Physicochemical Properties of Alkali-Treated Oat Globulin†

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Oat globulin dispersions (10% w/v) were incubated at an initial pH of 9.8 at 25, 37, and 55 °C over a period of 96 h. Turbidity increased at 25 and 37 °C with the formation of insoluble aggregates and decreased at 55 °C with little precipitation. The free SH content decreased progressively with time, while the surface hydrophobicity was increased at 25 °C and decreased at 37 °C. Differential scanning calorimetry showed progressive increases in denaturation temperature and decreases in width at half-peak height. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis demonstrated formation of soluble aggregates at 55 °C and degradation of oat globulin polypeptides at 25 and 37 °C, possibly due to proteolysis by protease(s) coextracted with the protein. No significant racemization of amino acids was observed in the alkali-treated protein.

INTRODUCTION

Alkaline conditions are occasionally encountered in the processing of foods and extraction of proteins. Proteins from many plant sources can be extracted with high yields at alkaline pH. Texturization of proteins by spin fiber technology requires the protein substrate be prepared in a highly alkaline dope (Smith and Circle, 1972). Sodium hydroxide is used in various food processing steps including peeling of potatoes and peaches, neutralizing casein preparations (sodium caseinate), and removal of toxic constituents such as aflatoxin and protease inhibitors in production of texturized foods and vegetable protein based whipping agents (Whitaker, 1982). Mild alkaline hydrolysis was used for deamidation of wheat vital gluten (Batey, 1980; Gebhardt et al., 1982), and several types of protein gels have been prepared by alkali (Ishino and Okamoto, 1975) or alkali–alcohol (Ishino and Kudo, 1977) treatments. Alkaline conditions also occur naturally in some foods; e.g., during storage of chicken egg, pH increases from approximately 6.5 to above 9.5 due to loss of carbon dioxide (Powrie and Nakai, 1986). Alkaline pH has also been shown to promote racemization of amino acids, leading to decreased digestibility and nutritional quality of dietary proteins (Hyashi and Kameda, 1980; Friedman et al., 1984).

Alkali extraction has been found to be the most effective procedure for preparing protein concentrates and isolates from oats (Ma, 1983a,b), and the formation of oat protein gels also required alkaline pH (Ma et al., 1988). The present investigation was conducted to study the changes in some physicochemical characteristics of oat globulin during incubation at alkaline pH.

MATERIALS AND METHODS

Oat globulin was prepared from pin-milled, defatted oat groats as described previously (Ma and Harwalkar, 1984).

Alkaline Treatments. Oat globulin dispersions (10% w/v) were prepared by mixing with distilled water and adjusting the pH to 9.7 with 2 M NaOH. The dispersions were kept in incubators controlled at 25, 37, and 55 °C for up to 96 h. A pH of 9.7 was selected because previous studies showed that pH around 9.7 was most suitable for extraction of protein from oat groats (Ma, 1983a) and for studying gelation of oat protein (Ma et al., 1988).

Turbidity. The turbidity of the oat globulin dispersions was determined by monitoring the absorbance at 500 nm using a Perkin-Elmer 320 spectrophotometer.

Sulfhydryl Content. The free sulfhydryl contents of oat globulin samples were determined according to the method of Beveridge et al. (1974).

Surface Hydrophobicity. The surface or effective hydrophobicity (S_0) of oat globulin samples was determined by the fluorescence probe method of Kato and Nakai (1980).

Differential Scanning Calorimetry. The thermal characteristics of oat globulin samples were examined by differential scanning calorimetry (DSC) using a Du Pont 1090 thermal analyzer equipped with a high-pressure cell. Aliquots (10 μL) of the samples were pipetted into DSC pans, sealed, and incubated at 25, 37, and 55 °C. An empty pan was used as a reference. The pans were heated at a programmed rate of 10 °C/min from 30 to 140 °C. Peak or denaturation temperature (T_d) and enthalpy (ΔH) were computed from the thermograms by the 1090 analyzer, and the width at half-peak height ($\Delta T_{1/2}$) was also recorded.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) of oat globulin samples was performed according to the method described by Ma (1983a). Bovine serum albumin, ovalbumin, carbonic anhydrase, and β-lactoglobulin were used as molecular weight markers.

Proteolysis. Proteolysis of oat globulin samples was measured by the o-phthaldialdehyde (OPA) spectrophotometric assay procedure (Church et al., 1983) with a slight modification. SDS was omitted from the OPA reagent, but an equivalent amount of SDS was added to the protein dispersions to enhance solubilization. Aliquots (20–60 μL) containing 5–100 μg of protein were mixed with 0.15 mL of 20% SDS, and 2.85 mL of OPA reagent (with OPA and mercaptoethanol but without SDS) was added. The mixtures were incubated for 2 min at ambient temperature, and the absorbance at 340 nm was measured. The α-amino groups released by proteolysis reacted with OPA and mercaptoethanol to form an adduct that absorbed strongly at 340 nm. Molar absorptivity of 600 M^{-1} cm^{-1} was used to determine the free amino groups released. The average number of peptide bonds broken was estimated by calculating the number of amino groups released per molecule of oat globulin subunit, on the basis of a mean molecular weight of 58 000.

Determination of Racemization in Alkali-Treated Oat Globulin. Racemization in alkali-treated oat globulin was determined by a procedure described previously (Paquet and Ma, 1989). Weighed protein sample (about 50 mg) was hydrolyzed in 50 mL of 6 N HCl at 110 °C for 23 h. The hydrolysate was

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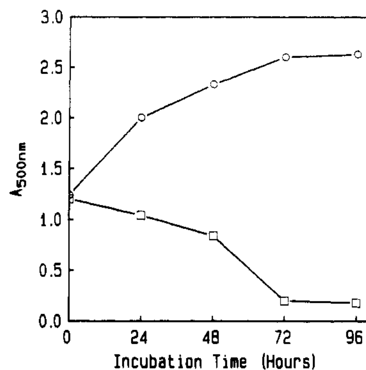

Figure 1. Turbidity of alkali-treated oat globulin dispersions incubated at 25 (○) and 55 °C (□). Data presented are averages of duplicate determinations.

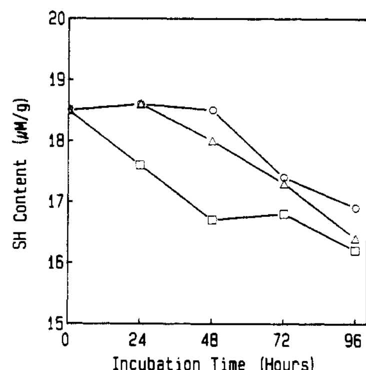

Figure 2. Effect of incubation at alkaline pH on the SH content of oat globulin. (○) 25 °C; (△) 37 °C; (□) 55 °C. Data presented are averages of duplicate determinations.

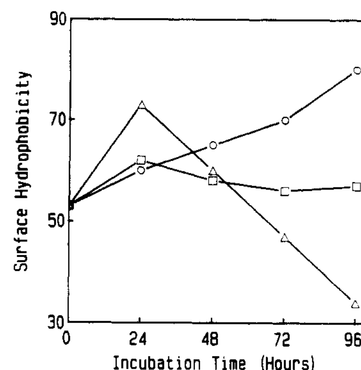

Figure 3. Effect of incubation at alkaline pH on the surface hydrophobicity of oat globulin. (○) 25 °C; (△) 37 °C; (□) 55 °C. Data presented are averages of duplicate determinations.

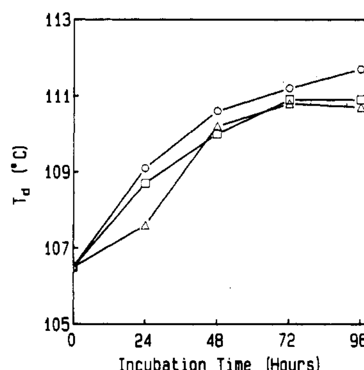

Figure 4. Effect of incubation at alkaline pH on the denaturation temperature of oat globulin. (○) 25 °C; (△) 37 °C; (□) 55 °C. Data presented are averages of triplicate determinations.

filtered and adjusted to 10 mL, and 1 mL was evaporated to dryness, resolubilized in 0.2 N sodium citrate buffer, pH 2.2, and analyzed for amino acid content. The remaining portion was reevaporated, and divided into two portions, and used for enantiomeric analysis. To a stirred solution of hydrolysate in 2 mL of the mixture of acetone–water (1:1) was added sodium bicarbonate (60 mg) and a solution of *N*-ethoxycarbonylphenylalanine *N*-hydroxysuccinimide ester (Eoc-Phe-ONSu) (150 mg), in 1 mL of acetone. The reaction mixture was stirred for 2 h, and the pH was brought to 2 with concentrated HCl. Acetone was evaporated, and the water layer was extracted with a mixture of chloroform and isopropyl alcohol (3:2). The combined organic layers were dried over sodium sulfate, evaporated, and used for high-performance liquid chromatographic analysis. Chromatographic conditions were those described previously (Paquet and Ma, 1989).

RESULTS AND DISCUSSION

Turbidity. The freshly prepared oat globulin dispersions were slightly turbid with an absorbance at 500 nm (A_{500nm}) of 1.20 (Figure 1). When the dispersions were incubated at 25 °C, the turbidity increased with time, with the formation of a small quantity of precipitate. At 37 °C, turbidity increased very rapidly (absorbance cannot be measured) and a large amount of precipitate was formed. When the dispersions were stored at 55 °C, turbidity deceased progressively (Figure 1), with little precipitation. The results indicate formation of insoluble aggregates when oat globulin was incubated at alkaline pH, and the aggregation was temperature dependent.

Sulfhydryl Content. The free SH contents of the alkali-treated oat globulin decreased at all incubation temperatures (Figure 2). At 25 and 37 °C, the SH content did not decrease until after 48 h. At 55 °C, SH content started to decrease after 24 h, and the extent of the changes was more pronounced at 55 °C than at lower temperatures. The decreases in SH contents may be attributed to linking of free SH groups to form disulfide bonds. Previous work (Ma and Harwalkar, 1987) showed that blocking of oat globulin SH groups prevented the formation of soluble aggregates by heat, but did not prevent the formation of insoluble aggregates. The present data suggest that soluble aggregates were formed in the alkali-treated oat globulin through disulfide linkages, particularly at 55 °C, since the changes in SH content were more extensive. Degradation of protein disulfide bonds in dilute alkali has been reported (Florence, 1980), and SH–SS exchange reaction may be enhanced. While these reactions would increase the net SH content, subsequent disulfide bond formation would lower the SH content in the alkali-treated oat globulin.

Surface Hydrophobicity. Figure 3 shows the effect of alkaline treatments on the surface hydrophobicity (S_0) of oat globulin. At 25 °C, there was a progressive increase in S_0 with storage time. At 37 °C, there was a sharp increase in S_0 at 24 h, followed by progressive decreases to values lower than that of the control. At 55 °C, S_0 was not markedly changed during incubation. The changes in surface hydrophobicity may be attributed to conformational changes in the alkali-treated oat globulin. The progressive increase in S_0 suggests unfolding of the protein to expose more hydrophobic groups. The decline in S_0 at 37 °C after 48 h of incubation could be due to extensive aggregation through interaction of the exposed hydrophobic groups. At 55 °C, hydrophobic interactions may be involved in the formation of soluble aggregates, and the observed changes in S_0 may indicate a balance between protein unfolding and aggregation.

DSC Characteristics. The changes in DSC characteristics of oat globulin during incubation at alkaline pH are shown in Figures 4–6. Progressive increases in T_d were observed at all three temperatures, and there were

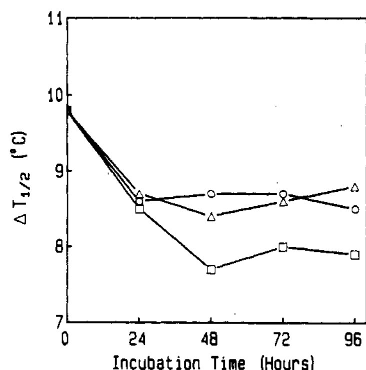

Figure 5. Effect of incubation at alkaline pH on the width at half-peak height of oat globulin. (○) 25 °C; (△) 37 °C; (□) 55 °C. Data presented are averages of triplicate determinations.

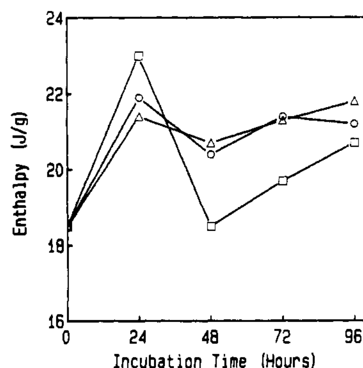

Figure 6. Effect of incubation at alkaline pH on the enthalpy of denaturation of oat globulin. (○) 25 °C; (△) 37 °C; (□) 55 °C. Data presented are averages of triplicate determinations.

no marked differences among the three treatments in the extent of increases (Figure 4). Progressive decreases in $\Delta T_{1/2}$ were observed in the alkali-treated oat globulin samples, leveling off after 48 h of incubation (Figure 5). The decreases were more marked at 55 °C. At both 25 and 37 °C, an initial increase in enthalpy at 24 was followed by a slight decrease (Figure 6). At 55 °C, a sharp increase in enthalpy was observed at 24 h, followed by a sharp drop at 48 h and slight increases afterward.

The denaturation temperature is a measure of the thermal stability of a protein (Ma and Harwalkar, 1988), and the width at half-peak height has been suggested as an index to evaluate the cooperativity of protein unfolding (Privalov et al., 1971). Increases in T_d and decreases in $\Delta T_{1/2}$ have been reported in heat-treated oat globulin (Ma and Harwalkar, 1988) and attributed to the formation of aggregates with a compact, ordered structure that would have higher thermal stability and denature in a highly cooperative fashion.

The initial enthalpy value of oat globulin at pH 9.8 was 18.5 J/g, considerably lower than that (26 J/g) recorded at neutral pH (Harwalkar and Ma, 1987), indicating partial denaturation since partially unfolded proteins require less heat energy (lower ΔH) for complete denaturation. Enthalpic changes observed by DSC are influenced by both endothermic and exothermic events including breakup of hydrogen bonding (endothermic), hydrophobic interactions (exothermic), and protein aggregation (exothermic) (Jackson and Brandts, 1970; Arntfield and Murray, 1981; Privalov, 1982). The initial increases in enthalpy at 24 h may be due to initiation of aggregation of oat globulin at alkaline pH, while subsequent changes reflect a balance of endothermic and exothermic reactions.

SDS-PAGE and Measurement of Proteolysis. The SDS-PAGE patterns of oat globulin incubated at alkaline pH are shown in Figure 7. Odd-numbered lanes show patterns recorded in the absence of mercaptoenthanol, and even-numbered lanes show patterns run under reducing conditions. Marker proteins (bovine serum albumin, ovalbumin, carbonic anhydrase, and β-lactoglobulin) and their molecular weights are shown on lane 21. The major band at lane 1 (mean M_r 58 000) corresponds to the oat globulin subunit (SU), and the two major bands at lane 2 (mean M_r 22 000 and 36 000) correspond to the basic (BP) and acidic polypeptide (AP) of the subunit. Oat globulin is a hexamer with a molecular weight of 320 000–370 000 and is composed of six subunits each containing an acidic and a basic polypeptide, two disulfide bonds, and one free sulfhydryl group (Brinegar and Peterson, 1982; Walburg and Larkins, 1983).

A gradual loss of subunit and the appearance of a band with mean M_r of 40 000 was observed in samples incubated at 25 (lanes 3, 5, and 7) and 37 °C (lanes 9, 11, and 13). These protein bands, when reduced by mercaptoethanol, showed predominantly BP and less AP (lanes 4, 6, 8, 10, 12, and 14). Increasing amounts of lower molecular weight components (16 000–18 000) were observed. The extent of changes was more pronounced at 37 °C than at 25 °C. At 55 °C, there was no marked loss in subunit and AP or appearance of low molecular weight components (lanes 15–20). There were progressive increases in the intensity of a high molecular weight (>100 000) band and a band that stayed at the origin (lanes 15, 17, and 19). These high molecular weight components disappeared in the presence of mercaptoethanol (lanes 16, 18, and 20). The high molecular weight (>100 000) band was also found in some samples incubated at 25 and 37 °C (lanes 3, 5, and 9), but was lost upon further incubation (lanes 7, 11, and 13). AP and BP were observed as minor bands in patterns run in the absence of mercaptoethanol. In a previous study (Harwalkar et al., 1989), incubation of oat globulin in buffer containing SDS was found to cause significant dissociation of the subunit to AP and BP. Such dissociation in the absence of reducing agent could be attributed to unfolding of the protein molecules to alter the proximity of the free SH groups to the disulfide bonds, promoting SH–SS exchange reaction. Since alkaline conditions caused partial denaturation of oat globulin as indicated by a decrease in enthalpy, SH–SS interchange may occur in the alkali-treated oat globulin, although the levels of subunit dissociation were much lower than those observed in SDS-treated samples (Harwalkar et al., 1989).

The SDS–PAGE data indicate degradation of alkali-treated oat globulin at 25 and 37 °C. Although mild alkaline hydrolysis has been used to deamidate wheat gluten (Batey, 1980; Gebhardt et al., 1982), the fact that no protein degradation was observed at 55 °C indicates that breakdown of oat globulin polypeptides was not directly caused by alkali.

The degradation could be due to proteolysis of oat globulin by endogenous proteolytic enzymes coextracted with the protein during its isolation. An experiment was conducted to determine the level of proteolysis in the alkali-treated oat globulin by OPA assay. Results show gradual increases in ΔA_{340nm} at 25 °C, marked increases at 37 °C, and no changes at 55 °C (Figure 8). The maximum absorbance increase at 37 °C after 72 h of incubation corresponded to approximately one to two peptide bonds per subunit, suggesting that the proteolysis was slow and not extensive. The proteinase(s) involved would seem to be highly temperature sensitive, being inactive at 55 °C.

The SDS–PAGE patterns suggest selective proteolysis of AP at 25 and 37 °C. The disappearance of both

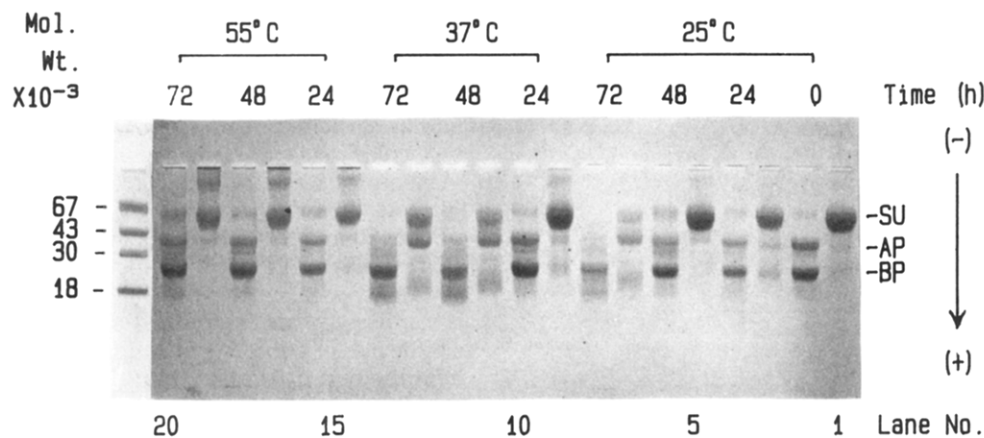

Figure 7. Sodium dodecyl sulfate–polyacrylamide gel electrophoretic patterns of alkali-treated oat globulin in the absence (odd-numbered lanes) and presence (even-numbered lanes) of mercaptoethanol. SU, subunit; AP, acidic polypeptide; BP, basic polypeptide.

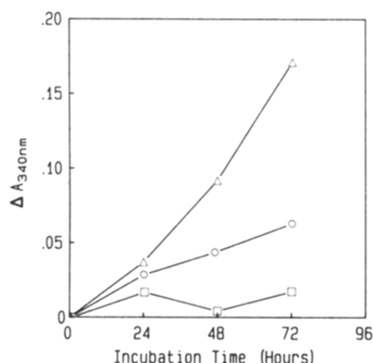

Figure 8. *o*-Phthaldialdehyde assay of proteolysis in alkali-treated oat globulin. (○) 25 °C; (△) 37 °C; (□) 55 °C. Data presented are averages of triplicate determinations.

residual subunit and the M_r 40 000 fraction under reducing condition shows that both fractions were still linked by disulfide bonds. Furthermore, the fact that AP was absent in the reduced samples suggests that the site of proteolytic cleavage was within the segment of the polypeptide chain held together by a disulfide bond. It is envisaged that initially the site of hydrolysis was mainly outside the region held by a disulfide bond. Under reducing conditions the intact subunit was separated into AP and BP, and the partially hydrolyzed subunit gave rise to BP, partially hydrolyzed AP, and a peptide fragment. Cleavage of some peptide bonds outside the disulfide-linked region of AP also occurred, giving rise to a "smaller" subunit (mean M_r 40 000).

The appearance of protein bands at and near the origin shows the formation of high molecular weight buffer-soluble aggregates, predominantly at 55 °C. The disappearance of these components under reducing condition indicates that these aggregates were linked by disulfide bonds. The absence of degradation products at 55 °C suggests that the soluble aggregates were formed from the subunits or dissociated polypeptides, whereas the occurrence of proteolysis and protein precipitation at 25 and 37 °C suggests that the insoluble aggregates were formed from hydrolyzed oat globulin molecules.

Racemization. Since the alkaline conditions used in this investigation were relatively mild compared to those (>0.1 N NaOH and >60 °C) used to study racemization of proteins (Friedman et al., 1984; Paquet and Ma, 1989), only the 55 °C incubated samples (for 96 and 192 h) were analyzed. The alkali-treated oat globulin samples were analyzed for the content of D enantiomers of the polar amino acids which are highly susceptible to racemization, such as Ser, Asp, and Glu. Only traces (less than 2%) of D-Asp were observed in both treated samples, while D-Ser and D-Glu were not detected. D-Ser was the most highly racemized amino acid in alkali-treated proteins and was suggested as a reliable indicator for the extent of racemization in proteins (Schwass and Finley, 1984; Paquet and Ma, 1989). Hence, the alkaline conditions used in this study were not harsh enough to cause significant racemization of oat globulin.

CONCLUSION

The present data show marked physicochemical changes in the alkali-treated oat globulin. Most of these changes were attributed to partial denaturation and alterations in the oligomeric structure (degradation and aggregation) of the protein. Degradation of oat globulin at 25 and 37 °C, preferentially the acidic polypeptide, was probably due to slow proteolysis by protease(s) coextracted with the protein. Since protein functionality is closely related to the physicochemical characteristics and conformational state of the protein, the present results suggest that alkali treatments and mild proteolysis may be used to modify the functional properties of oat protein. Previous study (Ma, 1985) showed that limited proteolysis by trypsin improved solubility, emulsifying, and water binding properties of oat protein. No significant racemization was observed in alkali-treated oat protein, suggesting that digestibility and protein quality would not be affected.

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