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SOLUBILITY OF PROTEIN COMPLEXED WITH GALLOYLGLUCOSES

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Key Word Index—Tannin; galloylglucose; protein-precipitation; two-stage mechanism; solubility; tannin structure; pH.

Abstract—Solubility of bovine serum albumin (BSA) (P) precipitated with tetragalloylglucose (T) was investigated by successive washing over the pH range of 3–7. The solubility was highly dependent on pH ($4 < 5 < 6 < 7 < 3$), and these results were comprehensible with the interrelation between T/P ratio required for the precipitation [T/P(R)] and number of the strong binding sites on a BSA molecule (NSB); when NSB > T/P(R) (pH 4 and 5), the precipitates were very stable, while when NSB < T/P(R) (pH 3, 6 and 7), precipitated BSA was easily solubilized by washing. Galloylglucose structure also affected the solubility of the precipitates at pH 4 (penta- < tetra- < 2,3,6-tri- < 2,3,4-tri-galloylglucose). These differences were explainable mainly with their NSBs, which increase dramatically with an increase in the number of galloyl groups in a galloylglucose molecule [35(penta) > 15(tetra) > 0(tri)].

INTRODUCTION

Tannins are classified into condensed and hydrolysable tannins from their chemical structures [1]. They can bind to protein strongly to form precipitates [1], and this property is important in their utilization and physiological properties. Tanning leather [2, 3] and protein-precipitation in the brewing industry [4, 5] directly use this property. Tannins determine the nutritional value of feed for herbivores by complexing with dietary protein and enzyme [6–8]. They frequently cause troublesome effects by complexation in isolating plant enzymes [9]. In ecosystems, tannins are related to the chemical defense of plants [11, 12].

We have reported that the co-precipitation of tannin with protein occurs in a two-stage mechanism which includes initial complexation and subsequent precipitation of the complexes [13, 14]. In the initial complexation, the number of galloyl groups in a galloylglucose molecule is important (penta > tetra > tri ≫ di ≫ mono), and at least three galloyl groups are necessary for effective complexation. In the precipitation stage, the precipitability of the complexes increases with an increase in the galloylglucose (T)/protein (P) molar ratio (more precisely in galloyl group (G)/P ratio) in complexes. Environmental factors such as pH, temperature, and ionic strength mainly affects the second precipitation stage, while the protein concentration mainly affects the initial complexation stage [15].

Stoichiometric investigations of the successive washing of the precipitates has revealed that at least two tannin-binding sites with high and low affinities, respectively, exist in proteins, and that the proportion of the strong binding sites varies depending on the protein structure [16]. This heterogeneity has been expected to affect the solubility of the galloylglucose-protein precipitates in aqueous solution. However, there are no systematic investigations between this heterogeneity and the solubility of the precipitates.

In this paper, effects of pH and galloylglucose structure on the solubility of galloylglucose-BSA precipitates in aqueous solution are discussed in terms of the interrelation between T/P ratio required for the precipitation and number of the strong binding sites on a BSA molecule.

RESULTS AND DISCUSSION

Effects of pH

Solubility of tetragalloylglucose-BSA precipitates was investigated by successive washing at various pHs. Methyl 2,3,4,6-tetra-*O*-galloyl-α-D-glucoside (**1**) and BSA were co-precipitated from solutions containing T/P ratios of 30 (pH 4–6) and 100 (pH 3 and 7), and the

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precipitates were washed with a 2 ml of the aqueous solution having the same pH as that from which they were precipitated. Both tetragalloylglucose and BSA in the resulting precipitates were determined by using HPLC [13]. Figure 1 shows changes of the amount of the precipitated BSA (1a) and the T/P ratio in the precipitates [T/P(P)] (1b) as a function of the number of washings. All of the T/P ratios described in this paper are molar ratios.

Figure 1(a) shows solubility of tetragalloylglucose-BSA precipitates at each pH. The solubility varies depending on pH ($4 < 5 < 6 < 7 < 3$) and number of washings. The precipitates are unstable at pH 3 and 7 and solubilized almost completely by 5 or 6 washings, while the precipitates are quite stable at pH 4 and 5, and no solubilization of BSA is observed by less than five washings.

Figure 1(b) gives information about binding strength between tetragalloylglucose and BSA as discussed in the previous paper [16]. The T/P ratio generally decreases greatly for a small number of washings and the decrease rate becomes smaller and constant as the washing increases. The initial large decrease rate indicates relatively weak binding, while the latter small decrease rate indicates relatively strong binding. The critical T/P ratio between strong and weak bindings means number of the strong binding sites on a BSA molecule, hereafter expressed as NSB. At pH 3, NSB could not be obtained, because the precipitates were dissolved completely at the T/P ratio larger than NSB.

Figure 2 shows NSB, obtained from Fig. 1(b), and the T/P ratio in the complexes required for 50%-BSA-precipitation [T/P(R)] [15] as a function of pH. Solubility of the complexes decreases with an increase in the T/P ratio, and T/P(R) is the ratio, where solubility of the complexes dramatically reduced. NSB increases with an increase in pH: pH 4 (15) < 5 (17) < 6 (19) < 7 (22), the value in parentheses is NSB. As discussed in the previous paper [14, 15], T/P(R) for BSA also varies depending on pH, and the effects of pH is different for various protein structures. Solubility difference observed in Fig. 1(a) is rationally comprehensible by interrelation of NSB with T/P(R). When NSB is greater than T/P(R) (pH 4 and 5), the precipitates are very stable because that tetragalloylglucose-BSA bonds are strong enough for maintaining the T/P ratio greater than T/P(R). On the other hand, when NSB is smaller than T/P(R) (pH 6 and 7), tetragalloylglucose is easily removed from the precipitates by washing until the T/P ratio becomes smaller than T/P(R), and consequently, the precipitates are solubilized easily.

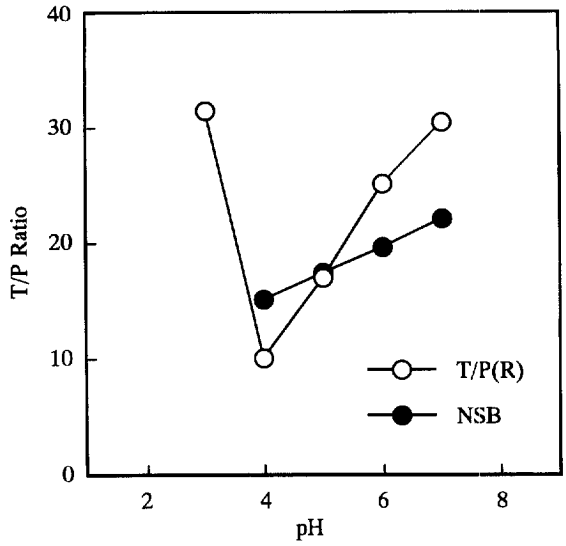


Fig. 2. Relationships between number of the strong binding sites on a BSA molecule (NSB) and the T/P ratio required for BSA-precipitation [T/P(R)] at various pHs.

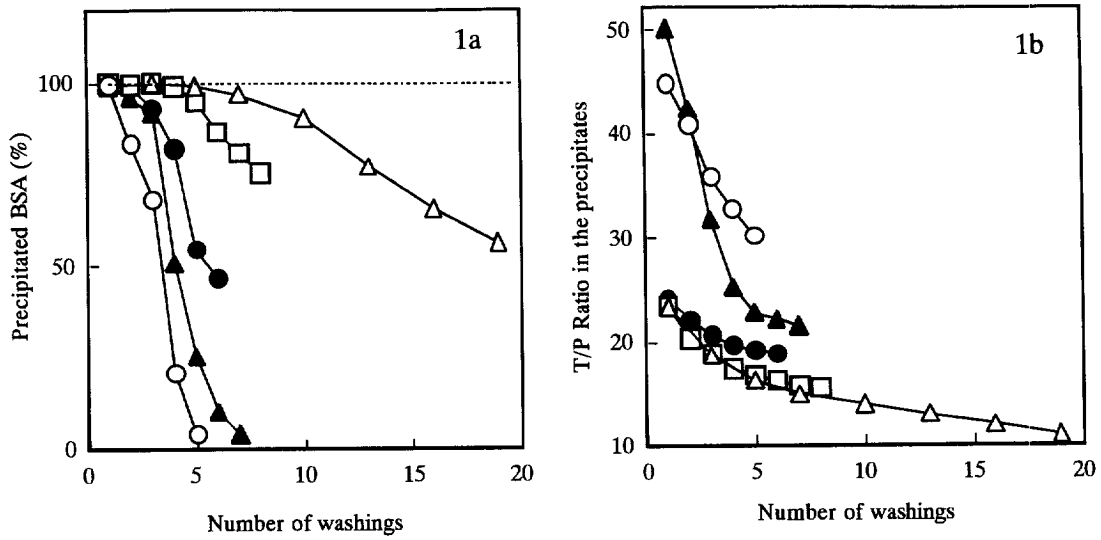
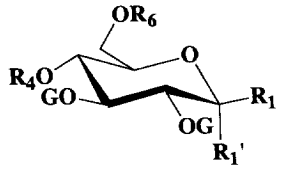


Fig. 1. Changes of the amounts of the precipitated BSA (a) and the T/P ratio in the precipitates (b) by successive washing at various pHs. –○–: pH 3; –△–: pH 4; –□–: pH 5; –●–: pH 6; –▲–: pH 7.



	R_1	R_1'	R_4	R_6
1:	H	OCH_3	G	G
2:	H	OCH_3	G	H
3:	H	OCH_3	H	G
4:	OG	H	G	G

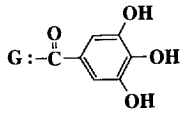


Fig. 3. Chemical structures of galloylglucoses used in the experiments.

Effects of galloylglucose structure

Effects of galloylglucose structure on solubility of the complexes were also investigated by using four galloylglucoses having three to five galloyl groups in a galloylglucose molecule, that is, 2,3,4-tri- (**2**), 2,3,6-tri- (**3**), 2,3,4,6-tetra- (**1**), and 1,2,3,4,6-penta-*O*-galloylglucose (**4**) (Fig. 4). Precipitates used for successive washing were prepared from solutions having T/P ratio of 30 (also 60 for galloylglucose **4**) at pH 4.

Figure 4 shows changes of the precipitated BSA (4a) and the T/P ratio (4b) during successive washing. Solubility of the precipitated BSA decreases with an increase in the number of galloyl groups in a galloylglucose molecule: 2,3,4-tri- (**2**) > 2,3,6-tri- (**3**) > tetra- (**1**) > penta-galloylglucose (**4**). The BSA precipitated with trigalloylglucoses **2** or **3** is very unstable and solubilized almost completely by five washings, while the BSA precipitated with pentagalloylglucose **4** is not solubilized at all, even after 20 washings. This order is opposite to those of relative affinity for BSA and BSA-precipitating ability of galloylglucoses.

NSB obtained graphically from Fig. 4(b) varies depending on the number of the galloyl groups in a galloylglucose molecule; penta- (**4**) (35) > tetra- (**1**) (15) ≫ 2,3,6-tri- (**3**) (0) and 2,3,4-tri-galloylglucose (**2**) (0), value in the parentheses is NSB. It should be noted that trigalloylglucoses **2** and **3** have no strong binding sites at all, even though these galloylglucoses have enough galloyl groups in a molecule for BSA-precipitation [14]. These results indicate that NSB increases with an increase in number of the galloyl groups in a galloylglucose molecule for galloylglucoses having four or more galloyl groups, and that cooperative association of at least four galloyl groups is required for the strong binding between galloylglucose and BSA.

A very similar decrease rate observed for the T/P ratio for pentagalloylglucose **4** and tetragalloylglucose **1**, are also interesting. Binding strength of the strong binding sites is almost the same between tetragalloylglucose **1** and pentagalloylglucose **4**. These observations suggest that an additional galloyl group to tetragalloylglucose does not enhance the strength of the strong binding sites in BSA but increases number of the strong binding sites.

Solubility differences of the complexes is also comprehensible with the interrelation between NSB and T/P(R). Precipitated BSA with trigalloylglucose **2** or **3** is easily solubilized, because NSB of 0 for trigalloylglucoses **2** and **3** is always smaller than their T/P(R)s. On the other hand, precipitated BSA with pentagalloylglucose **4** are very stable, because T/P(S) value of 35 is larger than its T/P(R), which was around 8.

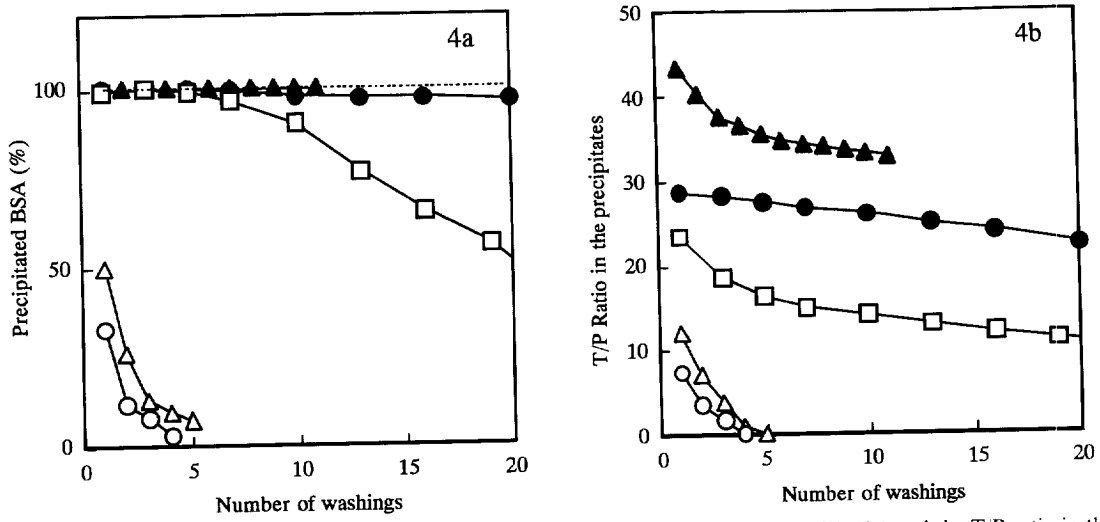


Fig. 4. Effects of galloylglucose structure on changes of the amounts of the precipitated BSA (4a) and the T/P ratio in the precipitates (4b) by successive washing. –○– 2,3,4-tri (2); –△– 2,3,6-tri (3); –□– tetra (1); –●– penta (4); –▲– penta (4) (T/P 60).

EXPERIMENTAL

Materials. A series of galloylglucoses, Me 2,3,4,6-tetra-*O*-galloyl-α-D-glucoside (**1**), Me 2,3,4-tri-*O*-galloyl-α-D-glucoside (**2**), Me 2,3,6-tri-*O*-galloyl-α-D-glucoside (**3**), 1,2,3,4,6-penta-*O*-galloyl-β-D-glucoside (**4**), were prepd by the method previously reported [17].

Successive washing of the galloylglucose-BSA precipitates. Compound **1**-protein ppts were obtained by mixing compound **1** (2.19 μmol for pH 4, 5, and 6, and 7.3 μmol for pH 3 and 7) in MeOH (0.2 ml) with BSA (0.073 μmol) in 0.2 M McIlvaine buffer of various pHs (1.4 ml) at 20° for 1 hr. The ppts were obtained by centrifugation (3000 rpm, 5 min) and washed with the buffer (1 ml). Successive washing of the ppts was carried out as follows: the ppts were stirred in the buffer (2 ml) at 20° for 5 min, and then recovered by centrifugation (3000 rpm, 2 min) after washing a stirring bar in the buffer (0.5 ml). After this procedure was repeated 1–20 times, the resulting ppts 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 [13]. For the investigation of the effects of galloylglucose structure, ppts were prepd at initial T/P ratio of 30 (also 60 for galloylglucose **4**), and the successive washing was carried out at pH 4.0 and 20°.

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