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BINDING NATURE AND DENATURATION OF PROTEIN DURING INTERACTION WITH GALLOYLGLUCOSE

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Key Word Index—Tannin; galloylglucose; protein-precipitation; two-stage mechanism; heterogeneity of binding; irreversible precipitation.

Abstract—Analysis of insoluble complexes between tetragalloylglucose and proteins following a series of successive washes with buffer indicated (1) heterogeneity of binding between galloylglucose and protein and (2) irreversible denaturation of protein during interaction with galloylglucose. Relatively large amounts of tetragalloylglucose were removed by initial washes, indicating weak, low affinity binding, whereas smaller amounts removed by subsequent washes suggest bonds with a higher affinity. Although the maximum number of bindings sites, calculated per 10 000 M_r of protein, was similar for BSA, myoglobin and lysozyme, the proportion of these sites that appeared to have high affinity, varied from 8 to 29%. The low proportion of strongly binding sites in lysozyme explains its relatively low tannin-complexing ability. Solubility decrease in protein during successive washing and decrease in the β-glucosidase activity indicate that irreversible denaturation of protein occurs, which progresses with an increase in the incubation time with galloylglucose and galloylglucose/protein molar ratio in the mixture. Relative affinity of galloylglucose is directly related to the ability to cause irreversible denaturation. © 1997 Elsevier Science Ltd

INTRODUCTION

Tannins, hydrolysable and condensed tannins, are plant polyphenols which have an unique protein-precipitating ability [1]. The mechanism of the co-precipitation has been of interest for a long time, and hydrogen bonding [2–5] and hydrophobic interactions [6, 7] have been reported as major binding modes. Hydrogen bonding between the phenolic hydroxyl group of tannin and the amide group of protein has been emphasized by the fact that the polyphenol was adsorbed on synthetic polymers such as nylon, which have only an amide functional group [2–4]. On the other hand, significance of the hydrophobic interactions has been suggested by the results that proteins, which were adsorbed on a Sepharose gel immobilized with polyphenols, were effectively eluted by anionic and non-ionic detergents [6]. Tannin has also been shown to interact quite selectively with proteins [8]. However, further binding character between tannin and protein has not been clarified.

Tannin may interact with protein in reversible and irreversible ways. Although many papers have described the reversible process, there are very few papers which describe the irreversible one. Some papers [9, 10] have described an irreversible quinone tanning, in which quinone formed by the oxidation of phenols binds to nucleophilic groups in a protein molecule covalently. Beart *et al.* [11] have indicated that proanthocyanidin polymer (condensed tannin) may form a covalent bond with nucleophilic species of protein. However, there are no papers concerning the irreversible changes during tannin–protein co-precipitation under the usual protein-precipitating conditions.

We have reported very accurate determination methods for galloylglucose and protein in their precipitates by using HPLC [12], and this method enabled us to measure very small changes in the precipitates. In the present study, stoichiometric changes of galloylglucose–protein precipitates during successive washing were studied and the results were found to be very useful for clarifying the binding character and solubility change of protein during interaction with galloylglucose.

In this paper, heterogeneity of the binding between tannin and protein and irreversible denaturation of protein during interaction with tannin, both of which were suggested by the stoichiometric study of the successive washing, are presented.

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RESULTS AND DISCUSSION

Successive washing of galloylglucose–protein precipitates

Co-precipitates of methyl 2,3,4,6-tetra-*O*-galloyl-α-D-glucoside (**1**) with BSA (p*I* 4.7, M_r 66000), myoglobin (p*I* 7.4, M_r 17600), and lysozyme (p*I* 11.4, M_r 14300) obtained at pH 4.0, 5.0, and 7.0, respectively, with a molar T/P ratio of 30, were washed with a fixed volume of the buffer having the same pH as the formation of the precipitates. After this washing was repeated 1–20 times, both tetragalloylglucose **1** and protein in the resulting precipitates were determined by the HPLC method, previously reported [12]. Figures 1 and 2 show changes of galloylglucose (T)/protein (P) molar ratio in the precipitates and the amounts of precipitated protein, respectively. For BSA, the results of the precipitates formed from a solution with an initial T/P ratio of 10 are also included in Fig. 1.

Although the T/P ratio in the precipitates decreases as the washing continues, the decrease rate (/a single washing) is not constant (Fig. 1). For BSA, a large decrease rate (*ca* 2.4/a single washing) is observed above the T/P ratio of 15, while the rate becomes comparatively small and constant (0.3/a single washing) as the number of washings increases. These observations suggest that there are at least two galloylglucose-binding sites with high and low affinities,

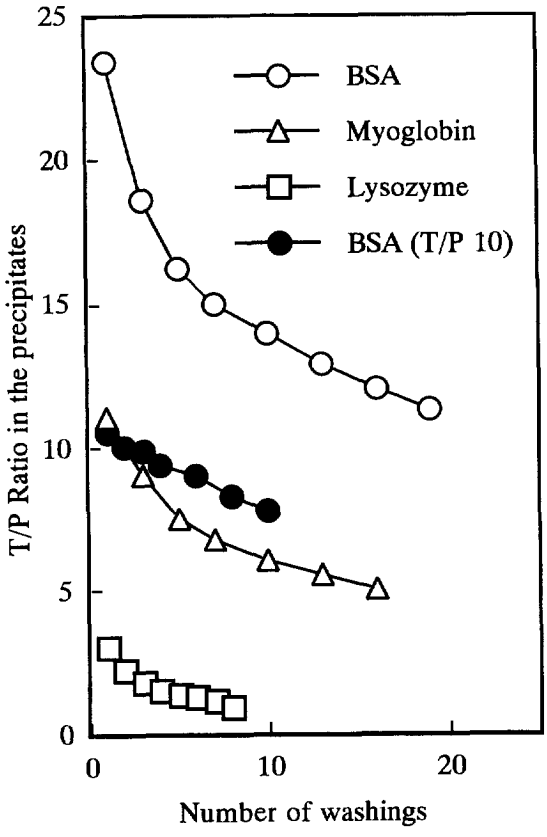


Fig. 1. Relationships between the T/P ratio in the precipitates and number of washings.

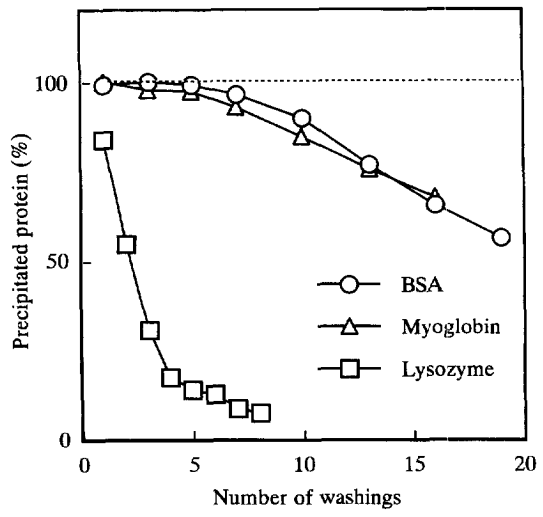


Fig. 2. Relationships between the precipitated protein and number of washings.

respectively. when the precipitates are stirred in buffer, the resulting suspension contains free galloylglucose, free protein, soluble complexes, and insoluble complexes (precipitates), and their composition is determined by two equilibria; one between insoluble complexes and soluble complexes and the other between soluble complexes and free protein and free galloylglucose. So, a large decrease rate in the T/P ratio indicates relatively weak binding, while a small decrease rate indicates relatively strong binding.

The critical T/P ratio of 15, hereafter expressed as NSB, probably indicates the number of strong binding sites on a BSA molecule. The results using a precipitate formed with an initial T/P ratio of 10, which show only small decrease rate (0.3/a single washing), are comprehensible as the initial T/P ratio in the precipitates of 11 is already smaller than the NSB of 15. In other words, all of the binding in these precipitates are strong. Similar relations are observed for myoglobin and lysozyme, and their critical T/P ratios are 7 and 1.5, respectively. Very similar decrease rates observed for the three different proteins in their strong binding regions indicate that the binding strength of the strong binding sites is almost independent of the protein structure.

Loss of the precipitated protein by a single washing depends strongly on the protein structure (Fig. 2). BSA and myoglobin do not dissolve at all after a few washings, but gradually do so as washing continues. On the other hand, precipitated lysozyme dissolves dramatically even after a few washings. The number of strong binding sites is closely associated with these observations. Solubility of the complexes decreases with an increase in the T/P ratio in the complexes and drops greatly around the T/P ratio when there was enough tetragalloylglucose to cause precipitation of 50% of the protein used [T/P-50(P)] [13]. These T/P ratios of BSA, myoglobin, and lysozyme under the present conditions are 10, 6, and 3, respectively [14].

Easy solubilization of lysozyme is explained by its smaller NSB (1.5) than the T/P-50(P) value of 3. On the other hand, NSBs of 15 and 7 for BSA and myoglobin, both of which are resistant to solubilization, are larger than the T/P-50(P) values of 10 and 6, respectively. Relative insolubility of the precipitates with lysozyme after 4–8 washings suggests that solubility of lysozyme is reduced by its irreversible denaturation.

Irreversible denaturation of protein

Figure 3 shows the amounts of the precipitated BSA during successive washing, compared with those during precipitation from the solution. These two relationships are different from each other. BSA is not solubilized effectively in successive washing even when galloylglucose is removed from the complexes until the T/P ratio becomes far less than 10, where only very small amount of the precipitates are formed from the solution. Furthermore, when successive washing was carried out using methyl 2,3,6-tri-*O*-galloyl-α-D-glucoside (**2**), which has a smaller relative affinity for BSA than tetragalloylglucose **1**, trigalloylglucose **2** was completely removed from the precipitates by five washings, while five percent of BSA still remained precipitated. These observations indicate that irreversible denaturation actually occurs during interaction with galloylglucose and does not require covalent bonding with tannin. Although irreversible quinone tanning, in which quinone formed by the oxidation of phenols binds to nucleophilic groups in protein molecule covalently, have been reported, the present results indicate denaturation without any formation of the covalent bonding even under the usual precipitation conditions.

In order to investigate the details of this denaturation process, a two-step precipitation of tetragalloylglucose **1** with BSA was conducted. After preincubation at various T/P ratios, the T/P ratios were adjusted to the final T/P ratio by adding BSA and/or tetragalloylglucose. Figure 4 shows the amounts of the precipitated BSA obtained at various preincubation times (4a) and various T/P ratios in the preincubation mixture (4b). Preincubation time 0 means direct coprecipitation under the final conditions. Although all of the final conditions are the same, the precipitated BSA varies because of the irreversible denaturation during preincubation. If the precipitation consisted of only a reversible process, the same amount of the precipitated BSA should have been observed under all conditions. Precipitated BSA increases with an increase in the preincubation time and leveled off after 30 min preincubation. Precipitated BSA also increases with an increase in the T/P ratio in the preincubation mixture. These results indicate that it takes 30 min to complete the irreversible reaction under the present conditions, and that galloylglucose is directly responsible for the irreversible solubility decrease of BSA.

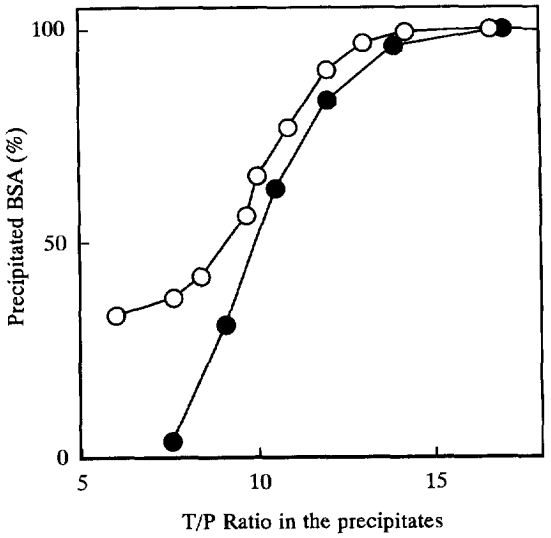


Fig. 3. Relationships between T/P ratio in the precipitates and the precipitated BSA during successive washing, compared with those during precipitation from the solution. –○–: Successive washing of the precipitates. –●–: Precipitation from the solution at various T/P ratios.

Effects of the preincubation time on the relative enzymatic activity were also investigated using penta-*O*-galloylglucose (**3**) and a synthetic condensed tannin for β-glucosidase. As expected, relative activity decreases with an increase in the preincubation time, and it takes several min and 30 min for pentagalloylglucose **3** and condensed tannin, respectively, before relative activity becomes constant. The extent of the decrease in the relative activity during preincubation is as large as 23 and 27% of the original enzymatic activity for pentagalloylglucose **3** and the condensed tannin, respectively. These results imply that irreversible inhibition, as well as a reversible one, should be important in the inhibitory effect of tannin on enzymatic activity.

Effects of galloylglucose structure on the irreversible denaturation investigated by using galloylglucoses **1**–**4** (Fig. 5) with 3–5 galloyl groups in a molecule are summarized in Fig. 6. Extent of the denaturation was evaluated by the solubility decrease as ΔBSA precipitated (%), which was obtained by subtracting the amount of the BSA without preincubation from that with preincubation. Galloylglucose having a larger number of galloyl groups in a molecule is more effective: penta- (**3**) > tetra- (**1**) > tri-galloylglucoses (**2** and **4**), and the galloylglucose, in which galloyl groups are less hindered by each other, is more effective: 2,3,6-tri- (**2**) > 2,3,4-tri-galloylglucose (**4**). These orders are similar to those of the relative affinity for BSA [12]. Galloylglucose having a higher relative affinity for BSA is more effective in irreversible denaturation. Binding of galloylglucose may cause conformational alteration of BSA.

Effects of the heterogeneous binding on the complexation ability of protein

Maximum number of binding sites on a protein molecule was determined by using excess of tetra-

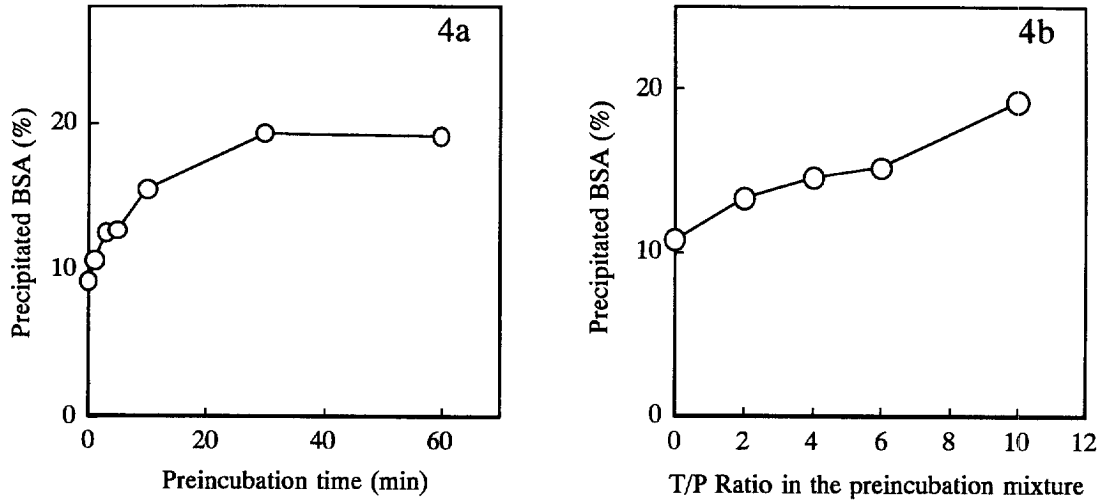
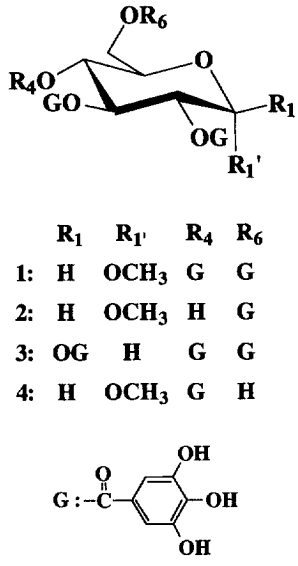


Fig. 4. Relationships of the precipitated BSA with preincubation time (a)* and the T/P ratio in the preincubation mixture (b)†. * Final T/P ratio: 2; T/P ratio in the preincubation mixture: 10. † Final T/P ratio: 2, preincubation time : 1 hr.



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

Fig. 5. Chemical structures of the galloylglucoses used in the experiments.

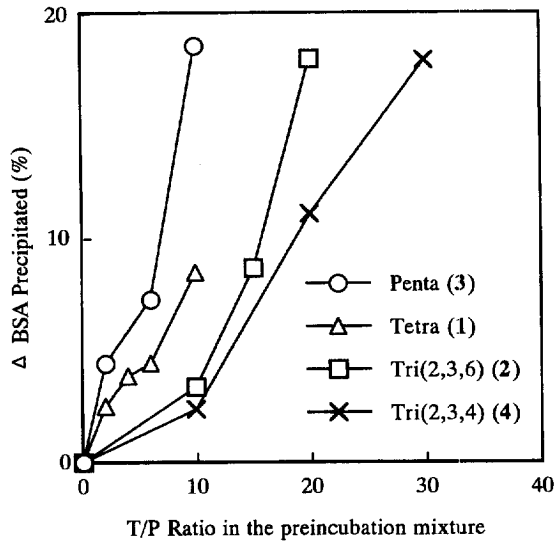


Fig. 6. Effects of galloylglucose structure on the increase in the amount of the precipitated BSA.

galloylglucose **1**. Figure 7 shows the T/P ratio in the precipitates plotted against the initial T/P ratio used in the experiment. The results of ovalbumin (p*I* 4.6, M_r 44 000) are also included. The T/P ratio in the precipitates increases with an increase in the initial T/P ratio and levels off at an initial T/P ratio less than 200. Thus, the T/P ratio in the precipitates at the initial T/P ratio of 250 was used as a measure of the maximum number of binding sites.

Figure 8 summarizes the maximum number of binding sites plotted vs M_r of protein. Interestingly, these plots give a straight line. The maximum number of binding sites is proportional to the M_r of protein. These results emphasize the significance of peptide amide groups in a protein molecule as binding sites with tetragalloylglucose, because the number of amide groups is roughly proportional to the M_r of protein. The amide group is considered to bind to the phenolic hydroxyl group in a tannin molecule by hydrogen bonding, as indicated by many workers [2–4].

Table 1 summarizes the maximum number of binding sites, number of strong binding sites, and content of the strong binding sites against maximum number of the binding sites. All of the results are based on protein M_r of 10 000. Although the number of strong binding sites varies from 1.0 to 4.0, the maximum number of binding sites is independent of protein structure and is always 11–14. As a result, the content of strong binding sites against the maximum number of binding sites varies from 0.08 for lysozyme to 0.29 for myoglobin.

It is known that lysozyme has a smaller complexation ability than BSA. Hagerman and Butler have reported that the amount of lysozyme, which

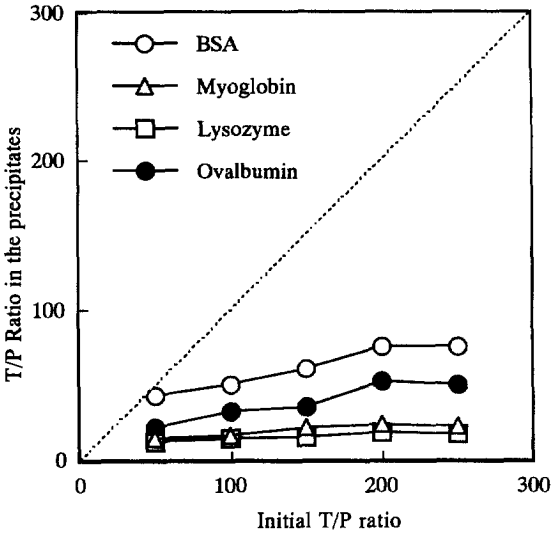


Fig. 7. Effects of the initial T/P ratio on the T/P ratio in the precipitates.

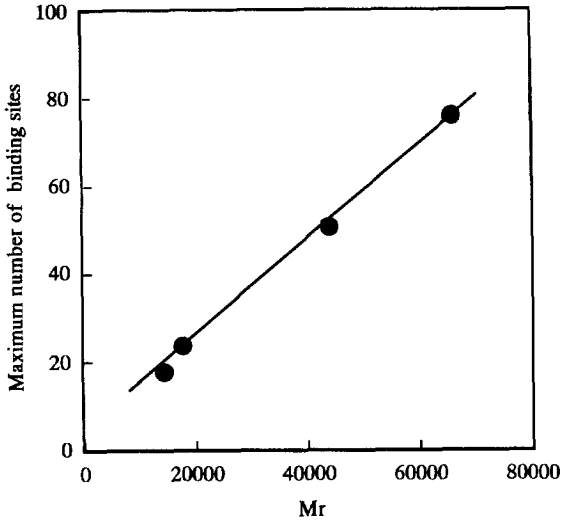


Fig. 8. Relationships between the maximum number of binding sites on a protein molecule and the M_r of protein.

cause 50% inhibition of tannin-^{125}I-labeled protein precipitation, are 45 and 1.4 times the weight of BSA at pH 4.9 and 7.8, respectively [7]. Low complexation ability of lysozyme, compared with BSA, is rationally explained by this different content of the strong binding sites. At pH 7.0, both the number of strong binding sites and the content of strong binding sites for lysozyme are only less than 1/3 of those for BSA. Thus, a large number of galloylglucoses preferentially bind to BSA, rather than to lysozyme, when the same weight of BSA and lysozyme are mixed with galloyglucose.

EXPERIMENTAL

Materials. Me 2,3,4,6-tetra-*O*-galloyl-α-D-glucoside (**1**), Me 2,3,6-tri-*O*-galloyl-α-D-glucoside (**2**), 1,2,3,4,6-penta-*O*-galloyl-β-D-glucoside (**3**), Me 2,3,4-tri-*O*-galloyl-α-D-glucoside (**4**), were prepd by the method of ref. [15]. Proanthocyanidin oligomer (DPn 3.7) was also prepd by the method of ref. [4].

Methods; (*i*) *Successive washing of compound* **1**-*protein precipitates.* Compound **1**-protein ppts were obtained by mixing compound **1**–MeOH (2.19 μmol/0.2 ml) with a protein–0.2 M McIlvaine buffer (0.073 μmol/1.4 ml) at 20° for 1 hr. pH 4.0, 5.0, and 7.0 were used for BSA, myoglobin, and lysozyme, respectively. The ppts obtained by centrifugation (3000 rpm, 5 min) were washed with the buffer (1 ml) before successive washing. Successive washing was carried out by stirring the ppts in the buffer with the same pH (2 ml) at 20° for 5 min, and the resulting ppts were isolated by centrifugation (3000 rpm, 2 min) after washing a stirring bar in the buffer (0.5 ml). This procedure was repeated 1–20 times, and the resulting ppts were dissolved in 1% aq. SDS soln containing Me gallate as an int. standard, and both compound **1** and protein were directly analysed by HPLC [12].

(*ii*) *Estimation of total tannin-binding sites on proteins.* Compound **1** (3.15–15.8 μmol)–MeOH (0.2 ml) was added to a soln of protein (0.073 μmol)–0.2 M McIlvaine buffer (1.4 ml) and incubated at 20° for 1 hr. Compound **1** and protein in the resulting ppts were analysed by the method described above.

(*iii*) *Two-step precipitation.* Galloylglucose (0–2.19 μmol)–MeOH (0.2 ml) was mixed with BSA (0.073 μmol)–0.2 M McIlvaine buffer (1.6 ml) at 20°. After incubation for 1–120 min, BSA (0.292 μmol) and galloylglucose dissolved in the buffer (0.8 ml) were added to the mix. in order to adjust the T/P ratio to 2 (4 and 6 for compounds **2** and **4**, respectively), and

Table 1. Number of strong binding sites, maximum number of binding sites, and content of the strong binding sites for four different proteins

Protein	pH	Number of strong binding sites*	Maximum number of binding sites*	Content of the strong binding sites†
Myoglobin	5.0	4.0	13.9	0.29
BSA	4.0	2.3	11.0	0.21
	7.0	3.3	12.1	0.27
Lysozyme	7.0	1.0	12.8	0.08
Obalbumin	4.0	—	11.5	—

* Based on protein M_r of 10 000.

† Number of the strong binding sites/maximum number of the binding sites.

the resulting mixt. was incubated for another 1 hr. The ppts obtained by centrifugation (3000 rpm, 5 min) were analysed by the method described above.

(*iv*) *Measurement of β-glucosidase activity.* Tannin (0.6 mg) and β-glucosidase (0.036 mg) were pre-incubated in MeOH–0.2 M acetate buffer (1:4, 1.6 ml) at 37° for 0.5, 1, 2, 5, 15, 25, or 40 min. The enzymatic reaction was initiated by the addition of *p*-nitrophenyl β-D-glucoside (1.49 mg)/MeOH–Buffer (0.8 ml) pre-incubated at 37° and monitored by measuring A_{360}.

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