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TANNIC ACID AND PROTEOLYTIC ENZYMES: ENZYME INHIBITION OR SUBSTRATE DEPRIVATION?

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Key Word Index Tannins; tannic acid; digestibility-reducing substances; bile salts; trypsin autolysis; allelochemics; chemical ecology.

Abstract—The direct effect of tannic acid (TA) upon trypsin (TRY) was examined using TRY-esterase activity to measure active TRY and the rate of its autolytic (self) degradation in the presence of TA, bovine serum albumin (BSA, a protein substrate), glycocholic acid (GCo, a bile salt) and combinations of these. Addition of TA to a standard TRY solution led to the formation of an insoluble complex but there was an enhanced rate of autolysis in the TRY remaining in solution, probably due to conformational changes in the TRY brought about through the formation of soluble complexes with TA. This phenomenon was not observed after addition of excess BSA to the TRY + TA system, indicating that here TRY does not complex with TA and so remains active as a protease. A similar result was seen where both BSA and GCo were added to produce conditions in which the surfactant solubilizes the otherwise insoluble TA–BSA complex. These findings confirm recent reports indicating the importance of tannin–binding to specific proteins for an understanding of the allelochemical activity of tannins and show that this specificity is not altered by the presence of gastrointestinal surfactants. In the ecologically relevant systems reported here TA would appear incapable of directly inhibiting TRY activity.

INTRODUCTION

Tannins have long been regarded as having digestibility-reducing effects when present in the diets of natural and agricultural populations of herbivores [1, 2] because of their ability to form insoluble complexes with proteins. The ecological interpretation of this complexation reaction has generally been that in the digestive tracts of herbivores protein so precipitated is rendered unavailable to the animal. This often manifests itself as enhanced levels of faecal nitrogen when tannin-rich diets are consumed [3].

The overwhelming majority of investigations into the biochemistry of the tannin–protein interaction have involved the *in vitro* formation of such complexes [4–7] and have been concerned with precipitation involving either the proteolytic enzymes of the gut or the substrate proteins on which proteases could act. Where workers have investigated the action of tannins on proteases and their protein substrates together [8–10], only the products of the proteolytic reaction have hitherto been measured. No account has, therefore, been taken of competitive effects due to variance in the affinity of tannins for the different proteins involved in a proteolytic system, although it is known [11] that a given tannin has a wide range of affinity (varying over three orders of magnitude) for different proteins. Thus we remain ignorant as to whether observed inhibition of a proteolytic system by a tannin relates to the direct action of the tannin on proteases or to the binding of that tannin to the substrate proteins, or to both these potential effects.

On the basis of published work by Hagerman and Butler [12] it does seem likely that tannins will bind preferentially to one protein only in a protein + protease system. This study sets out to examine whether the hydrolysable tannin tannic acid (TA) directly affects the enzyme or the substrate in the proteolytic system, using bovine serum albumin (BSA) as substrate and trypsin (TRY) as enzyme. The answer to this question will improve our understanding of the dose–response relationship between the quantities of tannins accumulated by plants and the allelochemical effects produced on herbivores.

RESULTS

The technique adopted in this study has been to examine the direct effect of TA upon the activity of TRY in the presence of BSA. TRY activity was followed by observing the rate of its autolytic (i.e. self) degradation (itself a proteolytic process) at pH 7.5. The active TRY remaining in a system at any given time can be estimated from the level of its esterolytic reaction with the artificial substrate (α-*N*-benzoyl-L-arginine ethyl ester; BAEE). Thus the decline of esterase activity of TRY with time can be used as a measure of the rate of autolysis of TRY. In this way TRY autolysis was studied in reactions containing TRY alone (control), TRY and TA, TRY and BSA, and TRY with BSA and TA. A second set of reactions using the same conditions but additionally incorporating the bile salt sodium glycocholate (GCo) was also performed in view of the recent observation [10] that GCo is able to

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influence the ability of tannins to interfere with proteolysis. These experiments allow (i) the measurement of changes in TRY esterolytic activity, which is presumed to relate to a change of proteolytic activity, owing to the addition of TA to the enzyme; and (ii) the effect of BSA and GCo upon the interaction between TRY and TA.

Figure 1 shows the change in the rate of esterolytic activity for TRY, TRY + BSA, TRY + TA and TRY + BSA + TA systems. Changes in tryptic (esterase) activity were measured by changes in absorbance (A) measured at 254 nm over the course of 6 hr autolysis experiments. Tryptic activity remaining at any time is expressed in terms of the absorbance of the control reaction (TRY alone) at time zero (A_0) divided by the absorbance (A_x) remaining at any given time. Therefore loss of activity results in a higher value for A_0/A_x, and the steeper the slope of the plot, the greater has been the rate of loss of esterolytic activity. For the experimental conditions employed, this method of data analysis gives linear functions for the control since it is a second-order reaction [13] and it will likewise give linear functions if the reactions containing a greater number of components remain second order.

As defined above, the control reaction has an A_0/A_x value of 1.0 at time zero (i.e. $A_0 = A_x$), and the increase in the ratio over time reflects the loss of tryptic activity due to autolysis. Addition of BSA to TRY leads to a slight reduction in the rate of autolysis, presumably due to interaction between the protein and protease reducing the opportunity for the autolytic process. By contrast, for the reaction containing TRY and TA there is a vertical shift upwards at time zero which is significantly greater than that of the control ($P < 0.001$). This can be explained by the immediate and observed loss of some trypsin by precipitation consequent upon the addition of TA at time zero. This vertical shift is eliminated by the inclusion of BSA in the TRY + TA system. This observation can be rationalized in terms of competition [11], i.e. of preferential binding between TA and BSA (BSA is present in sufficient amounts to complex with all the TA added) leaving the TRY free to act as in the TA-free solutions. An alternative explanation would be that both proteins have similar specificities for tannin binding but that the excess of BSA swamps the system removing TA from trypsin. As dietary proteins can be reasonably assumed to be present in marked excess of proteases in the gut, it is of no nutritional significance which of these two processes is operative in removing the inhibitory action of TA on trypsin, indeed both could be significant *in vivo*.

The rate of TRY autolysis in the TRY + TA system was observed to be markedly faster than that in the other systems (Fig. 1). This is despite the fact that the concentration of the enzyme in solution had been lowered owing to the initial loss of TRY by precipitation when TA was added. Two possible reasons can be put forward for this. (a) The apparently faster autolytic rate might be artefactual, caused by progressive enzyme inhibition through binding with TA which would prevent esterolytic activity in exactly the same way as autolysis. However, most authors regard tannin protein binding as being a rapid process occurring over 10–15 min [4, 6, 7, 12], which implies that all the loss of activity due to tannin binding should have taken place before the second reading of A_x was made after 40 min. (b) An alternative hypothesis to explain increased autolysis in the presence of TA would be that conformational changes have been induced in the

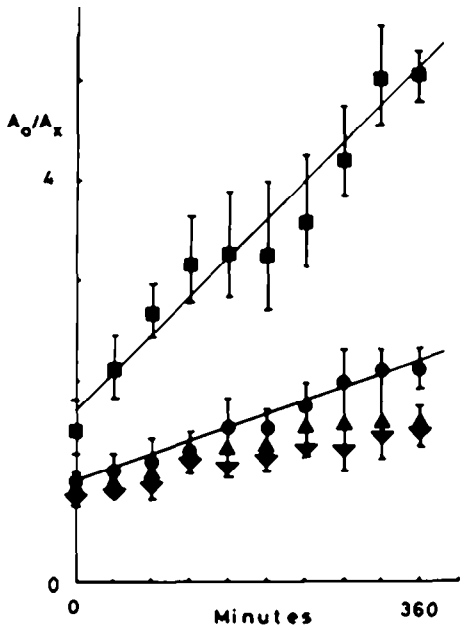

Fig. 1. Change in trypsin activity (A_0/A_x) against time plotted for (a) TRY + TA (■), (b) TRY (●, control), (c) TRY + BSA + TA (▲) and (d) TRY + BSA (▼). A_0 is the absorbance of the TRY control at 254 nm at time zero and A_x is the absorbance of the test solution at any given time. The concentrations of the reactants used were TRY, 1.82 mg/ml; BSA, 9.09 mg/ml; TA, 0.91 mg/ml. The regression equations for the four reactions are: (a) $y = 1.53 + 0.0098x$; (b) $y = 0.97 + 0.0033x$; (c) $y = 0.99 + 0.0019x$; (d) $y = 0.89 + 0.0018x$.

TRY by binding between TRY and TA in soluble complexes and this directly promotes autolysis. A similar phenomenon has been reported to occur when TRY is adsorbed on (negatively charged) silica surfaces [14]. This is a comparable explanation to that advanced by Mole and Waterman [10] to explain the increased rate of BSA proteolysis observed in TRY + BSA systems under conditions in which soluble tannin–protein complexes formed.

Figure 2 shows the results for TRY alone (control), TRY + TA and TRY + TA + GCo. The effect of adding GCo and then TRY to TA was to totally prevent the TRY TA precipitate formed in experiments where TRY was added to TA, leaving instead an optically clear solution. However, the failure of GCo to eliminate the initial inhibition of TRY by TA (A_0/A_x at time zero is significantly greater than for the control, $P < 0.001$) indicates that the surfactant effect of GCo is limited to preventing tannin protein complexes aggregating into a flocculum, and does not prevent TA/TRY complexation with consequent loss of esterolytic activity *per se*. Furthermore, although from Fig. 2 it would appear that some time is taken for this GCo and TA containing system to reach an equilibrium, a TA-induced increase in the rate of autolysis does still clearly become evident throughout the timespan of the experiment. A possible explanation for initial non-second-order reaction implied from the curve obtained for this experiment is that the GCo, whilst not significantly altering the initial degree of TA/TRY complexation does slow down the rate at which the soluble

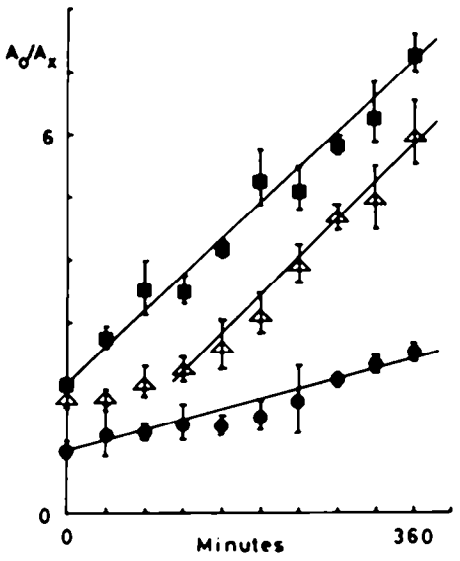

Fig. 2. Change in trypsin activity (A_0/A_x) against time plotted for (a) TRY + TA (■), (b) TRY + TA + GCo (Δ) and (c) TRY (●, control). A_0 is the absorbance of the TRY control at 254 nm at time zero and A_x is the absorbance of the test solution at any given time. The plots for TRY + BSA + GCo and TRY + BSA + TA + GCo did not differ significantly from those of the control. The concentrations of the reactants used were as in Fig. 1, plus GCo, 3.54 mg/ml. The regression equations for the three reactions are: (a) $y = 2.14 + 0.0135x$; (b) $y = 0.22 + 0.0015x$ (calculated from the fourth measurement of A_0/A_x); (c) $y = 0.92 + 0.0041x$.

complex, and hence denatured TRY, is formed and then destroyed by the autolytic process.

Reactions containing TRY + GCo and TRY + TA + BSA + GCo were found to be indistinguishable from the comparable reactions run without GCo. The latter result illustrates that even in the presence of a surfactant able to interrupt tannin protein binding BSA is still able to protect TRY from TA.

DISCUSSION

In the presence of an excess of BSA, TRY was found to be an ineffective competitor for complex formation with TA and remained able to act as a protease without any loss of activity. In terms of modelling the dietary situation in herbivores, the significance of this observation is to confirm that the effect of TA is to deprive TRY of substrate rather than to act directly upon it, so distinguishing TA from specific TRY inhibitors [15]. It is important to note that this state of affairs extends to systems in which tannin protein complexes are solubilized by bile salts. In these systems there was clear evidence that a BSA–TA interaction did still occur, thus preventing the TA-induced increase in TRY autolysis seen in the TRY + BSA + TA system in the absence of GCo. This provides complementary evidence to our earlier report concerning the tryptic digestion of soluble BSA-tannin complexes [10], in which we cautioned against the assumption [16] that gut surfactants which solubilize tannin–protein precipitates also eliminate any tannin–protein interaction.

The general ecological significance of these observations is to re-emphasize once again the complexity of the tannin–protein reaction in relation to the digestion processes. There is no such thing as a generalized tannin–protein interaction; the outcome of mixing tannins and proteins in the gut will depend upon the relative concentration of tannin and protein [10], the variable affinity of a tannin for different proteins in that system [12, 17] and, probably, the relative concentrations of individual proteins present. For these reasons the previous observation [18] that in rats consuming tanniniferous legume seeds there is an apparent inhibition of trypsin activity that can be reversed by the addition of polyvinylpyrrolidine to the diet should not necessarily be considered to be in contradiction of the results reported here.

Furthermore, with the possible exception of the rumen system, the effects of bile salts in mammals and gut surfactants in insects [16] need to be accounted for in the prediction of ecological effects arising from dietary tannin consumption. With regard to this last point, we believe that the recent report [19] that the toxic effects of high-tannin diets in rats can be reversed by addition of saponins (which are themselves toxic) represents a good example of the way a surfactant can effect the efficiency with which tannin and protein complex.

EXPERIMENTAL

The method employed for performing the basic autolysis reaction follows that of Whateley [13, 14], whilst the assay of TRY esterase activity follows Schwert and Takanaka [20].

A trypsin (TRY) stock soln (20 mg/ml, Sigma type III) was made up in 0.001 M HCl to prevent autolysis. At time zero, 1 ml of this soln was added to 10 ml of a Ca-free buffer (0.05 M NaCl, 0.01 M Tris, 0.002 M EDTA, pH 7.5) in order to initiate autolysis. Control reactions contained no other additions. The experimental variations made comprised the inclusion of the following substances in the 10 ml of buffer as detailed in the main text: BSA (Sigma, fraction V) to a final concn of 10 mg/ml; tannic acid (TA) (BDH) to 1 mg/ml, and glycocholic acid (GCo) (Sigma, grade I) to 8 mM. All glassware coming into contact with TRY was silanized using 'Repelcote' (Hopkin & Williams Ltd.) to prevent adsorption-catalysed autolysis. The reactions were contained in test tubes placed in a water bath set at 25°.

In order to assay the tryptic activity of the reactions, 50 μl samples were taken at timed intervals and added to 3 ml BAEE reagent (0.333 mM α-*N*-benzoyl-L-arginine ethyl ester·HCl, Sigma; 0.1 M Tris; 5.0 mM $CaCl_2$; pH 8.0) contained in a cuvette. The rate of esterolysis was measured immediately by recording the rate of change in the absorbance at 254 nm using a spectrophotometer coupled to a chart-recorder. All experimentally treated systems were run concurrently with a control reaction for comparison. Within the expts, each assay of tryptic activity was made in duplicate and each set of expts was replicated, and so the points plotted in the figures are derived from the mean values of these observations. The error bars surrounding the points indicate 95% confidence limits.

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