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Surfactants: their role in preventing the precipitation of proteins by tannins in insect guts

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Summary. Much more tannic acid or pin oak tannin is required to precipitate the abundant leaf protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC), from *Manduca sexta* gut fluid adjusted to pH 6.5 than is required to precipitate this protein from an aqueous buffer at the same pH. This finding demonstrates that some characteristic of *M. sexta* gut fluid, in addition to its basicity, counteracts the potential of tannins to precipitate ingested proteins. Gut fluid of *M. sexta* has a surface tension of 36–39 dynes/cm, indicating the presence of surfactants. Lysolecithin and linoleoylglycine, surfactants known to be present in insect gut fluids, also interfere with the precipitation of RuBPC by tannins at pH 6.5. It is concluded that detergency is a widespread property of insect gut fluids that counteracts the potential of tannins to precipitate dietary proteins, and it is argued that there is no longer any justification for continuing to refer to tannins as digestibility-reducing-substances. Finding that there has been no formidable barrier to the evolution of mechanisms that counter a generalized antidigestive action by tannins is difficult to reconcile with the idea that reduced digestibility is an evolved anti-herbivore adaptation of apparent plants.

Introduction

Tannins are water-soluble phenolic componds that occur widely in vascular plants. They have adverse effects upon organisms as diverse as viruses, bacteria, fungi, insects, reptiles, birds and mammals (Swain 1979), and they have been accorded an important role as defensive chemicals that protect plant tissues from herbivore attack (Feeny 1976; Rhoades and Cates 1976). Since tannins are known to precipitate proteins (van Sumere et al. 1975; Hagerman and Butler 1981; McManus et al. 1983), it has been suggested that they might reduce the digestibility of plant tissue, either by precipitating the proteins of the foliage or the digestive enzymes of an herbivore (Feeny 1976; Rhoades and Cates 1976). Since ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC) often makes up as much as 25% of the total protein and 25–50% of the soluble protein in photosynthetic tissues (Singer et al. 1952; Akazawa 1970; Lyttleton 1973; Jensen and Bahr 1977), it is a major dietary protein for any foliage-feeding insect. Its interactions with tannins are, therefore, particularly relevant to an evaluation of the potential role of tannins as digestibility-reducing substances. In an earlier study (Martin and Martin 1983), we showed that tannic acid, quebracho and pin oak (*Quercus palustris*) tannins precipitate many times their weight of RuBPC at pH values between 6.0 and 8.0, but that these polyphenols precipitate little or none of this protein at pH values above 8.0 in the presence of 0.17 *M* sodium chloride.

Since many tannin-protein complexes that are insoluble at neutral or slightly acidic pH values readily dissolve under more basic conditions (Hagerman and Butler 1978), it has been suggested that the high alkalinity of the gut fluids of many herbivorous lepidopteran larvae is a counter-adaptation to tannins (Feeny 1970; Berenbaum 1980). However, there are other mechanisms besides the maintenance of alkaline gut conditions by which herbivores avoid the potentially harmful effects of these compounds. Many polyphagous acridids, grasshoppers with neutral or slightly acidic gut fluids, are quite tolerant of tannins (Bernays and Chamberlain 1980; Bernays et al. 1980, 1981). The lack of any adverse effects of tannic acid on the locust, *Schistocerca gregaria*, has been attributed to the hydrolysis of tannic acid in the gut by β-glucosidases, adsorption of tannic acid on the peritrophic membrane, and "some other unknown factor" (Bernays 1981).

Insoluble tannin-protein complexes are readily solubilized by detergents (Goldstein and Swain 1965; Oh et al. 1980). While the surfactant properties of insect digestive fluids have received only limited study, the presence of surfactants has been established in the cabbageworm, *Pieris brassicae* (Turunen and Kastari 1979), the cricket, *Gryllus bimaculatus* (Collatz and Mommsen 1974), and the predatory beetle, *Dytiscus marginalis* (Vonk 1969).

In this study we have investigated the effects of detergents known to occur in insect gut fluids on the interaction of tannic acid and pin oak tannins with RuBPC at pH 6.5, a pH at which RuBPC ordinarily forms an insoluble complex with tannins. We have also explored the interaction of tannic acid and pin oak tannins with RuBPC in the gut fluid of the tobacco hornworm, *Manduca sexta*, and have discovered that even at a pH of 6.5 there is some factor present that interferes with precipitation. Finally, we have demonstrated the presence of surfactants in the gut fluid of this insect and propose that detergency, in addition to basicity, counteracts the potential of tannins to reduce the digestibility of ingested proteins.

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Materials and methods

Preparation of extracts of pin oak foliage

Pin oak leaf powder (40 mg dry wt) was extracted twice for 8 min with 1.6 ml of boiling 50% (v/v) aqueous methanol (Martin and Martin 1983). The extract was concentrated to dryness at reduced pressure, and the residue redissolved in water. Material that did not dissolve was removed by centrifugation. This method of extraction produced an extract with 5 times the protein-precipitating capacity of the 70% aqueous acetone extract recommended by Foo and Porter (1980).

Preparation of gut fluid

Gut fluid (pH 8.4–9.5), collected from an incision in the anterior portion of the midgut was centrifuged (32,000 × g, 15 min, 4° C) and deproteinized by mixing with 9 volumes of 95% ethanol. After 20 min at 5° C, the mixture was centrifuged (14,000 × g, 15 min, 4° C), solvent was removed from the supernatnat solution at reduced pressure, and the residue redissolved in a volume of water equal to 26.5% of the original. This concentration of gut fluid was used so that the final test solution (prepared by combining the RuBPC solution, gut fluid, buffer and tannin solution as described below) would have a concentration of gut fluid equivalent to the original.

Synthesis of linoleoylglycine

To 0.234 g of glycine in 1.5 ml of 2 *N* potassium hydroxide, cooled to 0° C, was added dropwise 0.5 g linoleoyl chloride and 1.0 ml of 2 *N* potassium hydroxide. The mixture was stirred for 30 min with a magnetic stirring bar, after which 20 ml of water was added. The mixture was acidified with concentrated hydrochloric acid and extracted with ether. The ether extract was washed with water until the extracts were neutral, dried over anhydrous sodium sulfate, and evaporated to dryness. The waxy residue was recrystallized from petroleum ether, giving 0.5 g of purified linoleoylglycine, m.p. 70–72° C; lit value, 64–67° C (Tsuchiki et al. 1965).

Precipitation of RuBPC by tannins

A stock solution of RuBPC and surfactant (or gut fluid) was prepared by combining 13 ml of a solution of RuBPC (2.5 mg/ml) in buffer (0.1 *M* MES, 0.17 *M* NaCl, pH 6.5) with 6.5 ml of surfactant solution (or concentrated deproteinized gut fluid), adjusting the pH to 6.5 with 0.5 *M* HCl, and adding buffer to a final volume of 22 ml. In the control, the surfactant (or gut fluid) was replaced by buffer. To a 1.35-ml aliquot of this stock solution was added 0.15 ml of water or of tannic acid or pin oak tannin solution. Following centrifugation (32,000 × g, 15 min, 20° C), the pellet was gently rinsed with 1.0 ml of buffer. The combined supernatant and rinse solution was applied to a 1.7 × 5.0-cm column of Sephadex G-25 (Pharmacia, PD-10) equilibrated in MES buffer. Proteins were eluted in 3.5 ml of the same buffer. Protein in the eluent was assayed by mixing a 50 µl aliquot with 2.5 ml of Coomassie Brilliant Blue G-250 dye reagent (BioRad Protein Dye Reagent, 1:4 dilution), and determining A_{595} after 6 min (Bradford 1976) using a blank of 50 µl of buffer plus 2.5 ml of the dye reagent. The absorbance at 595 was converted into mg RuBPC in solution by the use of a calibration curve constructed on the same day as the assay. Surfactant (or gut fluid) was included in the protein solutions used for calibrating those experimental solutions containing surfactant (or gut fluid). From a determination of the amount of protein in the original solution and the amount remaining after the addition of tannic acid or pin oak tannins, the amount precipitated was calculated.

Chemical sources

Glycine (Sigma G-7126), linoleoyl chloride (Sigma L-5753), lysolecithin (Sigma L-5379), MES (Calbiochem-Behring 475893), RuBPC (Sigma R-8000), sodium oleate (Sigma 0-7501), tannic acid (Sigma T-0125).

Surface tensions

Surface tension was measured on as little as 25 µl of sample using the horizontal thick-walled capillary apparatus of Ferguson (1943).

Results

Surfactant properties of M. sexta gut fluid

The surface tension of *M. sexta* gut fluid is 36–39 dynes/cm, and does not increase much above that value until the gut fluid has been diluted 10-fold or more (Fig. 1). This indicates that not only are surfactants present, but that they are present at about 10 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. Surface tension measurements on the gut fluids of other insect species suggest that surfactants occur widely in insect digestive fluids (Table 1).

Only two surfactants from insect gut fluids have been characterized. Lysolecithin, generated during the digestion

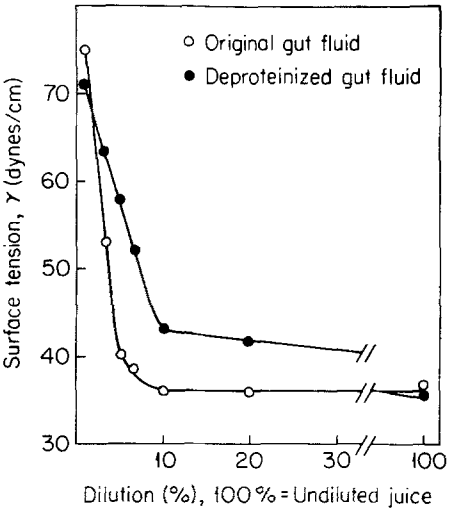

Fig. 1. Effect of dilution on surface tension (γ) of *M. sexta* gut fluid. Gut fluid was obtained from fifth instar larvae weighing less than 5 g, reared from eggs on an artificial agar-based diet (Yamamoto 1969) supplemented with wheat germ oil (2.99 g/1000 g diet)

Table 1. Surface tensions of gut fluids from *G. bimaculatus* adults, fifth instar nymphs of *M. sanguinipes*, *D. marginalis* larvae, and late instar larvae of three lepidopteran species

Species	γ (dynes/cm)	Source
Orthoptera		
Gryllus bimaculatus	37–42	Collatz and Mommsen (1974)
Melanoplus sanguinipes	33	This study
Coleoptera		
Dytiscus marginalis	35	Vonk (1969)
Lepidoptera		
Anisota senatoria	34	This study
Hyalophora cecropia	42	This study
Manduca sexta	36–39	This study

of phospholipids, is present in *P. brassicae* (Turunen and Kastari 1979), and long chain fatty acyl conjugates of amino acids are present in *G. bimaculatus* (Collatz and Mommsen 1974).

Precipitation of RuBPC from M. sexta gut fluid and surfactant solutions by tannins

When RuBPC is dissolved in *M. sexta* gut fluid adjusted to a pH of 6.5, much larger amounts of tannic acid or pin oak tannins are required to bring about precipitation of an insoluble complex than when this protein is dissolved in an aqueous buffer at the same pH (Table 2). While 300 µg of tannic acid precipitates all 2.0 mg of RuBPC from 1.5 ml of a buffer solution at pH 6.5, this amount of tannic acid precipitates only 0.3 mg of this protein when it is dissolved in 1.5 ml of *M. sexta* glut fluid, adjusted to a pH of 6.5. Even 500 µg of tannic acid fails to precipitate the entire 2.0 mg of RuBPC dissolved in the gut fluid. Likewise, a given amount of pin oak tannins precipitates much less RuBPC from *M. sexta* gut fluid adjusted to pH 6.5 than from a buffer at the same pH. These experiments clearly demonstrate that some characteristic of *M. sexta* gut fluid, in addition to its alkalinity, interferes with the formation and precipitation of insoluble complexes between RuBPC and tannins.

The precipitation of RuBPC-tannin complexes from dilute solutions of surfactants known to occur in insect gut fluids also requires larger quantities of tannin than are required to precipitate such complexes from aqueous solutions lacking surfactants (Table 2). Lysolecithin and linoleoylglycine at concentrations 10 and 7 times their CMC's, respectively, concentrations that are comparable to that of the surfactant in *M. sexta* gut fluid, are as effective as gut fluid at preventing the precipitation of RuBPC by tannic acid and pin oak tannins. These results strongly suggest that surfactants are responsible for interfering with the formation of insoluble RuBPC-tannin complexes in *M. sexta* gut fluid when the pH has been reduced to 6.5.

Sodium oleate, another surface active substance that would be generated during the digestion of lipids, is ineffective in preventing precipitation of RuBPC by tannic acid and much less effective than gut fluid or the two detergents in preventing precipitation by pin oak tannins.

Discussion

Since the diet of *M. sexta* does not include tannin-rich plants, and since the gut fluid of this species ordinarily has a pH between 8.4 and 9.5, it can hardly be argued that the presence of surfactants that interfere with the formation of insoluble RuBPC-tannin complexes at pH 6.5 is an evolved adaptation to cope with ingested tannins. The significance of this study lies rather in its demonstration that the detergency of insect gut fluid can serve to counteract the potential protein-precipitating capacity of tannins. Since every study that has tested for surfactants in insect gut fluids has confirmed their presence, there is reason to believe that detergency is a common and widespread characteristic of insect digestive juices. In species with neutral or slightly acidic guts, such as *M. sanguinipes* (Table 1) and other acridids, gut fluid detergency could play a major role in interfering with the precipitation of ingested plant protein

Table 2. Effects of deproteinized gut fluid and various surfactants on the precipitation of RuBPC from aqueous media (pH 6.5) by tannic acid and pin oak tannins. The assay mixture contained 2 mg RuBPC. Values are $\bar{x} \pm$SEM for the number of replicates given in brackets. CMC is the critical micelle concentration; MES is 2-(N-morpholino)ethanesulfonic acid

Medium (concentration)	RuBPC precipitated (mg)					
	Tannic acid (µg)			Pin oak tannins (mg dry leaf powder extracted)		
	210	300	500	1.8	2.5	5.0
Buffer (0.09 *M* MES, 0.15 *M* NaCl)	1.46±0.02 [16]	2.00±0.00 [3]	2.00±0.00 [3]	1.02±0.03 [13]	1.59±0.04 [10]	1.74±0.02 [3]
Deproteinized gut fluid (Full strength = 10 X CMC)	0.30±0.02 [3]	0.30±0.11 [7]	1.62±0.04 [6]	0.00±0.00 [3]	0.41±0.04 [7]	1.24±0.02 [3]
Lysolecithin (0.04% = 10 X CMC)	0.00±0.00 [3]	0.26±0.02 [5]	–	0.00±0.00 [3]	0.19±0.03 [5]	–
Linoleoylglycine (0.035% = 7 X CMC)	0.00±0.00 [3]	0.21±0.01 [3]	0.84±0.03 [4]	0.00±0.00 [2]	0.00±0.00 [2]	0.75±0.001 [3]
Sodium oleate (0.08% = 10 X CMC)	1.53±0.01 [3]	–	–	0.73±0.01 [7]	1.39±0.03 [6]	–

by tannins. Perhaps surfactants in the gut fluid are the "other unknown factor" hypothesized by Bernays (1981).

The hypothesis that tannins owe their effectiveness as defensive chemicals to generalized, dose-dependent antidigestive properties includes an assumption that counter-adaptations to such a mode of action are difficult to evolve. Finding that a widespread, possibly even ubiquitous, trait of the digestive systems of herbivores can interfere with the potential of tannins to precipitate a major dietary protein is a challenge to that assumption. Indeed, it is becoming increasingly evident that the digestive systems of insects possess a number of characteristics that could counteract the potential antidigestive properties of tannins. Not only does the detergency and/or alkalinity of gut fluid interfere with the formation of insoluble tannin-protein complexes, but in addition some species possess digestive β-glucosidases that are able to degrade hydrolyzable tannins, and peritrophic membranes that can adsorb tannins and remove them from the gut mileu (Bernays 1981). Whether any of these traits has evolved specifically in response to diets high in tannins is not at all clear, since they occur widely in species with low-tannin or tannin-free diets as well as species with high-tannin diets. However, what is clear is that the evolution of mechanisms that effectively counter a generalized antidigestive action by tannins is not such an unlikely event, whatever the selection pressures may have been that resulted in their evolution.

It is noteworthy that in those cases in which tannins have been shown to have an adverse effect upon the growth and survival of insect herbivores and in which the mode of action has been determined, it has been found that they act by inhibiting feeding or causing cell damage, not by interfering with digestion and assimilation (Bernays et al. 1980; Bernays 1981; Klocke and Chan 1982; Reese et al. 1982). Indeed, we are not aware of a single case in which ingested tannins have been demonstrated to reduce the efficiency of protein digestion in an insect herbivore. In our opinion, it is neither justifiable nor useful to continue to refer to tannins as digestibility-reducing-substances. A moratorium on the use of this unwarranted designation, at least until the appearance of an unambiguous demonstration of this mode of action, would allow discussions of the role of tannins in plant-insect interactions to focus upon the well-documented properties of these substances as feeding deterrents and toxins.

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