Percent yield of product from the different raw materials is indicated.

In Table II, grams of amino acids/100 g of protein expressed in percents are shown for the three different low free-gossypol products produced with the Raymond air classifier. Values are corrected to 100% recovery protein basis. No significant difference could be determined among the meals originated from the different sources. However, compared to wheat flour shown by Waggle et al. (1967), the nutritional attribution of low free-gossypol cottonseed meal is much higher.

Because of the higher fat content in the expelled meal, a larger number of coarse particles remained in the finely ground product. In general, the whizzer-type air classifier has a grinding effect on classified stock. In this case, it is possible that this grinding effect eliminated the coarser particles in the meal originating from the expelling process and increased the yield of low free-gossypol product.

On the basis of these findings, it might be suggested that particles below 50 μm contain higher levels of free gossypol. Air classifiers should be adjusted to have the cut point at this particle size.

The whole process can be assembled as a continuous one, starting with sieving out the hulls (if present in the raw meal), extrusion, cooling and drying, fine grinding, and air classification.

A process that can be adapted commercially should have adequate technical-processing tolerances at the various stages to handle variation in raw material. This would ensure final product quality to meet specifications. The results indicate that, even with variability in raw material and its origin, the free gossypol can be reduced efficiently to food standards.

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Registry No. Gossypol, 303-45-7.

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Study of Thermal Denaturation of Oat Globulin by Ultraviolet and Fluorescence Spectrophotometry[1]

Ching-Yung Ma* and Ventktesh R. Harwalkar

Thermal denaturation of dilute (<0.05%) oat globulin solutions at high ionic strength was studied by ultraviolet (UV) and fluorescence spectrophotometry. Ultraviolet spectra show a significant red shift of absorption maximum when the protein was heated at 110 °C. Second-derivative and difference-derivative spectra suggest exposure of tryptophan and tyrosine residues in the heated samples. Fluorescence emission spectra show a significant blue shift, indicating protein unfolding. When 1% oat globulin was heat aggregated and fractionated into soluble and insoluble fractions, UV and fluorescence spectra indicate no marked protein unfolding in the soluble fraction but extensive denaturation in the insoluble aggregates. The soluble fraction had significantly higher surface hydrophobicity than the soluble fraction and the unheated protein.

Heat treatments such as drying, roasting, and cooking are routinely used for the processing of oats and other cereal grains. These processes could cause substantial protein denaturation, which is critical to functionality such as gelation, emulsification, and foaming (Kinsella, 1976). Thermal denaturation (or unfolding) of proteins involves conformational changes from the native structure and can be monitored by spectrophotometric techniques such as circular dichroism (CD), ultraviolet (UV), and fluorescence spectroscopy. Spectroscopic studies have been conducted on soy 11S globulin (glycinin) heated in the presence of *N*-ethylmaleimide (NEM), which prevented protein aggregation by blocking sulfhydryl residues and hindering

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thiol disulfide interchange (Yamagishi et al., 1981, 1983). Oat globulin, the major oat protein fraction, is an oligomeric protein with quaternary and subunit structures very similar to that of glycinin (Brinegar and Peterson, 1982). However, the thermal aggregating behavior of the two proteins is significantly different, and NEM cannot prevent aggregation of 1% oat globulin solutions (Ma and Harwalkar, 1987). Preliminary data show that extensive aggregation or turbidity development can be prevented if oat globulin is heated at lower concentrations (<0.05%) with or without NEM. The present work was conducted to study the heat-induced denaturation of oat globulin by UV and fluorescence spectrophotometry. To assess the extent of unfolding, oat globulin was also denatured by 6 M urea, which has been shown to cause extensive unfolding of many proteins and may be used as a "standard" for comparing with other means of denaturation. Spectrophotometric and hydrophobicity measurements were also carried out on aggregated (insoluble) and dissociated (soluble) proteins fractionated from 1% oat globulin heated at 100 or 110 °C.

MATERIALS AND METHODS

Materials. Oat globulin was prepared from ground, defatted oat groats by the procedure described previously (Ma and Harwalkar, 1984). All reagents used were of analytical grade.

Heat Treatments. Oat globulin solutions (0.04% w/v) were prepared in 0.01 M phosphate buffer (pH 7.4) containing 1.0 M NaCl. The solutions were heated at either 100 or 110 °C. For experiments conducted at 100 °C, aliquots (1–2 mL) of globulin solution were pipetted into glass tubes, stoppered with glass marbles, and heated in a boiling water bath for predetermined time periods ranging from 5 to 60 min. After heating, the tubes were immediately cooled by immersing in an ice bath. For tests at 110 °C, resealable tubes and a hydrolysis heating block (both products of Pierce Chemical Co., Rockford, IL) were used. Heating experiments were also conducted on 1% oat globulin prepared in the same buffer. The dispersion was centrifuged at 4000*g* to remove insoluble materials, and more than 90% of protein was soluble in the buffer, as determined by the method of Lowry et al. (1951). This represented the maximum quantity of globulin that can be solubilized by the buffer. Aliquots (100 mL) of the soluble globulin were heated in 500-mL Erlenmeyer flasks covered with aluminum foil in either a boiling water bath for 60 min or a temperature-controlled autoclave at 110 °C for 10 min. Heating conditions were selected such that substantial quantities of both soluble and insoluble proteins can be obtained. The heated samples were centrifuged at 10000*g* for 20 min, and the residues were washed with distilled water while the supernatants were dialyzed extensively against distilled water at 4 °C. Both the residues and supernatants were freeze-dried to recovery the proteins.

Denaturation with Urea. Stock solutions of 6 M urea in 0.01 M phosphate buffer (pH 7.4) containing 1.0 M NaCl were prepared just before use. Oat globulin (0.04%) was solubilized in this solution and stored overnight at room temperature.

UV Spectrophotometry. Direct and second-derivative absorption spectra of 0.04% oat globulin were obtained on a Perkin-Elmer 320 spectrophotometer equipped with data handling system (Perkin-Elmer Corp., Oak Brook, IL). Difference second-derivative absorption spectra were obtained by placing the native (unheated) and denatured (by heat or urea treatment) protein solutions in the reference and sample compartments, respectively. The derivative wavelength difference, $\Delta\lambda$, was 2 nm for second-derivative and 4 nm for difference-derivative spectra.

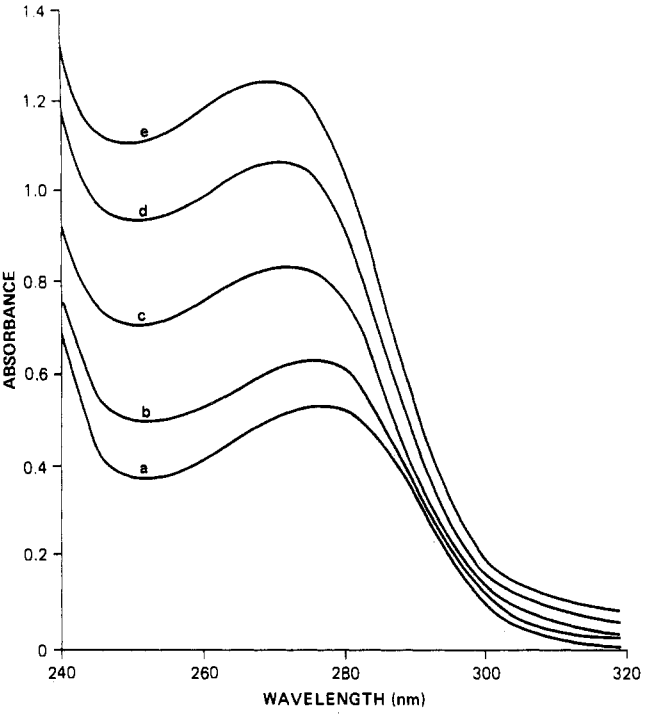

Figure 1. Absorption spectra of oat globulin heated at 110 °C: (a) unheated; (b) 5 min; (c) 10 min; (d) 30 min; (e) 60 min.

Fluorescence Emission Spectrophotometry. Fluorescence emission spectra were obtained with a Perkin-Elmer LS-5 spectrofluorimeter equipped with data handling system. The protein samples (0.02%) were excited at 280 nm, and emission was recorded at right angles to the excitation over the range of 280–420 nm. Corrections for the fluorescence of the buffer blanks were made.

Amino Acid Analysis. Amino acid analysis of oat globulin samples was performed according to Spackman et al. (1958).

Determination of Hydrophobicities. The surface or effective hydrophobicity (S_0) of oat globulin samples was determined by the fluorescence probe method of Kato and Nakai (1980). The Bigelow's hydrophobicity and the hydrophobic and hydrophilic amino acid contents were estimated from the amino acid compositions of the proteins (Bigelow, 1967).

RESULTS AND DISCUSSION

UV Spectra of Heated Oat Globulin. Figure 1 shows the direct absorption spectra of 0.04% oat globulin heated at 110 °C. The absorption maximum (277 nm for native protein) was shifted progressively to shorter wavelength with an increase in heating time, and the shift was more pronounced at 110 than at 100 °C (data now shown). There was also a progressive increase in absorbance over the wavelength region measured. Figure 2 shows the second-derivative absorption spectra of oat globulin heated at 110 °C. Between 250 and 300 nm, eight peaks (revealed as positive signals, $+d^2A/d\lambda^2$) and troughs (revealed as negative signals, $-d^2A/d\lambda^2$) were observed. Heating caused a progressive blue shift in all the peaks and troughs and the gradual disappearance of a peak at 279 nm (and a trough at 278 nm). Heating at 100 °C (data not shown) led to similar changes although the blue shift was less pronounced than at 110 °C. Denaturation by urea at room temperature overnight led to a more marked blue shift than by heating (dotted line in Figure 2). The difference second-derivative spectra of oat globulin heated at 110 °C

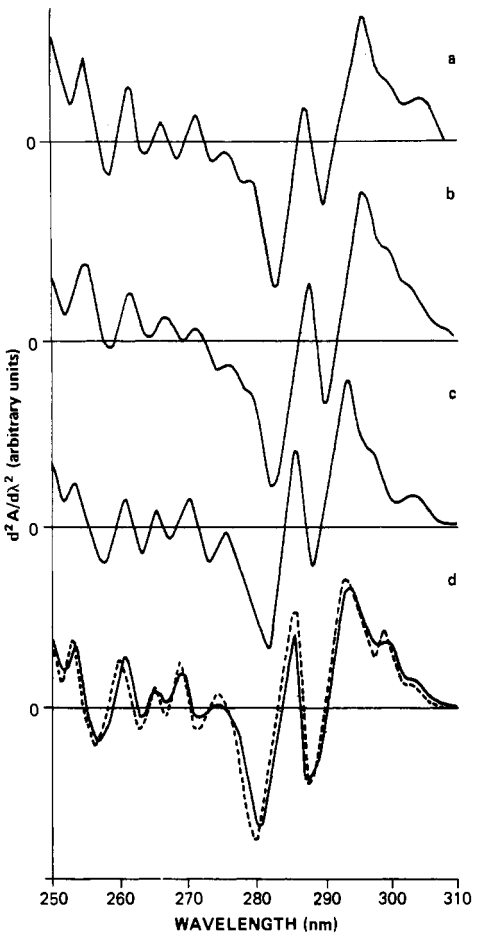

Figure 2. Second-derivative absorption spectra of oat globulin heated at 110 °C. The derivative wavelength difference, Δλ, was 2 nm. Key: (a) unheated; (b) 10 min; (c) 30 min; (d) 60 min. The dotted line in (d) represents the spectrum of protein denatured with 6 M urea at room temperature overnight.

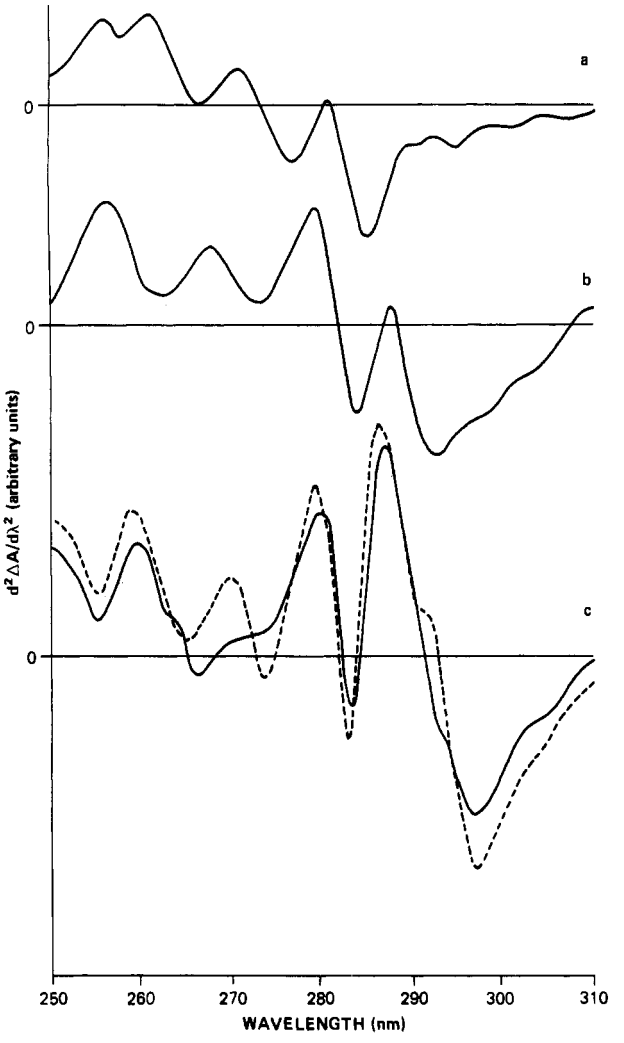

Figure 3. Difference-derivative absorption spectra of oat globulin heated at 110 °C. The derivative wavelength difference, Δλ, was 4 nm. Key: (a) 10 min; (b) 30 min; (c) 60 min. The dotted line in (c) represents the spectrum of protein denatured with 6 M urea at room temperature overnight.

are shown in Figure 3. Progressive blue shift in peaks and increase in derivative difference intensity ($d^2(\Delta A)/d\lambda^2$) were observed with an increase in heating time, and the extent of blue shift was again less pronounced at 100 °C (data not shown). Denaturation with 6 M urea at room temperature overnight caused a more marked blue shift and increase in intensity difference than heat treatment, and the appearance of a shoulder to the major peak near 290 nm (dotted line in Figure 3).

Changes in the environment around chromophoric residues in proteins caused by denaturation or solvent perturbation could lead to shifts in the peaks of the absorption spectrum to shorter wavelength (Solli and Herskovits, 1973). The blue shift was slight at 100 °C but quite significant at 110 °C, indicating that oat globulin was not extensively denatured at 100 °C. This is consistent with the exceptionally high thermal stability of oat globulin with a denaturation temperature near 110 °C (Ma and Harwalkar, 1984). The progressive increase in absorbance could be due to exposure of aromatic amino acid residues that absorb strongly in the UV wavelength range.

Derivative absorption spectrophotometry has been used to obtain information on the state and amount of aromatic acid residues in proteins (Balesterieri et al., 1978; Ichikawa and Terada, 1979; Servillo et al., 1982; Yamagishi et al., 1981, 1983). The peaks and troughs between 250 and 270 nm are due to phenylalanine while those between 270 and 300 nm are attributed to tyrosine and tryptophan. It has been recognized that the peak at 291–292 nm in derivative spectra is due to tryptophan and the transfer of its indole chromophore from the interior of the protein to surrounding water (Demchenko, 1978; Demchenko and Sandrovski, 1979). The present data show that apart from a blue shift in peak maxima (and trough minima) indicating denaturation, there were some qualitative changes in the derivative spectra of heated samples that could be related to changes in protein conformation protecting or exposing the chromophoric residues. The data show that denaturation caused by urea was more extensive than that by heat treatments.

Difference absorption spectrophotometry has been used to investigate conformational changes in proteins by denaturation (Donovan, 1969). However, slight turbidity development may mask the absorbance changes due to exposure of chromophoric residues, and the use of difference second-derivative spectrophotometry was found to be effective in overcoming disturbance by turbidity (Yamagishi et al., 1981, 1983). The blue shift in peaks in the heated and urea-treated oat globulin again indicate protein unfolding. The intensity difference between peaks and troughs is a measure of the degree of exposure of aromatic amino acid residues buried in the protein molecule. The data show that increases in heating temperature and time led to progressive exposure of the chromophoric residues, and urea perturbation again caused more extensive denaturation than heat treatments. The appearance of a shoulder to the major tryptophan peak in urea-

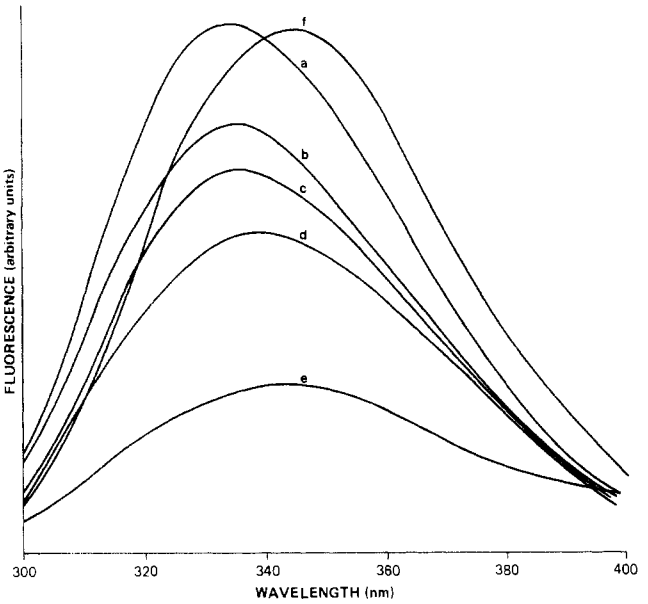

Figure 4. Fluorescence emission spectra of oat globulin heated at 110 °C. Excitation wavelength was 280 nm. Key: (a) unheated; (b) 5 min; (c) 10 min; (d) 30 min; (e) 60 min; (f) protein denatured with 6 M urea at room temperature overnight.

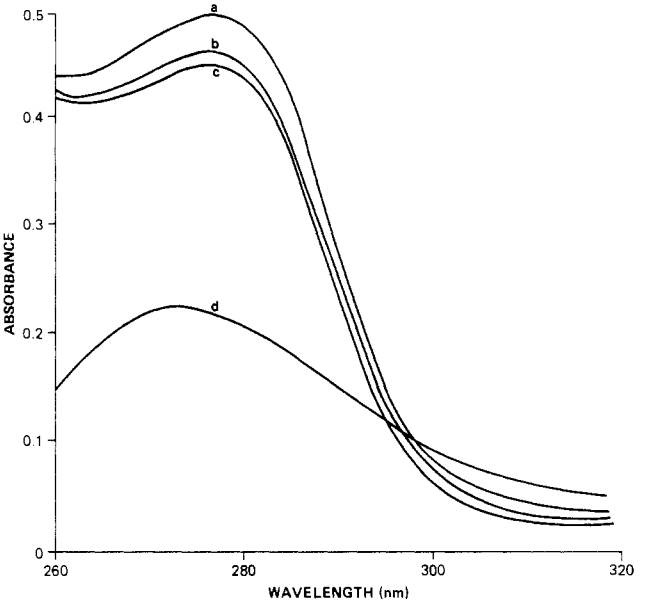

Figure 5. Absorption spectra of oat globulin fractionated after heat treatments. Samples (0.04% protein) were prepared in 0.01 M phosphate buffer (pH 7.4) containing 1.0 M NaSCN: (a) unheated; (b) 100 °C, soluble fraction; (c) 110 °C, soluble fraction; (d) 110 °C, insoluble fraction.

treated oat globulin suggests that some tryptophan residues were buried in the native and heat-denatured proteins but were exposed by 6 M urea. In soy glycinin, the tryptophan peak at 292 nm was not discerned in the difference-derivative spectra of the heated proteins but was observed in urea-denatured protein (Yamagishi et al., 1981).

Fluorescence Emission Spectra of Heated Oat Globulin. Figure 4 shows the fluorescence emission spectra of oat globulin heated at 110 °C. The results show a single peak at 336 nm in the unheated sample and a progressive shift of the peak to longer wavelength with increases in heating time and temperature. Heating also led to a gradual decrease in fluorescence intensity. Similar but less extensive red shift was observed in 100 °C (data not shown). Treatment with 6 M urea at room temperature overnight caused a more significant red shift of emission maximum than heating (Figure 4f).

The three aromatic amino acids in proteins (phenylalanine, tyrosine, tryptophan) could contribute to fluorescence, but fluorescence of most proteins is dominated by tryptophan residues with an emission maximum at 348 nm in water (Lackowicz, 1983). The fluorescence peak of oat globulin occurred at shorter wavelengths relative to the emission of tryptophan in water, and the blue shift was a result of shielding of the tryptophan residues from the aqueous phase by the protein matrix (Vladimirov and Burstein, 1960). Protein denaturation is generally accompanied by a red shift in fluorescence emission (Kronman and Holmes, 1971). The present data indicate a gradual loss of native structure in oat globulin with an increase in the extent of heat treatment, but denaturation was less extensive than in the presence of urea. The progressive decrease in fluorescence intensity in heated globulin may be due to exposure of tryptophan residues to a polar environment and the subsequent quenching of fluorescence (Yamagishi et al., 1981). Slight turbidity development in the heated samples may also result in quenched fluorescence intensity (Lackowicz, 1983).

UV and Fluorescence Emission Spectra of NaCl-Soluble and -Insoluble Fractions of Heated Oat Globulin. At higher protein concentration (≈ 1%), oat globulin formed insoluble aggregates when heated in buffer containing 1.0 M NaCl, and the extent of aggregation was dependent on heating time and temperature (Ma and Harwalkar, 1987). The heat-treated proteins can be separated into NaCl-soluble and NaCl-insoluble fractions by centrifugation. In order to study the spectral characteristics of these fractions, 0.01 M phosphate buffer (pH 7.4) containing 1.0 M NaSCN was used. Over 70% of the NaCl-insoluble aggregates were solubilzied by this treatment since thiocyanate is higher than chloride in the lyotropic series of salts with a greater protein perturbing and solubilizing effect (Damodaran and Kinsella, 1982). Figure 5 shows the absorption spectra of the NaCl-soluble fractions from 100 and 110 °C treated proteins and the NaCl-insoluble aggregates from the 110 °C treated globulin. The unheated control (Figure 5a) exhibited an absorption maximum at 276 nm, a negligible blue shift compared to globulin in 1.0 NaCl (Figure 1), indicating that the use of salt higher in the lyotropic series did not cause significant protein unfolding. The NaCl-soluble fractions (Figure 5b,c) had absorption maxima very close to that of the control, while a marked blue shift was observed in the NaCl-insoluble aggregates (Figure 5d). The NaCl-insoluble fraction from 100 °C heated globulin had an absorption spectrum (not shown) very similar to that of the 110 °C treated sample. The NaCl-soluble fractions also show no significant changes in the second-derivative absorption spectra when compared to the control (Figure 6a–c). The NaCl-insoluble fraction, however, showed a blue shift in derivative maxima and minima, and there was the appearance of a peak at 292 nm and the loss of a peak at 279 nm (Figure 6d).

Fluorescence spectral data (Figure 7) showed that the NaCl-soluble fractions had emission maxima close to that of the native protein, while the emission peak of the NaCl-insoluble fraction was observed at substantially longer wavelength. Thus, UV and fluorescence data show that the NaCl-soluble fractions contained essentially undenatured globulin while the proteins in the NaCl-insoluble fractions were substantially denatured, though less extensively than in the presence of urea.

Hydrophobicities of NaCl-Soluble and -Insoluble Fractions of Heated Oat Globulin. Table I shows the

Table I. Hydrophobicities of Native and Heated Oat Globulin[a]

globulin	surface hydrophobicity	Bigelow's hydrophobicity	hydrophobic amino acid content, %	hydrophobic/hydrophilic amino acids
native	60	825	29.9	0.71
soluble fraction, 100 °C	64	820	29.8	0.69
soluble fraction, 110 °C	68	815	29.6	0.68
insoluble fraction, 100 °C	125	828	30.0	0.72
insoluble fraction, 110 °C	135	830	30.2	0.73

[a] Average of duplicate determinations.

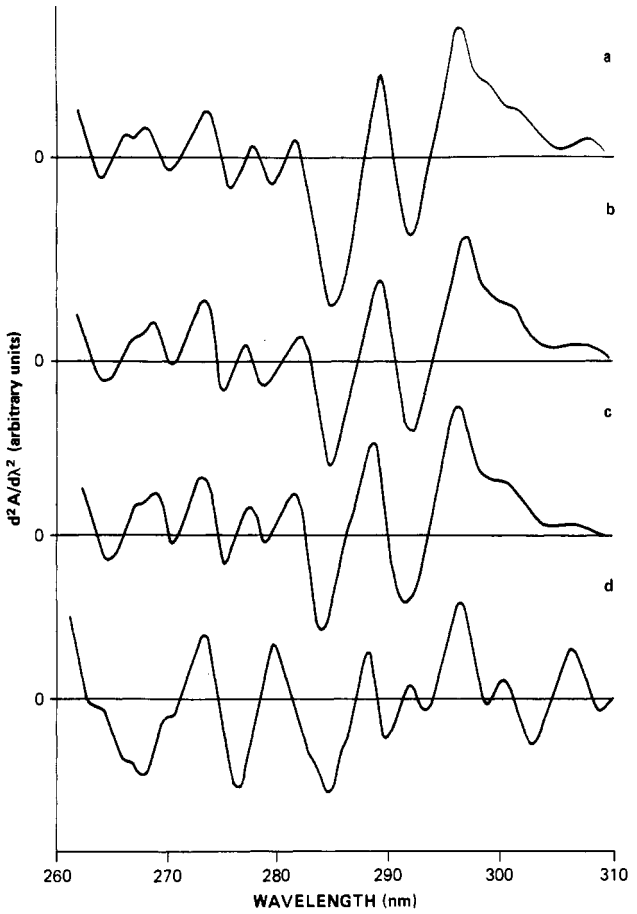

Figure 6. Second-derivative absorption spectra of oat globulin fractionated after heat treatments. Samples (0.04% protein) were prepared in 0.01 M phosphate buffer (pH 7.4) containing 1.0 M NaSCN. The derivative wavelength difference, $\Delta\lambda$, was 2 nm. Key: (a) unheated; (b) 100 °C, soluble fraction; (c) 110 °C, soluble fraction; (d) 110 °C, insoluble fraction.

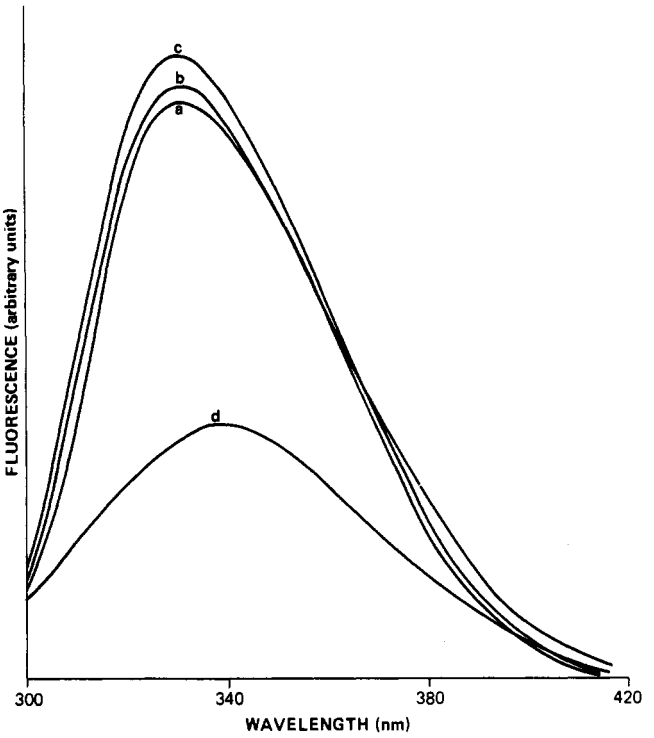

Figure 7. Fluorescence emission spectra of oat globulin fractionated after heat treatments. Samples (0.02% protein) were prepared in 0.1 M phosphate buffer (pH 7.4) containing 1.0 M NaSCN. Excitation wavelength was 280 nm. Key: (a) unheated; (b) 100 °C, soluble fraction; (c) 110 °C, soluble fraction; (d) 110 °C, insoluble fraction.

hydrophobicities of native and heated globulin fractions including surface hydrophobicity, Bigelow's hydrophobicity (Bigelow, 1967), hydrophobic amino acid content, and hydrophobic/hydrophilic ratio. The results show that the two NaCl-soluble fractions (100 and 110 °C treated) had hydrophobicities very close to those of the unheated control. The NaCl-insoluble fractions had a Bigelow's hydrophobicity, hydrophobic amino acid content and hydrophobic/hydrophilic ratio similar to those of the control, but the surface hydrophobicity was much higher. Oat globulin is composed of six pairs of acidic and basic polypeptides (Brinegar and Peterson, 1982), and polyacrylamide gel electrophoresis (in sodium dodecyl sulfate) of the soluble and insoluble fractions showed no marked redistribution of the two polypeptides (Ma and Harwalkar, 1987). The present data also suggest that the acidic and basic polypeptides were not segregated into soluble and insoluble fractions since the two fractions had similar hydrophobicities based on amino acid composition. In contrast, a significant difference between buffer-soluble and -insoluble fractions in hydrophobic amino acid content and hydrophobic/hydrophilic ratio was observed in heated glycinin and has been attributed to a redistribution of glycinin polypeptides into the soluble and insoluble fractions, with the insoluble fraction containing mainly the more hydrophobic basic polypeptides and the soluble fraction the more hydrophilic acidic polypeptides (Takagi et al., 1979). The marked increase in surface hydrophobicity in the insoluble oat globulin fraction could be due to extensive unfolding of the proteins, exposing buried hydrophobic amino acid residues (Kato and Nakai, 1980). The slight increase in surface hydrophobicity in the soluble fractions confirms that those proteins were not extensively unfolded.

CONCLUSION

The present data show that the state of chromophoric residues in oat globulin was altered by heat treatment, suggesting denaturation. The magnitude of spectral change was greatly influenced by the amount of heat (temperature/time) applied. This is consistent with previous data (Ma and Harwalkar, 1987) that show that the rate of thermal aggregation and degree of thermal denaturation (estimated from differential scanning calorimetric data) of oat globulin were closely related to the extent of heat treatment. The present results show that, even with

prolonged heating at temperature close to the denaturation temperature, oat globulin (in dilute solutions) seems to retain considerable native structure since the urea-perturbed protein exhibited further denaturation as indicated by more pronounced spectral changes. There was a segregation of native and denatured proteins into soluble and insoluble fractions, respectively, when oat globulin was heated at higher (1%) concentration. Spectral data and surface hydrophobicity measurements suggest that insoluble aggregates were formed from extensively denatured proteins. This is consistent with the generally accepted view that aggregation is preceded by denaturation, following the scheme $N \rightleftharpoons D \rightarrow A$, where N denotes native protein, D denatured protein, and A the aggregate (Ferry, 1948).

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Stability of Gliadin-Encapsulated Unsaturated Fatty Acids against Autoxidation

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The antioxidant effects at various A_w of spray-dried powders prepared from alcoholic solutions of gliadin, linoleic acid, and palmitic acid were compared against powders prepared by simple mixing of these components in the same portions and against gelatin or starch powders substituted for gliadin. The effectiveness of gliadin at low or moderate A_w in the simple mixture that was not as good as at high A_w was greatly improved via the process of spray-drying. This process caused a significant loss in the extraction efficiency of fatty acids by hexane but did not influence the in vitro digestion of gliadin. The outer surface of the spray-dried gliadin particles containing the lipid was characterized by the disappearance of deep dents otherwise observed by SEM. These findings imply that the lipid droplets must have been embedded in the gliadin matrix so as to avoid oxygen attack.

In the preceding paper (Iwami et al., 1987), we demonstrated that gliadin, a component of wheat gluten, functions as the best antioxidant among available food proteins against linoleate peroxidation in powder model systems and that its effectiveness continues longer at high A_w. Gliadin was never superior to other proteins in radical and sulfhydryl reactions. Since this protein is so abundant in glutamine and proline as to scarcely dissolve in water (Pomeranz, 1968), its physicochemical properties are of interest in connection with the antioxidant ability.

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