Precipitation of Protein in the Presence of Melanoidin[†]

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A melanoidin was prepared by the reaction of glycine-D-glucose system. The fraction of melanoidin between 10,000 and 50,000 in molecular weight was mainly used to clarify its interaction with protein. Remarkable precipitation was observed between the isoelectric points of melanoidin (pH 2.5) and ovalbumin (pH 4.6). An increase of ionic strength restricted those interaction. As the concentration of melanoidin rose against protein in the reaction system, the molar ratio (protein/melanoidin) in precipitate fell towards 1:1. When the melanoidin was in excess beyond a certain level, precipitation hardly happened. The composition in the reaction system to attain the maximum rate of precipitation was affected by molecular weight of melanoidin. Precipitation proceeded according to second order reaction in respect to both ovalbumin and melanoidin. Based on the analysis of Arrhenius plots, precipitation was found to be promoted by the two subsequent steps. A mechanism involved in this reaction was proposed.

It is well known that melanoidin obtained from Maillard reaction acts as an electrolytic polymer in an aqueous medium. Melanoidin resulting from a glycine-D-glucose system forms precipitate approximately at pH 2.5 and is characterized as an amphoteric electrolyte. And the titration curve for an aqueous solution of melanoidin discloses a number of dissociative groups different from each other in titration constant though they have not yet been identified.¹⁾

Its remarkable interaction with heavy metallic ions is brought about from the property as polyvalent electrolyte as presented by Kajimoto *et al.* who prepared a melanoidin-heavy metal complex in relation to investigations of antioxidant activity of melanoidin.^{2,3)} And authors inspected quantitatively that melanoidin is a hydrophillic colloidal electrolyte and remarkably active in an interaction with various electrolytes.¹⁾

Thus, melanoidin is reasonably assumed to interact with polyvalent electrolytic polymers such as protein under an appropriate condition and to play some roles in affecting the texture of foodstuff in general. Nakatani *et al.* studied

on the hardening of protein containing foods by the addition of Shoyu (soy sauce) during cooking.⁴⁾ This experiment attracted our attention because melanoidin as a pigment in Shoyu was expected to play an important role on hardening foodstuff. In fact, our preparatory test revealed that soybean became harder during cooking with Shoyu which was darker in tint. This hardening was supposed to be caused by combination of protein in soybean with a pigment in Shoyu. Kudo *et al.* have reported an interesting fact that beer yeast cells get together and form clusters in the presence of humic substance during fermentation. They suggested binding of humic substance as a kind of hydrophillic colloidal electrolytes to yeast cell wall.^{5,6)}

On the standpoint that melanoidin is an important factor to affect the physical state of components in foods, this paper describes an interaction between protein and melanoidin under various conditions and proposes a mechanism involved in these reactions.

EXPERIMENTAL

Preparation of melanoidin. An aqueous solution containing 2 M glycine, 2 M D-glucose and 0.2 M sodium bicarbonate was heated at 90°C for 24 hr. The resulting solution was applied to ultrafiltration.

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Fractionation of melanoidin according to its molecular weight.

Melanoidin was divided into six parts by means of an ultrafiltration apparatus (Model MC-2 of Bioengineering Co., Ltd.). The following filters were used; Diafilter G-05 H for 500, G-01 T for 1000, G-05 T for 5000, G-10 T for 10,000 and HFA-300 for 50,000 of molecular weight. The distribution of molecular weight of melanoidin obtained in this experiment is shown in Fig. 1.

FIG. 1. Distribution of Molecular Weight of Melanoidin.

An aqueous solution containing 2 M glycine, 2 M D-glucose and 0.2 M sodium bicarbonate was heated at 90°C for 24 hr. The resulting brown solution was fractionated by means of ultrafiltration.

Proteins

Ovalbumin; prepared from egg white by a salting out method. (3×crystallized)

Bovine serum albumin; purchased from NBC (1×cryst.)

Lysozyme; purchased from SIGMA Chemical Co. (3×cryst.)

Pepsin; purchased from SIGMA Chemical Co. (2×cryst.)

Standard conditions for interaction between protein and melanoidin. An aliquot of aqueous solution of melanoidin was mixed with one of protein, and the mixed solution was adjusted to pH 3.8 with hydrochloric acid or sodium hydroxide and kept standing at room temperature for 24 hr.

Estimation of precipitate

Protein. After formation of precipitate, centrifugation was carried out at 3000 rpm for 5 min. One ml of the supernatant was mixed with 2 ml of 20% trichloroacetic acid. The precipitate formed was

separated by centrifuge at 3000 rpm for 5 min and dissolved in 1 ml of 0.2 N sodium hydroxide. This treatment was repeated twice. The final precipitate was hydrolyzed in 1 ml of 5.7 N hydrochloric acid at 110°C for 16 hr and diluted five times with citrate buffer (pH 5.0, 0.2 M). One ml of this solution was submitted to colorimetry by Yemm and Cocking's method. The amount of protein was determined referring to a calibration curve.

Melanoidin. Estimation was carried out on the basis of O. D.₄₇₀ of the supernatant obtained above.

Measurement of initial velocity of formation of precipitate. Each of aqueous solutions of melanoidin and protein was adjusted to pH 3.8 prior to mixing, respectively. The mixed solution was applied to a spectrophotometer (Hitachi Model 124) within five seconds after mixing, and an increase of O. D.₅₀₀ was followed continuously.

RESULTS

Effects of various factors on an interaction between protein and melanoidin

pH. An effective formation of precipitate was attained in the pH range between the isoelectric point of protein and that of melanoidin as shown in Fig. 2. This fact revealed that the formation of precipitate was caused by an electrostatic interaction between protein and melanoidin.

FIG. 2. Effects of pH on Interaction between Melanoidin and Various Proteins.

A mixed solution containing 2 ml of 0.1% protein, 1.2 ml of 0.25% melanoidin (MW; 10,000~50,000) and 0.8 ml of distilled water was adjusted to various pH values with dil. HCl or NaOH, and kept standing at room temperature for 24 hr.

●, ovalbumin; ○, lysozyme; △, serum albumin; ▲, pepsin; ---, isoelectric point of melanoidin.

Ionic strength. The formation of precipitate was prevented in the higher region of ionic strength as shown in Fig. 3. This was in good agreement with the above presented result that an interaction between protein and melanoidin was based on an electrostatic attraction.

FIG. 3. Effect of Ionic Strength on Interaction between Melanoidin and Ovalbumin.

A mixed solution containing 4.0 ml of 0.5% ovalbumin and 5.0 ml of 0.2% melanoidin (unfractionated) was adjusted to various ionic strength with 1 M KCl, made up to 10 ml with distilled water and adjusted to pH 3.8. Precipitation was estimated after standing at room temperature for 1 hr.

Composition of the reaction mixture. Precipitation was remarkably affected by the ratio of melanoidin to protein (w/w) at any levels of concentration of protein as shown in Fig. 4. In the region of the lower ratio, the rate of precipitation rose with an increase of the ratio while it dropped in the region over a certain ratio. The lower the level of protein concentration, the higher the maximum rate of precipitation. On the other hand, precipitation was more effectively suppressed by the excess melanoidin when the level of protein was higher. The ratio required for complete inhibition of precipitation appeared to be proportional to the level of protein. Where protein was excess against melanoidin, the rate of precipitation is independent of the level of protein and proportional to the amount of melanoidin added. On the other hand, where melanoidin is excess against protein, the extent of precipitation decreased proportionally to the level of protein concentration. Probably,

FIG. 4. Effects of Composition on Interaction between Melanoidin and Ovalbumin.

A mixed solution containing 5.0 ml of 0.25, 0.5 and 1% ovalbumin and an appropriate volume of 20% melanoidin (unfractionated) was made up to 10.0 ml with distilled water, adjusted to pH 3.8 and kept standing at room temperature for 24 hr.

●, 0.125% ovalbumin; ○, 0.25% ovalbumin; ▲, 0.5% ovalbumin.

this is due to a high solubility of a protein-melanoidin complex formed in the excess of melanoidin.

Molecular weight of melanoidin. Figure 5 shows that the ratio (melanoidin/protein, mol/mol) required to attain the maximum rate of precipitation was lower when molecular weight of melanoidin was higher. No precipitation occurred below 1000 in molecular weight at

FIG. 5. Effect of Molecular Weight of Melanoidin on Precipitation of Protein.

A mixed solution containing 3.0 ml of 0.1% ovalbumin and an aliquot of 0.25% melanoidin (fractionated) was made up to 10.0 ml with distilled water, adjusted to pH 3.8 with dil. HCl or NaOH and kept standing at room temperature for 24 hr.

○, >50,000; ●, 10,000~50,000; △, 5,000~10,000; ▲, 1,000~5,000.

any ratio of melanoidin to protein. As the ratio corresponding to the maximum rate of precipitation was proportional to molecular weight to the power 2.5, melanoidin of M-1 ($> 50,000$) was estimated to be about 10^5 by the extrapolating method. In the absence of protein, each fraction of melanoidin (M-1, M-2, M-3 and M-4) precipitated between pH 2.5~2.8, which is considered to be the isoelectric point.

Interaction between M-2 fraction (10,000~50,000) of melanoidin and ovalbumin

M-2 fraction of melanoidin was used to elucidate the mechanism involved in an interaction between protein and melanoidin. This fraction is between 10,000 and 50,000 in molecular weight. The extent of precipitation was varied with the ratio of M-2 melanoidin to ovalbumin (mol/mol) in the reaction mixture in a way similar to the results obtained from Fig. 4 as shown in Fig. 6. Ovalbumin precipitated quantitatively by addition of melanoidin M-2 in the lower region ratio.

Figure 7 shows that the rate of precipitation approached to the constant level as the amount of protein added increased at any level of

FIG. 6. Effect of Composition on Interaction between Melanoidin and Ovalbumin. (Precipitation of Protein)

A mixed solution containing 3.0 ml of 0.1% or 0.25% ovalbumin and an aliquot of 0.25% melanoidin (M-2 fraction MW; 10,000~50,000) was made up to 6.0 ml with distilled water, adjusted to pH 3.8 with dil. HCl or NaOH and kept standing at room temperature for 24 hr.

Conc. of ovalbumin; O, 1.0×10^{-5} M; ●, 2.5×10^{-5} M.

FIG. 7. Effect of Composition on Interaction between Melanoidin and Ovalbumin. (Precipitation of Melanoidin)

A mixed solution containing 0.5 ml of 0.25% melanoidin (MW; 10,000~50,000) and an aliquot of 0.1% ovalbumin was made up to 6.0 ml, adjusted to pH 3.8 with dil. HCl or NaOH and kept standing at room temperature for 24 hr.

melanoidin concentration. However, sixty percent of the amount of melanoidin present in the system seemed to be resistant to precipitation even if ovalbumin was excess against melanoidin in amount. This suggests that melanoidin molecules belonging to M-2 fraction are considerably heterogeneous in charge, molecular shape and so on.

Composition in precipitate

The molar ratio of ovalbumin to M-2 melanoidin in precipitate approached to 1:1 as the concentration of melanoidin in the medium (soluble melanoidin) increased in the presence of a certain amount of protein as shown in Fig. 8. On the other hand, Fig. 9 shows that the ratio in the precipitate was brought to 1:1 as the concentration of protein in the medium (soluble protein) came near to zero in the presence of certain amount of melanoidin. The latter result is in good agreement with the former one. However, the ratio of protein in the precipitate appeared to rise without any limit as the concentration of a soluble protein increased. These facts make it evident that the ratio of protein to melanoidin in the precipitate is dependent on the concentration of protein and melanoidin in the medium, respectively.

FIG. 8. Relationship between Concentration of Melanoidin in Supernatant and Molar Ratio of Melanoidin to Ovalbumin in Precipitate.

A mixed solution containing 3.0 ml of 0.1% or 0.25% ovalbumin and an aliquot of 0.25% melanoidin (MW; 10,000~50,000) was made up to 6.0 ml with distilled water, adjusted to pH 3.8 with dil. HCl or NaOH, and kept standing at room temperature for 24 hr. Conc. of ovalbumin in the initial mixed solution; O, 1.0×10^{-5} M; ●, 2.5×10^{-5} M.

FIG. 9. Relationship between Concentration of Ovalbumin in Supernatant and Molar Ratio of Protein to Melanoidin in Precipitate.

A mixed solution containing 0.5 ml of 0.25% melanoidin (MW; 10,000~50,000) and an aliquot of 0.1% ovalbumin was made up to 6.0 ml with distilled water, adjusted to pH 3.8 with dil. HCl or NaOH and kept standing at room temperature for 24 hr.

Reaction order of formation of precipitate

The formation of precipitate was spectrophotometrically followed by turbidity in a continuous manner. The curves indicate that the reaction proceeded at the second order in respect to both ovalbumin and melanoidin as shown in Fig. 10 and Fig. 11. The plots, however, deviated from linearity beyond a

FIG. 10. Effect of Concentration of Protein on Initial Velocity of Formation of Precipitate.

An aliquot of 0.1% ovalbumin was quickly mixed with 0.3 ml of 0.25% melanoidin (MW; 10,000~50,000) and made up to 3.0 ml with distilled water. Each of aqueous solutions of melanoidin and protein was adjusted to pH 3.7 prior to mixing. The mixed solution was subjected to a spectrophotometer within five seconds, and an increase of O. D.₅₀₀ was followed continuously.

FIG. 11. Effect of Concentration of Melanoidin on Initial Velocity of Formation of Precipitate.

An aliquot of 0.25% melanoidin (MW: 10,000~50,000) was quickly mixed with 0.3 ml of 0.1% ovalbumin and made up to 3.0 ml with distilled water. Operations were carried out according to the manner shown in Fig. 10.

certain concentration. This suggests that the mechanism involved in the interaction between protein and melanoidin is substantially dependent on their concentrations.

Activation energy in an interaction between protein and melanoidin

The reaction mixture to satisfy the linear portions in the curves given in Figs. 10 and 11 was employed to draw Arrhenius plots. Figure 12 shows that the Arrhenius plots inflect at about 15°C. This reveals that the formation of precipitate is composed of two subsequent steps which are different from each other in reaction rate under this range of temperature. The activation energy as calculated from the Arrhenius plots is about 0.6 Kcal/mol in the high temperature region and about 7 Kcal/mol in the low temperature region. The former is considered to correspond to the activation energy for the formation of van der Waals' or hydrophobic bond, and the latter for that of ionic and/or hydrogen bond.

FIG. 12. Effect of Temperature on Interaction between Melanoidin and Ovalbumin.

A mixed solution containing 0.3 ml of 0.25% melanoidin (MW; 10,000~50,000), 0.3 ml of 0.1% ovalbumin and 2.4 ml of distilled water was subjected to a spectrophotometer, and an increase of $O.D._{500}$ was followed continuously. pH was adjusted to 3.7 prior to mixing. The initial velocity was estimated based on the increase of $O.D._{500}$ in five seconds after mixing at each temperature.

DISCUSSION

The above mentioned results were summarized into a simplified model as shown in Fig. 13. This model is based on a thought that the interaction between protein and melanoidin is influenced by two unit steps. The

FIG. 13. A Proposed Schema of Formation of Precipitate.

M: melanoidin, P: protein, MmPn: subunit.

first step is the formation of subunits through electrostatic combination and the second one is the aggregation of those subunits. It is probable that the formation of precipitate is dependent on facility of aggregation of subunits. On this point of view, the composition in precipitate should be equal to that in subunit.

Facility of aggregation is affected by the properties of subunit. Reasonably, the following two factors should be taken into consideration; electric charge and molecular size of subunit. These factors are dependent on a molar ratio between melanoidin and protein present in the reaction system and molecular weight of melanoidin as described in the next.

When melanoidin is excess against protein in the reaction system, the ratio between both components in subunit becomes to 1:1 if it is true of the above experimental results. This is probably owing to the configurational expanse of melanoidin molecule which is effective in covering up protein molecule enough to prevent an additional binding of melanoidin to protein. This speculation is considered to be valid according to recent work by Motai who assumed melanoidins from the glycine-, diglycine-, triglycine-xylose systems and the color of shoyu to be made up of a homologous series of symmetrically flexible chain polymers on the basis of viscometric data.⁷⁾ On the other hand, ovalbumin is well known to be globular protein, and it is considered to be remarkably compact in molecular shape in comparison with melanoidin. Subunits formed in excess of melanoidin will be hard to aggregate each other on account of its small molecular size and high electric charge.

On the other hand, subunits formed in excess presence of protein is thought to be high in the content of protein and large in molecular

size. Protein molecule is easy to bind to melanoidin one after another because the former is remarkably compact in size comparing with the latter. Therefore, a number of protein molecules must effectively counterveil the charges of melanoidin. Thus, these subunits should be facile in aggregation because of large molecular size and low electric charge.

Subunits formed in the above typical cases are visualized in Fig. 14. This model is also valid in explaining the dependence of the rate of precipitation on molecular weight of me-

FIG. 14. Formation of Subunit in the Two Cases. left: melanoidin excess, right: protein excess. M: melanoidin, P: protein.

Subunit	Molecular weight	Net charge	Aggregation
left	low	high	weak
right	high	low	strong

lanoidin which was shown in Fig. 5. That is to say, the rate of precipitation is high even in the excess of melanoidin against protein where molecular weight of melanoidin is small. This supports the thought that melanoidin is too small in molecular size to cover up protein molecule and to prevent an additional binding of melanoidin. Here, binding of both components must bring about an effective counter-veiling of charges.

The mechanism involved in an interaction between melanoidin and protein was mainly investigated in respect to one fraction of melanoidin (MW; 10,000~50,000) and ovalbumin as a globular protein, but the proposed schema will be valid for other fractions of melanoidin as assumed from the above description.

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