ARTICLES

BSA-Bound Persimmon Tannin Interactions with Other Proteins†

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The binding of persimmon tannin to immobilized bovine serum albumin (BSA) occurs at a much lower rate than to soluble proteins, while the precipitation of a second protein, e.g., alkaline phosphatase (APase), on BSA-bound tannin occurs at a normal rate. Tannin was also found to bind nonselectively to a polystyrene plate but surprisingly to a greater extent than to immobilized BSA. Although a minimum amount of APase binds to bound tannin, it exhibits very high activity, as demonstrated by a substantial increase of its turnover number upon its binding to the tannin. This observation seems to indicate a dramatic change in enzyme conformation, especially at the active site region. APase showed higher binding to bound tannin than BSA and was able to release it from tannin. The three-layer assemblage formed on the polystyrene plate surface is proposed as a simple model to study bezoars.

High concentrations of condensed tannins have been detected in certain fruits, including astringent cultivars of the persimmon (*Diospyros kaki* L.) (Ito, 1980; Lea, 1990). The ability of polyphenols to precipitate proteins was extensively exploited in numerous studies, e.g., to determine the tannin content of different plants (Bate-Smith, 1973; Gustavo, 1956), to investigate the influence of the nature of proteins on their rates of binding (Hagerman and Butler, 1980; McManus et al., 1980), and to inhibit enzymes (Goldstein and Swain, 1965; Mole and Waterman, 1987). However, little is known about the physical and structural features of the precipitated tannin–protein complex.

Recently, we reported a new method for tannin determination (Ittah, 1991), using a 96-well polystyrene plate. This very sensitive titration takes advantage of the multiple hydroxyl groups of tannin which can associate with more than one ligand, e.g., proteins. The method is based on the building, step by step, of an immobilized three-layer assemblage on the polystyrene plate: BSA, tannin, and alkaline phosphatase, the last being assayed with *p*-nitrophenyl phosphate (Bessey et al., 1946). The initial rates of the enzymatic reaction, and therefore the content of bound enzyme on the plate, were found to be proportional to the amount of tannin to be determined. This direct relation between bound enzyme and tannin concentration suggests the existence of a well-defined structure of this three-layer assemblage. In addition, the observation that the activity of this particular enzyme appeared to be unaltered upon its binding to the tannin was quite intriguing, pointing to the possibility that the active site had retained its quasi-original conformation.

Such a system, consisting of alternating layers of tannin and protein, might be formed in the stomachs of patients, initiating the buildup of bezoars. Theoretically, immobilization of soluble tannin on protein, e.g., from a food source, on the one hand, and its binding to another type of protein, e.g., digestive enzymes, on the other hand, can occur. In this perspective, the three-layer assemblage formed on the polystyrene plate surface can serve as a simple model to study factors influencing the formation of bezoars (Budd and McCreary, 1980; Sanderson et al., 1971).

The main objectives of this research were to study in depth some parameters that might affect the formation of the three-layered assemblage of BSA, tannin, and APase on the polystyrene plate and its behavior under various conditions.

MATERIALS AND METHODS

Materials. All chemicals were of reagent grade. Bovine serum albumin (BSA, fraction V powder) and *p*-nitrophenyl phosphate (*p*-NPP) were obtained from Sigma Chemical Co. (St. Louis, MO). Mammalian alkaline phosphatase (APase) was purchased from Rad Chemicals Co. (Nes-Ziona, Israel). Soluble tannin from persimmons (*D. kaki* L., var. Triumph) was purified as described elsewhere (Matsuo and Ito, 1980). Unprecoated polystyrene microtiter plates (96 wells, maxisorb, Catalog No. 442404), specially recommended for assays where proteins are bound to the plastic, were purchased from Nunc (Roskilde, Denmark) and read on an EIA reader 400 ATC (Salzburg, Austria).

Enzyme Assays. *1. General Procedure for Enzyme Assay (Mode 0).* Microplates were coated overnight (at 4 °C) with BSA (at variable concentrations, depending on the test performed) (200 μL) in sodium carbonate buffer (pH 9.6) and washed [all washings were with PBS (pH 7.4) after every incubation] twice; aqueous tannin (at variable concentrations, depending on the test performed) solution (100 μL) was added. After a 1-h incubation (all incubations were at ambient temperature, unless otherwise specified), the plates were washed twice, and APase (at variable concentrations, depending on the test performed) (100 μL) in phosphate-buffered saline (PBS, pH 7.4) was added. After a 1-h incubation at ambient temperature, the plates were washed twice and bound phosphatase was determined with *p*-NPP (100 μL, 0.5 mg/mL) in diethanolamine buffer (pH 9.4) at 405 nm after 15 min.

2. Effect of BSA Concentration (Used for Coating) on Tannin Binding. The microplate was divided into four compartments, each one coated with BSA (200 μL) at a different concentration (0.2%, 0.1%, 0.05%, and 0.0%). Serially diluted persimmon tannin solutions in PBS (pH 7.4) (from 1 to 20 μg/mL) were distributed into wells, and the assay was performed as above.

3. Effect of APase Binding Time on Its Activity. The coated plate (0.2% BSA), treated with serially diluted persimmon tannin solutions in PBS (pH 7.4) (from 1 to 12 μg/mL), was divided into

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four compartments, in each of which APase (0.8 unit/mL) was applied for a different period of time (15, 30, 45, and 60 min) and the assay performed as above.

4. Effect of Tannin Binding Time on APase Activity. The coated plate (0.2% BSA) was divided into five compartments, in each of which a serially diluted persimmon tannin solution in PBS (pH 7.4) (from 1 to 12 μg/mL) was applied for a different period of time (15, 30, 45, 60, and 90 min) and the assay performed as above.

5. Assay for Reused APase. The coated plate (0.2% BSA), treated with serially diluted persimmon tannin solutions in PBS (pH 7.4) (frm 5 to 40 μg/mL), was divided into four compartments. APase (0.8 unit/mL) was added to the first compartment. After a 1-h incubation, the enzyme, instead of being washed away, was transferred to the corresponding wells (bearing the same tannin concentration) of the second compartment. This process was repeated for the last two compartments. The assay was then conducted as usual.

6. Assay for Apparent Turnover Number. The coated plate (0.2% BSA) was divided into two compartments. Persimmon tannin solution (10 μg/mL) in PBS (pH 7.4) was added to the first compartment. After a 1-h incubation, the plate was washed and APase (0.8 unit/mL) was allowed to bind for 1 h to the tannin as usual. After the plate was washed, APase (0.8 unit/mL) was added to the second compartment and both free and bound enzyme were assayed with *p*-NPP in various concentrations.

7. Competition between APase and BSA for the Tannin. This was investigated under three different modes.

a. Mode 1. The coated plate (0.2% BSA) was treated with persimmon tannin solutions (8 μg/mL) followed by APase (MW ~ 150 000) (1 unit/mL or 0.4 μg/mL), as usual. After a 1-h incubation and washings, BSA (MW = 69 000) (100 μL) at different concentrations (0.2, 2, and 20 μg/mL) was distributed into wells. After a 1-h incubation, the assay was performed as usual.

b. Mode 2. The coated plate (0.2% BSA) was treated with persimmon tannin solutions (8 μg/mL) as in mode 1 but followed by BSA (100 μL) at different concentrations (0.2, 2, and 20 μg/mL). After a 1-h incubation and washings, APase (1 unit/mL or 0.4 μg/mL) was added. The assay was conducted as usual.

c. Mode 3. Persimmon tannin solutions (8 μg/mL) and BSA (100 μL) at different concentrations (0.2, 2, and 20 μg/mL) were mixed just before addition to the coated plate (0.2% BSA). After a 1-h incubation and washings, APase (1 unit/mL or 0.4 μg/mL) was added. The assay was conducted as usual.

RESULTS AND DISCUSSION

The immobilization of BSA on the polystyrene microplate takes place probably via its hydrophobic residues and appears to affect substantially its tertiary structure. The formation of hydrophobic pockets (Gaffney et al., 1986), essential for the binding of tannin molecules, seems to be reduced. Furthermore, this immobilization seems to have a profound effect on the flexibility of BSA conformation: The protein cannot readily bind the polyphenol molecules, as occurs in solution. Interaction with the immobilized BSA, therefore, becomes rather limited and time-consuming. The extent of binding of persimmon tannin was found to vary with time, reaching its maximum value around 1 h. For comparison, all known methods of tannin determination based on quantitative precipitation of protein–tannin complex from solution are completed within a few minutes (Hagerman and Butler, 1978). A binding profile of tannin with immobilized BSA, as expressed by the activity of APase bound to it, at different periods of time is shown in Figure 1. Tannins at concentrations from 2 to 6 μg/mL bind very poorly to immobilized BSA. As time progresses, more tannin molecules are bound to immobilized protein.

The concentration of BSA (0.2%) used for coating the microplates is high enough to give a dense monolayer to prevent any undesirable nonselective binding of proteinic

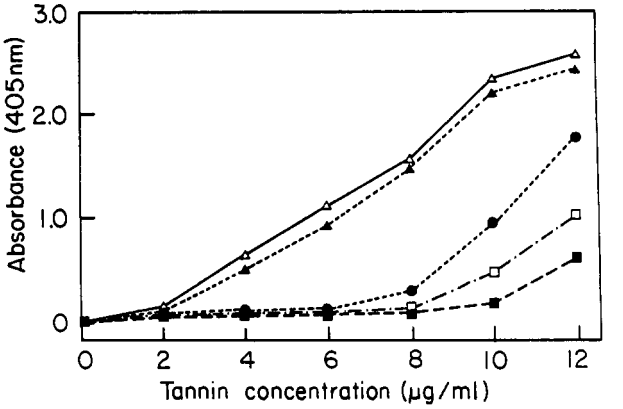


Figure 1. Alkaline phosphatase activity (after 20 min) vs concentration of tannin. Assays were performed after different binding periods of the persimmon tannin on immobilized bovine serum albumin, as described under Materials and Methods: (△) 90 min; (▲) 60 min; (●) 45 min; (□) 30 min; (■) 15 min. Tannin was serially diluted starting with 12 μg/mL in phosphate-buffered saline.

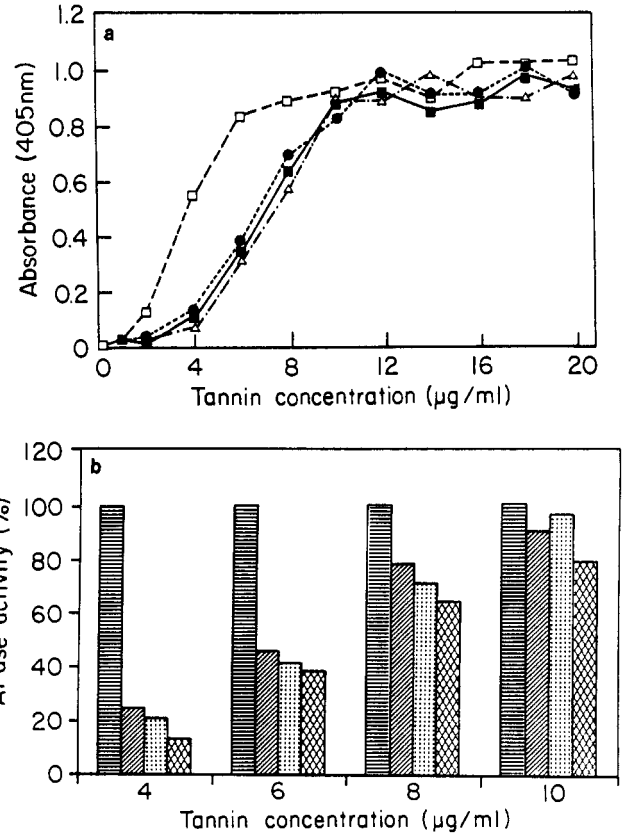


Figure 2. (a) Alkaline phosphatase activity (after 8 min) vs concentration of tannin. Assays were performed on a polystyrene plate coated with BSA at different concentrations, as described under Materials and Methods: (□) 0.0%; (●) 0.05%; (■) 0.1%; (△) 0.2%. (b) Diagram showing the percent of alkaline phosphatase activity vs coating bovine serum albumin concentrations: (horizontally lined bar) 0.0%; (diagonally lined bar) 0.05%; (dotted bar) 0.1%; (cross-hatched bar) 0.2%. Tannin was serially diluted starting with 20 μg/mL in phosphate-buffered saline.

species. We investigated the binding of tannin to BSA using various concentrations of the latter to coat the microplate. The results show no significant differences in the degree of binding within the range of concentrations tested. With no coating (0.0% BSA), the binding was surprisingly higher. A profile of this binding in shown in Figure 2a. In the absence of immobilized BSA, the tannin appears to bind in a nonselective way via hydrophobic

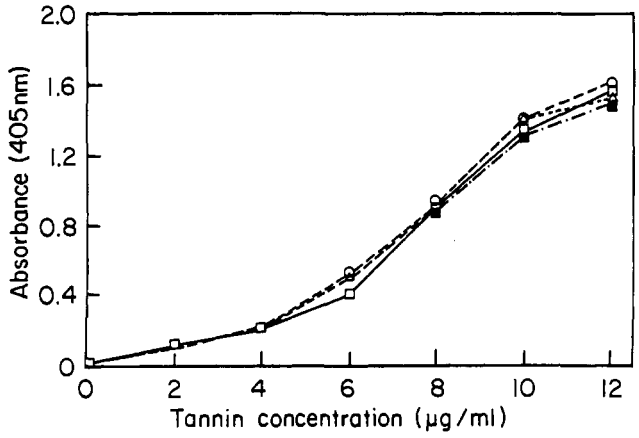


Figure 3. Alkaline phosphatase activity (after 12 min) vs concentration of tannin. Assays were performed after different binding periods of the enzyme to immobilized persimmon tannin, as described under Materials and Methods: (○) 60 min; (□) 45 min; (△) 30 min; (■) 15 min. Tannin was serially diluted starting with 12 μg/mL in phosphate-buffered saline.

forces, with possible formation of a π complex between the phenyl ring of the polyphenol and the aromatic backbone of the polystyrene. Another possibility is that the less dense the plate is with BSA, the more nonselective binding occurs, compensating for the decreasing number of binding sites on the protein. Without immobilized BSA, a saturation level of binding is reached for lower values of added tannin. As the amount of added tannin increases, the discrepancy in binding is reduced, as illustrated by the diagram in Figure 2b. We intend to study the effect of ionic strength and pH on this binding to elucidate its nature (whether hydrogen bonding or some other interaction is involved).

After the tannin has bound to immobilized BSA, it still possesses free hydroxyl groups which can bind another ligand, such as APase. The rate of this additional binding was studied by assaying the bound enzyme at different periods of time. A profile of time-dependent binding is presented in Figure 3. It appears that complete binding of APase to the bound tannin is rapid and resembles, in all aspects, the precipitation of proteins by tannins in solution.

Since the total amount of APase added in the assays is already very low (1 unit/mL or 0.4 μg/mL or 40 ng/well), the fraction of enzyme that remains bound to the tannin, after the washings, should be extremely small. To prove this point, we performed a simple experiment in which the enzyme solution was reused after the initial binding. The results, as shown in Figure 4, suggest that only some of the applied APase molecules are bound to the tannin and those that are not bound can be transferred and thus bind to tannin in the other wells, and so on, until free APase is totally exhausted. Therefore, the amount of enzyme remaining in each well after the washings (bound APase) is minimal by comparison with that contained in the initial aliquot. The enzyme was assayed for its concentration, before and after the binding, and no difference could be detected by conventional methods. However, a comparison of quantitative activities between APase solution (100 μL) at initial concentration vs bound APase revealed that about the same amount of substrate was hydrolyzed during the same period of time (5 min). The meaning of this observation is that probably the turnover number of APase (although apparent and not calculated) increased dramatically upon its binding to tannin (Figure 5). This result indicates that not only was

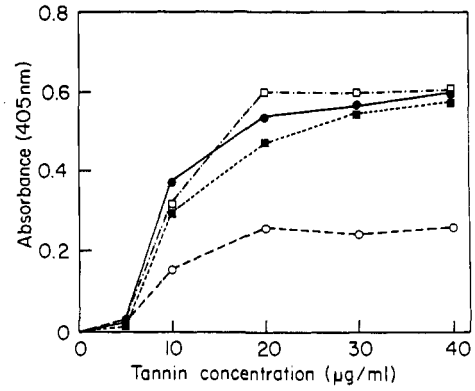


Figure 4. Alkaline phosphatase activity (after 5 min) vs concentration of tannin. Tannin was serially diluted starting with 50 μg/mL in phosphate-buffered saline. Alkaline phosphatase was reused as described under Materials and Methods: (●) first cycle; (□) second cycle; (■) third cycle; (○) fourth cycle.

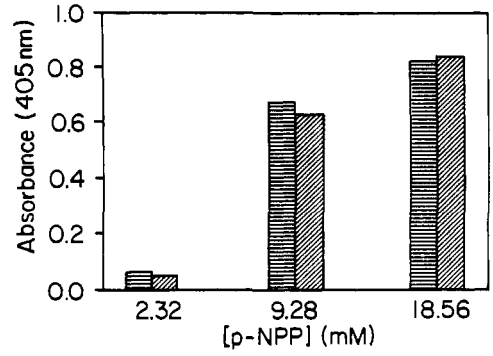
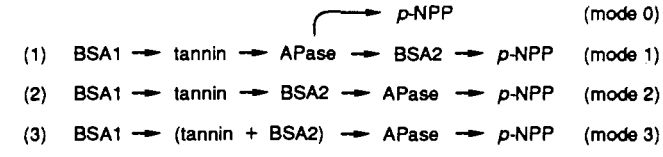


Figure 5. Alkaline phosphatase activity (after 5 min) vs concentrations of *p*-nitrophenyl phosphate in diethanolamine buffer (pH 9.4). Assays were performed with free enzyme (horizontally lined bars) and with bound enzyme (diagonally lined bars) as described under Materials and Methods.

the activity of the enzyme not lowered as generally expected from a bound enzyme but it actually increased. This could be due to a change in conformation of the enzyme in such a way that its active site became more easily accessible to the substrate. In studies dealing with interactions between tannin and enzymes (Mole and Waterman, 1987), emphasis was always put on the degree and nature of inhibition as reflected by the activity of the enzyme remaining in solution. No insight into the nature of the tannin-bound enzyme was ever reported. However, in a study of somewhat different character (McCracken and Meighen, 1979), immobilized *Escherichia coli* APase on Sepharose CL-4B (using cyanogen bromide) was reported to exhibit activity lower than or, at most, equal to that of free APase.

The competition between APase and another protein, e.g., BSA, was studied with three different sequential additions of the components to the wells of the microplate: In each mode, the ratio of BSA to APase concentrations



was varied from 1 to 100. A diagram summarizing the results of the mutual displacements of the two proteins is given in Figure 6. BSA seems not able to displace bound APase (mode 1), even at concentrations 10 times higher. APase activity remains practically the same as in the

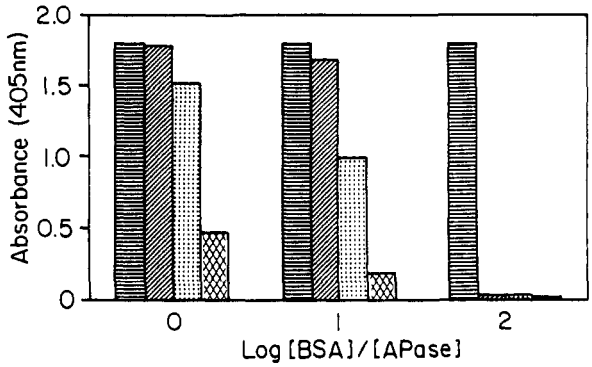


Figure 6. Alkaline phosphatase activity (after 15 min) vs the logarithm of ratio [BSA]/[alkaline phosphatase]. Assays were performed with enzyme bound to tannin (10 μg/mL) under different modes: (horizontally lined bars) mode 0; (diagonally lined bars) mode 1; (dotted bars) mode 2; (cross-hatched bars) mode 3, as described under Materials and Methods.

absence of additional BSA (mode 0). On the other hand, tannin-bound BSA was displaced easily by APase (mode 2): over 90% at a 1:1 ratio and approximately 60% at a 1:10 ratio. However, when given the possibility (mode 3), the tannin binds much more easily to soluble BSA than to immobilized BSA, and even at a BSA/APase ratio of 1, APase activity was dramatically reduced to about 25% of its original value. However, at a BSA/APase ratio of 100, under mode 1 conditions, BSA displaces completely the bound enzyme, and under mode 2 conditions it seems to bring the tannin into solution and to precipitate it, leaving no bound tannin with which the APase can interact. These results illustrate clearly the marked differences in degree of binding of various proteins to tannins. They also support the above observation that the precipitation of tannin is much faster with soluble than with immobilized protein.

This study suggests that the interactions between the tannin and the protein within the tannin–protein complex are dynamic. It is not just an insoluble complex which precipitates out, it can be constantly remolded. In the presence of an large excess of one ligand, e.g., BSA, it appears possible to release the already bound one. With regard to this last point, we believe that further research will lead to a specific, highly effective at low concentration, and nontoxic drug able ultimately to dissolve bezoars.

We are currently studying the kinetics of tannin-bound enzymes, with emphasis on proteases. Meanwhile, preliminary observations on alkaline phosphatase have shown no noticeable alteration in its biochemical parameters.

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