Effect of Acetylation on Turbidity of Glycinin

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Acetylation of ϵ-amino groups of lysine residues changed the aggregational properties of glycinin isolated from soybean. Soy glycinin with lysine residue modifications of 0%, 65%, and 95% were used for the experiment. Aggregation was detected by turbidimetry at 500 nm. There was a shift in isoelectric point of glycinin to the acidic region as the result of acetylation and a decrease in the turbidity of the protein above the p*I*. The solubilization effect of NaCl around the p*I* was diminished by acetylation. Aggregation of native glycinin by $CaCl_2$ above pH 6 was also suppressed by acetylation, the degree of which depended upon the degree of acetylation. Above neutral pH, thermal aggregation of glycinin was decreased as acetylation increased. The effects of NaCl and conglycinin, as suppressors of thermal aggregation, were also decreased as the result of modification.

INTRODUCTION

Chemical modifications of food proteins have recently received much attention as means for improving functional properties of the proteins for food processing (Li-Chan et al., 1979; Chobert et al., 1987; Childs and Park, 1976; Matheis and Whitaker, 1984).

Soy glycinin is known to exhibit some peculiar properties which in some cases impair its wider application in certain food systems. For example, soy glycinin is prone to cryoprecipitation (Wolf and Sly, 1967), aggregation by $CaCl_2$ addition (Shimada and Matsushita, 1981; Catsimpoolas et al., 1970), and extremely low solubility around acidic pH (Thanh and Shibasaki, 1976). The effect of heating on the turbidity of glycinin has been studied by numerous researchers (Hashizume et al., 1975; Utsumi et al., 1983). In our previous papers, we reported effects of acetylation on the conformation (Kim and Rhee, 1989a,b) and emulsifying properties (Kim and Rhee, 1990) of glycinin.

In this study, we investigated the effect of acetylation on the aggregation of glycinin and such factors as pH, heat, NaCl, $CaCl_2$, and conglycinin are discussed.

MATERIALS AND METHODS

Materials. Soybean flour was purchased from Sigma Chemical Co. (St. Louis, MO). Acetic anhydride, NaCl, $CaCl_2$, and other reagents used were of analytical grade.

Glycinin Purification. Glycinin (11S protein) rich fractions obtained according to the methods of Thanh and Shibasaki (1976) were further purified on a DEAE-Sephadex A-50 column. The purity of the glycinin fraction was found to be more than 93% by densitometer scanning of SDS-polyacrylamide gel electrophoresis of the protein. Conglycinin was also purified by isoelectric point precipitation and DEAE-Sephadex A-50 column chromatography.

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 of pH 7.6 (designated the "standard buffer" hereafter), and the pH was maintained by using a pH stat with 1 N NaOH. After the reaction, the sample was exhaustively ultrafiltered to eliminate salts before lyophilization. Lysine residues modified were determined by using ninhydrin reagent, and samples of 0%, 65%, and 95% lysine residue modification were used for the subsequent experiments. Samples of native and acetylated proteins were quantified by using micro-Kjeldahl nitrogen analysis.

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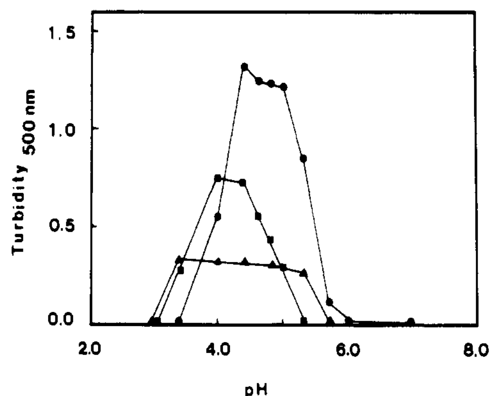

Figure 1. pH profiles of aggregation of 0% (●), 65% (■), and 95% (▲) acetylated glycinin.

pH Effect on Turbidity. Citrate–sodium citrate buffer was used in pH adjustment from 3 to 6, Tris-HCl buffer from pH 7.1 to 8.9, and KCl–NaOH for pH 12.5. The final concentration of buffer solution in which protein was dissolved was 0.06 M, and that of protein, unless stated otherwise, was 0.02%. Effects of NaCl (0.2 and 0.6 M) and $CaCl_2$ (0.006 M) on turbidity of differentially acetylated glycinin at various pHs were also determined.

Heating Effects on Turbidity. Heating experiments were carried out in a water bath with 2.5 mL of protein solutions in test tubes having screw caps to prevent evaporation during heat treatment. Concentration of the protein, unless stated otherwise, was 0.02%, and the buffer conditions were identical with those of the above experiment except that 0.01 M β-mercaptoethanol was added. At the end of the heating period, the tubes were removed and immediately cooled in running tap water. The turbidity of the solutions in the test tubes was measured at 500 nm. Before the turbidity was measured, each tube was shaken well to ensure uniform suspension of particle. Salt effects (NaCl and $CaCl_2$) and conglycinin effects on turbidity were also studied.

RESULTS AND DISCUSSION

Effect of Acetylation on Turbidity of Glycinin. The solubility behavior as a function of pH was changed with acetylation (Figure 1). The loss of positive charged ϵ-amino groups which resulted in the increase in the net negative charges of the protein should have resulted in a shift in the isoelectric point (4.64) to lower pH values, thus resulting in increased solubility of acetylated glycinin in the pH range above the isoelectric point but decreased solubility below the isoelectric point. It could be spec-

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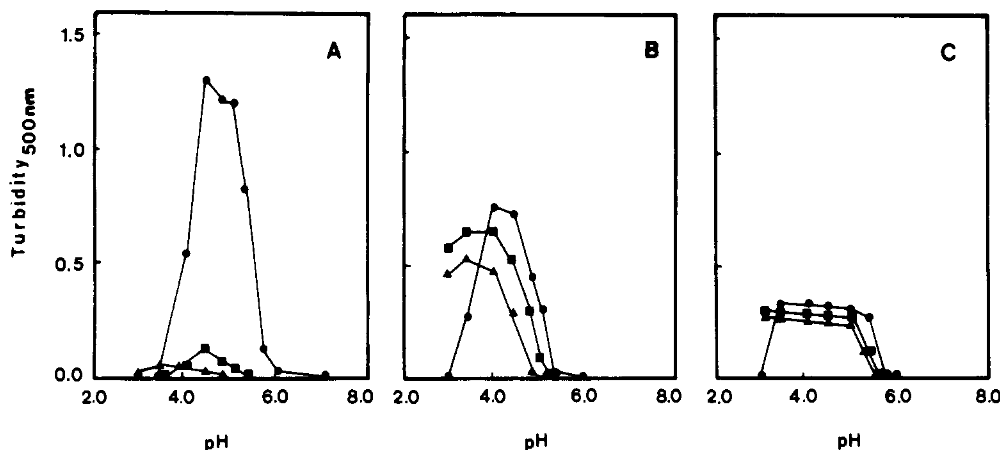

Figure 2. Effect of NaCl concentration on aggregation of (A) 0%, (B) 65%, and (C) 95% acetylated glycinin. Concentrations of NaCl used are 0 (●), 0.2 (■), and 0.6 M (▲).

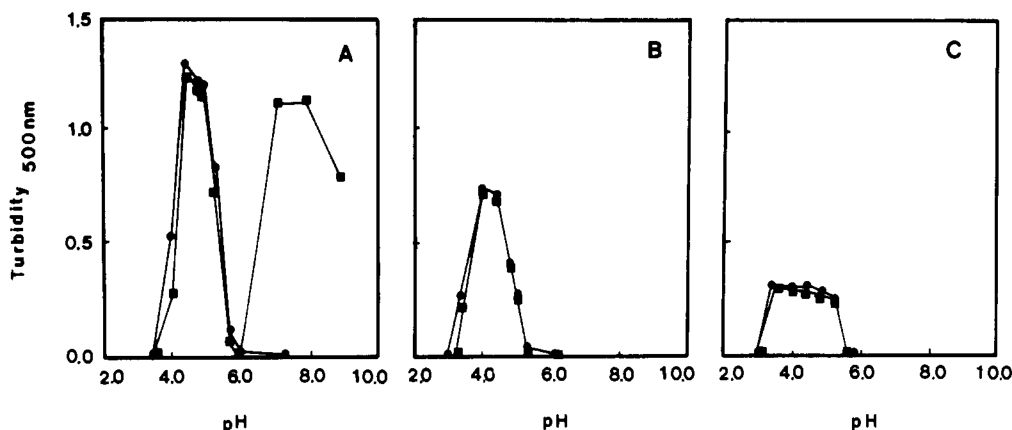

Figure 3. Effect of $CaCl_2$ (0.006 M) on aggregation of (A) 0%, (B) 65%, and (C) 95% acetylated glycinin: before $CaCl_2$ is added (●); after $CaCl_2$ is added (■).

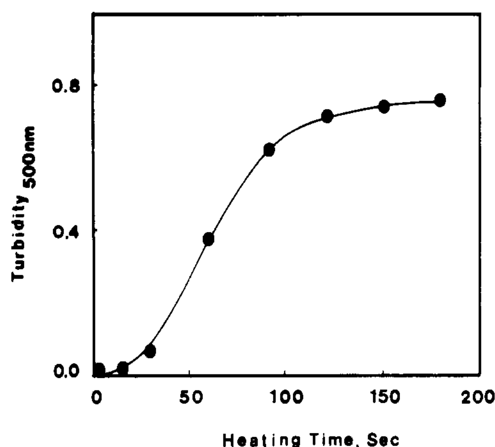

Figure 4. Effect of heating at 80 °C on 0.05% native glycinin solution.

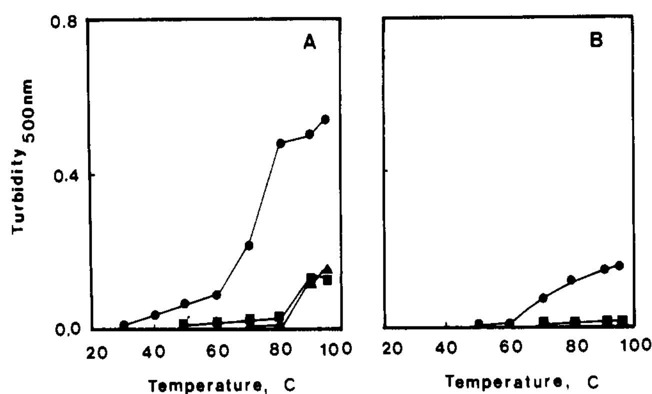

Figure 5. Effect of temperature on thermal aggregation of 0% (●), 65% (■), and 95% (▲) acetylated glycinin at (A) neutral (pH 7.1) and (B) basic (pH 8.9) condition.

ulated that native glycinin was aggregated mainly by hydrogen bonding at the isoelectric point, because the aggregates could be solubilized by heating. High amide content also supports the speculation. However, in the case of acetylated glycinin increase in hydrophobicity due to carboxymethylation of charged lysine residues and exposure of hydrophobic interior could be an additional force behind aggregation at around the isoelectric point (Kim and Rhee, 1989a,b). Generally, the turbidity profile of glycinin decreased with increase in the degree of modification.

Effect of NaCl and $CaCl_2$ on Turbidity. The effect of NaCl on the solubility of protein was significant in native glycinin, but the effect decreased with increase in acetylation (Figure 2). Sodium chloride is known to destabilize electrostatic bonding as well as hydrogen bonding between the proteins molecules, resulting in salting in of the native protein. But with increment of modification, with hydrophobic interaction being the predominant force behind aggregation, the solubilizing effect of NaCl progressively diminished. For 95% acetylated glycinin, the salting-in effect of NaCl was insignificant, while that of 65% modified glycinin was intermediate.

The calcium ion effect on aggregation of glycinin is shown in Figure 3. Appurao and Narasinga Rao (1975) and Sakakibara and Noguchi (1977) have described the ability of glycinin to bind calcium ion above the pK_a value of the

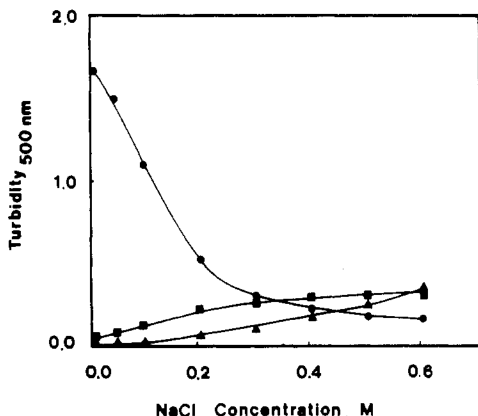

Figure 6. Effect of NaCl concentration on thermal aggregation of 0% (●), 65% (■), and 95% (▲) acetylated glycinin at pH 7.1. Concentration of protein solution was 0.06%.

histidine residue, the binding site for calcium ion. They also suggested that precipitability of the protein by calcium ion was also related to the conformation of the protein. In the experiments, addition of 0.006 M $CaCl_2$ to the 0.02% solution of native glycinin did not affect the aggregation pattern below pH 6, but turbidity developed around pH 7–8, as expected. However, for the acetylated glycinin, turbidity was not detected even above pH 6, and this could be due to the following two reasons: (1) conformational changes of glycinin subunits and (2) subunit dissociation, which might be important for the aggregation reaction to occur.

Effect of Heating on Turbidity. Figure 4 shows that thermal aggregation of 0.05% native glycinin at 80 °C was completed at 5 min, which was chosen as the heating condition for the subsequent experiments.

Figure 5 shows the effect of acetylation on thermal aggregation of glycinin at neutral and alkaline pH. Aggregation of native protein formed by heat treatment at pH 6–9 is due to interaction between basic units of glycinin as reported by many authors (Mori et al., 1981; Nakamura et al., 1984). They concluded that heating of glycinin solutions resulted in the formation of a transient soluble aggregate consisting of acidic and basic subunits. On further heating, this aggregate disappeared and complete dissociation into acidic and basic subunits took place. Because the basic subunits separated were more hydrophobic in nature, aggregation occurred even at pH 7.1, while acidic subunits remained soluble at this pH because of the low hydrophobicity and the repulsion between subunits. The turbidity of acetylated glycinin was much less than that of the native protein, probably because aggregation of basic subunits was overcome by repulsive forces resulting from the increased net negative charges.

Figure 6 shows the effect of ionic strength on the thermal aggregation of 0.06% protein solution. As the ionic strength of NaCl was increased, thermal aggregation decreased sharply for the native glycinin, but in the case of 65% and 95% acetylated samples turbidity actually increased slightly. Iwabuchi and Shibasaki (1981) reported that the nondissociated quaternary structure was main-

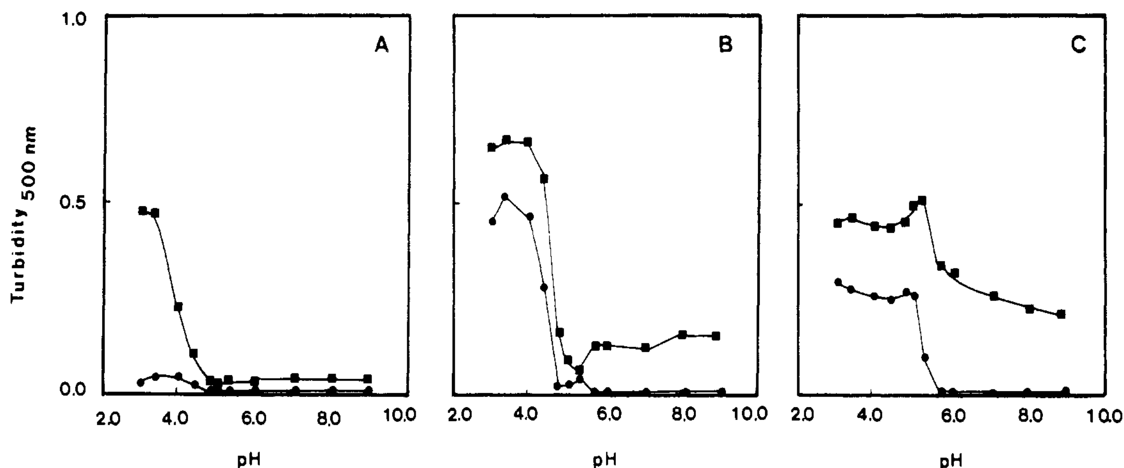

Figure 7. Effect of heating on thermal aggregation of (A) 0%, (B) 65%, and (C) 95% acetylated glycinin solution containing 0.6 M NaCl: before heating (●); after heating (■).

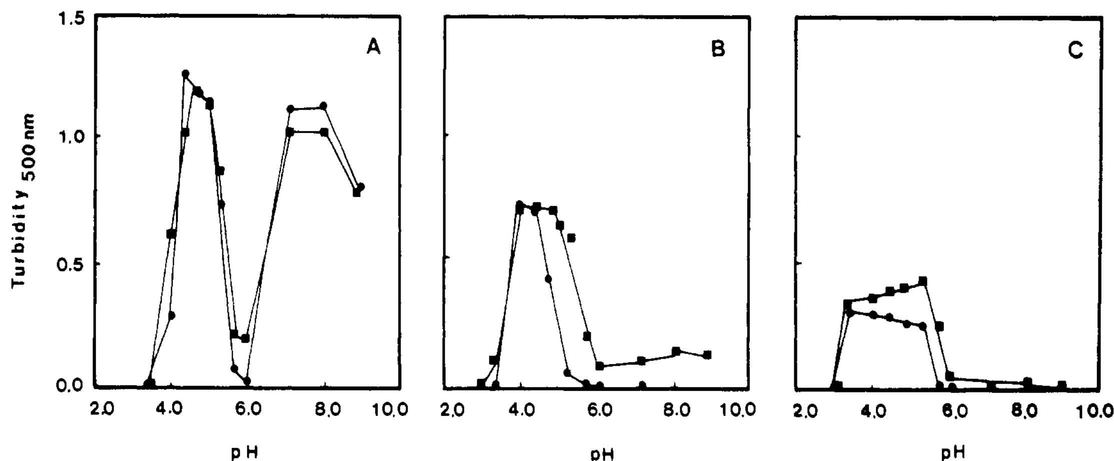

Figure 8. Effect of heating on thermal aggregation of (A) 0%, (B) 65%, and (C) 95% acetylated glycinin solution containing 0.006 M $CaCl_2$: before heating (●); after heating (■).

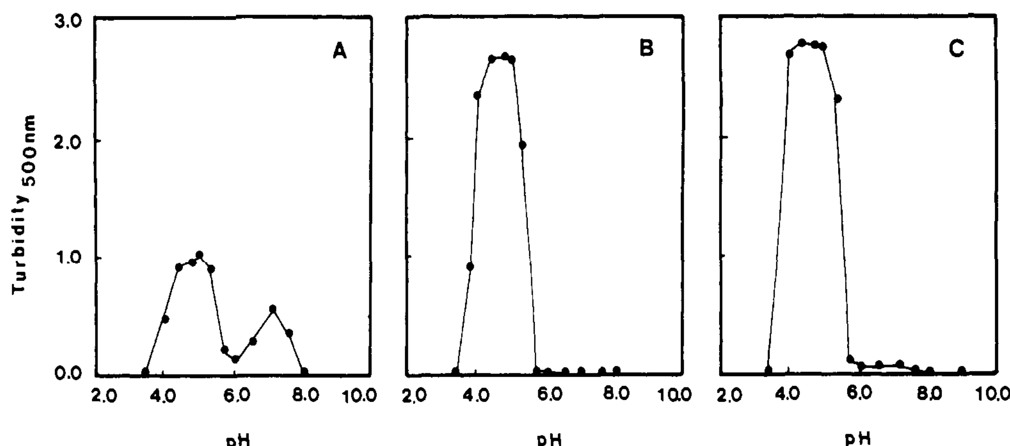

Figure 9. Effect of 0.1% conglycinin on the thermal aggregation of 0.02% glycinin over various pHs. Heating temperature and time of heating were 80 °C and 5 min, respectively. Pattern of thermal aggregation is of (A) glycinin only, (B) conglycinin only, and (C) mixture of conglycinin and glycinin.

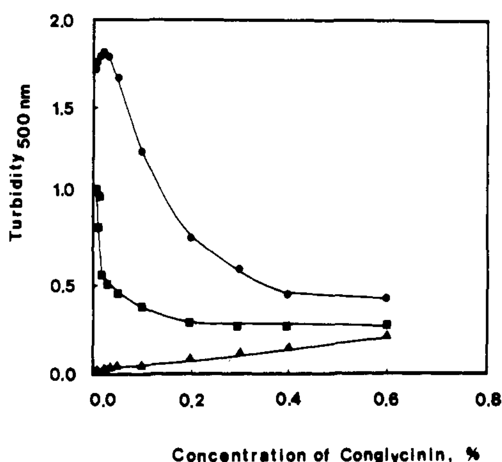

Figure 10. Effect of concentration of conglycinin on thermal aggregation of 0% (●), 65% (■), and 95% (▲) acetylated glycinin. Heating temperature and time of heating were 80 °C and 5 min, respectively.

tained during heat treatment at high ionic strength, using the method of immunoelectrophoresis and disc electrophoresis, implying resistance to thermal aggregation of glycinin in the presence of high ionic strength. However, the stabilizing effect of NaCl disappeared as the protein was modified, suggesting the importance of protein conformation. In the case of modified protein, turbidity actually increased with the increase in NaCl concentration. The results obtained are those expected, where electrostatic binding is more important for native glycinin and hydrophobic binding for the acetylated glycinin.

The effects of 0.6 M NaCl and 0.006 M $CaCl_2$ on thermal aggregation of native and acetylated glycinin at various pHs are shown in Figures 7 and 8, respectively. Sodium chloride suppressed turbidity of the native glycinin over the pH range in the absence of heating, but the effect disappeared in the acidic region upon heating. As for the excessively modified sample, NaCl did not exhibit a stabilizing effect in the acidic region even in the absence of heating and the turbidity increased over all the pH region when heated. The effect of NaCl on 65% acetylated glycinin was intermediate between that on the native glycinin and that on the 95% modified glycinin.

With native protein, the thermal aggregation pattern was affected by the presence of 0.006 M $CaCl_2$ above pH 6.0 (Figure 8). However, with acetylated glycinin, only a slight increase of aggregation by heating at pH 4.5–6.0 was detected.

Effect of Conglycinin on Thermal Aggregation of Glycinin. Utsumi et al. (1984) studied the thermal stabilization of glycinin by conglycinin and elucidated the mechanism of glycinin–conglycinin interaction. To investigate the pH range at which conglycinin contributed to thermal stabilization of glycinin, 0.1% conglycinin was mixed with 0.02% glycinin and the mixture was heated. Figure 9 shows that aggregation at pHs near 7 decreased when compared to the aggregation pattern of glycinin only. Therefore, pH 7.1 was selected for further study on the effect of conglycinin on the thermal aggregation of glycinin.

As shown in Figure 10, thermal aggregation of native glycinin decreased as the concentration of conglycinin increased. According to Utsumi et al. (1984), the acidic subunits with excessive net negative charge, dissociated from conglycinin by heat treatment, interact electrostatically with basic subunits dissociated from glycinin at pH 7.1. As a result, aggregation decreased because of negative charged repulsion between complexes composed of the basic subunits of glycinin and the acidic subunits of conglycinin. However, the slight increase of turbidity at low conglycinin concentration should be due to insufficient availability of negative charged conglycinin subunits. For acetylated glycinin, increase of negative charge of the basic subunits due to the modification reaction would have made the protein less reactive to negatively charged acidic subunits of conglycinin, accounting for the slight increase in turbidity as the concentration of conglycinin was increased.

This study revealed changes in the turbidimetric properties of glycinin upon acetylation. Such study is necessary in foods such as soy milk which lack calcium, as the metal causes precipitation of the protein. Acetylation, as revealed above, results in resistance of the protein to calcium precipitation, implying its applicability.

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