Journal of Chemical Ecology, Vol. 11, No. 4, 1985

EFFECTS OF SURFACTANTS, pH, AND CERTAIN CATIONS ON PRECIPITATION OF PROTEINS BY TANNINS

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(Received June 7, 1984; accepted August 7, 1984)

Abstract—Tannic acid and pin oak tannins precipitate large amounts of the abundant leaf protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC), over a wide pH range (6.15–9.30) in the presence of sodium, potassium, magnesium, and calcium ions at concentrations comparable to those reported in the gut fluids of lepidopteran herbivores. The presence of lysolecithin, a surfactant known to be present in the gut fluids of some insects, significantly reduces the amount of RuBPC precipitated under these conditions. We conclude that high detergency is far more effective than high alkalinity in countering the potential protein-precipitating properties of tannins. We further conclude that tannins do not deserve the status they were once accorded as general, all-purpose, dose-dependent, antidigestive defensive chemicals. We also describe the application of the Schaffner-Weissman protein assay for studying the protein-precipitating capacity of plant extracts. This method is far superior to the one we have used in our earlier studies.

Key Words—Tannins, digestibility-reducing substances, surfactants, detergency, RuBPC, herbivory, chemical defense, allelochemics.

INTRODUCTION

Tannins, which are water-soluble phenolic compounds that occur widely in vascular plants, have been accorded an important role in protecting plant tissues from herbivory (Feeny, 1976; Rhoades and Cates, 1976; Swain, 1979). The ingestion of tannins has been shown to interfere with normal growth and development in many foliage-feeding insects, although tannin-tolerant species are also well known (Bernays, 1981). Since tannins are known to be protein precip-

0098-0331/85/0400-0485$04.50/0

itants (van Sumere et al, 1975; Hagerman and Butler, 1981; McManus et al, 1983), it has been proposed that they might reduce the nutritive value of plant tissues by forming indigestible complexes with foliar proteins or by precipitating and inactivating digestive enzymes in the digestive tract of an herbivore (Feeny, 1976; Rhoades and Cates, 1976). While it is well-documented that tannins can act as feeding deterrents and toxins to some insect herbivores, the idea that they are digestibility-reducing substances is currently being challenged (Bernays, 1981; Martin and Martin, 1984).

Interactions between tannins and proteins are strongly influenced by pH, ionic strength, detergents, and the concentrations of certain specific ions (Goldstein and Swain, 1965; Feeny, 1970; van Sumere et al., 1975; Hagerman and Butler, 1978, 1981; Berenbaum, 1980; Oh et al., 1980; Martin and Martin, 1983, 1984; McManus et al., 1983). It would seem possible that an insect herbivore might counter the potential of tannins to reduce the digestibility of dietary protein if it were to maintain conditions in its gut that were unfavorable for the formation of insoluble complexes between the major proteins and tannins present in the ingested foliage. Indeed, it has been proposed that high gut alkalinity is an antitannin adaptation in lepidopteran larvae (Feeny, 1970; Berenbaum, 1980), and that detergency is a widespread characteristic of insect gut fluids that would counter the potential antidigestive properties of tannins (Martin and Martin, 1984).

It was the goal of this study to determine the influence of sodium, potassium, magnesium, and calcium ions, pH, and lysolecithin on the precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC) by tannic acid and pin oak extracts. Sodium, potassium, magnesium, and calcium were chosen for examination because they are the major cations present in foliage. Their effects were studied at concentrations similar to those reported in the gut fluids of two species of Lepidoptera, *Philosamia cynthia* and *Bombyx mori* (Giordana and Sacchi, 1978). Experiments were conducted over a pH range (6.15–9.30) that includes values typically recorded in the gut fluids of herbivorous insect species. The effect of lysolecithin was studied because this surfactant substance has been shown to be present in the midgut contents of *Pieris brassicae* (Turunen and Kastari, 1979). RuBPC was chosen as the test protein because it is a major dietary protein of any foliage-feeding insect, often making up as much as 25% of the total protein and 25–50% of the soluble protein in leaf tissue (Singer et al., 1952; Akazawa, 1970; Lyttleton, 1973; Jensen and Bahr, 1977). The study has been designed so that we may determine whether the conditions that prevail in the gut fluids of typical insect herbivores favor or disfavor the precipitation of RuBPC by tannins.

This paper also describes a procedure for determining the amount of protein in an insoluble protein–tannin complex that is far superior to the one we described earlier (Martin and Martin, 1982, 1983, 1984).

METHODS AND MATERIALS

Effects of Salts on Precipitation of RuBPC by Tannic Acid. A stock solution of RuBPC (0.6–0.7 mg/ml) in buffer (0.05 M) containing either sodium chloride (3 mM) and potassium chloride (160 mM) or magnesium chloride (30 mM) and calcium chloride (20 mM) was prepared by combining suitable volumes of a solution of RuBPC in buffer and a solution of the appropriate pair of salts in water. Chlorides were used because of their high solubility and because chloride is a major anion in the gut fluids of herbivorous insects. Any insoluble material was removed by centrifugation (15,000*g*, 15 min, 24°C), and the protein content of this stock solution was measured using the procedure described below. Controls were run in which no salts were included in the stock solution. To 1.8 ml of the stock solution, agitated on a vortex mixer, was added 100 μl of a freshly prepared solution of tannic acid (1.0 mg/ml or 3.0 mg/ml). After 10 min, the mixture was centrifuged (30,000*g*, 15 min, 24°C), the supernatant solution was removed, and the pellet was rinsed very gently two times with buffer and drained. The precipitated tannic acid–RuBPC complex was redissolved by stirring for 30 min at room temperature with 0.75 ml of a 1% SDS solution in 0.05 M Tris, pH 7.5. The protein content of this solution was measured using the procedure described below.

The RuBPC preparation used in these experiments (Sigma R-2000, lot 98C-7140) was found by HPLC analysis to be only 65–70% pure. The contaminant(s) absorbed in 282 nm were presumed to be proteinaceous. This RuBPC preparation was freely soluble in the salt-free buffers and in the buffers containing sodium and potassium chloride at pHs 6.90, 7.55, and 8.30. The presence of magnesium and calcium chloride in these buffers caused about 5% of the protein to precipitate out of solution. It was not established whether it was RuBPC or a contaminant that was precipitated. The RuBPC preparation was somewhat less soluble at pHs 6.15 and 9.30. About 25% of the protein precipitated from these buffers when magnesium and calcium chloride were present. At pH 6.15, even in the salt-free buffer or the buffer–sodium chloride–potassium chloride mixture, 10–15% of the protein either precipitated or failed to dissolve. As a result of these solubility characteristics, protein content varied slightly from one incubation mixture to another (0.900–1.213 mg/1.8 ml). This variability did not obscure the clear patterns that emerged from the study.

The buffers used in these experiments were 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.15), *N*-2-acetamido-2-aminoethanesulfonic acid (ACES, pH 6.90), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.55), *tris*-(hydroxymethyl)aminomethane (Tris, pH 8.30), and cyclohexylaminoethanesulfonic acid (CHES, pH 9.30).

Effect of Lysolecithin on Precipitation of RuBPC by Tannic Acid or Pin Oak Tannins in Presence of Salts. To an aliquot (1.3 or 1.7 ml) of a stock so-

lution, prepared as above, containing RuBPC (0.6–0.9) mg/ml), sodium chloride, potassium chloride, magnesium chloride, and calcium chloride in buffer (0.05 M) was added 100 μl of a solution of lysolecithin in buffer at a concentration that resulted in a final lysolecithin concentration of 0.06%. At pH 6.15 and 7.55, $[Na^+]$ = 2.5 mM, $[K^+]$ = 131.0 mM, $[Mg^{2+}]$ = 25.0 mM, and $[Ca^{2+}]$ = 16.0 mM; at pH 6.90, 8.30 and 9.30, $[Na^+]$ = 3.0 mM, $[K^+]$ = 160 mM, $[Mg^{2+}]$ = 30.0 mM, and $[Ca^{2+}]$ = 20.0 mM. In the controls, 100 μl of buffer replaced the lysolecithin. After 5 min, either 100 μl of a freshly prepared solution of tannic acid (3.0 mg/ml) or 100 μl of a solution of tannins extracted from mature pin oak foliage (2.5 mg dry wt) was added. After 10 min, any precipitate present was collected by centrifugation, rinsed, drained, and redissolved in 1% SDS in 0.5 M Tris (ph 7.5) as described above. The protein content was measured using the procedure described below.

Preparation of Foliage Extract. Mature pin oak (*Quercus palustris*) foliage was lyophilized and ground on a Wiley mill (60-mesh). Forty milligrams of leaf powder was extracted twice for 8 min with 1.6 ml of boiling 50% (v/v) aqueous methanol. The extract was concentrated to dryness at reduced pressure, and the residue redissolved in 1.6 ml of water. Material that did not dissolve was removed by centrifugation.

Protein Assay. Protein content was measured using the method of Schaffner and Weismann (1973). An aliquot of the test solution, containing 20–130 μg of protein, was made up to a volume of 0.75 ml by the addition of a solution of 1% SDS in 0.05 M Tris, pH 7.5. Protein was precipitated by adding 0.15 ml of 90% trichloroacetic acid and vortexing the mixture. After 2–5 min, the precipitate was adsorbed on a nitrocellulose membrane (0.45 μm) by vacuum filtration. The tube was rinsed once with 1.2 ml of 6% trichloroacetic acid, and the rinse was poured through the filter apparatus. The adsorbed protein was then stained by immersing the filter disk in a 0.25% solution of Amido black 10B in methanol–acetic acid–water (50:10:40 vol %) for 10 min. Excess, unbound Amido black 10B was removed from the stained disk by rinsing for 30–45 sec in water, then treating for a total of 4 min in three changes of destaining solvent (methanol–acetic acid–water, 90:2:8 vol %), rinsing again with water for 2–3 min, and finally blotting dry with filter paper. Protein-bound dye was then eluted by shaking for 10–15 min in 3.0 ml of eluting solution (25 mM NaOH, 0.05 mM EDTA in 50 vol % aqueous methanol). Absorbance was determined at 630 nm, and converted to micrograms of protein by the use of a calibration curve constructed from dilutions of a stock solution of RuBPC.

RESULTS

Improved Procedure for Determining Amount of Protein Precipitated by Tannin Solution. In earlier studies, Martin and Martin (1982, 1983, 1984) de-

termined the amount of protein precipitated by a tannin solution indirectly by measuring the amount of protein in solution before and after the addition of the tannin solution, using the dye-binding assay of Bradford (1976). It was not possible to measure the amount of protein precipitated directly because a detergent solution is required to redissolve the precipitated protein–tannin complex and detergents interfere with the Bradford assay. With the discovery that surfactants may play a major role in preventing the precipitation of proteins by tannins in insect guts (Martin and Martin, 1984), it became evident to us that a different assay would be required if we were to explore this effect and extend our studies to insects with gut fluids containing high concentrations of detergent substances. We have found that the method of Schaffner and Weissman (1973) circumvents the problems of the Bradford assay and can be used to measure the amount of protein present in a protein–tannin precipitate. In this procedure the insoluble protein–tannin complex is dissolved in a 1% SDS/Tris solution. Then protein is precipitated by the addition of trichloroacetic acid, adsorbed on a filter membrane, and stained with Amido black 10. This method is a significant improvement over the one we used earlier in the measurement of the protein-precipitating capacity of tannin solutions and plant extracts, and we urge its routine adoption.

Effect of Alkali Metal Ions and Alkaline Earth Metal Ions on Precipitation of RuBPC by Tannic Acid. The amount of RuBPC precipitated from solution by the addition of tannic acid is highly dependent upon pH and the presence of alkali metal ions or alkaline earth ions (Table 1). The concentrations of sodium (3 mM), potassium (160 mM), magnesium (30 mM), and calcium (20 mM) were selected to resemble the concentrations of these same cations in the midgut lumen contents of two herbivorous lepidopteran larvae, *Bombyx mori* and *Philosamia cynthia*. In these two species, Giordana and Sacchi (1978) have reported the following concentrations (mM): Na^+, 1.3 and 1.0; K^+, 149.5 and 196.8; Mg^{2+}, 27.4 and 8.6; and Ca^{2+}, 19.6 and 11.0 for *B. mori* and *P. cynthia*, respectively.

At pHs 6.15 and 6.90, the presence of sodium and potassium ions favors the precipitation of RuBPC by tannic acid. At pH 6.15, 100 μg of tannic acid precipitates 49% of the RuBPC present in a solution containing sodium and potassium chloride, but only 17% when these salts are absent (runs 1 and 2). At pH 6.90, 300 μg of tannic acid precipitates 84% of the RuBPC when sodium and potassium chloride are present, but only 10% of the RuBPC from the salt-free buffer (runs 4 and 5). At the more alkaline pHs, 7.55 and 8.30, however, very little RuBPC is precipitated (less than 5%) even by 300 μg of tannic acid (runs 7, 8, 10 and 11). This effect of pH is evident even at the lower pHs when the amount of RuBPC precipitated by a given amount of tannic acid is compared at pH 6.15 and 6.90 (runs 2 and 5). At pH 6.15, 100 μg of tannic acid precipitates 49% of the RuBPC from the salt-containing buffer, whereas at pH 6.90 this amount of tannic acid precipitates less than 5% of the RuBPC.

TABLE 1. AMOUNTS OF RuBPC PRECIPITATED FROM SALT SOLUTIONS AT VARIOUS pH's BY ADDITION OF SOLUTION OF TANNIC ACID

Run	pH	Cation conc. (mM)				RuBPC in incubation mixture (mg)	RuBPC precipitated (mg)[a]	
		Na^+	K^+	Mg^{2+}	Ca^{2+}		By 100 μg TA	By 300 μg TA
1	6.15	0.0	0.0	0.0	0.0	1.21	0.20 ± 0.004 (6)	1.11 ± 0.014 (6)
2		3.0	160.0	0.0	0.0	1.07	0.52 ± 0.010 (6)	0.99 ± 0.027 (6)
3		0.0	0.0	30.0	20.0	0.90	0.80 ± 0.008 (6)	ND[b]
4	6.90	0.0	0.0	0.0	0.0	1.18	0.03 ± 0.002 (6)	0.12 ± 0.002 (6)
5		3.0	160.0	0.0	0.0	1.20	0.06 ± 0.003 (3)	1.01 ± 0.011 (6)
6		0.0	0.0	30.0	20.0	1.13	0.85 ± 0.013 (6)	1.02 ± 0.008 (6)
7	7.55	0.0	0.0	0.0	0.0	1.18	0.02 ± 0.001 (6)	0.02 ± 0.001 (6)
8		3.0	160.0	0.0	0.0	1.15	0.02 ± 0.001 (6)	0.05 ± 0.002 (6)
9		0.0	0.0	30.0	20.0	1.09	0.32 ± 0.012 (6)	0.61 ± 0.007 (7)
10	8.30	0.0	0.0	0.0	0.0	1.18	ND	0.01 ± 0.001 (6)
11		3.0	160.0	0.0	0.0	1.18	ND	0.01 ± 0.001 (6)
12		0.0	0.0	30.0	20.0	1.13	0.31 ± 0.012 (6)	0.60 ± 0.009 (6)
13	9.30	0.0	0.0	30.0	20.0	0.92	0.38 ± 0.009 (6)	0.65 ± 0.009 (5)

[a]Values are the mean ± standard error, with the number of replicates given in the parentheses.
[b]ND, not determined.

The alkaline earth cations, magnesium and calcium, are even more effective than the alkali metal cations, sodium and potassium, at bringing about the precipitation of RuBPC by tannic acid. At pH 6.15 or 6.90, 100 μg of tannic acid precipitates 89% and 75% of the RuBPC from buffers containing magnesium and calcium chloride, respectively, but only 49% and 5% from the buffers containing sodium and potassium chloride (runs 2, 3, 5, and 6). Increased alkalinity does not nullify the effect of the alkaline earth ions in the same way it counters the effect of the alkali metal ions. Even at pHs 7.55, 8.30, and 9.30, significant quantities of RuBPC are precipitated from solutions containing magnesium and calcium chloride when tannic acid is added (runs 9, 12, and 13).

Effect of Lysolecithin on Precipitation of RuBPC from Salt Solutions by Tannic Acid and Pin Oak Foliage Extracts. Lysolecithin, a surfactant that has been detected in the gut fluids of *Pieris brassicae* (Turunen and Kastari, 1979), significantly reduces the amount of RuBPC precipitated by tannic acid (Table 2). Experiments were run in buffers containing all four cations at concentrations comparable to those reported in the gut fluids of *B. mori* and *P. cynthia*. The lysolecithin concentration (0.06%) was about 15 times the critical micelle concentration (CMC), which is the concentration at which there is a transition between the surfactant in the free, unassociated state and the micellar state. Sur-

TABLE 2. EFFECT OF LYSOLECITHIN ON AMOUNT OF RuBPC PRECIPITATED FROM SALT SOLUTIONS AT VARIOUS pH's BY ADDITION OF TANNIC ACID SOLUTION OR EXTRACT OF PIN OAK FOLIAGE

pH	Lysolecithin conc. (%)	RuBPC in incubation mixture (mg)	RuBPC precipitated (mg)[a]	
			By 300 μg TA	By foliage extract[b]
6.15[c]	0.00	0.96	0.82 ± 0.004 (4)	0.77 ± 0.009 (6)
	0.06	0.96	0.04 ± 0.001 (5)	0.03 ± 0.000 (6)
6.90[d]	0.00	1.18	1.09 ± 0.004 (5)	ND[e]
	0.06	1.18	0.05 ± 0.001 (6)	ND
7.55[c]	0.00	1.13	0.66 ± 0.019 (6)	0.51 ± 0.013 (5)
	0.06	1.13	0.15 ± 0.004 (6)	0.08 ± 0.004 (5)
8.30[d]	0.00	1.13	0.55 ± 0.016 (6)	ND
	0.06	1.13	0.18 ± 0.004 (6)	ND
9.30[d]	0.00	0.96	0.51 ± 0.006 (5)	ND
	0.06	0.96	0.21 ± 0.002 (6)	ND

[a]Values are the mean ± standard error, with the number of replicates given in the parenthesis.
[b]From 2.5 mg (dry wt) milled leaf material.
[c]$[Na^+]$ = 2.5 mM, $[K^+]$ = 131.0 mM, $[Mg^{2+}]$ = 25.0 mM, $[Ca^{2+}]$ = 16.0 mM.
[d]$[Na^+]$ = 3.0 mM, $[K^+]$ = 160.0 mM, $[Mg^{2+}]$ = 30.0 mM, $[Ca^{2+}]$ = 20.0 mM.
[e]ND, not determined.

face-tension measurements (Martin and Martin, 1984) have shown that in the gut fluids of *Manduca sexta* (on artificial diet), *Malacosoma sp.* (on black cherry foliate), and *Colias philodice* (on alfalfa foliage), surfactants are present at concentrations of 10, 20, and 30 times CMC, respectively. At pHs 6.15, 6.90, and 7.55, lysolecithin virtually prevents the precipitation of RuBPC by tannic acid. At pHs 8.30 and 9.30, lysolecithin reduces the amount of RuBPC precipitated from 49% to 16% and from 54% to 21%, respectively.

In earlier papers, Martin and Martin (1982, 1983) demonstrated that extracts of mature pin oak foliage have high protein-precipitating capacity, presumably due to the presence of high concentrations of tannins. In this study we demonstrate that at pHs 6.15 and 7.55, lysolecithin is also effective at preventing pin oak tannins from precipitating RuBPC from a solution containing alkali metal and alkaline earth cations (Table 2).

DISCUSSION

The objective of this study was to ascertain whether the conditions that prevail in the gut fluids of insect herbivores favor or disfavor the formation of insoluble complexes between RuBPC, the major protein of photosynthetic tissue, and plant tannins. Our results clearly indicate that conditions are unfavorable for the formation of such complexes. Our findings, therefore, are not supportive of the hypothesis that tannins reduce the digestibility of plant tissues by forming insoluble, indigestible complexes with ingested protein in an herbivore's gut.

Since potassium, magnesium, and calcium are the major cations in plant tissue, it is to be expected that they would also be the major cations in the midgut fluids of an insect herbivore. Potassium ions are also pumped into the midgut from the hemolymph. These ions favor the formation of insoluble RuBPC–tannin complexes. However, other characteristics of the gut fluid effectively counter the protein-precipitating potential of tannins, even in the presence of potassium, magnesium, and calcium ions.

Previous investigators have suggested that an elevated midgut pH may be an antitannin adaptation. Our results suggest that high detergency is far more effective than high alkalinity in reducing the amount of protein precipitated by tannins. While an increase in pH can virtually prevent the precipitation of RuBPC by tannins when only sodium and potassium ions are present, the amount precipitated is only moderately reduced by an increase in pH when magnesium and calcium ions are present. By contrast, lysolecithin significantly reduces the amount of protein precipitated when all four ions are present and is effective over a wide pH range.

It is becoming increasingly evident that the digestive systems of insects possess several characteristics that counteract the potential antidigestive properties of tannins (Bernays, 1981). This study and our earlier one (Martin and

Martin, 1984) suggest that the presence of surfactants in the gut fluid may be one of the most effective and widespread of these traits. Indeed, it is clear that tannins are not the general, all-purpose, dose-dependent, antidigestive, defensive chemicals they were once thought to be.

Acknowledgments—This research was supported by grants from the National Science Foundation (PCB78-22733 and DEB 80-22634). We thank Prof. Wayne Frasch for determining the purity of our RuBPC preparation by HPLC.

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