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Received for review February 13, 1989. Accepted December 4, 1989.

Effect of Acetylation on Emulsifying Properties of Glycinin

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Emulsifying properties of acetylated glycinin were checked on samples of 0%, 45%, and 90% lysine residue modification. Sonication at 15 W was employed for making emulsion. At an oil to protein solution volume ratio of 2.5:7 (v/v), pH 7.6, 90% acetylated glycinin showed an over 70% increase in emulsifying activity over native protein. Above the isoelectric point (p*I*) of the protein, emulsifying activity of acetylated protein was superior to that of native glycinin. Below the p*I*, native glycinin showed better emulsifying activity. Emulsion stability of the glycinin was significantly improved by acetylation. Changes in emulsifying properties of acetylated glycinin are discussed in terms of such intrinsic properties of the protein as surface hydrophobicity and flexibility. Effects of the presence of NaCl, $CaCl_2$, and β-mercaptoethanol and browning as the result of incubation with glucose are also discussed.

There have been numerous studies on functional properties of soy protein (Shiga and Nakamura, 1987; Townsend and Nakai, 1983; Deeslie and Cheryan, 1988), and chemical modification was proposed as one of the means for improving functional properties of the protein (Barman et al., 1977; Sung et al., 1983a; Kim and Kinsella, 1986). The primary objectives of modification are (a) to learn more about the functional properties in terms of the intrinsic properties of the protein and (b) to improve functionality for wider usage in the food industries. Acylation (Franzen and Kinsella, 1976; Barman et al., 1977; Brinegar and Kinsella, 1980; Sung et al., 1983a) and phosphorylation (Sung et al., 1983b) are known to increase solubility, emulsifying activity, and foaming capacity of the protein. Proteins showing poor functional properties could be effectively used in food processing following acylation (Barber and Warthesen, 1982; Eisele and Brekke, 1981).

More recently, attempts were made to correlate functional properties to physicochemical properties exhibited by the protein. In protein-based emulsion the protein forms an interfacial film. The physical properties of this film matrix and its surface characteristics determine its capacity to form and stabilize emulsion. Kato and Nakai (1980) reported that a significant correlation existed between surface hydrophobicity and emulsifying properties of food protein. Graham and Phillips (1979, 1980) attributed the higher rate of surface pressure development for β-casein than bovine serum albumin and lysozyme to greater flexibility of the β-casein, which increased its facility of unfold at the interface. Stability of protein emulsion was influenced by the properties of the interfacial film material and the viscosity of the continuous phase.

There have been numerous reports on emulsifying properties of soybean protein (Yasumatsu et al., 1972; McWatters and Cherry, 1977; Aoki et al., 1981, 1984; Voutsinas et al., 1983), but still more research is needed in order to apply the protein in the development for use as food. In our previous report (1989), we discussed the effect of acetylation on physicochemical properties of glycinin, such as conformation, surface hydrophobicity, and flexibility. In this paper, emulsifying properties of native and acetylated glycinin are discussed in the absence and presence of NaCl, $CaCl_2$, and β-mercaptoethanol. The effect of storage with glucose, which results in a Maillard reaction, was also determined. Glycinin was chosen because of ease in purification of a large quantity, availability of

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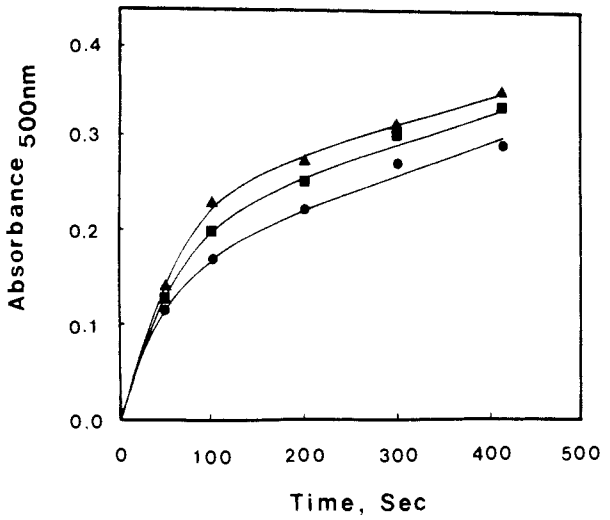

Figure 1. Absorbance at 500 nm of the protein-stabilized emulsion as a function of sonication time. Emulsion was stabilized by 0.5% glycinin, phosphate buffer (pH 7.6), and the oil to protein solution ratio was 1.5:7. Degree of acetylation: 0% (●), 45% (■), 90% (▲).

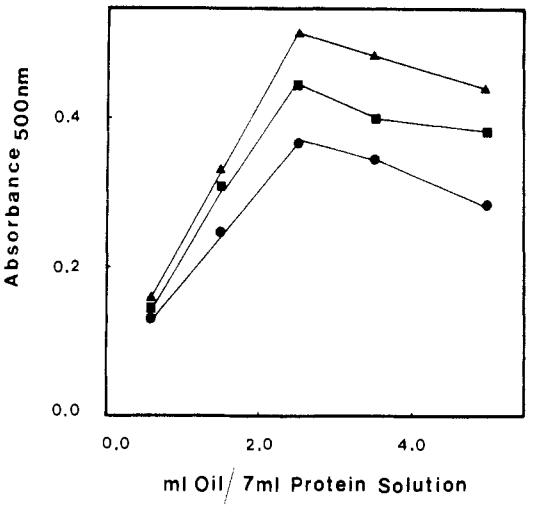

Figure 2. Absorbance at 500 nm of the protein-stabilized emulsion at different oil to protein solution ratios. The sonication time was 5 min at the constant power of 15 W. Degree of acetylation: 0% (●), 45% (■), 90% (▲).

large amount of literature (Kitamura et al., 1976; Moreira et al., 1979; Badley et al., 1975; Iyengar and Ravestein, 1981), and the need for improvement in emulsifying properties for use as food.

MATERIALS AND METHODS

Methods. Soybean flour was purchased from Sigma (St. Louis, MO). Acetic anhydride, 8-anilinonaphthalenesulfonic acid (ANS), and olive oil were also from Sigma. Other reagents used were of analytical grade.

Glycinin Purification. The glycinin-rich fraction obtained by Thanh and Shibasaki (1976) was applied to a DEAE-Sephadex A 50 column. Purity of the fraction was found to be more than 93% by densitometer scanning of SDS-polyacrylamide electrophoresis of the protein.

Acetylation. The acetylation procedure used was similar to that of Riordan and Vallee (1971). Acetic anhydride was added slowly to the protein dissolved in 30 mM potassium phosphate buffer (pH 7.6) (designated as the standard buffer), and the pH was maintained by using a pH-stat with 1 N NaOH. After the reaction, the sample was exhaustively ultrafiltered with distilled water to remove salts before lyophilization. Lysine residue modified was determined with use of ninhydrin reagent (Fraenkel-Conrat, 1957), and samples of 0, 45, and 90% lysine

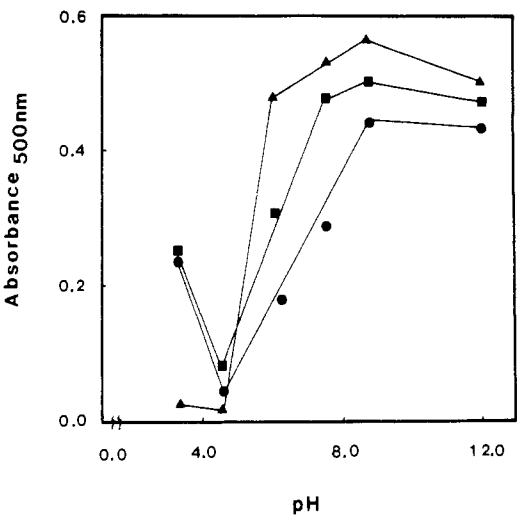

Figure 3. Effect of pH on emulsifying activity at an oil to protein solution ratio of 2.5:7 and sonication time of 5 min. The buffers used were as in Materials and Methods. Degree of acetylation: 0% (●), 45% (■), 90% (▲).

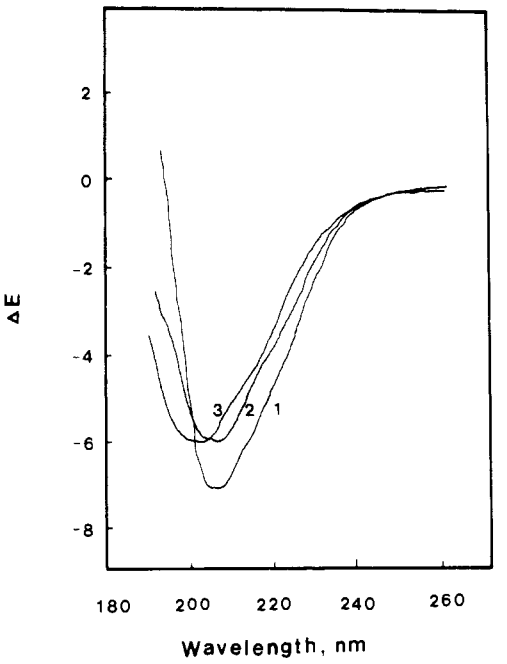

Figure 4. CD spectra of native and acetylated glycinin. Degree of acetylation: (1) 0%, (2) 65%, (3) 95%.

residue modification were used for the subsequent experiments. Native and acetylated protein samples were quantified by micro-Kjeldahl nitrogen analysis.

Circular Dichroism (CD). CD spectra of native and acetylated glycinin were made with a Jasco spectropolarimeter equipped with a xenon lamp. Measurements were made at room temperature. Protein concentrations were 0.01% in the standard buffer.

Viscosity. The specific viscosity of 1% protein solution in the standard buffer was determined on an Oswald-type viscometer. Measurement was made at 25 °C, and the time for the sample to flow through the capillary tube under gravity was used to calculate specific viscosity.

Emulsifying Activity. Emulsifying activities of native and acetylated protein samples were evaluated by spectroturbidimetry according to Pearce and Kinsella (1978) with slight modification. Emulsification was done with use of a sonicator (Branson Sonic Power Co., Model 350) at the constant power output of 15 W. In order to maintain a constant temperature, the vial used for emulsification was equilibrated in the ice solution. Protein concentration and volume were fixed at 0.5% and 7 mL,

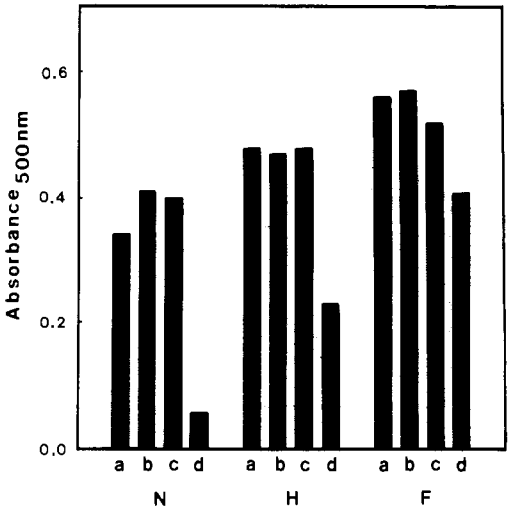

Figure 5. Effects of NaCl, $CaCl_2$, and β-mercaptoethanol on emulsifying activity. NaCl concentration was 0.6 M, $CaCl_2$ concentration was 0.006 M, and that of β-mercaptoethanol was 0.01 M. Key: N, native protein; H, 45% acetylated protein; F, 90% acetylated protein. a–d represent protein in buffer only, buffer containing NaCl, buffer containing β-mercaptoethanol, and buffer containing $CaCl_2$, respectively.

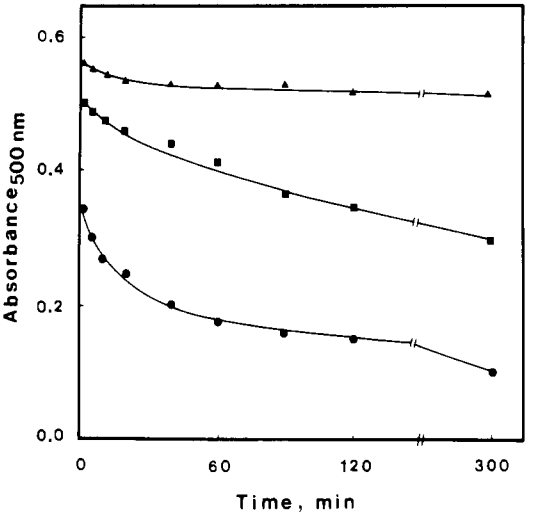

Figure 6. Emulsion stability at an oil to protein solution ratio of 2.5:7 and sonication time of 5 min, 30 mM phosphate buffer. Degree of acetylation: 0% (●), 45% (■), 90% (▲).

respectively. The volume of olive oil used was generally either 1.5 or 2.5 mL.

After sonication, a 5-μL aliquot was immediately pipetted from the emulsion with a Hamilton syringe and diluted 4000-fold into 0.1% SDS solution. The tubes were inverted three times, and absorbance at 500 nm was recorded with a Beckman DU 6 spectrophotometer. Effects of such factors as pH, salts (NaCl, $CaCl_2$), β-mercaptoethanol, and browning on emulsifying properties of the protein samples were tested. Emulsion stability of native and acetylated glycinin was checked as time dependence of emulsifying activity.

Maillard Reaction. Protein samples were incubated with glucose in order to evaluate effects of the acetylation on browning. Native and modified samples were mixed with glucose (2:1, w/w) and placed in desiccators preequilibrated with saturated sodium bromide solution. Incubation was done at 65 °C for 0, 1, 2, 4, and 6 days to obtain different degrees of browning. Degrees of browning of the differentially modified and incubated samples were detected according to Clark and Tannenbaum (1970). Samle portions of 0.8 g were dispersed in 12 mL of distilled water (pH 8). After the mixture was equilibrated at 37 °C, 0.25% Pronase was added to extract brown pigments. The reaction was allowed to continue for 24 h, and brown pigment eluted

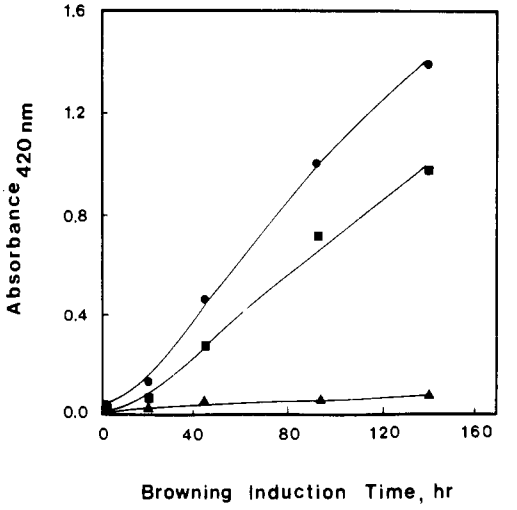

Figure 7. Effect of acetylation on browning of glycinin incubated with glucose. Degree of acetylation: 0% (●), 45% (■), 90% (▲).

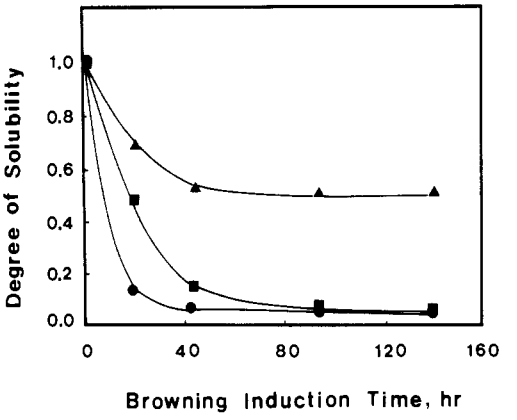

Figure 8. Effect of browning on solubility of acetylated glycinin, at pH 7.6. The buffer used was 30 mM phosphate buffer. Degree of acetylation: 0% (●), 45% (■), 90% (▲).

was detected at 420 nm. Effects of browning on emulsifying activity as well as on solubility of the native and acetylated protein were investigated. The buffer used in this experiment was 30 mM phosphate buffer (pH 7.6). For the solubility test, 0.5% protein was used and the protein remaining in the supernatant after centrifuging at 10000g for 30 min was determined by the biuret–phenol method (Brewer et al., 1974).

RESULTS AND DISCUSSION

Effects of Acetylation on Emulsifying Activity of Glycinin. Effects of sonication time at the constant power of 15 W on emulsifying activity of native and acetylated glycinin are shown in Figure 1. The oil to protein solution ratio was 1.5:7. Emulsifying activity of the samples increased in the same pattern regardless of the acetylation: sharply initially for 100 s and then gradually after that. After 500 s of sonication, the emulsifying activity actually decreased, partly due to generation of heat at the sonicator tip. For further experiments on emulsifying activity, a sonication time of 5 min was chosen.

The ratio of olive oil to the protein solution giving the maximum emulsifying activity was investigated (Figure 2). When the ratio of oil to the protein solution was 2.5:7, the emulsifying activity was maximum. Therefore, in subsequent experiments, the oil to protein solution ratio and sonication time were fixed at 2.5:7 and 5 min, respectively.

Effect of pH on Emulsifying Activity. Emulsion was prepared from solution of protein having a range of

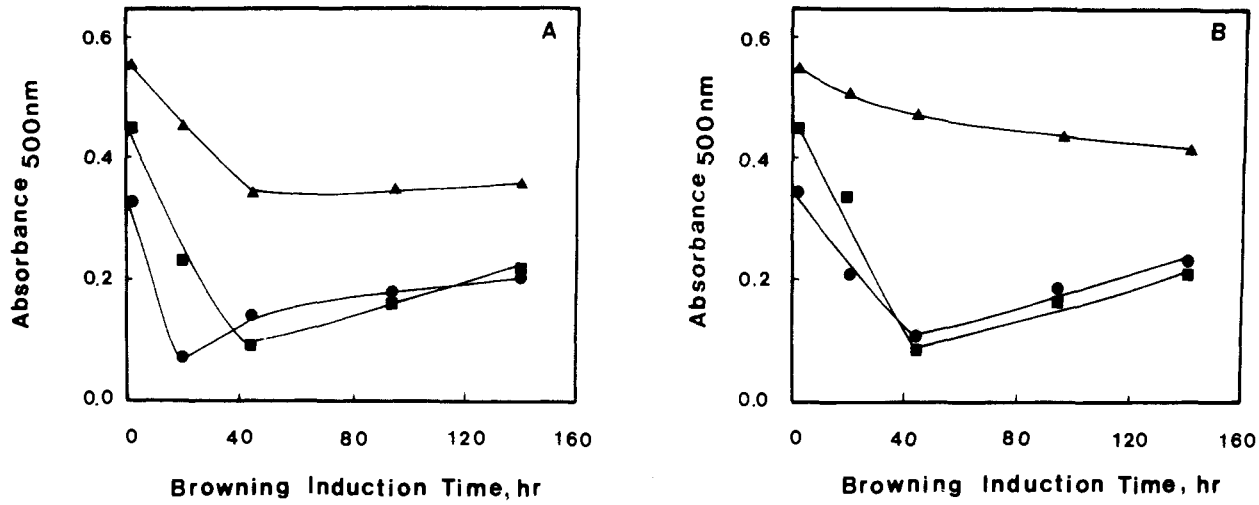

Figure 9. Effect of browning on emulsifying activity of native and acetylated glycinin (A) with and (B) without centrifuging off insoluble aggregates. Degree of acetylation: 0% (●), 45% (■), 90% (▲).

pH values (Figure 3). Turbidity of the protein emulsions increased with pH above the isoelectric point of the glycinin. Emulsifying activity of acetylated protein was higher than that of native glycinin in the range of pH 4.5–12, but below the isoelectric point of the protein, the emulsifying activity of the native glycinin was higher.

At the interface of oil and water, the protein orients hydrophobic residues to the oil phase and hydrophilic residues to the aqueous phase, reducing surface tension at the interface. Emulsifying activity is dependent upon various intrinsic physical properties of the protein such as shape, hydrophobicity, flexibility, and surface charge (Shukla, 1982). Our previous experiment on surface hydrophobicity indicated an increase in surface hydrophobicity from less than 90 to more than 190 upon excessive acetylation of lysine residues. Also, the flexibility of the protein, which was investigated on a differential scanning calorimeter, increased as the result of dissociation and denaturation of subunits (Kim and Rhee, 1989). The enthalpy, which is thought to be inversely proportional to flexibility of the protein, dropped from 3.6 to 0 cal/g of protein when lysine residue modification was above 65%. Figure 4 shows circular dichroism spectra of native and acetylated glycinin. Native glycinin is reported to consist of 5% α-helix, 35% β-sheet, and 60% random structure (Koshiyama and Fukushima, 1973). Figure 4 indicates the strong negative band at 216 nm, which is characteristic of β-sheet conformation, is decreased as a result of acetylation reaction and random structure is increased at the expense of it. This result supports the increase of glycinin in flexibility as a result of modification at lysine residues.

Thus, acetylated glycinin with higher surface hydrophobicity and flexibility showed improved capacity to disperse and stabilize oil droplets more efficiently than the native protein with lower surface hydrophobicity and flexibility.

Effects of Various Salts on Emulsifying Activity. Figure 5 shows the effects of NaCl, β-mercaptoethanol, and $CaCl_2$ on emulsifying activity of native and acetylated glycinin. Acetylation improved emulsifying activity of glycinin regardless of the salts present.

Sodium chloride substantially improved emulsifying activity of native glycinin while its effects on acetylated glycinin were less pronounced (Figure 5).

Addition of β-mercaptoethanol improved emulsifying activity of the native protein only. Addition of β-mercaptoethanol should have reduced disulfide bonds in the native protein making it more flexible and exhibiting higher emulsifying activity. A DSC thermogram of acetylated glycinin revealed that the modification reaction caused the protein to be highly denatured and flexible, and thus the effect of β-mercaptoethanol would not be pronounced as in the case of native glycinin.

The effect of calcium ion on emulsifying activity of the proteins was more apparent than either NaCl or β-mercaptoethanol. Emulsifying activity of native glycinin decreased almost 70% while that of the 90% acetylated glycinin was about 25%, when 0.006 M calcium chloride was added. A decrease in solubility (data not shown) due to calcium ion, which affected the native protein the most, accounts for the corresponding decrease in the emulsifying activity of the protein samples.

Effects of Acetylation on Emulsion Stability. For stable emulsion dispersion, a protein must have appropriate hydrophobicity, hydrophilicity, and sufficient repulsive charge to overcome coalescence and coagulation between oil globules, respectively. Generally, the stability of the protein-based emulsions is influenced by the properties of the interfacial film material and the viscosity of the continuous phase. Figure 6 shows emulsion stability of native and acetylated glycinin in the standard buffer. Emulsion stability of native glycinin decreased rapidly, while that of 90% acetylated glycinin was virtually unchanged even for 300 min. Stability of 45% modified protein was intermediate between the two. Blocking of the positive charge of lysine residues to neutral charge resulted in net increase of negative charge of the protein, with the increased ability to repulse lipid globules in the emulsion stabilized by the protein. Also an increase in flexibility of the acetylated protein could have further contributed to emulsion stability by preventing coalescence of lipid globules. Finally, the increase in viscosity of the continuous phase of acetylated protein solution could partly account for the improvement in emulsion stability as the reduced viscosities of native glycinin and 90% acetylated glycinin were found to be 0.048 and 0.1, respectively.

Effects of Browning on Emulsion Activity. Figure 7 shows the effect of acetylation on browning of the glycinin incubated with glucose at 65 °C with relative humidity of 58%. Browning occurred rapidly for the native protein, but that of 90% acetylated protein was negligible even after incubation of 6 days.

The solubility profile of the native and acetylated glycinin upon incubation with glucose is shown in Figure 8.

Solubility of the native protein dropped rapidly even at an incubation time of 20 h. Initially, hydrophobic aggregation is strongly suggested because of high surface hydrophobicity of the protein, and browning was not significant at this stage of incubation. However for longer incubated proteins of 0% and 45% acetylation, the solubility drop should be due to cross-linking between protein globules as the result of Maillard reaction. As for 90% modified glycinin, hydrophobic aggregation should be mainly responsible for the decrease in solubility since browning did not occur even at prolonged incubation time of 6 days. However, the hydrophobic interaction would not be large since charge repulsion between the protein molecules will be substantial due to acetylation.

The effects of browning on emulsifying activity of the protein samples with and without centrifuging insoluble aggregates are shown in Figure 9. Figure 9A shows that emulsifying activity of native glycinin dropped rapidly at 20 h of incubation but increases slightly at further incubation. This result indicated that solubility of protein is important in emulsion formation and that hydrophobic and hydrophilic products of Maillard reaction could somehow enhance emulsifying activity of the protein. The drop in emulsifying activity of 90% acetylated glycinin with incubation time was not so significant as compared with the native glycinin. Drop in the emulsifying activity of 45% acetylated protein was slightly less than the native sample.

When the insoluble aggregates were not centrifuged off, the emulsifying activity of the samples generally improved, particularly the native and 45% acetylated glycinin incubated at 20 h and 90% modified protein samples (Figure 9B). Hydrophobic force is speculated to be weak enough to be dirupted by sonication, thereby solubilizing an insoluble aggregate that could be used in emulsion formation. But as the browning reaction began to predominate, the native and 45% acetylated glycinin would be coagulated by covalent bonding that would not be solubilized by sonication, thus accounting for similar emulsifying activity as the centrifuged samples.

These results indicate that the acetylation improved emulsifying properties of glycinin. Less susceptibility of acetylated glycinin to calcium ion and browning is especially encouraging, since these are two of the most serious problems in decreasing the quality of soy products such as soy milk.

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Received for review July 12, 1989. Accepted December 10, 1989.

Role of Ferritin as a Lipid Oxidation Catalyst in Muscle Food†

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Iron was released from ferritin by both cysteine and ascorbate at the pH found in muscle foods (5.5–6.9). The rate of iron release from ferritin was influenced by temperature and ferritin and reducing agent concentrations. Storing beef muscle at 4 °C for 11 days resulted in a decrease in the concentration of ferritin antibody precipitatable iron, suggesting that iron is released from ferritin in situ. Physiological concentrations of ferritin catalyzed lipid oxidation in vitro, and heating ferritin increased the rate of lipid oxidation. These data suggest that ferritin could be involved in the development of off-flavors in both cooked and uncooked muscle foods.

Lipid oxidation is one of the major causes of the chemical deterioration of muscle foods. The primary catalysts of lipid oxidation in skeletal muscle have been suggested to be hemoproteins and ferrous iron (Igene et al., 1979; Pearson et al., 1977). Soluble low molecular weight chelates of iron are thought to be the form of iron responsible for enzymic and nonenzymic lipid oxidation in vivo (Halliwell and Gutteridge, 1986). Hazell (1982) and Decker et al. (1989) reported that 4–14% of the soluble iron in beef, lamb, chicken, pork, and flounder have molecular weights of less than 12 000.

Muscle foods are subjected to several processes that may increase the low molecular weight or "catalytic" iron. Storing muscle at refrigerator temperatures increases low molecular weight (<10 000) iron concentrations in mackerel ordinary muscle 1.4-fold in 7 days (Decker and Hultin, 1989). Kanner and co-workers (1988) also reported "catalytic" iron, as measured by the bleomycin method, to increase over 3-fold in turkey muscle stored at 4 °C for 7 days.

Cooking affects the distribution of iron in muscle foods. Schricker and Miller (1983) observed increases in nonheme iron in baked and microwaved ground beef. They also reported that addition of ascorbic acid and hydrogen peroxide to ground beef resulted in increased nonheme iron concentrations. Although storage, cooking, and chemicals change the iron distribution of muscle, little direct evidence is available to identify the source of iron affected by these treatments.

Ferritin, a soluble iron storage protein found in liver, spleen, and skeletal muscle, has a molecular mass of 450 000 Da and contains 4500 iron molecules when fully loaded (LaCross and Linder, 1980). Ferritin releases iron in the presence of reducing agents such as superoxide anion, ascorbate, and thiols (Boyer and McCleary, 1987). Iron released from ferritin by ascorbate (Gutteridge et al., 1983) and superoxide anion (Thomas et al., 1985) catalyzes lipid oxidation in vitro. These data suggest that ferritin could be partially responsible for the observed changes in iron distribution and catalysis of lipid oxidation in stored and processed muscle foods.

The objectives of this research were to (1) determine whether iron is released from ferritin by ascorbate and cysteine at the pH and temperatures expected in muscle foods, (2) determine whether iron is released from ferritin by heat, and (3) determine whether physiological concentrations of ferritin in beef muscle could catalyze lipid oxidation in vitro.

MATERIALS AND METHODS

Materials. Ascorbate, cysteine, Ferrozine [4,4′-[3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl]bisbenzenesulfonic acid], horse ferritin serum antibodies, soybean phosphatidylcholine, and 2,2′-dipyridyl were purchased from Sigma Chemical Co. Ferritin (horse spleen) was obtained from Boehringer Mannheim Biochemicals. All other chemicals were reagent grade or purer.

Methods. Ferritin was separated from loosely bound and unbound iron by dialysis against Chelax resin for 12 h. The iron concentration of dialyzed ferritin was determined by using dipyridyl as described by Drysdale and Munro (1965). The concentration of iron bound to ferritin in beef psoas major muscle was determined by antibody precipitation of ferritin according to the method of Linder and Munro (1972). The psoas major muscle used in these experiments was removed from the animals within 2 h of slaughter, chopped in a food processor, and stored at 4 °C for the indicated time. A 10% muscle homogenate was prepared by blending coarsely chopped muscle for two, 1-min bursts in 0.015 M NaCl. The extract was then heated in a water bath (70 °C) for 10 min, cooled on ice for 15 min, and centrifuged at 14000*g* for 20 min. The supernatant was collected and used for the determination of ferritin-bound iron concentration using antibody precipitation as described by Linder and Munro (1972). Although this method can result in proteins other than ferritin being precipitated by the ferritin antibodies, over 95% of the iron precipitated by the antibodies is bound to ferritin (Linder and Munro, 1972). Existence of fer-

† This paper (89-5-182) is published with the approval of the director of the University of Kentucky Agriculture Experiment Station.

0021-8561/90/1438-0674$02.50/0