Pergamon

0031-9422(95)00451-3

Phytochemistry, Vol. 40, No. 5, pp. 1503–1505, 1995

0031-9422/95 $9.50 + 0.00

QUANTITATIVE DETERMINATION OF TANNIN AND PROTEIN IN THE PRECIPITATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HARUO KAWAMOTO,* FUMIAKI NAKATSUBO and KOJI MURAKAMI

Faculty of Agriculture, Kyoto University, Sakyo-ku Kyoto 606-01, Japan

(*Received in revised form* 25 *April* 1995)

Key Word Index—Tannin; hydrolysable tannin; protein-precipitation; HPLC; quantitative determination; relative affinity for protein.

Abstract—High performance liquid chromatography was applied for the determination of both tannin and protein in tannin–protein coprecipitates and the relative affinities of a series of galloylglucoses for bovine serum albumin were determined. Relative affinity is affected mainly by the number of the galloyl groups in the galloylglucose molecule with penta > tetra- > tri- ≫ di- ≫ monogalloylglucose. The position of the galloyl group in the glucose core also affects the relative affinity but the effect is smaller than that of the number with 2,3,6- > 2,3,4-tri-*O*-galloylglucose and 4,6- > 2,3-di-*O*-galloylglucose.

INTRODUCTION

A characteristic ability of tannins (i.e. hydrolysable and condensed tannins) widely distributed in plant [1] is their strong interaction with protein followed by precipitation. Tannery [1] and brewery [2] utilize this ability for tanning hide and removal of protein from beverages, respectively. Many tannin properties, such as astringency [3], inhibition of enzymatic activity [4] and anti-fungal activity [5], are also based on this ability. Thus, clarifying the mechanism of tannin–protein interaction is important for understanding the properties of tannins.

Stoichiometric study of tannin–protein coprecipitation is useful to clarify the mechanism. But, it is difficult to determine both tannin and protein in precipitates precisely, due to the lack of a suitable analytical method. Many methods have been used for their quantitative determination, which include turbidimetry [6], Folin–Denis [7], ferric chloride [8], vanillin–HCl [9], *n*-butanol–HCl [10], rhodanin [11] and iodate [12] assays for tannin, and Kjeldahl [13], Lowry [14] and ninhydrin [15] assays for protein. These methods, however, are not specific for tannin or protein. Furthermore, when more than two kinds of tannins or proteins are included in the precipitates, these methods cannot distinguish between structures. Dye- [16] or ^{125}I- [17] labelled protein has been used to distinguish between unlabelled proteins. However, these methods are complex in their procedures and there is no proof that the labelled protein has the same properties as the natural protein.

In this paper, an analytical method for both tannin and protein in the tannin–protein coprecipitates is described using high-performance liquid chromatography (HPLC). Relative affinities of a series of galloylglucoses for protein (bovine serum albumin (BSA)) obtained by competitive precipitation are also presented.

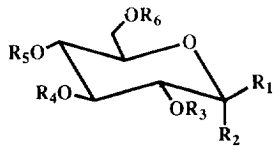
RESULTS AND DISCUSSION

HPLC is an excellent technique for analysis of a mixture which contains several kinds of compounds. Recently this technique has also been applied to biological macromolecules, such as proteins [18]. In particular, reverse-phase is becoming the predominant HPLC method for the separation of peptides and proteins, and the eluent currently employed for this purpose is mostly aqueous trifluoroacetic acid (TFA)–acetonitrile [19]. The mechanism of the separation is considered to be based on the hydrophobic interactions between peptide or protein and the hydrophobic stationary phase [20].

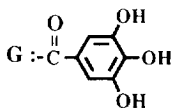
Generally, tannin is known to form soluble complexes with protein even in homogeneous solution [21]. In order to determine tannin and protein independently by HPLC, tannin and protein should not exist as soluble complexes during the analysis. So, first, we confirmed that the galloylglucoses **1**–**7** and BSA do not form soluble complexes under the chromatographic conditions (0.1% aqueous TFA–acetonitrile) generally used for the separation of protein.

After forming the precipitates by mixing galloylglucose **1** (0.5–3 mg in 0.5 ml of 0.2M acetate buffer (pH 4.5)) with BSA (F-V, M_r: 69000, 5 mg in 0.5 ml of buffer), the resulting suspension, including the precipitates, was solubilized again in 1.0% aqueous sodium dodecyl sulphate (SDS) solution with catechol (1 mg ml^{-1}) as an internal standard. The resulting clear solution was

*Author to whom correspondence should be addressed.



	R_1	R_2	R_3	R_4	R_5	R_6
1:	H	OCH_3	G	G	G	G
2:	H	OCH_3	H	H	H	G
3:	H	OCH_3	G	G	H	H
4:	H	OCH_3	H	H	G	G
5:	H	OCH_3	G	G	G	H
6:	H	OCH_3	H	G	G	G
7:	OG	H	G	G	G	G



analysed by HPLC. SDS was used as a detergent for solubilizing galloylglucose–protein precipitates [22].

A chromatogram of the solution is shown in Fig. 1a, together with those of compound **1** (Fig. 1b) and BSA (Fig. 1c). Chromatogram **2a** consisting of three peaks of catechol (3.1 min), compound **1** (7.3 min) and BSA (10.6 min) is a mixed one of **2b** and **2c**; no other peaks are observed. Furthermore, the contents of compound **1** and BSA determined by HPLC are also very close to the amounts used in the experiment (Table 1). These results indicate that compound **1** and BSA are analysed independently without any formation of soluble complexes under the chromatographic conditions. SDS may act by inhibiting the formation of the soluble complexes. Galloylglucoses **1**–**6** were also analysed independently of BSA. Thus, galloylglucose and BSA in the precipitates can be determined independently by HPLC under the chromatographic conditions described.

One of the advantages of the HPLC method is that more than two kinds of tannins or proteins in a mixture can be determined simultaneously. As an example, the relative affinities of galloylglucoses for BSA were determined by obtaining the ratio of galloylglucoses (**2**–**7**) to methyl 2,3,4,6-tetra-*O*-galloyl-α-D-glucoside (**1**) (T(**2**–**7**)/T(**1**) ratio) in the precipitates formed by competitive precipitation with BSA. This ratio is considered to be directly related to the relative affinity of galloylglucoses (**2**–**7**) compared with galloylglucose **1**. The relative affinity of galloylglucose for BSA has been studied at pH 2.2 by equilibrium dialysis [23, 24]. The present method gives the relative affinity from the amount of the galloylglucose directly incorporated in the galloylglucose–BSA precipitates at pH 4.5 (near optimal pH for precipitation). Under such experimental conditions, almost all of the BSA added was precipitated.

Figure 2 shows the relationships between the T(**2**–**7**)/T(**1**) ratio in the precipitates and the T(**2**–**7**)/T(**1**) ratio used for the competitive assay. The T(**2**–**7**)/T(**1**) ratio in the precipitates increases linearly with an increase in T(**2**–**7**)/T(**1**) ratio used for the competitive assay and it is related to the structure of the galloylglucose. Monogalloylglucose, **2**, was not detected in the precipitates even when ten times of compound **2** was used against compound **1**. Relative affinity, defined as the slope of each line, is in the order penta (**7**) (2.3) > tetra (**1**) (1.0) > tri(2,3,6) (**6**) (0.31) > tri(2,3,4) (**5**) (0.16) > di(4,6) (**4**) (0.018) > di(2,3) (**3**) (0.0042) > mono (**2**) (0); the values in the parentheses show the slope. Relative affinity is affected mainly by the number of the galloyl groups with

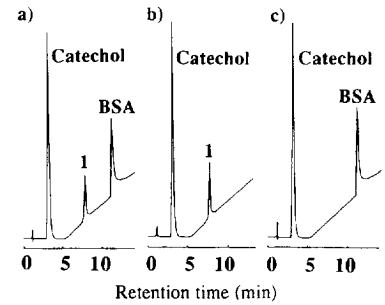


Fig. 1. Chromatograms of galloylglucose **1** and BSA. (a) galloylglucose **1**–BSA complexes dissolved in 0.1% SDS solution; (b) galloylglucose **1**; (c) BSA.

Table 1. Quantitative determination of galloylglucoses **1** and BSA in the suspensions including galloylglucose **1**–BSA precipitates

Experimental conditions (mg)		Measured/used	
Galloylglucose **1**	BSA	Galloylglucoses **1**	BSA
0.5	5.0	0.96	1.04
1.0	5.0	1.06	1.03
2.0	5.0	1.03	1.01
3.0	5.0	0.99	1.03

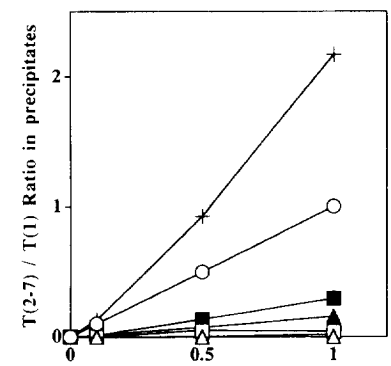


Fig. 2. Relationships between T(**2**–**7**)/T(**1**) ratios in precipitates and T(**2**–**7**)/T(**1**) ratios used in competitive precipitation. ○, compound **1**; △, compound **2**; □, compound **3**; ●, compound **4**; ▲, compound **5**; ■, compound **6**; +, compound **7**.

penta > tetra > tri ≫ di ≫ mono. The galloyl group interacts synergetically with protein and galloylglucoses having less than three galloyl groups shows only very weak affinity for BSA. Position of the galloyl group is also a factor affecting the relative affinity, even if the same number of galloyl groups are present in the molecule, tri(2,3,6) > tri(2,3,4); di(4,6) > di(2,3). Galloyl groups, which are less sterically hindered with each other, can interact with protein more effectively than more hindered ones.

EXPERIMENTAL

Materials. A series of galloylglucoses, methyl 2,3,4,6-tetra-*O*-galloyl-α-D-glucoside (**1**), methyl 6-*O*-galloyl-α-D-glucoside (**2**), methyl 2,3-di-*O*-galloyl-α-D-glucoside (**3**), methyl 4,6-di-*O*-galloyl-α-D-glucoside (**4**), methyl 2,3,4-tri-*O*-galloyl-α-D-glucoside (**5**), methyl 2,3,6-tri-*O*-galloyl-α-D-glucoside (**6**) and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucoside (**7**), were prepd by galloylation of glucose derivatives using tri-*O*-benzylgalloyl chloride and subsequent debenzylation. BSA was purchased from Sigma. SDS, MeCN, TFA and catechol were purchased from Nakarai tesk Co. Ltd.

HPLC analysis. Galloylglucose–BSA ppts were washed with 0.2M acetate buffer (pH 4.5, 1 ml) and dissolved in 1% aq. SDS soln containing catechol (1 mg ml^{-1}). The resulting clear soln was analysed by HPLC. HPLC was carried out using the following conditions. Column: COSMOSIL C_{18} reverse-phase column; eluent: 0.1% aq. TFA/0.1% TFA in MeCN (4:1 to 2:3, 10 min); flow rate: 1 ml min^{-1}; detector: UV 220 nm; retention time: catechol (3.1 min), BSA (10.6 min), **1** (7.3 min), **2** (1.4 min), **3** (1.8 min), **4** (2.1 min), **5** (5.4 min), **6** (6.5 min) and **7** (5.3 min).

Competitive precipitation of galloylglucoses for BSA. A mixt. of tetragalloylglucose **1** (3 mg, 3.74 μmol) and galloylglucose (**2**–**7**) (0.129–35.6 mg, 0.374–37.4 μmol) in 0.8 ml of 0.2 M acetate buffer (pH 4.5) were mixed with BSA (5 mg/0.8 ml of buffer) at 20° for 1 hr. After sepn of ppts by centrifugation (3000 rpm, 10 min), the two galloylglucoses and BSA were determined by HPLC.

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