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Interactions of gallotannins with proteins, amino acids, phospholipids and sugars

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Abstract

Gallotannins play contrasting roles in food quality. They exhibit strong antioxidative and antibacterial properties, and at the same time show certain antinutritional effects. To explore this possible effect of gallotannins, the interaction mechanism between gallotannins and typical food components was investigated. The molecular structure of gallotannins and their interactions with amino acids (glycine, alanine, proline and leucine) were first studied. It is proved that galloyl groups of gallotannins are hydrophobic sites and that these groups can interact with aliphatic side chains of amino acids through hydrophobic association. Further, the binding of gallotannins (1,2,6-tri-*O*-galloyl-D-glucose (TGG) and 1,2,3,4,6-penta-*O*-galloyl-D-glucose (PGG)) to typical proteins, phospholipids and sugars was examined quantitatively. It is indicated that gallotannins bound more to proteins (histone, bovine serum albumin, casein and gelatin) and phospholipids (L-α-lecithin, L-α-cephalin and sphingomyelin) than to sugars, and that PGG had stronger binding affinity to proteins, phospholipids and sugars than did TGG. The gallotannin–protein and gallotannin–phospholipid interactions were the result of cooperative effects of hydrogen bonding and hydrophobic association, and hydrogen bonding was the predominant effect in the interactions between gallotannins and sugars.

Keywords: Gallotannins; Binding; Food component; Hydrophobic association

1. Introduction

Gallotannins (see Fig. 1) are naturally occurring phenolic compounds, which primarily consist of a glucose core esterified with gallic acid (GA) or its derivatives. They are mainly obtained by isolation from Chinese gallnuts, and are also found existing in many fruits and vegetables, such as grape, strawberry, raspberry, pecan, etc. Over the last decades, gallotannins have exhibited contrasting properties in foods. Their antioxidative and antibacterial activities have been described in several reports (Hong, Wang, Huang, & Hsu, 1995; Otake, Makimura, Kuroki, Nishihara, & Hirasawa, 1991; Salah, Miller, & Paganya, 1995; Scalbert, 1991), showing good prospects for use as antioxidant and preservatives in food processing. For example, 1,2,3,4,6-penta-*O*-galloyl-D-glucose (PGG) shows strong antibacterial effects at lower content (<0.5 g/l) under which 100% inhibition is observed on many bacteria, such as *Bacillus subtilis*, *Streptococcus cremoris*, *Staphylococcus aureaus* and *Bacillus thuringiensis* (Shi & Di, 2000). In addition, the antioxidative effects of gallotannins, BHA and BHT on lipid peroxidation were elucidated by (Kimura, Okuka, & Okuda, 1984). They found that the antioxidative abilities followed the sequence: PGG > TeGG > TGG > DiGG > GA > BHA ≈ BHT, suggesting a possible replacement of synthetic antioxidants BHA and BHT by gallotannins, which are natural products, in food processing.

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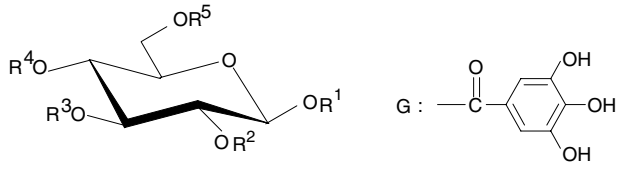

Fig. 1. The structural model of gallotannins [DiGG: two of R^1-R^5 are galloyl group (G); TGG: three of R^1-R^5 are G; TeGG: four of R^1-R^5 are G; PGG: R^1-R^5 are G].

On the other hand, gallotannins figure importantly in protein precipitation, enzyme inhibition and metal chelation through forming various complexes (Baxter, Lilley, Haslam, & Williamson, 1997; Petersen & Hill, 1991; Salvador, Erdman, & Sherman, 1990; Shi, He, & Haslam, 1994). Further studies (Takechi & Tanaka, 1987; Cartriona, Cai, Russell, & Haslam, 1988; Mortin, Lilley, & Bailey, 1986) reported that similar complexes could be formed between gallotannins and many food-derived proteins, polysaccharides and alkaloids. Such complexes might result in a decrease in bio-conversion, enzyme deactivation, and in shortages of some microelements in human body. All of these are responsible for the antinutritional effect of gallotannins in food processing (He, Yao, & Shi, 2001; Weder & Telek, 1997). To minimize this effect and make full use of gallotannins in the food industry, knowledge of the interaction mechanisms between gallotannins and food components is desirable. Although the occurrence of gallotannins in human foods is restricted so far, mainly involving ellagitannins and gallagitannins (Clifford & Scalbert, 2000; Cerda, Ceron, Tomas-Barberan, & Espin, 2003; Cerda, Llorach, Ceron, Espin, & Tomas-Barberan, 2003), the results of this work should be significant nevertheless with reference to human foods.

Some studies (Hagerman & Butler, 1978; Petersen & Hill, 1991; Shi et al., 1994; Takechi & Tanaka, 1987) describing the binding of gallotannins to proteins and polysaccharides have been reported. A recognition that hydrogen bonds participate in most interactions between gallotannins and food components was accepted by researchers, and the binding sites were explained by a further study (Cartriona et al., 1988). In addition, it was reported that PGG appeared to bear a stronger ability to interact with basic proteins and lipids than those with acidic or neutral compositions, and that ionic bonding appeared to participate in the PGG binding (Takechi & Tanaka, 1987). However, some other observations in these studies can not be explained either by hydrogen or ionic bond theory. For example, compared with 1,2,6-tri-*O*-galloyl-D-glucose (TGG), PGG has a lower solubility, but it exhibits a stronger association with food components in aqueous solutions. In addition, some proteins containing more aromatic groups or hydrocarbon side chains have strong association with gallotannins. Therefore, as one important mode of chemical interaction, hydrophobic association between gallotannins and typical food components is investigated in this paper.

2. Materials and methods

2.1. General methods

3,6-Di-*O*-, 1,2,6-tri-*O*-, 2,3,4,6-tetra-*O*- and 1,2,3,4,6-penta-*O*-galloyl-D-glucose (DiGG, TGG, TeGG and PGG) were available from previous work (He, Shi, Yao, Luo, & Ma, 2001; Shi & He, 1993). Glycine, alanine, proline and leucine were purchased from Mingzhu Chemical Co. (Shanghai, China). Proteins, phospholipids and sugars tested were from Sigma (St. Louis, MO). Other chemicals were of the highest purity available. Centrifugation was performed at room temperature on a preparative MSE centrifuge (Model GF-8, 1400–10,000 rpm). The residual free PGG or TGG in the supernatant was separated by chromatography on a column of Sephadex LH-20 using 9:1 EtOH–water as eluant. The eluted compounds were concentrated on a rotary evaporator under reduced pressure at 30–35 °C. TLC analyses were carried out on DC: Alutolien Cellulose F plates that were developed with solvent systems: (A) 6:94 AcOH–water, and (B) 14:1:5 *n*-butanol–AcOH–water. Fluorescent spots on TLC plates were detected by UV light (260–300 nm).

2.2. Solubilities of gallotannins

Gallotannins (DiGG, TGG, TeGG and PGG, 40 mg for each) were put into 10 ml volumetric flasks individually and distilled water was added to scale. Saturated solutions were obtained after samples were shaken for 24 h at 22 °C since there still was insoluble part of gallotannin at the bottom of every sample. 2.0 ml of saturated solutions were taken and diluted to the extent suitable for UV spectrophotometric measurement. The absorbance values of diluted solutions were determined at 275 nm. The concentrations of saturated solutions, that is, the solubilities of gallotannins were obtained by calculations based on dilution times and the standard curves of four gallotannins. Amino acid solutions with different concentrations were prepared with distilled water and solubilities of PGG in the solutions were determined by the method similar to experiment above.

2.3. Binding assay to food compositions

5.0 ml of PGG (or TGG) solution (0.2 mg/ml 9:1 EtOH–water) was mixed with each 5.0 ml of 9:1 EtOH–water in which the testing substance content was 1.0 mg/ml, and shaken at 30 °C for 20 h. Then the incubation mixtures were centrifugated at 2000 rpm

for 15 min. For each sample, the supernatant was applied to a Sephadex LH-20 column (20 × 2 cm i.d.) equilibrated with 9:1 EtOH–water. The elution was carried out with 9:1 EtOH–water at a flow rate of 0.50 ml/min at room temperature. Based on TLC analysis (PGG R_f 0.10 (A), R_f 0.45 (B); TGG R_f 0.13 (A), R_f 0.40 (B)), the fractions containing free PGG (or TGG) were collected and the content was measured by UV determination. The amount of bound PGG (or TGG) was obtained by subtracting the amount of free PGG (or TGG) from that of original PGG (or TGG). The binding affinity of gallotannins to food compositions was expressed by PBP (percentage of bound PGG) and PBT (percentage of bound TGG) values.

3. Results and discussion

3.1. Hydrophobicity of galloyl groups of gallotannins

Solubility experiments were designed to explore the hydrophobicity of the galloyl groups of gallotannins. In a previous work (Shi et al., 1994), the UV spectra of 3,6-di-*O*-, 1,2,6-tri-*O*-, 2,3,4,6-tetra-*O*- and 1,2,3,4,6-penta-*O*-galloyl-D-glucose (DiGG, TGG, TeGG and PGG) in aqueous solution were recorded, respectively. The data indicated that these species had nearly identical spectra, with $\lambda_{max\ 1}$ around 215 nm and $\lambda_{max\ 2}$ around 275 nm. In a UV analysis of interactions between gallotannins and food components, the absorbance of the reaction solution was measured at 275 nm where food components had very little absorbance. Based on this knowledge, the solubilities of the four gallotannins (DiGG, TGG, TeGG and PGG) in aqueous solution were determined by the spectrophotometer at 275 nm and the results are shown in Table 1. The solubility of DiGG, TGG, TeGG and PGG in aqueous solution is 40, 18, 12 and 10×10^{-4} M, respectively. It could be deduced that the more galloyl groups the gallotannin contains, the lower solubility it has. These observations are consistent with the conclusion that the galloyl group is a hydrophobic site of gallotannins.

To explore the hydrophobic association of gallotannins with food components, model interactions between PGG and four amino acids (glycine, alanine, proline and leucine) containing different aliphatic side chains were investigated. Fig. 2 shows the change of solubility (S) of PGG against different amino acid concentration

Table 1
Solubility of gallotannins in aqueous solution (measurements were made at 22 °C)

Gallotannin	DiGG	TGG	TeGG	PGG
Number of galloyl group	2	3	4	5
Solubility ($\times 10^{-4}$ M)	40	18	12	10

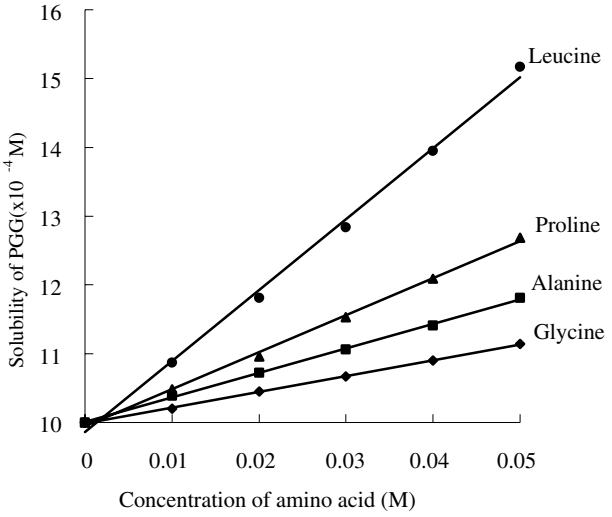

Fig. 2. Influence of amino acids on solubility of PGG (curve equation is as follows: Glycine, $S = 29.914C + 0.987$, $R^2 = 0.9994$; Alanine, $S = 35.571C + 10.009$, $R^2 = 0.9991$; Proline, $S = 53.857C + 9.945$, $R^2 = 0.9979$; Leucine, $S = 103.2C + 9.860$, $R^2 = 0.9962$; measurements were made at 22 °C).

(C), and each curve reflects that a linear trend is followed when the concentration of amino acid is less than 0.05 M. It is obvious that the solubility of PGG is increased in each amino acid solution as compared with that in aqueous solution ($S = 10$, see Table 1). In the case of leucine, the solubility of PGG is up to 15.2×10^{-4} M when the concentration of leucine is 0.05 M, an increase of more than 50%. Therefore, it could be deduced that aliphatic side chains of amino acids could associate with hydrophobic groups (e.g., the galloyl group) of PGG through hydrophobic association, while polar groups (carboxylic and amino groups) of amino acids are well dissolved in water, thereby leading to the increase of solubility of PGG. The higher concentration of each amino acid raises the likelihood of forming hydrophobic associations with PGG, which, in turn, increases the solubility of PGG. On the other hand, it is indicated in Fig. 2 that the slope of the curve is directly connected with the ability of amino acids to promote solubility of PGG. For glycine, alanine, proline and leucine, the carbon number of the aliphatic side chain is 1,2,4 and 5, respectively, and their ability to promote solubility of PGG follows the sequence:

glycine < alanine < proline < leucine

Therefore, it is reasonable to draw the conclusion that the abilities of amino acids to form hydrophobic association with PGG are proportional to the number of aliphatic carbons in their side chain. Leucine contains the largest aliphatic group among the amino acids tested, so it could effectively interact with the galloyl groups of PGG through the formation of hydrophobic association so as to most remarkably increase the

solubility of PGG. Glycine has only one aliphatic carbon in its side chain, so it has the weakest ability to promote solubility of PGG. Keeping in mind that amino acids are the fundamental components of proteins, these results above permit us to obtain more information about gallotannin–protein interactions. That is, the hydrophobic association between hydrophobic groups of gallotannin and hydrophobic regions of proteins should be one of the important ways of strengthening gallotannin–protein interactions. For further understanding of the association of gallotannin with food components, the interactions between gallotannins (PGG and TGG) and proteins (α-amylase, pepsin, histone, bovine serum albumin BSA, casein and gelatin), phospholipids (L-α-lecithin, L-α-cephalin and sphingomyelin) and sugars (glucose, maltose, maltotetraose, maltohexaose and starch) were explored.

3.2. Interaction of gallotannis with proteins, phospholipids and sugars

The general procedure for studying the interactions of gallotannins with food components, such as proteins, phospholipids and sugars, was to mix solutions of the two substances for a certain time. After the elapsed time, the sample was centrifuged and the supernatant was purified by Sephadex LH-20 to obtain a solution containing the free gallotannin. The gallotannin content in the eluent was measured by the spectrophotometric method. The binding affinities of gallotannins to protein, phospholipid and sugar were calculated based on the amount of gallotannin bound to these components, expressed as percentage of bound PGG (PBP) and percentage of bound TGG (PBT). It was observed in experiment that when PGG (or TGG) react with histone there was little precipitate after centrifugation, suggesting that the binding affinity of PGG (or TGG) to histone is very weak. However, the PBP and PBT values of histone were as high as that of BSA, which formed much precipitate with PGG and TGG. Thus the amount of precipitate is only related to the solubility of the complex formed and not to the binding affinity. Therefore, the gallotannin binding affinity cannot be determined by the amount of precipitate or by direct spectrophotometric analysis of the supernatant for the reason that the formed complex might be soluble in the supernatant, although the two methods were conventionally used by previous researchers. Indeed, the procedure employed in this research is closer to the fact.

As shown in Table 2, both PGG and TGG display different binding affinities to food components. From the data obtained, it appears that the binding affinity of gallotannins to proteins does not depend on the molecular weight of the proteins, and that histone, casein, BSA and gelatin bind to PGG and TGG with stronger affinity than doα-amylase and pepsin. The reason is

Table 2
PBP and PBT values of proteins, phospholipids and sugars (the mixture of gallotannin and testing substance was first shaken at 30 °C for 20 h and then used for analysis)

Substance	Molecular weight ($\times 10^3$)	PBP (%, w/w)	PBT (%, w/w)
Proteins			
α-amylase	97	47	34
Pepsin	34	30	19
Histone	15	78	58
BSA	69	80	59
Casein	>100	82	62
Gelatin	100	87	65
Phospholipids			
L-α-lecithin	0.83	77	59
L-α-cephalin	0.79	69	55
Sphingomyelin	0.75	66	49
Sugars			
Glucose	0.18	8	6
Maltose	0.34	11	8
Maltotetraose	0.67	19	12
Maltohexaose	0.99	30	19
starch	100	41	30

that in the polypetide chains of histone, casein and gelatin there are more amino acid residues containing aromatic groups and aliphatic side chains. For example, casein and gelatin contain in weight 16.8% and 20% of prolyl residues, respectively (Takechi & Tanaka, 1987). With the recognition that hydrophobic association is one mode of gallotannin–protein interaction, such amino acid residues can form certain hydrophobic environment in the solution and lead to a stronger association with PGG and TGG. BSA has only 4% of prolyl residues (Takechi & Tanaka, 1987), but it has PBP and PBT values similar to that of casein which contains 16.8% (Takechi & Tanaka, 1987) of prolyl residues. The possible reason is that in the gallotannin–protein interaction hydrogen bonds between phenolic groups of gallotannins and polar groups (guanidine, amide, peptide, amino and carboxyl groups) of proteins play an important role. The conclusion could be drawn that the stronger binding affinity of BSA to gallotannins is mainly due to hydrogen bonds rather than hydrophobic association.

Both phospholipid and PGG appear to have very low solubility in 9:1 EtOH–water solution, implying that their ability to form hydrogen bonds is limited. This will result in weaker hydrogen bond interaction between the phenolic groups of PGG and the carbonyl and phosphorous groups of phospholipids. However, all the phospholipids tested have higher PBP values as shown in Table 2, reflecting strong interactions between phospholipids and PGG. Therefore, it could be confirmed that hydrophobic association between the galloyl groups of the gallotannins and the hydrophobic hydrocarbon chains of phospholipids takes part in the gallotannin–phospholipid interaction, and that the stronger binding

affinity of phospholipids to PGG mainly results from hydrophobic association but not from hydrogen bonds.

Sugars tested show higher solubility in 9:1 EtOH–water solution, since they contain many hydroxyl groups which can participate in their interaction with gallotannins through hydrogen bonds. It is shown in Table 2 that the binding affinities of sugars are generally lower than those of proteins and phospholipids. This could be explained if hydrogen bonding between hydroxyl groups of sugars and phenolic residues of gallotannins governs the association of sugars with gallotannins. The sugars with higher degree of polymerization exhibit stronger binding affinity because they possess more hydroxyl groups to form hydrogen bonds with PGG and TGG. On the other hand, PGG has more phenolic residues, so it can form more hydrogen bonds with sugars and shows higher binding affinity to sugars than does TGG.

So, gallotannin–protein and gallotannin–phospholipid interactions appear to be the result of cooperative effects of hydrogen bond and hydrophobic association. However, hydrogen bonding is the predominant mode in the interaction between gallotannins and sugars. PGG and TGG generally show stronger binding affinity to proteins and phospholipids than to sugars. PGG appears to have a stronger capacity to interact with proteins, phospholipids and sugars than does TGG, because the former contains more galloyl groups and phenolic groups, which are responsible for the formation of hydrophobic associations and hydrogen bonds, respectively.

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