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Characteristics of Proline-rich Salivary Proteins Induced in Rat Parotid Glands by Tannins in Bean Hull

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ABSTRACT

Feeding rats a diet containing bean-hull causes hypertropy in their parotid glands due to the high tannin content. The amount of feed intake of rats fed bean-hull was higher than that of rats fed a standard diet. However, the increase in body weight of rats fed bean-hull was lower than that of rats fed a standard diet, which resulted in significantly low feed efficiency of the bean-hull containing diet. Within one week, parotid glands significantly enlarged and a series of proline-rich proteins (PRPs) were produced, which were similar to those induced by feeding high-tannin sorghum with slight differences in molecular weights. Even though the direct comparison between PRPs produced by the bean-hull containing diet and those induced by the high sorghum diet is not appropriate due to laboratory inconsistences, several new PRPs were produced by high tannin diets in both experiments. Differences in molecular weights of PRPs induced in two different tannin sources must be further investigated to be fully characterized. These morphological and biochemical changes have now been demonstrated to occur in response to the ingestion of tannins, presumably to diminish the anti-nutritional effects of tannins.

KEY WORDS: proline-rich proteins (PRPs), tannin, feed efficiency, parotid glands, rats.

INTRODUCTION

Proline-rich proteins are major components of saliva in humans as well as other animals. The macromolecules in saliva consist almost exclusively of protein, and it has for some time been apparent that this contains a complex mixture of proteins.[1] Amino acid analysis of human salivary proteins have demonstrated an unusually large amount of proline which varies from 16 to 33% of total amino acids. As a result of work by several investigators, it is now clear that saliva contains a group of highly unique proteins which usually are referred to as proline-rich proteins (PRPs).[2] Amsterdam *et al.*[3] found that a single injection of isoproterenol to rats results in a rapid secretion of PRPs and consequent loss of secretory granules from the salivary glands. Subsequently, Selye *et al.*[4] demonstrated that repeated administration of the drug causes a marked hyperplasia and hypertrophy of the parotid and submandibular glands. Prolonged treatment of rats with isoproterenol led to the appearance of a PRP in saliva which was not observed in untreated animals.[5]

Feeding of high-tannin sorghum to rats mimics some of the phenotypic changes observed after treatment of rats with isoproterenol. Within 3 days of feeding high-tannin sorghum, Mehansho *et al.*[6] observed about a 3-fold enlargement of parotid glands and about a 12-fold increase in the synthesis of a series of PRPs. These rats showed decreased weight gains compared to rats fed normal diets. However, the resumption of body weight gain by rats feeding on high-tannin diets was found to coincide with the maximum increase in parotid gland size and PRP synthesis. It was further observed that enlarged parotid glands and increased synthesis of PRPs in rats fed high-tannin diets returned to normal levels within 1 week when the rats were fed tannin-free, sorghum diets. Cell free translation experiments indicate that mRNAs coding for the PRPs are greatly increased after feeding high-tannin sorghum, similar to results obtained with parotid glands from isoproterenol-treated rats. Propranolol or alprenolol, an antagonist, was added to the high-tannin diet and observed to prevent the tannin-mediated induction of PRPs and parotid hypertrophy.[7]

PRPs exhibit a very high relative affinity for condensed tannins.[6] Tannins would bind selectively to these high affinity proteins even in the presence of a large proportion of other proteins with marginal or average affinities for tannins. A common characteristic of proteins and polypeptides with high affinity for tannin is their high pro-

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line content. Calf-skin gelatin (18 mol % proline and hydroxyproline) and rat parotid PRPs (43 mol % proline) were the most effective at binding tannins of all naturally occurring polymers tested.[8] Thus, these proteins rich in proline that are synthesized by the parotid glands in response to a high dietary tannin intake may function as tannin-binding agents and defend the animal against dietary tannin which would otherwise show even greater harmful effects.[9] The biological significance of PRPs has not been clearly established other than as a possible defense mechanism against dietary tannins. A similar defensive response in higher animals, including man, can be expected. Bennick[2] reported that human parotid secretions contain more than 70% PRPs. Butler *et al.*[10] observed that humans would select diets containing sufficient tannins to maintain the parotid glands in the induced state so that the contents of PRPs in human saliva could be kept at a certain amount.

Tannins, by definition, exhibit the ability to complex and precipitate proteins. Hundreds of millions of people in the semi-arid tropics depend heavily upon sorghum and legumes as sources of calories and proteins. Food legumes are important in the diets of people in most developing countries and are considered leading candidates to combat protein malnutrition since they are rich in proteins. They are also good sources of minerals, calories, and vitamins. Legumes contain high amounts of tannins, especially in legume hulls. If legumes are consumed without dehulling, the nutritional quality of legume could be reduced since tannins have several anti-nutritional effects such as diminished net energy intake, reduced digestibility, impaired growth rate and decreased food intake.[10] If PRP production can be induced by tannins from legumes and reduce the anti-nutritional effects, legumes can be used more widely as a good and economic protein source in many developing countries. In this study, PRP production from rat parotid glands and the characteristics of PRPs induced by tannins from bean-hull were examined.

MATERIALS AND METHODS

1. Animals and diets

Fifteen male Sprague-Dawley rats weighing 130 - 143 g (6 weeks old) were housed individually in stainless steel, mesh-bottomed (mesh #25) cages in a temperature and humidity controlled room. The rats were maintained on a commercial rat ration, Prolab 1000 (Agway Inc., Ithaca, NY) for 2 days after their arrival in order to adjust to the new environment. A bottle of distilled water was hung on each cage as well as an aluminum feed cup. The cups were refilled every day to assure ad libitum feeding. On the third day, rats were weighed and divided into 2 treatment groups. One group was control and fed AIN-76A standard diets, the other group was fed AIN-76A containing 5% dry ground bean hulls (Table 1). Hulls of dry bean seeds were removed from the black turtle soup variety after soaking in 95% ethanol for 24 hours. Hulls were removed and defatted by soaking them for 30 min in diethyl ether (10 ml per g of hulls). Tannin content in dry bean hull was measured after isolation of tannins by extraction using 50% acetone. After extraction, acetone was removed by bubbling with nitrogen gas and residue was freeze-dried. Adsorption chromatography using Sephadex LH-20 (Pharmacia, Inc., Piscataway, NJ) was conducted to further characterize and purify isolated tannins. Condensed tannin content in hulls of black turtle soup was calculated by dividing amounts of extracted tannins by amount of dried hulls. Hulls contain about 10% tannins. Therefore the diet with ground bean hulls was expected to contain approximately 0.5% tannin. Approximate tannin concentration in hulls of black turtle soup is as in Table 2.

Rats were fed a standard diet (control group) or the bean-hull containing diet (treatment group) for a week and euthanized by over-exposure to carbon dioxide. For one week of the experimental period, diet intake and weight increases were monitored every day to compare

Table 1. Compositions of experimental diet

Ingredient	g/Kg diet
Casein	200
Corn oil	50
Starch	150
Sucrose	500
Cellulose	45
Choline	2
AIN-76A vitamin mix[1]	10
AIN mineral mix[2]	35
DL-methionine	3
Dried black bean hull	5
Total	100%

1) AIN Vitamin Mixture (g/kg): Thiamin hydrochloride 600 mg, riboflavin 600 mg, pyridoxine hydrochloride 700 mg, nicotinic acid 3 g, D-calcium pantothenate 1.6 g, Folic acid 200 mg, D-biotin 20 mg, cyanocobalamin 1 mg, retinyl palmitate pre-mix (250,000 IU/g) 1.6 g, DL-alpha-tocopherol acetate (250 IU/g) 20 g, Cholecalciferol (400,000 IU/g) 250 mg, Menaquinone 5 mg, sucrose, finely powered 972.9 g

2) AIN Mineral Mixture (g/kg): Calcium phosphate dibasic 500 g, sodium chloride 74 g, potassium citrate monohydrate 220 g, potassium sulfate 52 g, Magnesium oxide 24 g, manganous carbonate (43 - 48% Mn) 3.5 g, Ferric citrate (16 - 17% Fe) 6 g, Zinc carbonate (70% ZnO) 1.6 g, Cupric carbonate (53 - 55% Cu) 0.3 g, potassium iodate 0.01 g, sodium selenite 0.01 g, chromium potassium sulfate 0.55 g, sucrose finely powdered 118 g.

Table 2. Amounts of tannin isolated from black turtle soup bean using extracting solvent system with 50% acetone and adsorption chromatography with sephadex SH-20 and results of vanillin assay of tannin from black turtle soup for 50% acetone and column chromatography

	50% Acetone extraction	Column chromatography
Tannin (mg) isolated from 100 g bean (black turtle soup)	835	356
[a]Catechin equiv. (mg) / 100 mg isolated tannin	91.6 ± 2.1	69.5 ± 1.9

[a]Values are means ± SD, n = 3

Table 3. Initial and final weight of animals and amount of average daily diet consumption for the experiment. Feed efficiency was calculate as dividing weight gain by average daily feed intake

	Control group (n=7)	Treatment group (n=8)
Initial body weight (g)	161.32 ± 6.00[1)]	163.75 ± 4.30
Final body weight (g)	210.66 ± 16.77	208.12 ± 6.94
Weight increase (g)	49.33 ± 16.57	44.38 ± 3.34
Feed intake (g)	15.65 ± 1.19*	18.81 ± 1.13
Feed efficiency	3.45 ± 0.93*	2.36 ± 0.19

1) Mean ± SD

*$p < 0.05$ significantly different between control group and treatment group by Student's t-test.

the feed efficiency of the two different diets. After euthanization, parotid salivary glands were removed and connective tissue or fat were stripped. After washing with saline solution, the weighed glands were stored frozen at −20 ℃ for future assay.

2. PRP isolation and characterization

The glands were first extracted with 25 mM Tris-HCl (pH 7.4 with 0.14 M NaCl) buffer. This extract presumably contained most salivary proteins produced in parotid glands, including PRPs.

For analysis of proteins from parotid glands, the methods of Mehansho *et al.*[6] were modified to optimize the extraction conditions. Some modifications such as concentration of extraction medium and extraction time maximized the amount of extracted proteins. Protein extraction with buffer was conducted as follows. Rat parotid glands were thawed and placed in 5 volumes (w/v) of 25 mM Tris-HCl, pH7.4 with 0.14 M NaCl buffer. Tissues were homogenized in an Omni-mixer (Fulktork, Fisher Scientific, U.S.A) at top speed for 60 seconds. After centrifugation at 100,000 × g for 1 hour, polyacrylamide gel electrophoresis was carried out as described in Laemmli[11] with the supernatant fluid, using 12% polyacrylamide.

The methods of Mehansho *et al.*[6] were modified and used for PRP isolation and characterization. Mehansho *et al.*[6] found that PRPs showed high solubility in trichloroacetic acid (TCA), unlike other proteins, which means TCA extracts contain the largest amount of PRPs. Therefore, PRPs were extracted using TCA as an extraction solvent. Frozen parotid glands from rats in each group (7 rats in control and 8 rats in treatment group) were pooled together, subsequently thawed and homogenized with the Omni-mixer as described above in 5 volumes (w/v) of cold 10% of trichloro acetic acid (TCA). The homogenate was centrifuged at 17,000 × g for 20 minutes. TCA was removed from the soluble fraction of the supernatant by extraction with water-saturated ether 4 times, 4 volumes each time. The aqueous phase, designated the TCA-soluble fraction, contains PRP.

The fluid was chromatographed on a Bio-Gel A 1.5 m column (1.5 × 90 cm) for further characterization. The column was equilibrated and eluted with 25 mM tris-HCl, pH 7.4, containing 0.14 M NaCl. The fractions with high absorbance at 230 nm were pooled and the SDS electrophoresis was performed to confirm the PRPs. Proteins were fixed in the gel and stained by the procedure of Steck *et al.*,[12] except the formaldehyde concentrations of the second staining solution and destaining solution were increased by 5- and 2.5-fold, respectively. Destained gels were soaked in 10% acetic acid and 1% glycerol for about 20 min before drying.

RESULTS AND DISCUSSION

Table 3 showed feed intake and weight gain of animals for a one-week experimental period. Feed efficiency was calculated by dividing weight gain by feed intake. The hull-containing diet group consumed significantly more diet compared to standard diet group, although the weight increase difference was not statistically significant. Therefore, the feed efficiency of treatment group was significantly lower than that of the control group.

Weights of animals and parotid salivary glands after feeding diet containing hulls for 1 week are presented in Table 4. The weight of glands per gram body weight between two groups were significantly different. Tannin intake showed hypertrophic effect on the rat parotid glands as in previous studies with rats[13-15] and mice.[16] However, the magnitude of hypertrophy of glands in the present study was not as dramatic as the previous studies since those studies showed roughly 5- to 10-fold increases in sizes of the glands. This difference in increase of size could be due to the amount of tannins in the feed, or the different tannin source. The bean-hull containing diet of the present study contained about 0.5% tannins. The previous study of Mehansho *et al.*[6] used high tannin sor-

ghum as a sole diet and the tannin content of Savanna sorghum grain, which is a high tannin breed of sorghum, contained 2% condensed tannins. However, the investigator found that 1% bean-hull tannin containing semi-purified diet was very toxic to weanling rats since 3 rats died within a week of feeding trial in the pilot study. Therefore, in the present study, 0.5% tannin level was selected. Since high tannin sorghum contained about 4-fold more condensed tannin than the bean-hull containing diet of the present study, size increases in the parotid glands could be different. Another possible explanation for this difference is the duration of this experiment. Parotid glands seem to enlarge at a maximum rate after 3 days of feeding tannin.[6] Afterwards, the enlarged glands get smaller as the animals adapt to the tannin-containing diet.[6,7] In the present study, feeding tannin (bean-hull containing diet) lasted for a week. If animals were adapted to the tannin diet, the size of glands could return to normal size by the time of termination. The smaller magnitude of gland hypertrophy in the present study could be explained by different tannin sources, less tannin intake, or the duration of experimentation.

SDS-polyacrylamide electrophoretic patterns of proteins induced in parotid salivary glands of rats treated with bean-hull tannin are shown in Fig. 1. Tris buffer extracts from control group (A) and treatment group (B) were compared with three standard proteins (BSA-66K, ovalbumin-43K and cytochrome C-12K). Treatment group showed 2 more bands around 40 – 50K when compared to the control group. When parotid glands were extracted with TCA, 5 ml of extract were chromatographed. Fig. 2 showed the chromatography of TCA soluble proteins of extract and 5 peaks were detected when the fraction was analyzed with spectrophotometer of 230 nm wave length. Fractions of peak II, peak IV lacked absorbance at 280 nm, which is consistent with results obtained with other PRPs.[13,14,17] Each peak was pooled and analyzed with SDS-polyacrylamide gel electrophoresis. Fig. 3 is the result of electrophoretic patterns of acid-soluble proteins which presumably represented proline-rich salivary proteins. The proteins eluted from chromatography were compared with standard proteins of BSA (66K), ova-

Table 4. Weight of animals and parotid salivary glands after feeding either standard diet or diet containing dried bean hulls for 1 week

	Control group (n = 7)	Treatment group (n = 8)
Final body weight (g)	210.66 ± 16.77[1]	208.12 ± 6.94
Weight of parotid glands (g)	0.252 ± 0.02758	0.285 ± 0.08018
mg glands / g body weight	0.958 ± 0.156*	1.371 ± 0.389

1) Mean ± SD
*$p < 0.05$ significantly different between control group and treatment group by Student's t-test.

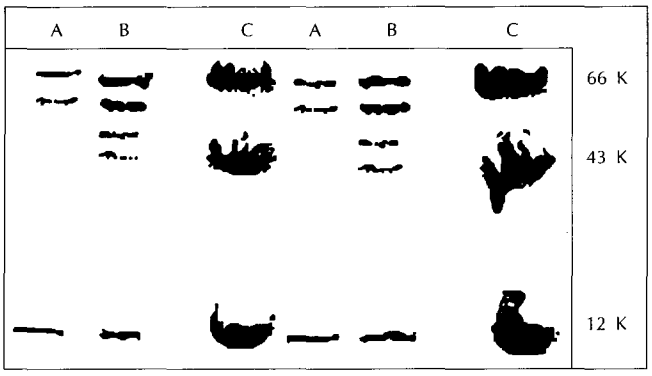

Fig. 1. SDS-polyacrylamide gel electrophoresis of buffer (25 mM Tris-HCl, pH 7.4 with 0.14 M NaCl) extracts from parotid glands. (A) control group (B) 7 week old rat with bean-hull containing diet (C) standard proteins (BSA-66 K, Ovalbumin-43 K, cytochrome C-12 K).

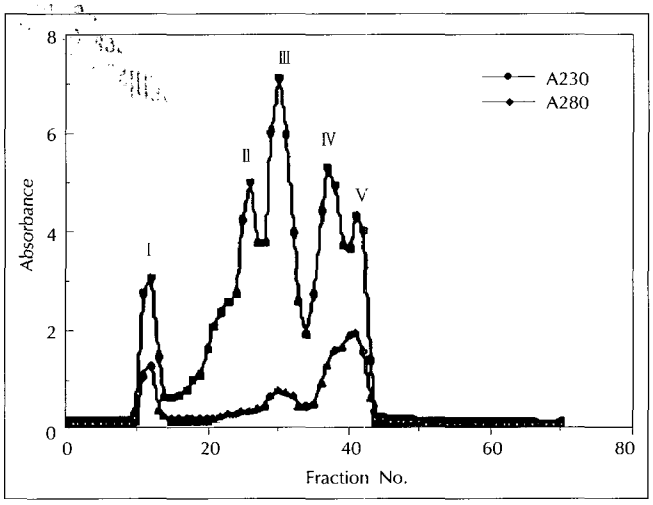

Fig. 2. Chromatography of parotid gland extract on Bio-Gel A-1.5 m. Five ml of extract were chromatographed on a column (1.5 × 90 cm) of Bio-Gel A-1.5 m. The column was equilibrated and eluted with 25 mM Tris-HCl, pH 7.4, containing 0.14 M NaCl. Eight ml fractions were collected with flow rate of about 50 ml/hr. Each peak was pooled and analyzed with SDS-polyacrylamide gel electrophoresis (Fig. 3).

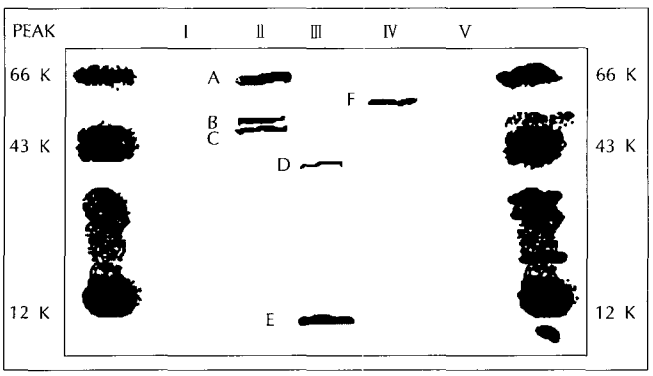

Fig. 3. SDS-polyacrylamide gel electrophoresis of protein from each peak of Bio-Gel A chromatography (Fig. 2). Standard proteins used: BSA-66 K, Ovalbumin-43 K, Cytochrome C-12 K.

Table 5. Comparison of PRPs isolated from parotid glands of rats fed bean hull containing diet in the present study and rats fed high and low tannin sorghum (Mehansho *et al.* 1992).

Scaled movement of standard proteins	[1]Proteins found in rat parotid glands when extracted with Tris buffer		[2]PRPs found in rat parotid glands	[3]PRPs found in rat parotid glands (Mehansho *et al.*)	
	Rats fed standard diet	Rats fed tannin containing diet	Rats fed tannin containing diet	Rats fed low tannin sorghum	Rats fed high tannin sorghum
				200 K	200 K
65 K	66 K	66 K	66 K		
55 K	54 K	54 K	54 K		
45 K		47 K	48 K		
35 K			33 K	38 K	38 K
25 K				27 K	27 K
15 K	10 K	10 K	11 K		15 K

1) Results of electrophoresis shown in Fig. 1.
2) Results of electrophoresis shown in Fig. 3.
3) Results of elctrophoresis of tricholoroacetic acid soluble fractions from rat parotid glands conducted by Mehansho *et al.* (1992).

lbumin (43K) and cytochrome C (12K). Proteins from the parotid glands eluted from the chromatography showed one band around 66K, four bands around 30 to 60K and one band around 12K.

Table 5 presents estimated molecular weights of the proteins found in the present paper of gel electrophoresis in Fig. 1 and 3. The results were compared in scaled table with the results of the previous study conducted by Mehansho *et al.*[7] of rat feeding with high and low tannin sorghum. Mehansho *et al.*[7] found 200K, 4 proteins of 32 to 38K, 27K and 15K proline rich glycoproteins from rat parotid salivary glands when feeding rats with high tannin sorghum. When low tannin sorghum was used, protein patterns were 200K, 38K and 27K. Four more proteins of 32K, 33.7K, 36K and 15K were found with high tannin sorghum.

When rats were fed bean-hull containing diet, more proteins of 30K to 40K were produced from parotid glands than when standard diet was used. TCA extracts showed two more proteins when compared to 25 mM Tris-HCl buffer extracts. The electrophoresis of TCA extracts showed more specifically separated patterns of proteins from parotid glands than the electrophoretic results of Tris-HCl buffer extracts since electrophoresis of TCA extracts were conducted after column chromatography. Since TCA soluble proteins were assumed as PRPs, bands found around 30 – 48K could be PRPs induced by bean-hull tannins. Four different kinds of PRPs were found in the present study. The number of proteins induced by tannins contained in diet were the same in the two studies of Mehansho *et al.*[7] and the present study, but the molecular weights were quite different. Discrepancies in protein patterns between the two studies are explained in previous section. Since direct comparison between PRPs produced by the bean-hull containing diet and those induced by the high sorghum diet is not appropriate due to the laboratory inconsistences, analysis of amino acid compositions of a series of proteins found from rats fed bean-hull containing diet would be necessary. At any rate, similar patterns of protein production as found in the study of Mehansho *et al.*[7] were shown from rats fed the bean-hull containing diet. Further investigation will be conducted to fully characterize and explain the differences in molecular weights of PRPs induced in two different tannin sources.

The morphological and biochemical changes have now been demonstrated to occur in response to the ingestion of tannins presumably to diminish the anti-nutritional effects of tannins. However, the results of this study failed to prove such a hypothesis since even when PRPs were induced by tannins in rats fed the bean-hull containing diet, they showed a lower feed efficiency. Other anti-nutritional effects of tannin such as inhibition of mineral absorption could be improved by PRP production but further study would be necessary to prove this hypothesis.

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