Activities of enzymes of the pancreas, and the lumen and mucosa of the small intestine in growing broiler cockerels fed on tannin-containing diets

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Diets containing vegetable tannins, predominantly hydrolysable gallotannins, at levels of 13·5, 25 and 50 g/kg were fed to growing broiler cockerels to examine their effect on enzymes in the pancreas, the intestinal lumen and the intestinal mucosa. Pancreas weight per unit live weight showed a significant ($P < 0·05$) increase with increasing level of dietary tannin while that of the liver remained unaffected. Trypsin (*EC* 3.4.21.4) and α-amylase (*EC* 3.2.1.1) activities in the pancreas of birds fed at the highest level of tannins were more than double those from birds fed on a tannin-free control diet. In the intestinal lumen inhibition of trypsin activity increased with increasing level of dietary tannin; α-amylase activity was inhibited at intermediate tannin levels but was restored at the highest level. Dipeptidase (*EC* 3.4.13.11) and sucrose α-glucosidase (disaccharidase) (*EC* 3.2.1.48) in the intestinal mucosa were both inhibited by tannins. Growth of the birds and digestibility of nitrogen were adversely affected by the tannin-containing diets.

Tannin: Pancreatic enzymes: Brush border enzymes: Cockerel

It is well established that tannins are potential protein precipitants (Hagerman & Butler, 1980; Hagerman & Klucher, 1986; Makker *et al.* 1987) and that they reduce the digestibilities of proteins (Mohammed & Ahmed, 1987) when present in animal feeds. Elevated faecal nitrogen excretion associated with ingestion of tannin-containing feeds is ascribed largely to interactions between either tannins and dietary protein or tannins and digestive enzymes, or both. However, Mitjavilla *et al.* (1977) concluded that the excess faecal N is mostly a result of mucus hypersecretion.

Studies in vitro (Griffiths, 1981; Lumen & Salamat, 1980; Horigome *et al.* 1988) and in vivo (Griffiths & Moseley, 1980; Horigome *et al.* 1988) have demonstrated the formation of tannin–enzyme complexes. However, adopting a technique in vitro more relevant to the situation in vivo, Mole & Waterman (1987) showed that trypsin retained all its activity in the presence of tannic acid when the system included a substrate protein (bovine serum albumin; BSA) and glycocholic acid, indicating that under such conditions tannins have a preferential affinity for dietary protein over enzyme protein. In previous work Mole & Waterman (1985) had shown that the formation of complexes between tannins and BSA, used as a substrate for proteolysis, could result in either enhancement or inhibition of proteolysis, or no effect at all depending on the tannin:BSA ratio in the complex.

In contrast to what had been reported with rats (Griffiths & Moseley, 1980; Horigome *et al.* 1988), tannins were shown to exert no effect on protein digestion by insect herbivores (Martin *et al.* 1985, 1987).

The present study was undertaken to investigate the response of the pancreas to tannins

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Table 1. *Ingredients and chemical composition of experimental diets*

	Diet			
Total tannins* (g/kg)...	50·0	25·0	13·5	0·0
Ingredients (g/kg)				
Barley	—	280·0	410·0	557·5
Salseed meal	516·0	257·0	137·0	—
Soya-bean meal	255·0	247·5	245·0	240·0
Rice	120·0	120·0	120·0	120·0
Wheat bran	50·0	50·0	50·0	50·0
Fish meal	41·0	33·0	28·0	25·0
Vegetable oil	18·0	12·5	10·0	07·5
Composition				
Crude protein (nitrogen × 6·25; g/kg)	201·5	201·5	201·2	201·4
Metabolizable energy (MJ/kg)	12·66	12·64	12·60	12·64

* Estimated from condensed and hydrolysable tannin content of salseed meal.

in terms of changes in activities of enzymes in the lumen of the small intestine, in order to provide further insight into the mode of action of dietary tannins. A further aim was to examine the effect of dietary tannins on the activity of membrane-bound enzymes which are considered to take part in nutrient absorption (Miller & Grane, 1961).

In a series of experiments, extracted meal from the seed of the Sal tree (*Shorea robusta*) was used as a source of tannins. The seed is mainly collected from tropical forests for its oil. The seed residues after oil extraction have a crude protein (nitrogen × 6·25; CP) and a carbohydrate content similar to that of cereals but have a much higher content of tannins, particularly hydrolysable tannins.

MATERIALS AND METHODS

Experimental animals and diets

The broiler cockerels (*Cobb*), supplied by Hamish Morison Ltd, Earlston, used in the present experiment were received on the day of hatching. They were reared in a group for 2 weeks on a commercial broiler starter mash (Varley Feeds, Darlington). Rearing and experimental treatments were conducted in a controlled environment room (temperature $22 \pm 2°$, lighting period 23 h/d).

The formulation and estimated chemical composition of four isonitrogenous (20 g CP/kg) and isoenergetic (12·6 MJ/kg) diets containing salseed meal (diet 1), barley (diet 4) or mixtures of salseed and barley (diets 2 and 3) are shown in Table 1. Vitamin–mineral premix (Nutec Ltd, Lichfield) was added to the feed at a rate of 25 g/kg. The estimated composition is based on analysis of the components used in the preparation of the diets.

Analysis of tannins

The vanillin method of Price *et al.* (1978) was used to estimate the content of condensed tannins which were extractable only in acidified methanol (10 ml hydrochloric acid/l). The vanillin reagent gives positive reactions with non-tannin free flavanoids (Sarker & Howarth, 1976). Polyvinylpyrrolidone was used to bind tannins and, thereby, provide a blank which compensated for the contribution of the non-tannin free flavanoids to the colour development with the vanillin reagent. The dye-labelled protein precipitation

method (Asquith & Butler, 1985) for total tannin content was applied to extracts made with aqueous methanol (500 ml/l). Since the condensed tannins were not extracted with this solvent the result was taken to be the content of hydrolysable tannins.

Digestibility trial

After the adaptation period, birds were weighed and allocated to forty individual battery cages. Each cage was equipped with a feeding trough, a drinker and a tray for the collection of excreta. Each of the four experimental diets were fed to ten birds. Throughout the experiment diets were moistened just before feed was offered to minimize losses of feed and marker. Experimental diets to which chromic oxide (20 g/kg) had been added were fed for the 15 d. Feeding was restricted (65 g/d at start, rising to 95 g/d on day 15) so that all birds, irrespective of diet, consumed the same amount of feed each day. No collections of excreta were made for the first 7 d in which the passage of Cr_2O_3 was allowed to stabilize (Kotb & Luckly, 1972); collection of excreta was started after day 7. Some birds (two birds per treatment) were fed on experimental diets without Cr_2O_3 to provide gut tissue samples for histological examination and to provide sample blanks for use in Cr_2O_3 analysis. Collection of excreta was carried out at least six times each day over a 7 d period; collected excreta were immediately frozen, subsequently freeze-dried, ground and stored at $-4°$ in sealed plastic containers while awaiting analysis. Excreta samples were analysed for total N, Cr_2O_3 (Fenton & Fenton, 1979), uric acid (Terpstra & De Hart, 1974) and ammonia (Agricultural Development and Advisory Service, 1986). N digestibility was calculated by the method of Terpstra & De Hart (1974). After the digestibility trial, birds were kept on the same diets but without Cr_2O_3 for a further 2 weeks during which feeding was *ad lib.*

Sample collection for determination of enzyme activities

After 4 weeks on the experimental diets the birds were killed by cervical dislocation. The digestive tract was promptly exposed and each segment was tied to prevent further movement of the gut contents. The distal duodenum was removed and carefully washed with ice-cold saline (9 g sodium chloride/l). The contents of a length (about 150 mm) of jejunum were extruded by gentle pressure between the thumb and forefinger into a preweighed specimen tube and stored at $-20°$. The pancreas was also removed, freed from connective tissue and fat, frozen in liquid N_2 and stored at $-70°$.

Preparation of tissue homogenates and determination of enzyme activities

The duodenal sections were cut open longitudinally, laid on the base of an ice-cooled Pyrex oven dish and the mucosa was scraped off using a microscope slide. The weighed mucosa was homogenized in ice-cold distilled water (Lasheras *et al.* 1980), centrifuged (15200 ***g*** for 20 min at 5°), diluted with distilled water and analysed immediately for dipeptidase (*EC* 3.4.13.11; Nicholson & Kim, 1975) and sucrose α-glucosidase (disaccharidase) (*EC* 3.2.1.48; Dahlqvist, 1968) activities.

Portions of intestinal chyme (1 – 1·5 g) were homogenized in ice-cold saline and centrifuged (70000 ***g*** for 20 min at 4°). After dilution the supernatant fractions were assayed for trypsin (*EC* 3.4.21.4) (Hamerstrand *et al.* 1981) and for α-amylase (*EC* 3.2.1.1); dye-labelled starch (Starch Azure; Sigma) was used as the α-amylase substrate.

Each pancreas was homogenized in potassium chloride (0·154 mol/l)–Tris-HCl (50 mmol/l), adjusted to pH 7·4 and centrifuged (70000 ***g*** for 20 min at 4°). Trypsin and α-amylase activities were determined as described previously; pancreatic trypsin was activated using purified enteropeptidase (enterokinase) (*EC* 3.4.21.9; Gertler & Nitsan, 1970).

Table 2. *Live-weight gain (age 14–42 d), feed conversion efficiency (age 28–35 d), apparent nitrogen digestibility (age 21–38 d), and liver and pancreas weights (age 42 d) in fowl fed on diets containing different levels of tannins*

(Results are means with their standard errors for eight animals)

	Diet*							
Total tannins (g/kg)....	50·0		25·0		13·5		0·0	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Live-wt (g)	392·8[a]	6·2	933·0[b]	17·2	1210·8[c]	16·5	1475·0[d]	11·9
Feed conversion efficiency (g gain/g feed intake)	0·16[a]	0·01	0·21[b]	0·020	0·27[c]	0·01	0·37[d]	0·01
Apparent digestibility* of crude protein (N × 6·25)	0·391[a]	0·026	0·615[b]	0·020	0·698[c]	0·011	0·772[d]	0·013
Liver wt (g/kg live wt)	21·0[a]	0·18	19·5[a]	1·0	20·0[a]	0·5	21·0[a]	0·14
Pancreas wt (g/kg live wt)	3·8[a]	0·2	2·6[b]	0·1	2·0[c]	0·5	1·8[c]	0·1

[a, b, c, d] Values in the same row with different superscript letters were significantly different ($P < 0·05$).

* For details, see p. 190 and Table 1.

Statistical procedure

Results were subjected to analysis of variance to detect differences between treatments. Means were then compared using Duncan's multiple range test.

RESULTS

Tannin content of salseed meal

The condensed tannins of salseed meal were present at a level of 26 g catechin equivalent (CE)/kg and the hydrolysable tannin content was estimated to be 73 g/kg.

Animal performance and protein digestibility

All performance variables presented in Table 2 were adversely affected ($P < 0·05$) by increasing levels of dietary tannins. Birds which received tannins in their diets at levels of 13·5, 25·0 and 50·0 g/kg achieved live-weight gains of 0·82-, 0·63- and 0·27-fold those achieved by the birds fed on the tannin-free diet between weeks 1 and 4 of the treatment period. This effect amounted to a decrease in live-weight gain of between 197 and 234 g for every 10 g/kg increase in tannin content of the diet. Feed conversion efficiency, recorded in week 3, of birds which received the highest amount of tannins was reduced by more than half compared with the controls. Apparent digestibility of protein was negatively correlated with tannin content (g/kg) of the diet (digestibility $= 0·79 - 0·0076 \times$ tannin content, R^2 0·91, n 32). The effect on protein digestibility, of tannins at the highest level, was disproportionately greater than at the lower levels.

Pancreas and liver weights

Tannins exerted a significant ($P < 0·05$) effect on the weight (g/kg live weight) of the pancreas but the liver weight remained unaffected. No lesions were observed in either the pancreas or the liver. The weight of the pancreas from birds which received the highest amount of tannins was more than twice that of the control birds. Significant enlargement of the pancreas was detectable only at tannin concentrations above 13·5 g/kg. As the tannin

Table 3. *Trypsin* (EC *3.4.21.4*) *activity* (*units* × 10^{-2}) *and α-amylase* (EC *3.2.1.1*) *activity* (*units* × 10^{-3}) *in pancreas of fowl fed on diets containing different levels of tannins*

(Results are means with their standard errors of samples from eight animals)

	Diet*							
Total tanins (g/kg) ...	50·0		25·0		13·5		0·0	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Trypsin								
Units/g pancreatic tissue	124[a]	13	98[a]	8	106[a]	11	109[a]	11
Units/kg body-wt	428[a]	66	255[b]	28	249[b]	29	206[b]	17
α-Amylase								
Units/g pancreatic tissue	1165[a]	119	1166[a]	156	937[a]	27	855[a]	84
Units/kg body-wt	3840[a]	270	2740[b]	200	2340[b]	200	1620[c]	120

[a, b, c] Values in the same row with different superscript letters were significantly different ($P < 0.05$).

* For details, see p. 190 and Table 1.

content increased from 13·5 to 25 g/kg and from 25 to 50 g/kg the pancreas increased in weight by 33 and 110% respectively compared with the control birds. The effect of tannin content on pancreatic hypertrophy, like the effect on protein digestion, was disproportionately greater at the highest tannin level.

Pancreatic enzyme activities

When trypsin and α-amylase activities (Table 3) were expressed as units per g pancreas fresh weight there was no significant difference between treatments, although there was a trend towards increasing α-amylase activity with increasing content of tannin in the diet. When these activities were related to live weight, significant differences were found. The pancreas from birds which received the highest amounts of tannins accumulated 1·1-fold more trypsin activity and 1·4-fold more α-amylase activity than that observed in the pancreas from the control birds.

Lumen enzyme activities

The activities of both trypsin and α-amylase in the gut (Table 4) suffered increasing inhibition as the level of salseed tannins in the diet increased from 0 to 25 g/kg. However, at the highest level (50 g/kg), whilst the trypsin activity was lowest, the α-amylase activity had been restored to a level near that of the control birds.

Membrane-bound enzyme activities

The activities (Table 5) of mucosal dipeptidase (L-leucylglycine substrate) and sucrose α-glucosidase were both sensitive to tannins. At the highest level of tannins the activities of both enzymes were reduced to about one-third of that found in the control birds.

DISCUSSION

The differences in growth rate observed in the present experiment were consistent with previous observations on diets containing tannins (Mohammed & Ahmed, 1987). However, in the later stage of the *ad lib.* feeding period a rapid increase in the growth rate was noted among the birds fed on the diets containing 50 and 0 g tannins/kg but not in the birds on the other treatments. It is possible that this was a consequence of the restriction on feeding

Table 4. *Lumen enzyme activity in the mid-gut of fowl fed on diets containing different levels of tannins*

(Results are means with their standard errors of samples from eight animals)

	Diet*							
Total tannins (g/kg)...	50·0		25·0		13·5		0·0	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Trypsin (*EC* 3.4.21.4; Units × 10^{-2})								
/g wet chyme	6·42[a]	0·30	9·12[a]	0·76	14·14[b]	1·44	24·78[c]	2·53
/g dry chyme	47·42[a]	5·26	60·67[a]	5·93	102·71[b]	13·24	245·20[c]	17·27
α-Amylase (*EC* 3.2.1.1; Units × 10^{-2})								
/g wet chyme	66·71[a]	4·69	34·60[b]	1·85	61·46[a]	4·53	66·64[a]	2·50
/g dry chyme	454·62[a]	19·79	238·60[b]	17·00	435·43[a]	27·33	603·83[c]	49·98

[a, b, c] Values in the same row with different superscript letters were significantly different ($P < 0·05$).

* For details, see p. 190 and Table 1.

Table 5. *Membrane enzyme activity in distal duodenal mucosa of fowl fed on diets containing different levels of tannins*

(Results are means with their standard errors of samples from eight animals)

	Diet*							
Total tannins (g/kg)...	50·0		25·0		13·5		0·0	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Dipeptidase (*EC* 3.4.13.11; Units × 10^{-3})								
/g mucosa	1·55[a]	0·23	2·40[b]	0·28	2·91[b]	0·26	4·50[c]	0·12
/g protein	43·3[a]	7·0	73·5[b]	8·6	92·6[b]	10·00	139·2[c]	10·0
Sucrose α-glucosidase (*EC* 3.2.1.48; Units)								
/g mucosa	12·6[a]	1·0	13·2[a]	1·6	25·7[b]	3·4	42·4[c]	1·7
/g protein	347[a]	37	397[a]	5·3	620[b]	47	778[c]	37

[a, b, c] Values in the same row with different superscript letters were significantly different ($P < 0·05$).

* For details, see p. 190 and Table 1.

during the digestibility period of the experiment since no similar observation has been made in a subsequent experiment using the same four diets as used here but where diets were given *ad lib.* throughout.

The elevated faecal N excretion observed when birds were fed on diets containing tannins could have resulted from either higher levels of endogenous protein in faeces, impaired digestion of dietary protein, or both. The observation is consistent with many similar observations (Griffiths & Moseley, 1980; Mohammed & Ahmed, 1987) which clearly demonstrate the adverse effect of dietary tannins.

The pancreatic enlargement induced by the tannin-containing diets in this experiment may have been an adaptive growth response to the presence of tannins in the gut, probably

mediated by hormones transported in the blood. Several investigators (Rothman & Wells, 1967; Johnson & Guthrie, 1974; Solomon *et al.* 1978; Dembinski & Johnson, 1980) have demonstrated the roles of gastrointestinal hormones, particularly cholecystokinin (CCK) and secretin, on pancreatic growth. Pancreatic enlargement similar to that obtained in the present experiment has been reported previously in response to soya-bean trypsin inhibitor (Levison *et al.* 1979) and lectins (Abbey *et al.* 1979; Grant *et al.* 1987). This may indicate a common mode of action of these antinutritional substances, at least at a cellular level. Brand & Morgan (1981) have demonstrated that oral administration of soya-bean trypsin inhibitor to the rat elicits a rapid fall in the concentration of CCK in gut tissue, probably as a result of secretion of CCK into the circulation; it is possible that tannins may exercise a similar influence.

Pancreatic synthesis and secretion is predominantly influenced by gastrointestinal hormones; CCK plays a major role by direct action on pancreatic acini (Rothman & Wells, 1967; Mainz *et al.* 1973; Liddle *et al.* 1985). The presence of tannins in the diet, through their effect on enzymes in the gut, may augment the secretion of CCK. In chickens it is possible that there is an additional factor involved in the regulation of pancreatic function. In contrast to other simple-stomached animals, the nerve endings in the pancreas of the domestic fowl terminate in direct contact with the acinar cells (Watanabe & Yasuda, 1977). This may indicate more involvement of neurocontrol in synthesis and secretion of pancreatic enzymes in the domestic fowl.

The ratio, α-amylase:trypsin activity accumulated within the pancreas was higher for the treatment groups (9·7:1 average) than for the control birds (7·8:1) and activities of both enzymes in pancreatic tissue were increased. This does not accord well with the effect of CCK on enzyme synthesis by rat pancreatic cells in vitro (Case *et al.* 1988) which resulted in a 42% increase in trypsinogen 1 and virtually no change in amylase synthesis (−4%). On the other hand administration of CCK by injection to rats over a 9 d period resulted in both pancreatic hypertrophy and increased activities of α-amylase and trypsin (Barrowman & Mayston, 1974). In a series of experiments (A. E. Ahmed and R. Smithard, unpublished results) we have noted that responses in the activities of α-amylase and trypsin in the pancreas to the presence of tannins in the diet may parallel one another or may differ quite markedly, depending on the period over which the birds have been fed on tannins.

At the highest level of dietary tannin the α-amylase activity in the gut was restored whilst that of trypsin remained severely inhibited. In this group of birds it appears that the hypertrophied pancreas, which was able to accumulate the highest amount of α-amylase, was able to secrete sufficient α-amylase to combine with free tannins and to restore amylolytic activity to a normal level in the gut. Our recent unpublished work (A. E. Ahmed and R. Smithard) has revealed the presence of high levels of trypsinogen but low trypsin activity in the intestinal chyme of birds fed on diets with high levels of tannins. Activation of this chyme with enteropeptidase resulted in 1·3-fold higher trypsin activity. It is therefore possible that mucosal enteropeptidase needed for the activation of trypsinogen, like the other mucosal enzymes assayed in the present experiment, is inhibited by tannins in the gut; also, tryptic autolysis may have been inhibited as found in vitro (Mole & Waterman, 1985). It might be expected that inhibition of enteropeptidase, as the activator of trypsin, might have a more pronounced effect on proteolytic activity than direct tannin inhibition of trypsin itself.

The inhibition of brush-border enzymes observed in the present study may have resulted from either direct complex formation between the enzymes and the tannins or through secondary effects which led to diminished synthesis. There is a close association between membrane digestion and the absorption of nutrients and it is likely that the tannins exert an indirect effect on absorption through this association. It is also possible that the

unabsorbed nutrients may affect osmoregulation of gut fluids, resulting in enhanced food passage (Launiala, 1968).

The findings reported here support the contention that the excessive excretion of faecal N associated with ingestion of tannins may largely be ascribed to the formation of tannin–endogenous enzyme complexes which inhibit digestion of dietary protein. There is also the possibility that tannins may play a role in the absorption of nutrients and this aspect is at present under further examination.

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