubin was obtained by hand-washing the insoluble layer as in the preparation of gluten (*5*). The material has been characterized, and the results have been reported (*5*). The wheat gluten sample was purchased commercially from Sigma for the NMR experiments. For the microscopic studies thin sections of the wheat grain cultivars Avalon were used.

Scanning Electron Microscopy. A Petri dish was filled to the brim with distilled water, and wheat grain section or caroubin was sprinkled onto the water. After 30 min, a few drops of 25% glutaraldehyde fixative were added to the water to cross-link the spread protein fibrils overnight. The samples were picked up from underneath onto pieces of glass coverslip and allowed to air-dry. The pieces of glass were then attached to aluminum specimen stubs with double-sided sticky tabs and coated with a 25 nm thick layer of gold in an EMITECH K550

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gold-coating unit. The samples were then examined and photographed in a Leica Stereoscan 360 scanning electron microscope (Leo, Cambridge, U.K.) at an accelerating voltage of 10 kV.

NMR Experiments. Caroubin or wheat gluten (10−15 mg) was hydrated in an excess of D_2O in 5 mm NMR tubes. After the samples had been stirred, the tubes were sealed and left at 278 K overnight. ^2H transverse relaxation measurements were then carried out using the Carr−Purcell−Meiboom−Gill (CPMG) sequence over a temperature range of 298−373 K at a frequency of 46.05 MHz on a Bruker MSL-300 spectrometer (*7*). Ninety degree pulse lengths were 10 μs. Pulse spacing was 4 ms, and the receiver dead time was 15 μs. A total of 64 scans and 1K data points were accumulated. The time between the scans was 5 s. The experimental data were analyzed using WinDXP software (Resonance Instruments Ltd.) to obtain the distribution of the T_2 values and corresponding populations. This fitting procedure is called "zeroth order regularization", which introduces a smoothing parameter into the fitting algorithm.

FTIR Experiments. FTIR spectra of the caroubin were recorded on a MagnaIR 860 FTIR spectrometer (Nicolet) equipped with am HgCdTe detector. A thermostatic horizontal attenuated total reflectance (HATR) cell with a Ge crystal (Specac) was used to measure the spectra of the insoluble caroubin samples. The fully hydrated caroubin sample was placed into the cell, and spectra were measured at different temperatures ranging from 298 to 368 K. At each temperature the empty cell was used as reference. For gluten, FTIR experiments of dried and hydrated samples were carried out for comparison. Water bands in the spectra of the hydrated samples were compensated by digitally subtracting a corresponding water spectrum recorded under the same conditions. For all experiments, 256 scans at 2 cm^{-1} resolutions were averaged. When necessary, traces of water vapor bands were removed by subtraction of a vapor spectrum. For the comparison of the structure of dried and hydrated gluten and caroubin, the spectra were Fourier deconvoluted using spectrometer software (resolution enhancement factor of 2.0, bandwidth = 16 cm^{-1}).

DSC Experiments. DSC was carried out using a Perkin-Elmer DSC 7 robot system fitted with an IntraCooler II cooling unit. The instrument was calibrated for temperature using the melting of β-naphthyl ether (300.5 K) and indium (426.6 K). The samples of gluten and caroubin were hydrated with an excess amount of distilled water and left overnight at 5 °C. About 10 mg of hydrated sample was weighed into a preweighed aluminum sample pan with a 2 atm limit. The sample pan was sealed, and DSC was performed in the temperature range 283−393 K at a heating rate of 10 K/min. After the initial heating run, the sample was rapidly cooled and reheated to observe any reversible transitions.

RESULTS AND DISCUSSION

Scanning Electron Microscopy. Figure 1 shows scanning electron micrographs of caroubin and wheat grain section spread on a water surface. Wheat flour interacts rapidly with water and forms fibrils on the surface of water. The hydrated gluten fibrils extended from the flour fragments and quickly developed into a linked network. The particles associated with the fibrils are starch granules, which are carried outward from the flour fragment as the fibrils extend. The adherence of starch granules to the surface of protein fibrils is clearly shown (Figure 1), but the fine fibrils are not as easily distinguished because they are dried down onto the glass support. In addition, sheets can also be observed, although they are obscured by starch granules. Fibril structures derived from wheat endosperm tissue have been reported by Seckinger and Wolf (*8*) and Bernardin and Kasarda (*9*). Caroubin forms protein fibrils and sheets, but caroubin fibrils are finer in appearance than

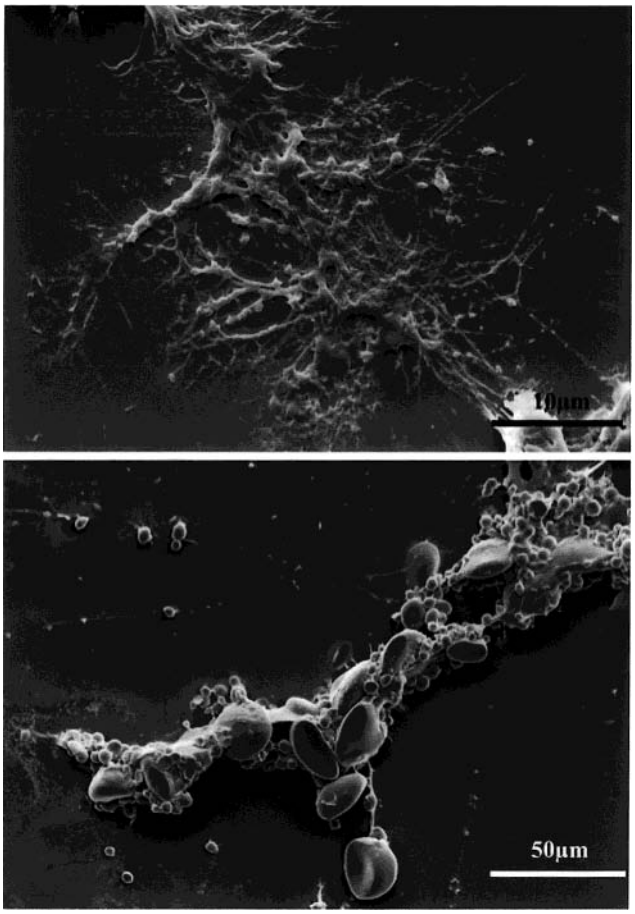

Figure 1. Scanning electron micrographs of spread caroubin (above) and gluten (below).

those formed by gluten. This is the first report that caroubin forms both sheets and fibrils on hydration. Although both caroubin and gluten can form fibrillar structures and sheets, differences can be observed under microscopy on protein hydration. This might be due to the differences of the hydration properties of the two proteins. On addition of water to caroubin a homogeneous mass is formed more readily than is with gluten.

NMR. The ^2H NMR transverse relaxation of caroubin and gluten hydrated with D_2O could be fitted by a double-exponential model. This appears to be different from the previous ^2H NMR studies on gluten in which three components were found (*10*). This is because different fitting procedures were used. The present results were obtained using "zeroth order regularization", which expresses the relaxation rates as means about which there are finite distributions. The relaxation value represents the center of the distribution. In the previous study, the relaxation curve was forced to fit into three discrete delta function components, that is, short, intermediate, and long components. The differences in relaxation times of the short and intermediate components were small and are effectively fitted in the current method as values within a distribution width. Long component values are similar in relaxation rate and population to those found previously for gluten (*10*).

Multiexponential transverse relaxation has been observed in hydrated polymer systems such as gluten (*10*). In such a system, two regions may be distinguished. Close to the polymer there will be chemical exchange

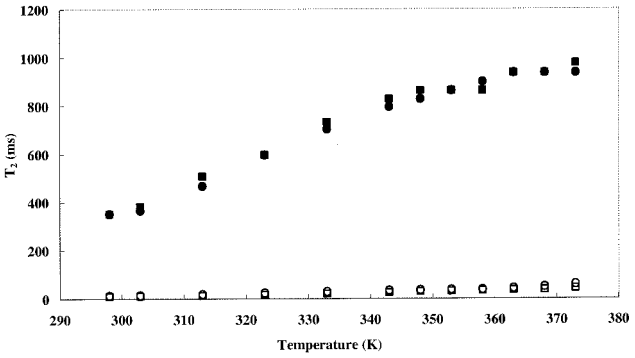

Figure 2. Effect of temperature on the ^{2}H transverse relaxation times (T_2) of D_2O-hydrated gluten and caroubin: (●) long T_2 component of caroubin; (○) short T_2 component of caroubin; (■) long T_2 component of gluten; (□) short T_2 component of gluten.

between exchangeable protons or deuterons on the polymer and water. Near the polymers, there will also be a diffusive exchange between distant bulk water and water near the polymer. Such a system is expected to exhibit multiple relaxation processes if (*11*)

$$2\sqrt{2}(A^2/\pi^2 D)|\Delta\gamma| > 1 \qquad (1)$$

$\Delta\gamma$ is the difference in relaxation rates of water between the two regions, and A represents a dimension characteristic of the region containing polymers and water. The slow-decaying component of the transverse relaxation time is 350 ms, which is close to that of pure D_2O. The diffusion coefficient (D) of pure water is 2.5×10^{-9} m^2/s. The fast-decaying component of the transverse relaxation time, which is assigned to water close to the protein, is 9.9 and 12.6 ms for gluten and caroubin, respectively. For condition 1 to be met the characteristic dimensions are 9 and 11 μm for gluten and caroubin, respectively. This implies that water greater than these distances away from the hydrated protein mass will be measured in this experiment as bulk water and contribute to the long relaxation time component. Using this assignment, the hydration levels of both caroubin and gluten at 298 K can be estimated by adding a known amount of D_2O into an NMR tube containing known amounts of protein. The values obtained are 3.17 and 1.86 g of water/g of dry solid for caroubin and gluten, respectively. Caroubin seems to absorb more water per unit weight than gluten, and this may be related to the observations of the differences in spreading behaviors observed by microscopy (Figure 1).

^{2}H NMR transverse relaxation of caroubin and gluten in an excess of D_2O was also measured over a 298−373 K temperature range. The effect of increasing temperature resulted in increases in the T_2 values of the two components as shown in Figure 2. There are no significant differences in T_2 values between the caroubin and gluten. Both proteins also show the trends of increasing populations of the short component and decreasing populations of the long component on heating (Figure 3). To compare the relative changes in hydration with temperature, the water uptake per gram of protein was calculated and plotted against temperature in Figure 4. It is clear that caroubin has a greater increase in hydration with temperature than gluten.

On heating, it is expected that a hydrophilic matrix will expand and absorb water, whereas a hydrophobic one will contract and expel water (*12*, *13*). Effects of

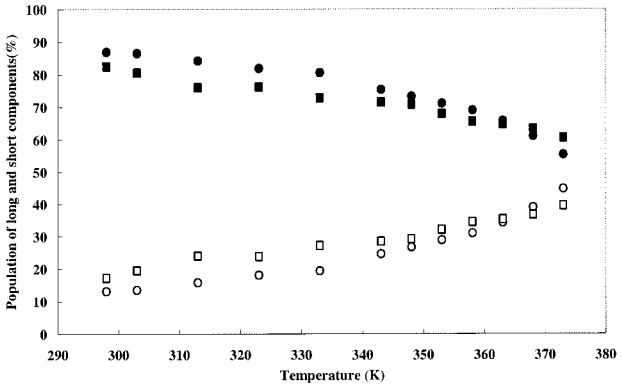

Figure 3. Effect of temperature on the populations of long and short T_2 components of D_2O-hydrated caroubin and gluten: (●) long T_2 component of caroubin; (○) short T_2 component of caroubin; (■) long T_2 component of gluten; (□) short T_2 component of gluten.

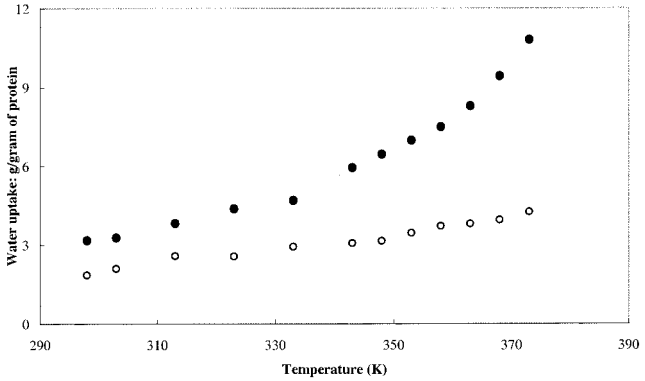

Figure 4. Effect of temperature on the uptake of water by caroubin and gluten proteins: (●) caroubin; (○) gluten.

temperature on water sorption by wheat gluten have been reported (*10*). The present results are consistent with the previous findings and imply that both gluten and caroubin have a positive coefficient of expansion, consistent with a hydrophilic character. The increase in the population of the short-decay component when the proteins were heated in water indicates a sorption of additional water by the proteins. This suggests that hydrogen bonds were broken by the heating process. In effect, the secondary structure of the proteins opened up and released more accessible polar groups. The fact that the caroubin absorbs more water than the gluten on heating suggests that the caroubin network expands more readily than gluten and that heating has a greater effect on the structure.

FTIR. The FTIR spectra of dry and hydrated caroubin and gluten are shown in Figure 5 A,B. Fourier deconvolution of the amide region showed that the amide I region of the dry caroubin consisted of two components with identical intensities: a peak at 1654 cm^{-1}, which may be assigned to α-helix structure, and one at 1641 cm^{-1}, due to internal β-sheet. (This assignment is confirmed by an additional shoulder at 1689 cm^{-1}.) On hydration of caroubin the positions of both peaks shifted downward by 4 cm^{-1} due to the hydration. The β-sheet bands became slightly weaker, but overall the effect of hydration on the secondary structure appeared to be limited. The center of the amide II band shifted from 1534 cm^{-1} in the dry sample to 1544 cm^{-1} in the fully hydrated sample, indicating an increased amount of protein−water hydrogen bonds and, thus, a considerable exposure of the protein backbone to water. However, the

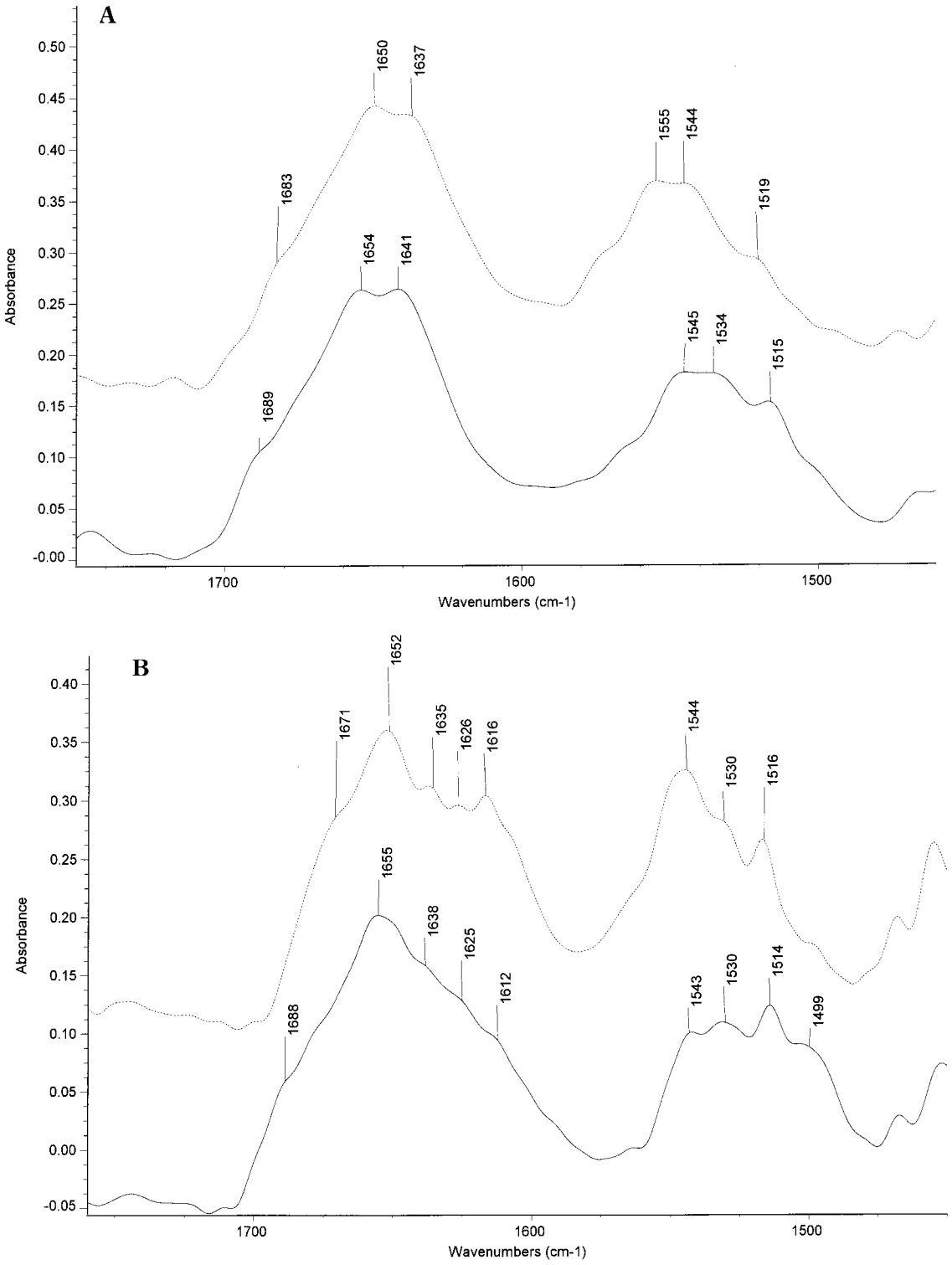

Figure 5. FTIR spectra of dried and fully hydrated caroubin (A) and gluten (B): (top line) hydrated samples; (bottom line) dried samples.

absence of major changes in the amide I band showed that overall there was not much change in the secondary structure. This was in contrast to the hydration behavior of gluten (Figure 5B). A significant increase of intermolecular β-sheet and extended structures was observed on hydration as suggested in increasing intensities at 1626 and 1616 cm^{-1}. This is typical for gluten and is consistent with previous observations on whole gluten for the high molecular weight subunit of glutenin (*10*, *14*). The differences in the hydration behaviors of gluten and caroubin suggest a more ordered and stable structure in caroubin than in gluten.

The effect of heating fully hydrated caroubin was investigated by variable-temperature FTIR as shown in Figure 6. There was no marked difference in the amide I band between the spectra at 293 and 313 K. At 337 K the spectrum was somewhat broadened and the maximum shifted to 1636 cm^{-1}. However, between 333 and 353 K a strong increase in relative intensity around 1627 cm^{-1} shifted the band maximum to 1629 cm^{-1}. At

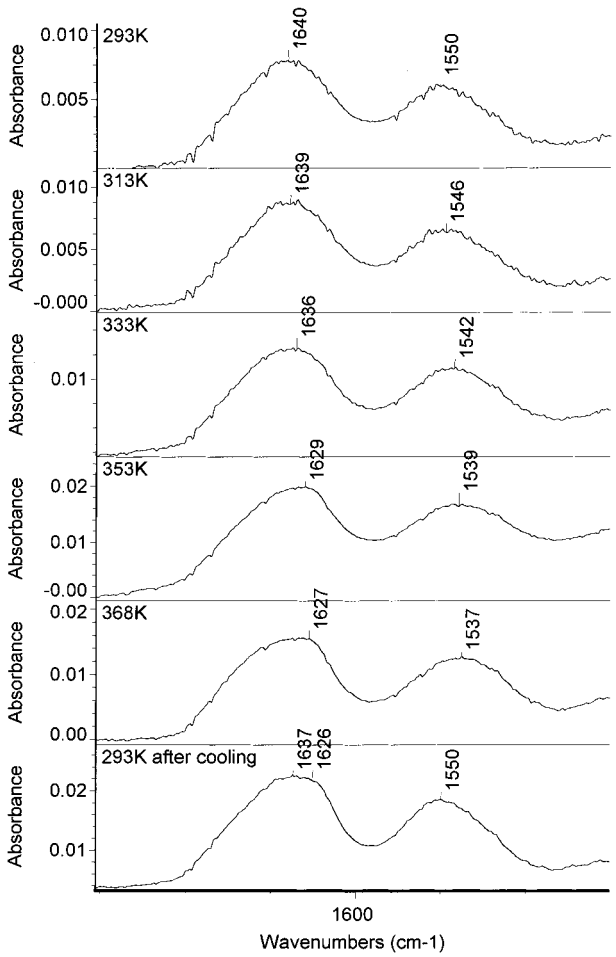

Figure 6. FTIR spectra of hydrated caroubin recorded at various temperatures.

368 K this 1627 cm^{-1} band was clearly the strongest component in the amide I band, and there was also a visible broadening around region 1670−1680 cm^{-1}. The 1625/1680 cm^{-1} band pair has generally been used as an indicator for protein aggregation via intermolecular β-sheet structure (*15*). The associated loss around 1640 cm^{-1} suggests that this was formed at the expense of unordered or α-helical structures. After the sample had cooled to 293 K, a strong shoulder at 1626 cm^{-1} remained, which suggested that the aggregation process was largely irreversible. Significantly it is above 330 K that the slope of the water uptake curve of caroubin deviates from that of gluten (Figure 4), suggesting that the observed structural changes result in enhanced water absorption.

The peak maximum of the amide II band was shifted continuously from 1550 cm^{-1} at 293 K to 1537 cm^{-1} at 368 K, and the band shape became more symmetrical at higher temperatures. However, this shift was reversible and, after cooling, the band shape and maximum were similar to those of the original spectrum. This suggests that the shift is the result of reversible hydrogen bonding effects.

DSC. Figure 7 shows the difference in first and second scans of the fully hydrated caroubin and gluten when heated over a temperature range of 283−393 K. No transition was observed in the rescanning. The curve of hydrated caroubin showed a broad asymmetric endothermic peak starting from 366 K. The maximum peak temperature is 374 K, and enthalpy is 11 J/g. In

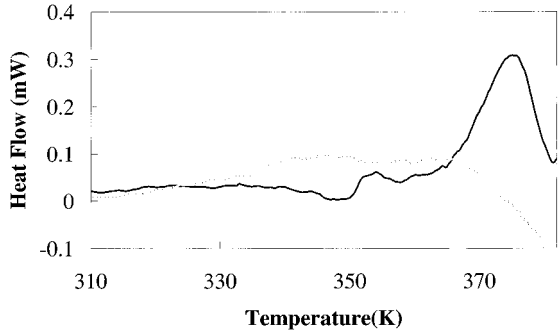

Figure 7. DSC thermograms at 10 °C/min for fully hydrated caroubin and gluten: (bold solid line) caroubin; (light line) gluten.

contrast, fully hydrated wheat gluten gave no peak when heated in a DSC within the same temperature range. This has been observed previously and explained by assuming that the wheat gluten has disordered structure and appears to be more like an amorphous polymer (*16*, *17*). The origin of the transition observed on the caroubin sample is likely to be due to some partial denaturation and aggregation and is consistent with the FTIR studies of the effect of hydration and heating of caroubin.

Conclusions. Significant differences were found between caroubin and gluten even though they possess similar rheological properties. Caroubin, like gluten, can form both fibrillar and sheet structures when hydrated. NMR showed that the caroubin network is more open and more easily accessible to water and expands more on heating. Results from FTIR, NMR, and DSC are consistent with a structural change in caroubin above 330 K. Although there are rheological similarities between caroubin and gluten in the uncooked state, the results obtained here suggest that there may be significant differences between the materials after cooking. More research is needed to understand the structure−function relationships and to determine to what extent caroubin might be used as a gluten substitute.

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