Raman Spectroscopic Study of Oat Globulin Conformation

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Analysis of Raman spectra of oat globulin showed that extreme pH values caused an increase in the amide and C−H stretching band intensity, indicating changes in the secondary structures of the protein due to denaturation. Similar changes were observed when oat globulin was treated with chaotropic salts and several protein perturbants. Sodium dodecyl sulfate, β-mercaptoethanol, and ethylene glycol also caused a shift in the amide III′ band, suggesting a transition from β-sheet to a random coil conformation. Heating at temperatures near the denaturation temperature of oat globulin led to increases in the amide and C−H band intensity, indicating unfolding of the protein. The data indicate that FT-Raman spectroscopy is suitable for studying the secondary structure of plant proteins such as oat globulin.

Keywords: *Raman spectroscopy; oat globulin; protein conformation*

INTRODUCTION

Oat globulin is an oligomeric protein with a quaternary structure very similar to that of soy 11S globulin (glycinin), a heat-coagulable protein. It is made up of six pairs of acidic and basic polypeptides, with each acidic and basic polypeptide linked by disulfide bonds to form a subunit. The six subunits are linked by noncovalent forces to form a hexamer (Derbyshire et al., 1976; Neilsen, 1985; Brinegar and Peterson, 1982). Despite the similarities in quaternary structure, the thermal aggregating and gelation properties of oat globulin were found to be quite different from those of soy glycinin (Ma and Harwalkar, 1987; Ma et al., 1988), which may partly be attributed to differences in secondary and tertiary structures between the two proteins. Oat protein products (concentrates and isolates), with globulin as the predominant protein fraction, have been shown to possess functional properties comparable to those of commercial soy protein isolates (Ma, 1982, 1983), the most widely used vegetable protein ingredient in foods. A detailed understanding of the structure−function relationship in oat globulin is essential in predicting and controlling the functional performance of oat protein in fabricated foods. Studies of protein conformation under different buffer conditions can provide valuable information for improving specific functional performance of protein ingredients, such as gelation and emulsification. The conformation of oat globulin has been studied by differential scanning calorimetry (DSC) (Harwalkar and Ma, 1987) and UV and fluorescence spectrophotometry (Ma and Harwalkar, 1988a) under the influence of heating and different buffer conditions. However, changes in the secondary structures cannot be assessed by these techniques.

The secondary structures of 21 seed globulins from monocotyledonous and dicotyledonous plants, including oat globulin, have been studied by circular dichroism (CD) spectroscopy (Marcone et al., 1998). Results show that similar to most plant globulins, oat globulin has a low α-helix content and a large quantity of β-sheet and random coil structures. The secondary structures of these proteins were determined at low concentrations (~1.0 mg mL^{-1}) and only at one buffer condition (pH 7.5, ionic strength 0.5). The requirement for clear samples in CD analysis limits its application to dilute protein solutions or transparent gels (Matsuura and Manning, 1994). Interference due to absorbance of various salts and buffer substances in the far-UV region (Stanley and Yada, 1992) also limits the use of CD spectroscopy in studying the effects of environmental conditions such as chaotropic salts and protein perturbants on protein conformation. The secondary structure of oat globulin has not been studied by other methods such as X-ray diffraction, multidimensional NMR, or vibrational spectroscopy including infrared (IR) and Raman spectroscopy.

The applicability of Raman spectroscopy to both solid and liquid samples makes it a useful tool to investigate in situ protein structural changes during denaturation and aggregation/gelation (Li-Chan et al., 1994; Li-Chan, 1996a). However, fluorescence continues to be a major problem in Raman spectroscopic analysis of plant materials including vegetable proteins when visible laser excitation is used. This is mainly due to phenolic compounds coextracted with the plant substances. A significant advance in solving the fluorescence problem has been the development of Fourier transform (FT) Raman spectroscopy (Schrader et al., 1991; Li-Chan et al., 1994). The use of a diode-laser pumped Nd:YAG laser radiation at 1064 nm in conjunction with interferometers and Fourier transform have resulted in improved suppression of fluorescence, good signal-to-noise ratios, and marked shortening in data acquisition time. The advantages of near-infrared (NIR)-FT Raman spectroscopy over conventional visible laser Raman spectroscopy have been demonstrated in the study of soy proteins, including the effects of heating, gelation, and pressure, and in the study of soy protein−phospho-

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lipid interactions (Li-Chan et al., 1994). NIR-FT Raman spectroscopy has also been used in the study of polymeric biomaterials (Davies et al., 1990), and its potential in food analysis has been demonstrated (Ozaki et al., 1992), including quantitative analysis of oils and fats (Sadeghi-Jorbachi et al., 1991) and the determination of the level of acetylation in modified starches (Phillips et al., 1999). However, despite the advantages of the FT-Raman instrument, there are few reports on its use in the study of proteins from either animal and plant sources.

In the present investigation, changes in oat globulin conformation under the influence of different environmental conditions and heating will be studied by FT-Raman spectroscopy.

MATERIALS AND METHODS

Oat seeds (variety Hinoat) were grown at the Central Experimental Farm, Agriculture and Agri-Food Canada, Ottawa, and were dehulled in a pin-mill. Oat globulin was extracted from defatted oat groats with 1 M NaCl (Ma and Harwalkar, 1984), and the isolated oat globulin was stored at −20 °C. The protein content of oat globulin, determined according to a micro-Kjeldahl method (Concon and Soltess, 1973) using a nitrogen to protein conversion factor of 5.80, was 98.9%, as reported previously (Ma, 1983). The purity of the oat globulin preparation was checked by SDS−polyacrylamide gel electrophoresis (Laemmli, 1970) and found to be highly homogeneous. All chemicals used were of analytical grade.

Raman Spectroscopy. Oat globulin dispersions (10−15% w/v) were prepared in different solvents. For the control, protein was dispersed in distilled water and stirred at room temperature for 30 min. To prepare wet protein pellet, the protein dispersion was centrifuged in a benchtop microcentrifuge at 10000*g* for 5 min, and the supernatant was discarded. After either the protein dispersions or the wet pellets had been packed into glass capillary tubes, both ends of the tubes were carefully heat-sealed.

Chaotropic salts were added to oat globulin dispersions (≈10% in distilled water) to give a final salt concentration of 1.0 M. A previous study (Harwalkar and Ma, 1987) showed that significant unfolding of oat globulin can be observed only at a higher salt concentration of 1.0 M. Protein perturbants including sodium dodecyl sulfate, β-mercaptoethanol, urea, and ethylene glycol were added as solid or liquid to oat globulin dispersions to give the desirable final concentrations of reagents and protein (10−15%). The pH of the dispersions was controlled by the use of 0.01 M phosphate buffer, pH 7.4. The selected perturbant concentrations were also based on previous studies (Harwalkar and Ma, 1987; Ma and Harwalkar, 1988a), which showed that conformation of oat globulin was markedly affected under these levels of perturbants.

To study the effect of pH on the Raman spectral characteristics of oat globulin, protein dispersions (≈10% in distilled water) with desirable pH were prepared by the addition of 0.1 N HCl or 0.1 M NaOH with mixing in a magnetic stirrer (Harwalkar and Ma, 1987). The protein samples were mixed for an hour to allow for pH equilibration.

For the heating experiments, oat globulin dispersions (10% w/v) were prepared in 0.01 M phosphate buffer, pH 7.4. The protein samples, in stoppered glass tubes, were heated in an autoclave at 110 °C for the prescribed time periods. Raman spectra of heated oat globulin dispersions or pellets were found to be noisy with very poor signal-to-noise ratio. The heated protein samples were therefore freeze-dried, and Raman spectra of solid samples were recorded. Preliminary experiments showed that freeze-dried protein samples exhibited Raman spectra identical to those in dispersions (Figure 1) or wet pellets (not shown), indicating that freeze-drying did not affect the conformation of oat globulin.

Raman spectra were collected on a Bio-Rad FTS-60 FT-NIR Raman spectrometer equipped with an Nd:YAG laser at 1064 nm (Bio-Rad Laboratories, Cambridge, MA). Raman spectra were recorded at room temperature under the following conditions: laser power, 500 mW; spectral resolution, 4 cm^{-1}; number of scans, 1000. The spectral data were baseline-corrected and normalized to the intensity of the phenylalanine band at 1004 ± 1 cm^{-1}. The Raman spectra were plotted as intensity (arbitrary units) against Raman shift in wavenumber (cm^{-1}). Protein dispersions containing urea were spiked with 0.2 M KNO_3 because urea vibrates at the phenylalanine region, and the KNO_3 peak (1046 ± 1 cm^{-1}) was used instead for normalization.

All analyses were performed in duplicates or triplicates, and the results are reported as the average of these replicates.

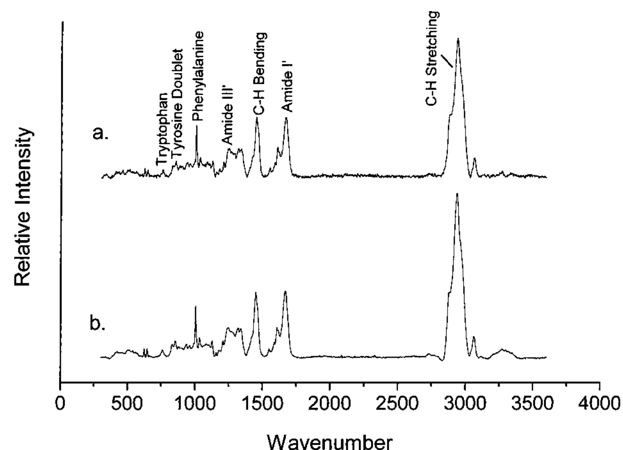

Figure 1. Raman spectra (450−3500 cm^{-1}) of (a) oat globulin dispersion (≈10%) in distilled water and (b) freeze-dried oat globulin powder.

Table 1. Tentative Assignment of Some Bands in the Raman Spectrum of Oat Globulin (in Distilled Water)

wave-number, cm^{-1}	assignment	structural information
760	tryptophan	sharp intense line for buried structure
830, 850	tyrosine	state of phenol-OH (exposed or buried, H donor or acceptor)
>1275	amide III′	α-helix
1235 ± 5	amide III′	antiparallel β-sheet
1245 ± 4	amide III′	disordered structure
1450	C−H bending	microenvironment, polarity
1655 ± 5	amide I′	α-helix
1670 ± 3	amide I′	antiparallel β-sheet
1665 ± 3	amide I′	disordered structure
2800−3000	C−H stretching	microenvironment, polarity

RESULTS AND DISCUSSION

Spectral Assignment. Parts a and b of Figure 1 show the typical Raman spectrum of 10% oat globulin dispersion in distilled water and freeze-dried powder, respectively. The two spectra were almost identical, except that the freeze-dried sample exhibited a slightly smoother baseline and a small unidentified peak at ∼3300 cm^{-1} (Figure 1b). The results suggest that freeze-drying did not affect the conformation of oat globulin as determined by Raman spectroscopy. The spectrum of a wet pellet (not shown) was also identical to those of the dispersion and dry solid. Table 1 shows the tentative assignment of some major bands based on comparison with Raman spectral data reported by previous workers (Tu, 1986; Li-Chan and Qin, 1998; Li-Chan et al., 1994; Peticolas, 1995). The locations of the amide I′ and III′ peaks indicate that β-sheet and random coils are the major secondary structures in oat globulin. This is in agreement with circular dichroism (CD) data,

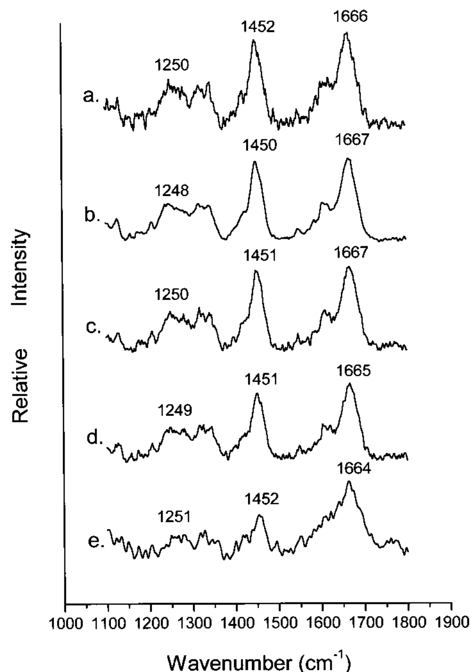

Figure 2. Raman spectra of oat globulin dispersions (≈10%) under different pH conditions: (a) pH 3; (b) pH 5; (c) pH 7; (d) pH 9; (e) pH 11.

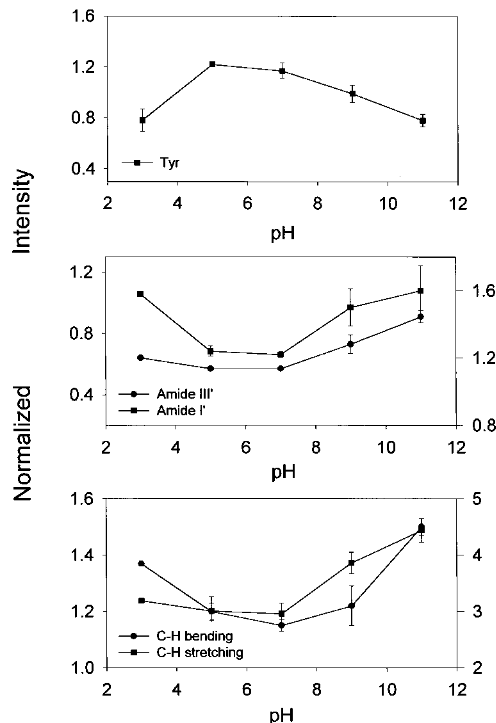

Figure 3. Effect of pH on normalized intensity of several regions in Raman spectrum of oat globulin dispersions (or pellets). Error bars represent standard deviations of the means.

which indicate that oat globulin and many plant globulins have a relatively small quantity of α-helical structure but a large amount of β-sheet and random coil structures (Marcone et al., 1998). The small intensity of the transitions near 500 cm^{-1} makes it difficult to analyze the disulfide peaks. This is due to the relatively low content of disulfide and sulfhydryl groups in oat globulin, similar to that of legume globulins (Derbyshire et al., 1976; Brinegar and Peterson, 1982; Neilsen, 1985).

Effect of pH. Figure 2 shows the Raman spectra of oat globulin at different pH values. There was a shift in the amide I′ vibration, which suggests a transition from a β-sheet structure near neutral pH to a random coil structure at extremely acidic (pH 3) and alkaline (pH 9−11) pH values. Extreme pH values also led to decreases in the tyrosine doublet band intensity, I_{850}/I_{830} (Figure 3), and this indicates an increased "buriedness" or participation of the tyrosine phenolic groups as hydrogen bond donors (Li-Chan, 1996a,b). The intensities of the amide I′, amide III′, and C−H stretching bands were increased by highly acidic (pH 3) and alkaline (pH 9−11) pH values (Figure 3), and this indicates protein denaturation. These results are consistent with our previous DSC data (Harwalkar and Ma, 1987) which showed that the denaturation temperature (T_d), an index of thermal stability, and enthalpy, an index of the amount of ordered structure, were highest at pH 5−7, the isoelectric pH range for oat globulin (Ma, 1983), suggesting a native conformation. The T_d and enthalpy were markedly decreased at pH values <4.0 and >9.0, indicating reduced heat stability and partial protein unfolding in oat globulin (Harwalkar and Ma, 1987). Most proteins are stable over a certain pH range, normally near their isoelectric pH, where repulsive forces are low and the proteins remain in their native state. At pH far from the isoelectric point, large net charges are induced and proteins will be partially unfolded due to intramolecular side-chain charge repulsion leading to rupture of hydrogen bonds and a breakup of hydrophobic interactions (Morrissey et al., 1987).

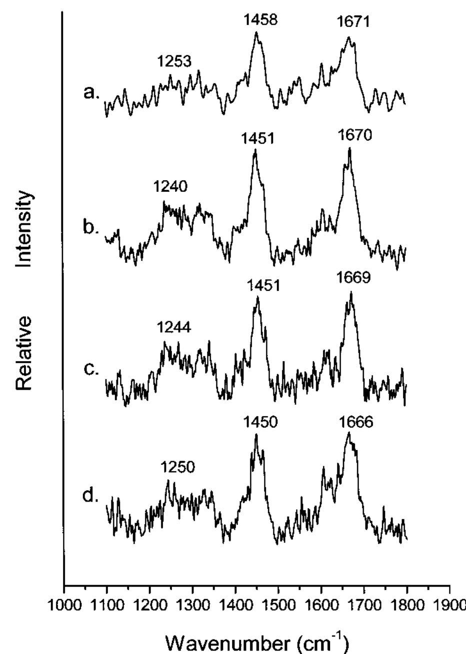

Figure 4. Raman spectra of 10% oat globulin dispersions in 1.0 M sodium salts: (a) NaCl; (b) NaBr; (c) NaI; (d) NaSCN.

Effect of Chaotropic Salts. Figure 4 shows the Raman spectra of 10% oat globulin dispersions in the presence of 1 M sodium salts, and the spectra displayed much more noise than spectra in distilled water (Figure 1). There were shifts in the amide I′ and amide III′ regions (Figure 4) and increases in the intensity of the amide and C−H bending vibrations (Figure 5). The progressive shifts and changes in band intensity, indicating progressive protein unfolding, followed the order

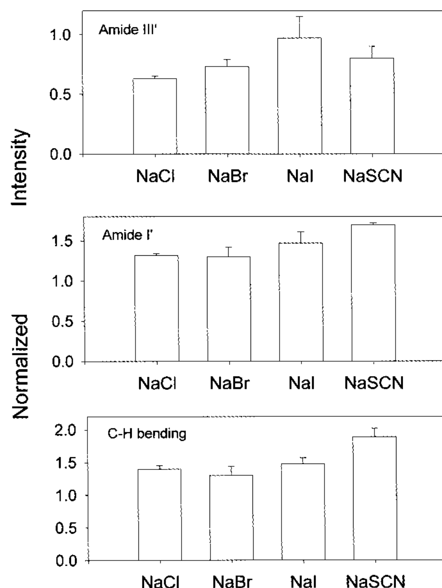

Figure 5. Effect of chaotropic salts on normalized intensity of several regions in Raman spectrum of oat globulin dispersions (or pellets). Error bars represent standard deviations of the means.

$SCN^- > I^- > Br^- > Cl^-$ according to the lyotrophic or chaotropic series of anions (Hatefi and Hanstein, 1969). Protein conformation can be perturbed by the addition of salts, which influence the electrostatic interactions with the charged groups and polar groups and affect the hydrophobic interactions via a modification of water structure (von Hippel and Schleich, 1969; Damodaran and Kinsella, 1982). The degree to which water structure is affected depends on the nature of anions or cations, following the lyotropic series (Hatefi and Hanstein, 1969). Anions (e.g., I^- and SCN^-) and cations (e.g., Li^{2+} and Ca^{2+}) higher in this series could reduce the free energy required to transfer the nonpolar groups into water and could weaken intramolecular hydrophobic interactions and enhance the unfolding tendency of proteins (von Hippel and Wong, 1965). Furthermore, Cl^- and Br^-, which are lower in the anion series, could promote salting-out and aggregation due to their higher molar surface tension and help to stabilize the protein conformation. I^- and SCN^-, on the other hand, are destabilizing agents because of their higher hydration energy and steric hindrance, which promote unfolding, dissociation, and salting-in of proteins (Boye et al., 1997). The Raman data are consistent with a previous DSC study that showed a progressive decrease in the enthalpy value of oat globulin when the anion was changed from chloride to bromide, iodide, and thiocyanate, which implies progressive protein denaturation (Harwalkar and Ma, 1987). The DSC data also showed that the relative effectiveness of these anions in perturbing the heat stability of oat globulin again follows this lyotropic series.

Effect of Protein Perturbants. The effects of a number of protein structure perturbants on the Raman spectral characteristics are shown in Figures 6 and 7. Sodium dodecyl sulfate (SDS), β-mercaptoethanol (ME), and ethylene glycol (EG) all caused a marked shift in the amide III′ band to higher frequency, which indicates a transition from β-sheet to random coil conformation (see Table 1). EG (40% v/v) and 6 M urea also led to shifts in the C−H bending peak (Figure 6). The tyrosine doublet intensity either increased or decreased depending on which perturbant was used (Figure 7). This indicates changes in the tertiary structure (Li-Chan, 1996b). The intensity of the amide I′ and C−H stretching transitions was increased in the presence of these perturbants (Figure 7), suggesting protein denaturation.

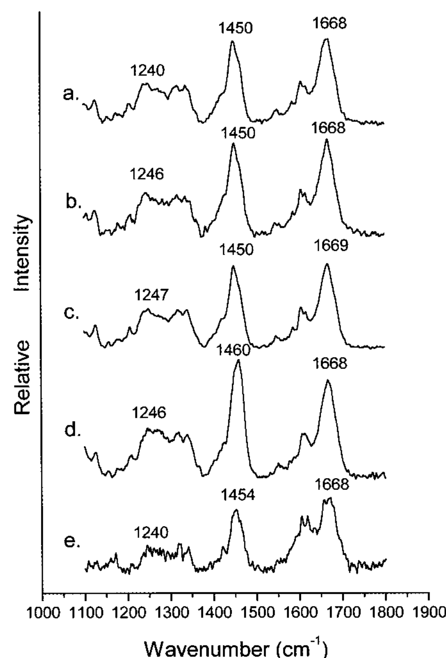

Figure 6. Effect of different protein perturbants on the Raman spectrum of oat globulin dispersions (≈10−15%): (a) control (distilled water); (b) 40 mM SDS; (c) 5% ME; (d) 40% EG; (e) 6 M urea.

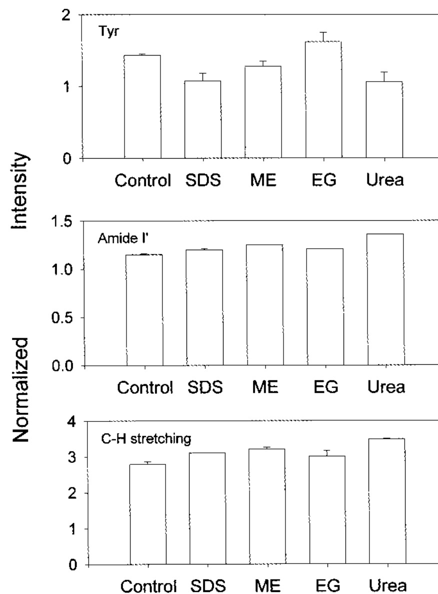

Figure 7. Effect of protein perturbants on normalized intensity of several regions in Raman spectrum of oat globulin dispersions (or pellets). Error bars represent standard deviations of the means.

SDS is an anionic detergent that can bind to protein by noncovalent forces to increase the net charge and hence lead to ionic repulsion and unfolding of polypeptides (Steinhardt, 1975). ME is a reducing agent and can break up disulfide bonds to create destabilization of oligomers such as oat globulin, which contain polypeptides linked by disulfide bonds. EG could lower the dielectric constant of water and weaken the nonpolar interactions between protein molecules, thereby causing

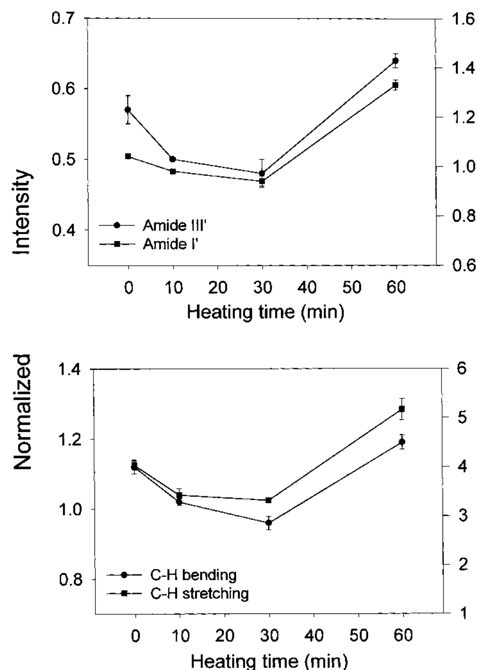

Figure 8. Effect of heating (at 110 °C) on normalized intensity of several regions in Raman spectrum of oat globulin (freeze-dried powder). Error bars represent standard deviations of the means.

destabilization. Urea effectively disrupts the hydrogen-bonded structure of water and facilitates protein unfolding by weakening hydrophobic interactions (Kinsella, 1982). Urea also increases the "permittivity" of water (Franks and England, 1975) for the apolar residues, causing loss of protein structure and heat stability. The present Raman data showed that these reagents caused marked changes in the secondary and tertiary structures of oat globulin. This may be attributed to the perturbation of the tertiary and quaternary structures of the oligomeric protein by destabilizing some primary (hydrogen bonds, hydrophobic forces) and secondary (disulfide bonds) chemical forces that are important in the stabilization of oat globulin conformation. The results are consistent with our previous DSC data (Harwalkar and Ma, 1987) which showed that in the presence of these perturbants, thermal stability and enthalpy of oat globulin were decreased, indicating conformational changes and partial denaturation of the protein.

Effect of Heating. Figure 8 shows changes in the Raman band intensity in several spectral regions when oat globulin was heated at 110 °C for different periods of time. There was an initial slight decrease of the amide I′ and amide III′ regions in the first 30 min, followed by marked increases in peak intensity at 60 min (Figure 8), suggesting some protein unfolding. Similar trends were observed in the C−H bending and stretching vibrations (Figure 8), again indicating protein denaturation when oat globulin was heated for extended periods of time.

The T_d of oat globulin was ∼110 °C (Harwalkar and Ma, 1987), and heating at or near the T_d has been shown to lead to progressive decreases in the enthalpy (Ma and Harwalkar, 1988b), which indicates that some protein denaturation takes place. Heating at 110 °C also led to a progressive red shift in the UV absorption spectra and a blue shift in the fluorescence emission spectra, which also suggest that protein unfolding occurs (Ma and Harwalkar, 1988a). The Raman data are in agreement with these results, although the DSC and the UV/fluorescence spectroscopic results showed more rapid protein unfolding than the present Raman data. This may be due to the fact that Raman spectroscopy is more sensitive to secondary structural changes, whereas DSC and UV/fluorescence spectroscopy are sensitive to changes in tertiary and quaternary structure. These sensitivities suggest that during the course of thermal denaturation of oat globulin, alterations in the tertiary and quaternary structure take place before changes in secondary structure.

Conclusion. The present Raman data show that the conformation of oat globulin is influenced by pH, chaotropic salts, and some protein perturbants and by heating. The Raman results are consistent with previous DSC and UV/fluorescence data. This suggests the involvement of both primary and secondary forces in stabilizing the protein structure.

The present results indicate that FT-NIR Raman spectroscopy is an appropriate technique for studying the secondary and tertiary structure of vegetable proteins such as oat globulin. The minimization of fluorescence is essential for plant protein preparations that may contain phenolic compounds coextracted with the solvents. However, the FT instrument seems to be less sensitive than the conventional visible laser Raman spectroscopy (Dr. E. Li-Chan, personal communication), and a higher protein concentration is required to obtain reproducible data.

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