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EFFECTS OF ENVIRONMENTAL FACTORS ON TWO-STAGE TANNIN-PROTEIN CO-PRECIPITATION

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Key Word Index—Tannin; galloylglucose; protein-precipitation; two-stage mechanism; pH; temperature; concentration; ionic strength.

Abstract—Effects of environmental factors on two-stage co-precipitation of tetragalloylglucose with three different proteins [bovine serum albumin (BSA), lysozyme, and myoglobin] were investigated. Factors such as pH, temperature, and ionic strength, mainly affected the second precipitation stage. On the other hand, protein concentration mainly affected the initial complexation stage. Precipitability of the galloylglucose-protein complexes is directly related to the solubility of the original protein under each environmental condition.

INTRODUCTION

Tannins are polyphenolic substances having characteristic protein-precipitating ability, and they are widely distributed in almost all plant tissues [1]. Scientists in many fields have been interested in their protein-precipitating ability, because this ability is closely related to leather making [2], physiological activity of herbal medicines [3, 4], taste of foodstuffs and beverages [5, 6] and nutritional value of feeds [7, 8].

Several environmental factors have been reported to affect tannin–protein co-precipitation. pH is one of the most important factors [9–13]. Although Gustavson has reported that the binding of tannic acid on insoluble gelatins was independent on pH [11], many workers have reported that maximum precipitation of tannin with soluble protein occurred near the isoelectric point (p*I*) of protein [9, 10]. Hagerman and Butler have compared the precipitability of sorghum tannin with five different proteins (pepsin, ovalbumin, BSA, trypsin, and lysozyme) under various pHs [10]. They have reported that the maximum precipitation occurred at pH 3 [pepsin (p*I*: 1.0)], 3–5 [BSA (p*I*: 4.9) and ovalbumin (p*I*: 4.6)], and >8 [trypsin (p*I*: 10.1) and lysozyme (p*I*: 11.0)]. Ionic strength [10, 12] and temperature [13] also have been reported to affect the precipitation.

We have reported several lines of evidence which suggest a two-stage precipitation mechanism including initial complexation and subsequent precipitation [14, 15]. It is very important to clarify which process is affected by environmental factors in order to understand the details of the co-precipitation mechanism. However, the above question has not been answered yet.

In this paper, effects of pH, temperature, protein concentration, and ionic strength on the two-stage tannin-protein co-precipitation are discussed from the results of the stoichiometric investigations.

RESULTS AND DISCUSSION

Effects of pH

Co-precipitation of methyl 2,3,4,6-tetra-*O*-galloyl-α-D-glucoside (**1**) and three proteins, that is, BSA, myoglobin, and lysozyme having different p*I*s (4.7, 7.4, and 11.4, respectively) were carried out at pH 3–8. The amount of galloylglucose and protein in the precipitates were determined by the HPLC method previously reported [14].

The amount of the precipitated BSA (%) is shown in Fig. 1 as a function of galloylglucose (T)/protein (P) ratio in the initial reaction solution [T/P(I); Fig. 1(a)], and as the T/P ratio in the precipitates [T/P(P); Fig. 1(b)]. As observed previously at pH 4.5 [15], the amount of the precipitated BSA is related to both T/P ratios. BSA starts to precipitate at certain T/P ratios, and the amount increases linerly as both T/P ratios increase. Similar results were obtained also for myoglobin and lysozyme. In order to compare these results, T/P ratios required for the precipitation of 50% of

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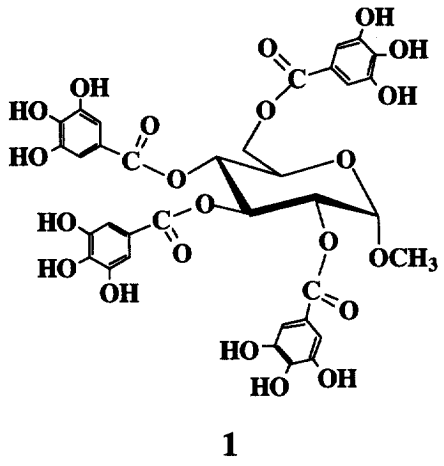

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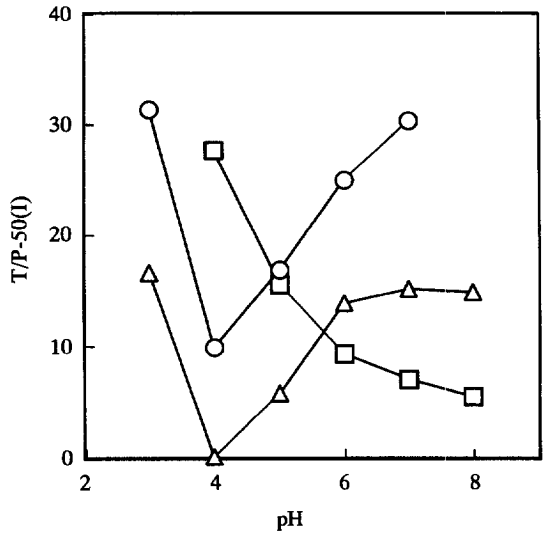

Fig. 2. Effects of pH on T/P-50(I) for BSA, myoglobin, and lysozyme. -○- BSA; -△- myoglobin; -□- lysozyme.

the BSA [T/P-50(I) and T/P-50(P), respectively] were obtained graphically from Fig. 1(a) and (b). These T/P-50 values are used for discussing the effects of pH on the two-stage protein precipitation.

Figure 2 shows the relationships between T/P-50(I) and pH for three proteins. T/P-50(I) Value is a good indicator for protein-precipitating ability under each condition; large T/P-50(I) indicates low precipitating ability, while small T/P-50(I) indicates high ability. As expected, T/P-50(I) is highly dependent on both protein structure and pH. For BSA with p*I* 4.7, T/P-50(I) is the lowest at pH 4, and the value increases greatly as the pH increases or decreases from pH 4. T/P-50(I) of lysozyme with p*I* 11.4 increases as the pH decreases from 8 to 4. The most effective pH for myoglobin with pI 7.4 is more acidic than its pI. Thus, protein-precipitating ability is highly pH-dependent.

Two reasons are possible for this pH-dependence in view of the two-stage precipitation mechanism; one derived from the changes in the initial complexation ability and the other derived from the changes in the solubility of the complexes.

T/P-50(P) and adsorptivity of galloylglucose on insolubilized protein help to answer the above question. T/P-50(P) is a good indicator for precipitability of the complexes, because precipitability of the complexes increases with an increase in the T/P ratio in the complexes at each pH [15]. Large T/P-50(P) indicates low precipitability, while small T/P-50(P) indicates high precipitability. On the other hand, the adsorptivity directly indicates the initial complexation ability. Figure 3 summarizes the T/P-50(P) and the amount of the galloylglucose adsorbed on the insolubilized proteins as a function of pH. Relationships between T/P-50(I) and pH shown in Fig. 2 are also included.

The adsorptivity varies depending on pH but is almost independent of protein structure. For all three proteins, the adsorptivity gradually increases with an increase in pH from 2.2 to 7 and drops at pH 8. Low adsorptivity observed at pH 8 may be due to the

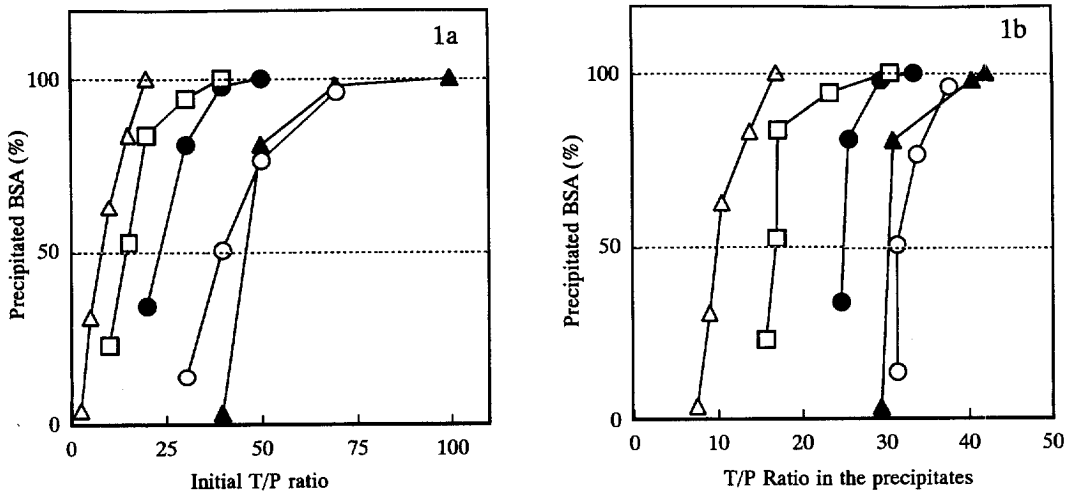

Fig. 1. Relationships of the amount of the precipitated BSA with the initial T/P ratio (1a) and the T/P ratio in the precipitates (1b). -○- pH 3.0; -△- pH 4.0; -□- pH 5.0; -●- pH 6.0; -▲- pH 7.0.

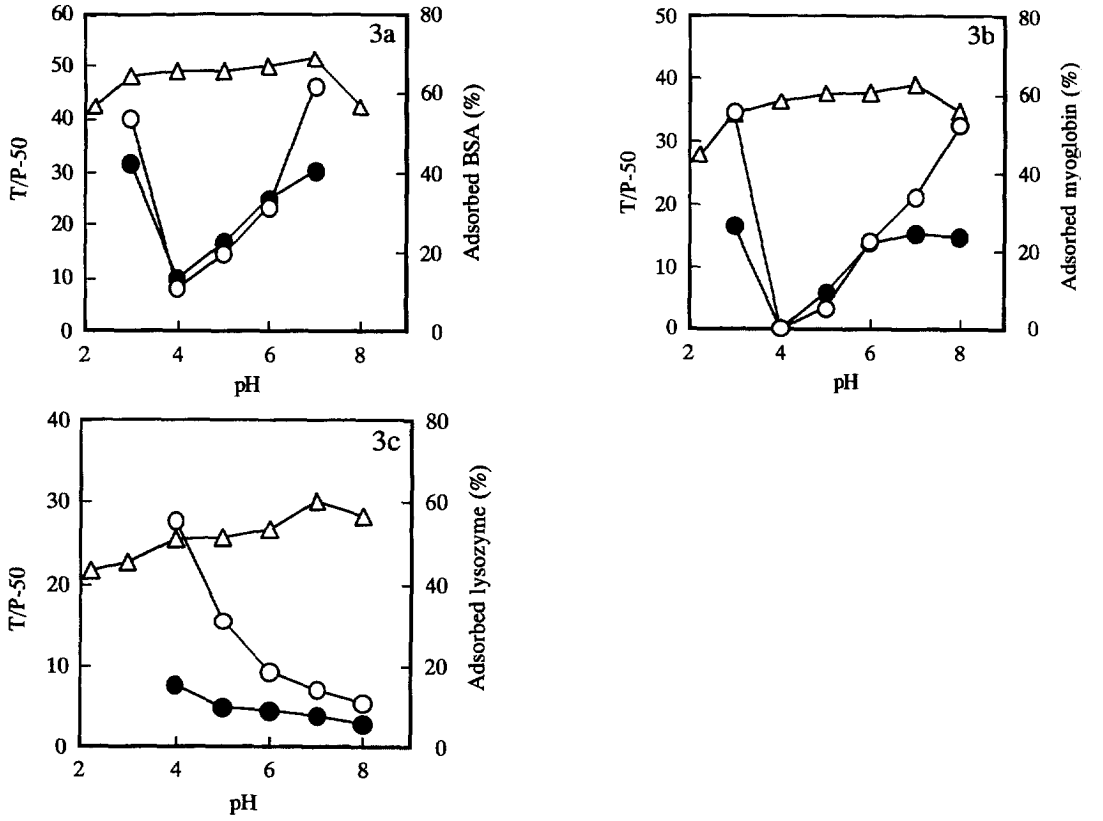

Fig. 3. Effects of pH on T/P-50(I), T/P-50(P), and adsorptivity of compound 1 on insolubilized proteins. (a) BSA; (b) myoglobin; (c) lysozyme, -○- T/P-50(I); -●- T/P-50(P); -△- adsorbed protein.

ionization of the phenolic hydroxyl groups in galloylglucose molecule, as the phenolate anion is not active in hydrogen bonding. These results indicate that chemical structures of amino acid residues of protein, including ionized and unionized forms, have little effect directly on the initial complexation. If chemical structure of amino acid residues directly affects the complexation ability, the adsorptivity should change dramatically around the pK_as of their functional groups. Furthermore, it should be noted that the relationships between pH and the adsorptivity are not correlated with those between pH and the protein-precipitating ability indicated by T/P-50(I). These observations suggest that the complexation ability does not play a critical role in highly pH-dependent co-precipitation.

On the other hand, relationships between T/P-50(P) and pH are very similar to those between T/P-50(I) and pH. This indicates that precipitability change of the complexes is mainly responsible for the pH-dependent protein-precipitating ability.

There are some deviations observed in the relationships between T/P-50(I) and T/P-50(P). Although these values are almost the same at pH 4, 5 and 6 for BSA and myoglobin, T/P-50(I) is larger than T/P-50(P) under other conditions. These observations are comprehensible by heterogeneity of tannin-binding sites shown in the previous paper [16]. When T/P-50(P) is smaller than the number of strong bindings sites, all bonds in the complexes with the T/P ratio required for precipitation are strong, and, consequently, all of the galloylglucose bind to protein. On the other hand, when T/P-50(P) is larger than the number of strong binding sites, some of the bonds in the precipitates are weak, and some galloylglucose molecules exist as free galloylglucose. These results indicate that pH also affects the complexation stage when T/P-50(P) is smaller than the number of strong binding sites on a protein molecule.

Effects of protein concentration, temperature, and ionic strength

Figure 4 shows the results of the effect of BSA concentration investigated at pH 4. BSA-Precipitating ability decreases as BSA concentration decreases from 4.53×10^{-5} to 2.27×10^{-6} mol l^{-1}, as shown by the large increase in the T/P-50(I) from 7 to 44. On the other hand, effect of BSA concentration on T/P-50(P) is very small, and T/P-50(P) is almost constant at all concentrations. These results suggest that BSA concentration mainly affects the initial complexation ability, not the precipitability of the complexes.

Low complexation ability under dilute conditions is explained by the law of mass action. When a galloylglucose molecule binds to a BSA molecule, concentration of the resulting complexes is inversely proportional to [galloylglucose] × [BSA] ([] means con-

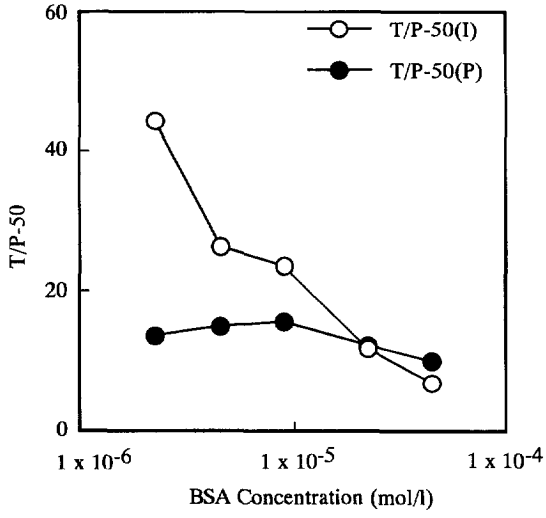

Fig. 4. Effects of BSA concentration on T/P-50(I) and T/P-50(P).

centrations). As BSA concentration is reduced to half while maintaining the same T/P ratio, concentration of the complexes is reduced to a quarter. It has been frequently observed that protein still remains soluble in the supernatant solution even though the supernatant solution has a very high T/P ratio. These observations are rationally understood by the reduced complexation ability under dilute conditions.

Figures 4 and 5 show the results of reaction temperature and ionic strength, respectively. BSA-precipitating ability increases dramatically as the temperature decreases. Low temperature is favourable for the precipitation. The ability decreases as NaCl concentration increases from 0.005 to 0.1 mol l^{-1}, and increases dramatically as NaCl concentration increases from 0.1 to 1.0 mol l^{-1}. In both figures, relationships between pH and T/P-50(P) are very similar to those between pH and T/P-50(I). These very similar relationships suggest that precipitability

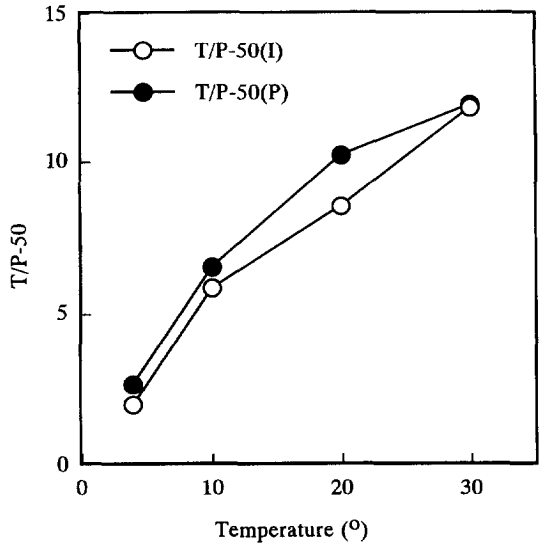

Fig. 5. Effects of temperature on T/P-50(I) and T/P-50(P).

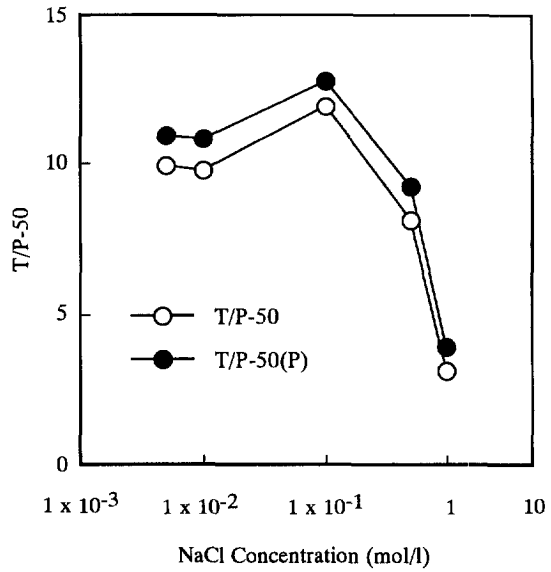

Fig. 6. Effects of NaCl concentration on T/P-50(I) and T/P-50(P).

change of galloyglucose-BSA complexes is mainly responsible for different BSA-precipitating ability under various temperature and ionic strength conditions.

In conclusion, effects of environmental factors on the two-stage protein-precipitation are summarized in Fig. 7. Protein concentration mainly affects the initial complexation, while pH, temperature, and ionic strength mainly affect the precipitability of the complexes. All the results presented here indicate that precipitability of galloylglucose-protein complexes is directly reflected by the solubility of the original protein under various environmental conditions. It is generally recognized that solubility of protein is minimal at the pH around pI of protein, because the electrostatic repulsion between protein molecules becomes minimal around the pI [17]. As for the effect of ionic strength, generally, solubility of protein increases at low ionic strength and decreases at high ionic strength as the ionic strength increases [17]. This is the principle of the salting-out technique for protein purification.

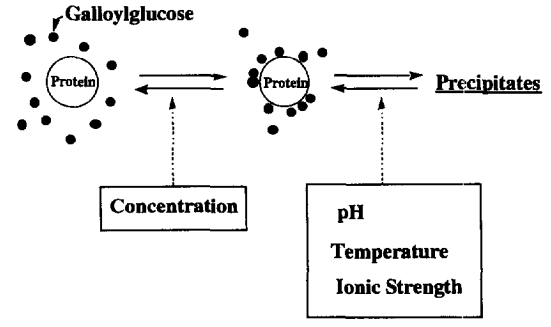

Fig. 7. Effects of the environmental factors on two-stage galloyglucose-protein precipitation.

EXPERIMENTAL

Materials. Me 2,3,4,6-tetra-*O*-galloyl-α-D-glucoside (**1**) was synthesized by the method previously reported [18]. Protein used in the experiments are BSA (M_r 66000, p*I* 4.7), myoglobin (M_r 17600, p*I* 7.4), and lysozyme (M_r 14300, p*I* 11.4). Insolubilized proteins were prepd by the following method [19]. Protein soln (100 mg 50 ml^{-1}) in 0.2 M NaOAc buffer (pH 5.0) for BSA, 0.2 M McIlvaine buffer (pH 5.0) for myoglobin, and 0.2 M McIlvaine buffer (pH 7.0) for lysozyme was mixed with 4% aq. glutaraldehyde soln (50 ml), and the resulting mix. was stirred at room temp. for 0.5–3 hr. The resulting ppts were washed with H_2O (50 ml × 5) and centrifuged at 3000 rpm for 5 min (recovery: *ca* 110% based on the protein used). They were used in the adsorption experiment without drying.

Methods. (i) *Protein precipitation.* Compound **1** (0.5–50 molar equivalents of protein) in 0.2 ml of MeOH was added to a protein soln of 0.2 M McIlvaine buffer (1.6 ml, pH 3–8) (final protein concn: 4.53×10^{-5} M) at 20°. After incubation at 20° for 1 hr, the resulting ppts were sepd by centrifugation (3000 rpm, 5 min), and the ppts were washed with the buffer (1 ml) followed by centrifugation. The ppts obtained were dissolved in 1% SDS soln containing methyl gallate as an int. standard, and both compound **1** and protein were directly determined by using HPLC [14]. BSA concn (2.27×10^{-6}–4.53×10^{-5} M) and temp. (4–30°) were used for the effects of BSA conc and temp., respectively. Ionic strength was changed by using 0.2 M McIlvaine buffer containing NaCl at various concns (final concn: 0–1.0 M).

(ii) *Adsorption of compound* **1** *on insolubilized protein.* Compound **1** in MeOH (0.5 mg 0.2 ml^{-1}) was added to a suspension in 0.2 M McIlvaine buffer (1.2 ml) containing 2 mg of insolubilized protein (dry basis), and the resulting mixt. was incubated at 20° for 1 hr. The amount of the adsorbed compound **1** was determined by obtaining the amount of the unbound compound **1** with *A* 280 of the supernatant soln in 2% aq HCl, from the initial amount used.

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