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STOICHIOMETRIC STUDIES OF TANNIN–PROTEIN CO-PRECIPITATION

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Key Word Index—Tannin; hydrolysable tannin; galloylglucose; tannin-protein co-precipitation; stoichiometry; complexation; relative affinity; mechanism.

Abstract—Co-precipitation of a series of galloylglucoses (hydrolysable tannins) with bovine serum albumin (BSA) was studied stoichiometrically by analysing both galloylglucoses and BSA in the precipitates using HPLC. BSA-precipitating ability increased mainly with an increase in the number of galloyl groups in a galloylglucose molecule but was also affected by the position of the galloyl group (penta- > tetra- > 2,3,6-tri- > 2,3,4-tri- ≫ di- ≫ monogalloylglucose). The precipitated BSA increased linearly with an increase in the number of galloyl groups bound to a BSA molecule. BSA-precipitating abilities of the galloylglucoses were closely related to their relative affinities for BSA. These results suggest a two-stage mechanism: initial complexation of galloylglucose with BSA and subsequent precipitation, as a mechanism of the co-precipitation.

INTRODUCTION

Protein-precipitating ability is a characteristic property of hydrolysable and condensed tannins which are widely distributed in plants [1]. Many tannin properties, such as astringency [2], fungitoxicity [3,4] and inhibition of enzymatic activity [5] are considered to be based on this ability. Because of these properties, it is also believed that tannins play important roles in plant physiology [6, 7]; they also have roles in tanning hides [8], removal of protein [9], medicine [10] and so on. Thus, clarifying the mechanism of tannin–protein co-precipitation is particularly important.

Two mechanisms for tannin–protein co-precipitation have been proposed [11]. One is a cross-linking mechanism based on association, in which one tannin molecule binds to two or more protein molecules. Another mechanism is a two-stage mechanism, which involves initial complexation stage of tannin with protein and subsequent precipitation of the complexes. However, there is still little evidence supporting these precipitation mechanisms.

A stoichiometric study of the precipitation process is one of the most important approaches in order to clarify the mechanism, Van Buren and Robinson [12] reported the amount of tannic acid (hydrolysable tannin) and gelatin in their co-precipitates under various conditions, but their results did not lead to discussions of mechanism. Heterogeneity of tannic acid, which is a mixture of many kinds of galloylglucoses, was the main reason.

Haslam reported the relationships between initial β-glucosidase galloylglucose (hydrolysable tannin) ratio and the amount of the precipitated β-glucosidase using a series of galloylglucoses with definite chemical structures [13]. He estimated precipitated β-glucosidase by measuring enzymatic activity in the supernatant solution and evaluated the β-glucosidase-precipitating ability as a galloylglucose concentration causing 50% β-glucosidase precipitation. He observed that monogalloylglucose did not have the ability to cause precipitation and that the precipitation capacity of galloylglucoses having two to five galloyl groups increased with the number of galloyl groups in the molecule. However, relationships between amount of the precipitated β-glucosidase and number of the galloylglucose bound on β-glucosidase were not clarified. Consequently, a stoichiometric relation between tannin, protein and co-precipitation was not clear. This is because of the lack of an analytical method to determine tannin and protein in the precipitates formed.

We have reported an analytical method for tannin and protein in co-precipitates by direct analysis using HPLC [14]. This method enabled us to discuss the co-precipitation stoichiometrically. In the present paper, mechanisms of co-precipitation of galloylglucoses with known chemical structure with bovine serum albumin (BSA) is discussed from the results of a stoichiometric investigation.

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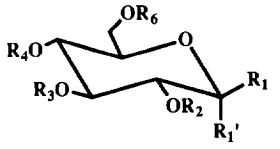
RESULTS AND DISCUSSION

BSA-precipitating abilities of galloylglucoses

We studied relationships between chemical structure of condensed tannin- and protein-precipitating abilities and concluded that both phenolic hydroxyl groups on A- and B-rings in the flavanol repeating unit, play an important role in protein-precipitation [15–17]. However, hydrolysable tannins, especially galloylglucoses, which have much simpler chemical structure than condensed tannins, are more appropriate compounds for stoichiometric investigation; galloylglucoses have only galloyl groups in the molecule and it is easy to synthesize a series of galloylglucoses with systematically changed chemical structures. The finding, as reported by Haslam [13], that the number of galloyl groups in a galloylglucose molecule is a major factor affecting the protein-precipitating ability is an important clue for understanding the mechanism of tannin–protein co-precipitation. Thus, a series of galloylglucoses (**1**–**6**) having different number of galloyl groups at different positions in a glucose core were selected for the model compounds (Fig. 1); they were prepared by galloylation of glucose derivatives [18].

Precipitated BSA (%) plotted as a function of initial galloylglucose (T)/BSA (P) ratio (T/P ratio) used in the experiment is shown in Fig. 2. All of the ratios hereafter used in this paper are molar ratios. BSA-precipitating ability was evaluated as an initial T/P ratio required for 50% BSA-precipitation (T/P-50) obtained from Fig. 2; penta-(**6**)(8) > tetra-(**5**)(11) > 2,3,6-tri-(**4**)(21) > 2,3,4-tri-(**3**) (34) ≫ digalloylglucose (**2**) (400) (number in the parentheses is T/P-50). The ability increased with an increase in the number of galloyl groups in the galloylglucose molecule, as reported by Haslam [13].



	R_1	$R_{1'}$	R_2	R_3	R_4	R_6
1:	H	OCH_3	H	H	H	G
2:	H	OCH_3	H	H	G	G
3:	H	OCH_3	G	G	G	H
4:	H	OCH_3	G	G	H	G
5:	H	OCH_3	G	G	G	G
6:	OG	H	G	G	G	G

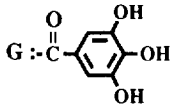


Fig. 1. Galloylglucoses used in stoichiometric investigations.

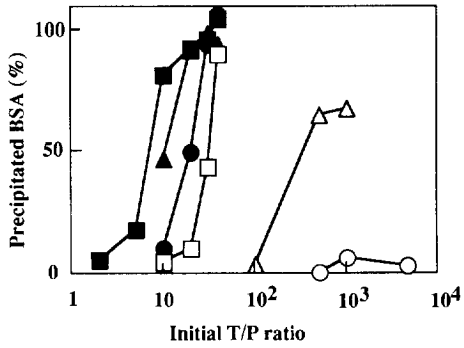


Fig. 2. Relationships between initial T/P ratio and amount of precipitated BSA: ○, compound **1**; △, compound **2**; □, compound **3**; ●, compound **4**; ▲, compound **5**; ■, compound **6**.

Monogalloylglucose is not a multidentate agent but also has BSA-precipitating ability (several per cent precipitation at an initial T/P ratio of 1060 and 4860). This is an interesting observation related to the precipitation mechanism; co-precipitation between monogalloylglucose and BSA must take place other than by cross-linking.

The BSA-precipitating ability of monogalloylglucose is very low and the T/P-50 of digalloylglucose (**2**) (400) is very large, compared with those (8–34) of tri-pentagalloylglucoses (**3**–**6**). These results indicate that more than three galloyl groups in a galloylglucose molecule are required for substantial BSA-precipitating ability.

Although trigalloylglucoses **3** and **4** have the same number of galloyl groups in their molecules, 2,3,4-trigalloylglucose (**3**) (T/P-50 = 34) has a lower precipitating-ability than 2,3,6-trigalloylglucose (**4**) (T/P-50 = 21). These results suggest that the distribution of galloyl groups in a galloylglucose molecule also affects such ability even though this effect is lower than that of the number. The lower precipitating ability of compound **3** is considered to be due to its more sterically hindered galloyl groups compared with those in compound **4**. Thus, the steric environment of the galloyl groups in a galloylglucose molecule is also important for galloylglucose–BSA precipitation. These results are also supported by BSA-precipitating abilities of several phenol gallates measured under similar conditions (inital T/P ratio: 25; BSA concentration: 2.72×10^{-5} mol l^{-1}; pH 4.5; 20°) (Kawamoto, H., Nakatsubo, F. and Murakami, K., personal communication); the amounts of the BSA precipitated by 1,2-, 1,3-, and 1,4-digalloyloxybenzenes are 0, 0.5, and 8.0%, respectively, the amounts precipitated by 1,2,3-, and 1,3,5-trigalloyloxybenzenes are 28, and 79%, respectively. Thus, compounds, in which galloyl groups are located far from each other, have a high BSA-precipitating capacity.

Relationships between amount of precipitated BSA and composition in precipitates

Figure 3 shows the relationships between T/P ratio in the precipitates and precipitated BSA (%) obtained for

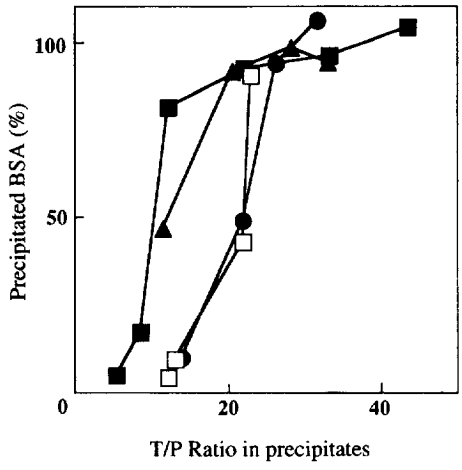


Fig. 3. Relationships between T/P ratio in precipitates and amount of precipitated BSA: □, compound **3**; ●, compound **4**; ▲, compound **5**; ■, compound **6**.

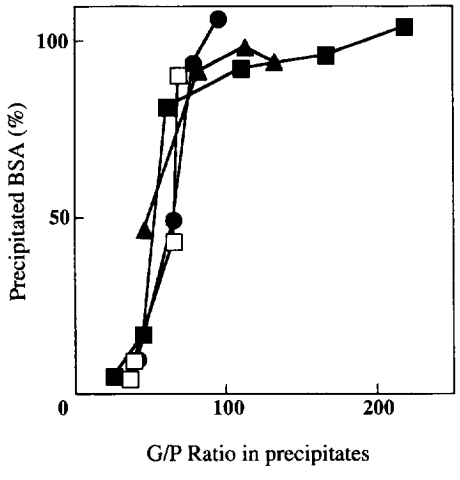


Fig. 4. Relationships between G/P ratio in precipitates and amount of precipitated BSA: □, compound **3**; ●, compound **4**; ▲, compound **5**; ■, compound **6**.

tri-pentagalloylglucoses **3–6** with high BSA-precipitating abilities. The amount of precipitated BSA increases with the increase in the T/P ratio in the precipitates. Thus, the amounts of precipitated BSA are related to the number of galloylglucose bound on a BSA molecule.

There are two other interesting observations from Fig. 3. One is that two trigalloylglucoses (**3** and **4**) have different BSA-precipitating abilities but that the relation between the T/P ratio in the precipitates and the amount of precipitated BSA is the same. The other is that a galloylglucose with several galloyl groups can precipitate the same amounts of BSA at a smaller T/P ratio in the precipitates than a galloylglucose with fewer galloyl groups. These results suggest that the amount of precipitated BSA is related more to the number of galloyl groups bound on BSA rather than the number of galloylglucose molecules bound on BSA.

Figure 4 shows the precipitated BSA plotted against G/P ratio in the precipitates obtained by multiplication of the T/P ratio in the precipitates by the number of galloyl groups (G) in a galloylglucose molecule. Interestingly, all of the galloylglucoses **3–6** show the same relationship between G/P ratio in the precipitates and precipitated BSA (%) and show no influence of the structural differences between galloylglucoses. Under the conditions used (BSA concentration: 4.53×10^{-5} mol l^{-1}, pH 4.5, 20°), there are no precipitates formed at G/P ratio in the precipitates less than 30; precipitated BSA increases linearly with an increase in G/P ratio from 30 to 85 (precipitated BSA (%) = $1.82 \times$ G/P − 54.4, $r = 0.91$, cf. $r = 0.76$ in Fig. 3 at T/P ratio of 5-25); all of the BSA used precipitated at G/P ratios in the precipitate more than 85. Thus, the amount of precipitated BSA is determined by the number of galloyl groups bound on a BSA molecule, regardless of the chemical structure of the galloylglucose. These results also suggest that galloyl groups in a galloylglucose molecule are the sites interacting with BSA. Galloyl groups bound on BSA may change its surface character and/or conformation resulting in a decrease in its solubility.

Mechanism of BSA–galloylglucose co-precipitation

We have reported the relative affinities of galloylglucoses (**1–6**) for BSA determined by competitive precipitation of galloylglucoses (**1**, **2**, **3**, **4**, or **6**) with tetragalloylglucose (**5**) for BSA; penta- (**6**) (2.3) > tetra- (**5**) (1.0) > 2,3,6-tri- (**4**) (0.31) > 2,3,4-tri-(**3**) (0.16) ≫ di-(**2**) (0.018) ≫ monogalloylglucose (**1**) (0) (number in parentheses is relative affinity) [14]. These relationships are very similar to those obtained for BSA-precipitating ability; both BSA-precipitating ability and relative affinity increase with an increase in the number of galloyl groups in a galloylglucose molecule. Mono- and digalloylglucoses (**1** and **2**) have only a very small precipitating ability and relative affinity. The trigalloylglucoses, **3** and **4**, have different precipitating abilities and relative affinities: 2,3,6-tri-(**4**) > 2,3,4-trigalloylglucose (**3**).

Figure 5 shows the relationships between initial T/P ratio and T/P ratio in the precipitates. At the same initial T/P ratio above 20, galloylglucoses with higher relative affinities form precipitates with T/P ratios larger than those with lower relative affinity. These results indicate that galloylglucoses with higher relative affinities can bind to BSA more effectively than ones with lower relative affinity. Thus, the resulting galloylglucose–BSA complexes with a higher T/P ratio (higher G/P ratio) form larger amounts of precipitates.

The results of BSA-precipitating ability and relative affinity indicate that cross-linking is not an important mechanism for galloylglucose–BSA co-precipitation. For cross-linking, one galloylglucose molecule should bind to more than two BSA molecules simultaneously. This requires at least two separate binding sites in a galloylglucose molecule and each of them should bind to BSA effectively. For example, trigalloylglucose has two binding sites; one consists of one galloyl group and the

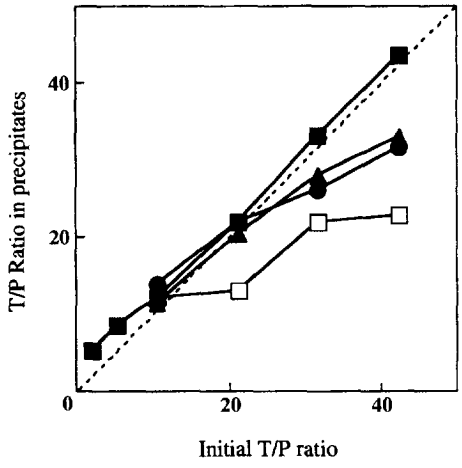


Fig. 5. Relationships between initial T/P ratio and T/P ratio in precipitates: □, compound 3; ●, compound 4; ▲, compound 5; ■, compound 6.

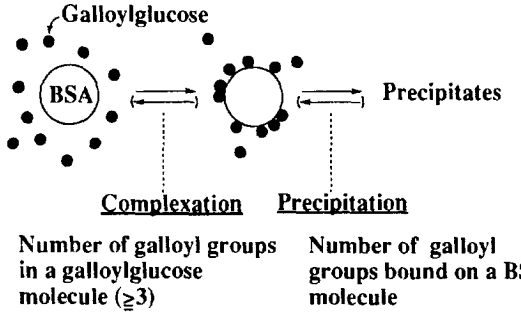


Fig. 6. Two-stage mechanism for galloylglucose–BSA co-precipitation.

other consists of two galloyl groups. However, the binding between each binding site and a BSA molecule is considered to be very weak because of the very low relative affinities of mono- (**1**) (0) and digalloylglucose (**2**) (0.018).

Consequently, all of the results described in this paper can be explained by adopting a two-stage mechanism for galloylglucose–BSA co-precipitation (Fig. 6). The initial stage is complexation of galloylglucose with BSA. In this stage, the relative affinity of galloylglucose for BSA is very important for effective complexation and the relative affinity is determined by the number of galloyl groups and the steric environment around the galloyl group. Effective complexation requires more than three galloyl groups, which may interact with a BSA molecule synergetically. The subsequent stage is a precipitation of galloylglucose–BSA complexes. The amount of BSA precipitated is directly related to the number of galloyl groups bound on a BSA molecule. Under our experimental conditions, BSA complexed by 30 galloyl groups starts to precipitate, the amount of precipitated BSA complexed by 30–85 galloyl groups increases linearly with an increase in the number of galloyl groups bound on a BSA molecule, and BSA complexed by more than 85 galloyl groups precipitates completely.

EXPERIMENTAL

Materials. Me 6-*O*-galloyl-α-D-glucoside (**1**), Me 4,6-di-*O*-galloyl-α-D-glucoside (**2**), Me 2,3,4-tri-*O*-galloyl-α-D-glucoside (**3**), Me 2,3,6-tri-*O*-galloyl-α-D-glucoside (**4**), Me 2,3,4,6-tetra-*O*-galloyl-α-D-glucoside (**5**) and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucoside (**6**) were prepd by galloylation of glucose derivatives using tri-*O*-benzylgalloyl chloride and subsequent debenzylation [18]. Sodium dodesylsulphate, acetonitrile, trifluoroacetic acid and catechol were purchased from Nakarai tesk Co. Ltd. BSA (F-V) was purchased from Sigma Co. Ltd.

Method. Galloylglucoses (**1**–**6**) (0.145–122 mg, 0.152 μmol–353 μmol, 2–4860 molar equivalents of BSA) in 0.8 ml of 0.2 M acetate buffer (pH 4.5) was mixed with a soln of BSA (M_r 69 000) (5 mg, 0.0725 μmol) in 0.8 ml of the buffer at 20°. After stirring for 1 hr, the resulting ppts were sepd by centrifugation (3000 rpm, 5 min) and washed with buffer (1 ml) followed by centrifugation (3000 rpm, 5 min). The ppts obtained were solubilized again by adding 1 ml of 1.0% aq. Na dodesylsulphate soln containing 2 mg of catechol as int. standard and the resulting clear soln analysed by HPLC [14]. CC: column: COSMOSIL C_{18}; eluent: 0.1% aq. TFA/0.1% TFA in MeCN (4:1 to 3:2, 10 min); flow rate: 1 ml min^{-1}; detector: UV 220 nm. R_t (min) catechol (3.1), BSA (10.6) **1** (1.4), **2** (2.1), **3** (5.4), **4** (6.5), **5** (5.3) and **6** (7.3).

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