Kinetics of Alkaline Phosphatase Immobilized, via Persimmon Tannin, on a 96-Well Polystyrene Microplate†

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The kinetics of *p*-nitrophenyl phosphate (*p*-NPP) hydrolysis by tannin-immobilized alkaline phosphatase (APase) was followed at 405 nm using a 96-well polystyrene microplate. The Michaelis constant (K_m) was measured and found to be comparable to that of soluble APase. A change in the conformation of the catalytic site is proposed to explain the high activity of tannin-bound APase. An additional amount of tannin added to the three-layer assemblage had no effect on the activity of the bound enzyme. The initial rates of bound enzyme (and therefore its relative amount bound to tannin) were affected by the addition of different concentrations of bovine serum albumin (BSA) to the APase solution; APase exhibited a much higher affinity than BSA to tannin. Tween 80, a nonionic detergent, broke down the BSA–tannin complex more efficiently than it did the APase–tannin one.

Recently, we reported a new method for tannin determination in various fruits (e.g., persimmon) using a 96-well polystyrene plate (Ittah, 1991a). The method is based on the construction, step by step, of a three-layer assemblage on the polystyrene plate: bovine serum albumin (BSA), tannin, and alkaline phosphatase, the last being assayed with *p*-nitrophenyl phosphate (Bessey et al., 1946). This method was found to be comparable with other well-known methods (Hagerman and Butler, 1980). Although it is based essentially on the same principle as the other methods, precipitation of tannins with proteins (Goldstein and Swain, 1965), no isolation of the protein–tannin complex is needed in its investigation. The use of a solid phase ensured a safe and convenient handling of the fragile complex. Several interesting features of the complex were noticed (Ittah, 1991b). For instance, the binding of tannin to immobilized BSA occurs at a much slower rate than in soluble BSA. Moreover, this binding was found to take place at higher rates and to greater extents in acidic pHs than in neutral or basic ones (Ittah, 1991a). A maximum precipitation was observed at the isoelectric point of the protein (Hagerman and Butler, 1980). Alkaline phosphatase bound more strongly to tannin than BSA and allowed the release latter from tannin under some specific conditions. However, the most surprising feature found refers to the activity of the immobilized APase compared with that of the soluble enzyme. The activity of the enzyme increased instead of being lowered, as would generally be expected from a bound enzyme (Gabel and Hofsten, 1970). One explanation for such an observation is that upon immobilization on the persimmon tannin the conformation of the enzyme might be altered, causing considerable changes in its activity.

The main objectives of this research were to study the kinetics of the persimmon tannin-bound APase, to investigate quantitatively the competition between two proteins (viz. BSA and APase) for the bound tannin, and finally to follow the behavior of tannins and proteins under the influence of a detergent (e.g., Tween 80). A deeper insight into parameters which influence the building of alternating layers of tannin and proteins (or other food constituents) might lead to a better understanding of the formation (or the dissolution) of bezoars in the stomachs of patients (Sanderson et al., 1971).

MATERIALS AND METHODS

Materials. All chemicals were of reagent grade. Mammalian alkaline phosphatase (APase) was purchased from Rad Chemicals Co. (Nes-Ziona, Israel). Soluble tannin from persimmons (*Diospyros kaki* L. var. Triumph) was purified as described elsewhere (Matsuo and Ito, 1980). Bovine serum albumin (BSA, fraction V powder), *p*-nitrophenyl phosphate (*p*-NPP), and Tween 80 were obtained from Sigma Chemical Co. (St. Louis, MO). Unprecoated polystyrene microtiter plates (96 wells, Maxisorb, cat. no. 442404) were purchased from Nunc (Roskilde, Denmark) and read on an EIA 400 ATC reader (Salzburg, Austria). These plates are especially recommended for assays of proteins bound to the polystyrene.

Enzyme Assays. *1. Kinetics.* A microplate was coated overnight (at 4 °C) with 0.2% BSA in 15 mM sodium carbonate buffer (pH 9.6) and washed twice [all washings were with 10 mM PBS (pH 7.4) after every incubation], and aqueous tannin solution (100 μL, 4 μg/mL) was added. After 1 h of incubation (all incubations were at ambient temperature, unless otherwise specified), the plate was washed twice and divided into two equal compartments, each of which was subdivided into two columns for the blanks (in duplicates) and into four columns for the enzymatic reaction (in quadruplicates). APase (100 μL, 0.8 unit/mL) in phosphate-buffered saline (PBS, pH 7.4) was distributed into all wells, except for the blanks. After 1 h of incubation, the plate was washed twice, and *p*-NPP at different concentrations (100 μL, 0–2 mg/mL) was added to the corresponding wells, including those for the blanks. The plate was then read on an ELISA reader at 405 nm; the readings were repeated every 15 min for 9 h.

2. Effect of Additional Tannin on APase Activity. A 96-well microplate was sequentially treated as above (BSA, tannin, and APase). After 1 h of incubation, the plate was washed twice, and an additional tannin solution (100 μL, 6 μg/mL) was distributed to all of the wells. After 1 h of incubation and the usual washings, the assay was completed as before. The experiment was repeated with an additional tannin solution of 8 μg/mL.

3. Competition between APase and BSA for the Tannin. To a BSA-coated plate treated with persimmon tannin solution (100 μL, 4 μg/mL) in PBS (pH 7.4) were added premixed solutions (100 μL) of BSA (0–4 μg/mL) and APase (1 unit/mL) in quadruplicates to the corresponding wells. After 1 h of incubation, the plate was washed twice, and the bound APase was assayed with *p*-NPP (1 mg/mL) as usual.

4. Effect of a Detergent (Tween 80) on APase Binding. A BSA-coated plate (0.2%) was divided into four compartments,

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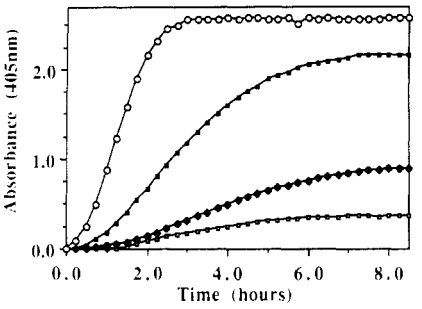

Figure 1. Tannin-immobilized alkaline phosphatase activity vs hydrolysis time; effect of substrate (*p*-NPP) concentration on the retardation of product appearance in the wells: (□) 0.1 mM; (♦) 0.5 mM; (■) 1 mM; (○) 4 mM. The wells were read at 405 nm every 15 min.

and the influence of the detergent was tested under four different sequential additions of the components:

a. The wells in the first compartment were treated in triplicate with a premixed solution (100 μL) of tannin (4 μg/mL) and Tween 80 (0–40 ppm). After 1 h of incubation and washings, APase (1 unit/mL, 100 μL) was added and the assay completed as above.

b. The wells in the second compartment were first treated in triplicate with a tannin solution (4 μg/mL). After 1 h of incubation and washings, Tween 80 solutions (0–40 ppm) were distributed into the wells. After 1 h of incubation and washings, APase solution (100 μL, 1 unit/mL) was added, and the assay was performed as usual.

c. The wells in the third compartment were first treated in triplicate with a tannin solution (4 μg/mL) and then followed, after the usual incubation and washings, by an APase solution (100 μL, 1 unit/mL). After 1 h of incubation and washings, Tween 80 solutions (0–40 ppm) were distributed into the wells. After 1 h of incubation and washings, bound APase was assayed as above.

d. The wells in the fourth compartment were treated in triplicate with a tannin solution (4 μg/mL). After 1 h of incubation and washings, a premixed solution (100 μL) of APase (1 unit/mL) and Tween 80 (0–40 ppm) was added, and the assay was completed as above.

RESULTS AND DISCUSSION

Mammalian alkaline phosphatase was immobilized on BSA-bound tannin. We postulated in a recent study (Ittah, 1991b) that BSA probably uses most of its hydrophobic pockets to bind to the polystyrene surface, leaving only a few of them available for the binding of the persimmon tannin (proanthocyanidin molecule). Therefore, the latter might use most of its hydroxyl groups to bind with APase. This binding seems to affect strongly the conformation of the immobilized enzyme, conferring upon it a catalytic activity with a much more efficient turnover than that of the free enzyme. The interactions between this enzyme and the bound tannin, on the one hand, and the substrate, on the other hand, appear to differ substantially from those existing in solution. To quantify these interactions, experimental studies were conducted to establish biochemical parameters of the bound enzyme.

When product formation is followed as a function of time, there exists a time lapse during which no product is detected in solution. Figure 1 shows the effect of substrate concentrations on this lag phases: very short for high concentrations and much longer for lower ones. This behavior is probably due to diffusion limitation of the substrate, as generally observed with immobilized enzymes on a solid phase (Hornby et al., 1968). When the initial rates of the reaction, measured following this lag phase, were plotted as a function of a wide range of *p*-NPP concentrations, the kinetics of bound enzyme appears to follow Michaelis–Menten closely (Figure 2a). The K_m

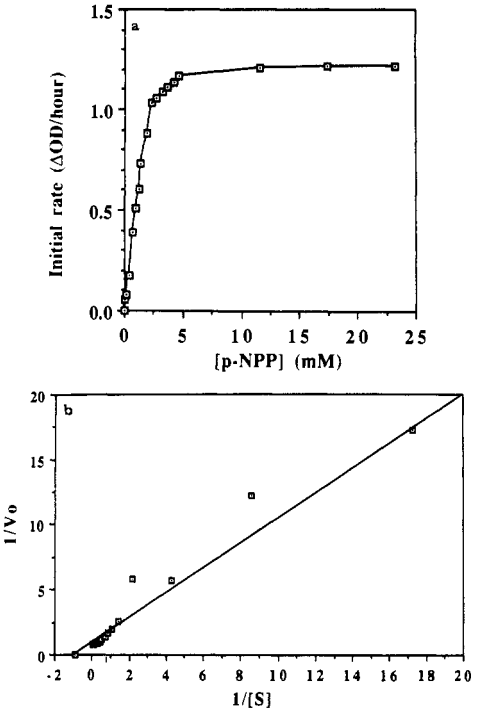

Figure 2. Kinetics of *p*-NPP hydrolysis with tannin-immobilized alkaline phosphatase: (a) initial rates vs substrate concentration (Michaelis–Menten plot); (b) Lineweaver–Burk plot. Assays were performed with *p*-NPP concentrations ranging from 0.1 to 23 mM, and the wells were read at 405 nm as described under Materials and Methods. The regression equation for the hydrolysis was $y = 0.9702 + 1.0309x$ with $R^2 = 0.956$.

(0.94 mM), as derived from the corresponding Lineweaver–Burk plot, was found to be nearly the same as that of the free enzyme (K_m = 0.92 mM) measured at the same ambient temperature. A very similar value (K_m = 0.986 mM) was obtained from statistical provisional estimates (Wilkinson, 1961). Therefore, the immobilized enzyme appears not to have changed its affintiy for the substrate. Interestingly, when APase was immobilized on a rendzina soil (Perez-Mateos et al., 1991), its K_m was reported to be increased (K_m = 6.72 mM) compared to that of the soluble enzyme (K_m = 1.70 mM) under the same assay conditions, suggesting that accessibility of the substrate to the binding site was restricted. In another study where APase was entrapped within an active sol–gel glass of tetramethoxysilane polymer (Braun et al., 1990), two kinetically different immobilized forms of the enzyme (K_m = 0.8 mM, 10%; K_m = 7 mM, 90%) were defined. Here, too, alterations of the conformation seem to affect greatly the affinity of the enzyme for *p*-NPP.

The amount of enzyme bound to the tannin is very small (presumably a monolayer) compared with that contained in the whole aliquot (100 μL). Nevertheless, the amount of substrate hydrolyzed per unit of time was almost the same in both cases (Ittah, 1991b). It appears that conformational changes might have occurred in the vicinity of the catalytic site. This result raises the possibility that binding of the enzyme to BSA-bound tannin does not take place at random but occurs in a well-defined orientation. But, could addition of excess tannin have any effect on this orientation and cause inhibition of the bound enzyme? Figure 3 shows two examples [at high (a) and low (b) concentrations of the substrate] of such an effect. No change in the activity of the enzyme, at any substrate concentration, was detected upon addition of more tannin. A possible explanation for this behavior might be that the additional tannin binds at hydrophobic pockets far from

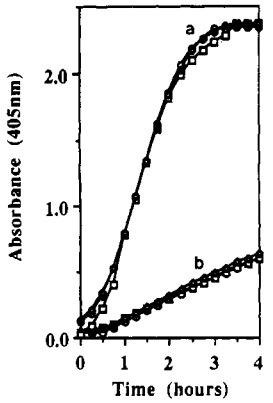

Figure 3. Tannin-immobilized alkaline phosphatase activity vs hydrolysis time at high (a, 4 mM) and low (b, 0.1 mM) substrate concentration; effect of additional tannin on enzymatic activity. Assays were performed with different concentrations of additional tannin (100 μL) as described under Materials and Methods: (□) 0 μg/mL; (○) 6 μg/mL; (■) 8 μg/mL.

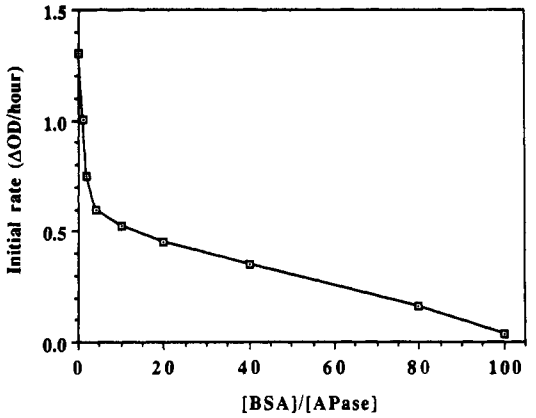

Figure 4. Change in initial rate of tannin-immobilized alkaline phosphatase against the ratio [BSA]/[APase]. Premixed BSA and APase were added to bound tannin in ratios varying from 0 to 100, and immobilized APase was assayed as described under Materials and Methods.

the catalytic site, causing no disturbance in the enzyme activity. It could also be that the bound tannin saturated the enzyme and, therefore, no hydrophobic pockets would be available for the binding of additional tannin. In any case, the catalytic site appears to remain free of tannin.

The competition for the tannin between APase and another protein, i.e., BSA, was studied quantitatively by adding a mixture of the two proteins to the bound tannin. The initial rates, which actually express the relative amount of the enzyme bound to the tannin, were measured for molar BSA/APase ratios varying from 0 to 100. The results, as illustrated in Figure 4, show that the higher the ratio of BSA/APase, the less APase will bind to the tannin. However, the relationship is not linear. Although at low ratios the decrease of bound-APase activity is relatively rapid, it still exhibits about half of the original bound enzyme activity (free of BSA) at a ratio of 10. At higher ratios, the activity of the bound enzyme slowly and linearly decreases, becoming insignificant only at a ratio of 100. These results illustrate the large differences in binding abilities between BSA and APase. Since the bonds between proteins and tannins are reversible (hydrophobic and hydrogen bonds) and involve dynamic equilibria, a ligand (e.g., APase) possessing a high binding affinity is able to displace another (e.g., BSA) that has a lower binding affinity. The rate of displacement depends on the relative concentrations of both ligands. In our case, a huge excess of BSA is needed to prevent completely the binding of

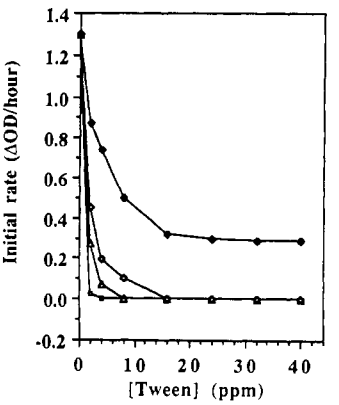

Figure 5. Effect of the detergent Tween 80 on the extent of binding of alkaline phosphatase to BSA-bound tannin. Assays were performed under different protocols (described under Materials and Methods): (□) protocol 1; (△) protocol 2; (♦) protocol 3; (◇) protocol 4.

APase. This confirms our previous findings (Ittah, 1991b), in which APase displaced tannin-bound BSA more easily than BSA displaced tannin-bound APase.

Another way to study the relative binding strengths of various ligands to tannin involves the use of detergents, e.g., Tween 80. As a nonionic detergent, Tween 80 competes with the tannin for the hydrophobic pockets of the protein. Therefore, the stronger the binding between the protein and the tannin, the more energy (or more detergent molecules) is needed to take them apart. The influence of Tween 80 was investigated under four different protocols which might illustrate possible interactions between tannins and proteins in the stomachs of patients suffering from bezoars. In the first one, Tween 80 at various concentrations, premixed with tannin (4 μg/mL), is supposed to interfere with tannin binding to the protein, thus reducing the number of binding sites for APase. As shown in Figure 5, at a concentration of Tween 80 as low as 2 ppm, the initial rate of bound APase declined to zero, indicating that no bound tannin was available to bind the enzyme. In a second protocol, Tween 80 at various concentrations was added to the already bound tannin to test its ability to cause partial or total disruption of the immobilized BSA–tannin complex, thereby reducing the number of binding sites for APase. As shown in Figure 5, very small amounts of Tween 80 were sufficient to completely interfere with the binding. From the results of these two experiments, it is concluded that Tween 80 competes strongly with the tannin for the hydrophobic pockets of BSA or, alternatively, that the binding of BSA to tannin is quite weak. Furthermore, it appears that Tween 80 prevents the binding of tannin in solution to immobilized BSA as easily as it disrupts the already bound tannin from the protein. In a third protocol, the disruptive effect of various concentrations of Tween 80 on the three-layer assemblage (BSA–tannin–APase) was investigated. As shown in Figure 5, even at Tween 80 concentrations of 40 ppm, complete breakdown was not observed. Moreover, the bound APase seems to be released slowly as Tween 80 concentration increases, finally reaching a constant level which is not affected by the detergent (up to 40 ppm). This behavior illustrates clearly the strong binding which exists between APase and bound tannin. In a fourth protocol, a premixed solution of APase and Tween 80 at various concentrations was added to the already bound tannin. As shown in Figure 4, as much as 16 ppm was needed to prevent completely the binding of the enzyme to the tannin. Although more Tween 80 is needed in this case than in protocols 1 or 2, it is clear that the detergent

competes more easily with APase in solution than with immobilized enzyme (protocol 3).

The results of this study suggest that, upon immobilization of the enzyme on the tannin, changes in its conformation take place preferentially at its catalytic site only, with no change of its binding site. It has also been quantitatively established that the binding of different proteins to BSA-bound tannin occurs at different initial rates.

Our ultimate goal in this field remains in the search for a highly effective drug capable of dissolving bezoars by taking apart proteins and tannins or, alternatively, preventing the formation of bezoars. In this context, we are currently studying the effect of various compounds (e.g., low molecular weight amines, peptides, proteolytic enzymes, other detergents) on the formation and the stability of the three-layer assemblage, BSA–tannin–APase.

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