THE EFFECT OF VARYING LEVELS OF RAW LIMA BEAN (*Phaseolus lunatus*) ON THE RAT'S PANCREATIC AND INTESTINAL TRYPSIN (EC 3.4.21.4) AND CHYMOTRYPSIN (EC 3.4.21.1) ACTIVITIES

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SUMMARY

Two series of assays involving a total of 120 growing rats were carried out to investigate the effect of varying levels of raw lima bean (RLB) on pancreatic and intestinal trypsin EC 3.4.21.4 and chymotrypsin (EC 3.4.21.1) activities.

Experiment one indicates significant ($P < 0.01$) inhibition of both pancreatic trypsin and chymotrypsin due to RLB feeding. Enzyme activities in both the small and large intestine were also significantly ($P < 0.01$) depressed while enzyme values in the caecum were not. Age x Diet interaction was non-significant with respect to these enzymes.

Pancreatic trypsin and chymotrypsin correlated significantly ($P < 0.01$; $P < 0.05$) with RLB with respective R^2 , coefficient of multiple determination, of 0.94 and 0.67. Trypsin activity in both the small and large intestine was also significantly ($P < 0.01$) correlated with RLB in a negative quadratic fashion with respective R^2 of 0.78 and 0.96. Chymotrypsin activity in both the small and large intestine was also significantly ($P < 0.01$) correlated with respective R^2 of 0.79 and 0.92.

The second study suggests a less than 10% replacement of cooked lima bean by the raw to avert significant pancreatic proteinase inhibition, and a less than 15% replacement of the cooked lima bean by the raw to avert significant inhibition of the proteinases especially in the small intestine.

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INTRODUCTION

The enhanced growth-promoting effect of cooked for autoclaved versus raw soba beans was first reported by Osborne and Mendel (1917). In spite of the numerous investigations and improved technology during the last couple of decades, the explanation for this observation has remained unclear. The demonstration by Ham *et al.* (1944, 1945) of a substance from raw soybean that inhibited intestinal proteolysis in chicks and the ultimate isolation of a crystalline trypsin inhibitor from

raw soybean by Kunitz (1945) have stimulated numerous investigations on the role of the proteinase inhibitors in the depression of nutritional value of certain plant proteins.

Chernick *et al.* (1948), Abbey *et al.* (1979), Schingeothe *et al.* (1970) noted that chicks and rats fed raw soybean and field bean in their diets had enlarged pancreases accompanied by excessive elaboration of pancreatic proteinase, lipase and amylase into the small intestine. They suggested that trypsin inhibitor in soybean and field bean might produce growth retardation through the stimulation of increased enzyme secretion, with some of the endogenous nitrogen being incompletely reabsorbed and ultimately lost to the animal.

Recent reports (Aletor and Fetuga, 1984a & b) have indicated that part of the growth depressing effect of raw lima bean feeding to the rat is a consequence of the impaired digestion and/or absorption of dietary carbohydrates as well as the marked alterations in the normal activities of certain hepatic and extra-hepatic enzymes.

The present report comprises two experiments with the twin-objective of ascertaining the effect of raw lima bean on trypsin and chymotrypsin activities in various segments along the rat's alimentary tract; and to establish, in relation to these enzymes, the tolerance level of raw lima bean in cooked lima bean based diets. This is considered of veterinary importance since grazing livestock often stray into plots of growing legume seeds.

MATERIALS AND METHODS

The International Institute of Tropical

Agriculture (IITA), Ibadan supplied the lima bean used in the present study. The matured seeds were cooked with three changes of water, being a simulation of the mode of preparation in most homes, prior to consumption. The cooked bean was thereafter oven-dried at 55–59°C for 3 days followed by sun-drying for another 3 days. The dried samples were finely milled and put in screw-capped bottles. Raw lima bean (RLB) was similarly milled, kept in screw-capped bottles and stored in deep freezer before use.

Management of experimental animals

A total of 72 rats (36 males + 36 females) of the Wistar strain obtained from the pre-clinical rat colony of the College of Medicine, University of Ibadan, Ibadan was used for the first experiment. The rats, weaned at 21 days were fed standard laboratory animal stock diet until they were 28 days old weighing 61.73 — 65.67g.

The basal diet whose composition is given in earlier studies (Aletor and Fetuga, 1984a & b) was formulated to furnish 16% crude protein on dry matter basis with the cooked lima bean providing from the basal by replacing 0 (control), 20, 40, 60, 80 and 100% of the cooked lima bean with raw lima bean.

Experiment two which was designed to ascertain the tolerance of the rat's pancreatic and intestinal proteinases to RLB was essentially a follow-up of the first. It was based on the observation that even when RLB replaced only 20% of the cooked bean, pancreatic and intestinal trypsin and chymotrypsin activities were significantly inhibited. Therefore, the levels of replacement of the cooked bean with the raw was reduced to 0, 5, 10 and 15%. These diets were assigned to another set of 48 rats (24 males + 24 females) obtained from the same litter origin as the first experiment. They had similarly been weaned at 21 days, fed standard laboratory stock diets until they were 28 days weighing between 62–65g.

The two experiments lasted for 16 days during which water and food were offered *ad libitum* in individual metabolic cages.

Preparation of pancreatic and intestinal homogenates

At 8-daily interval, 6 rats (3 males + 3 females) per treatment were randomly selected and killed by neck decapitation. The pancreases and intestines were carefully dissected out, freed of any adhering mesentery and blotted free of blood. The intestinal loop was divided into the small and large intestine and the caecum employing the technique of Sidons (1969) to prevent the loss of intestinal fluid. The pancreases and intestines plus contents were homogenized in four parts of ice-cold double distilled water using an all-glass of teflon head homogenizer surrounded by melting ice. All cellular debris were got rid of by centrifugation at 2000 rev. per min. for 10 minutes with an angle 13 refrigerated centrifuge at 4°C.

Activation of Pancreatic trypsinogen and chymotrypsinogen

Both trypsinogen and chymotrypsinogen occur in the pancreas and pancreatic secretions as in inactive precursors of trypsin and chymotrypsin. Therefore, prior to their assays, the precursors were activated with enterokinase (EC 3.4.21.19) using the method of Howard and Yudkin (1963).

Enzymatic Assays

Pancreatic and intestinal trypsin activities were assayed for using the spectrophotometric technique of Rick (1962) in which the hydrolysis per unit of the substrate, Na-p-Toluenesulfonyl-L-Arginine Methylester (TAME) was followed spectrophotometrically.

Chymotrypsin activity was similarly assayed for using the technique of Rick (1962) in which the hydrolysis of the substrate, N-Benzoyl-L-Tyrosine Ethylester (BTEE) per unit time was followed spectrophotometrically.

Statistical Analysis

All data were subjected to factorial analysis of variance and regressions using the IBM-computer SPSSH (1976 version).

RESULTS

Results for experiment one are summarized in Tables 1, 2 and 3. Table 1 shows that there were significant ($P < 0.01$) decreases (when compared with the control) in the levels of pancreatic trypsin and chymotrypsin due to raw lima bean (RLB) feeding. Age x Diet interaction was non-significant with respect to these enzymes.

Activities of intestinal trypsin (Table 2) and chymotrypsin (Table 3) were

significantly ($P < 0.01$) depressed in both the small and large intestine while values in the caecum were not.

Prediction equations for the relationship between enzyme activity (dependent variable) and RLB levels were obtained by fitting a quadratic equation ($Y = a + bx + cx^2$) to the data after it was established that this function gave the equation of best fit as judged by the magnitude of their R^2 , the coefficient of multiple determination, the F-test and correct signing of the explanatory or independent variables which reflects conformity with logic of physiologic basis of animal nutrition. Regression of pancreatic trypsin activity (Y_1) and chymotrypsin activity (Y_2)

TABLE 1

Effect of Raw Lima Bean on Pancreatic Trypsin and Chymotrypsin Activities (Mean + SEM) in the Rat

Deits	Age (Days)	TRYPSIN	CHYMOTRYPSIN
		(x10u mole TAME MIN ⁻¹ LITRE ⁻¹)	(x10 u mole BITEE MIN ⁻¹ LITRE ⁻¹)
1. 0% RLB	36	291.90 ± 35.88	147.95 ± 32.67
	44	296.07 ± 36.42	162.49 ± 26.06
	36	236.30 ± 71.56*	136.35 ± 44.58*
2. 20% RLB	44	239.08 ± 82.21*	128.28 ± 21.30*
	36	201.53 ± 61.64*	133.52 ± 43.42*
3. 40% RLB	44	236.30 ± 61.16*	133.73 ± 29.93*
	36	204.87 ± 12.12*	122.85 ± 22.92*
4. 60% RLB	44	214.63 ± 20.57*	126.90 ± 18.37*
	36	148.73 ± 47.25**	82.42 ± 14.47**
5. 80% RLB	44	176.53 ± 23.75**	91.75 ± 20.39**
	36	134.83 ± 26.62**	92.02 ± 9.64**
6. 100% RLB	44	161.09 ± 21.62**	91.52 ± 15.91**

Significant difference between the control diet and RLB levels * = $P < 0.05$ ** = $P < 0.01$; RLB = Raw lima bean; TAME = Na-p-Toluene sulfonyl-L-Arginine Methyl Ester; BTEE = N-Benzoyl-L-Tyrosine Ethyl Ester.

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TABLE 2

Effect of Raw Lima Beans on Intestinal Trypsin Activity (Mean + SEM) in the Rat

		TRYPSIN (μ mole TAME MIN ⁻¹)		
Diets	Age (Days)	Smal	Large	Caecum
1. 0% RLB	36	529.59 $\pm$ 53.01	405.91 $\pm$ 129.76	212.66 $\pm$ 57.83
	44	364.18 $\pm$ 57.51	258.53 $\pm$ 88.09	175.13 $\pm$ 16.66
2. 20% RLB	36	394.80 $\pm$ 145.04*	300.24 $\pm$ 87.55	170.97 $\pm$ 29.87
	44	304.37 $\pm$ 52.35*	238.78 $\pm$ 123.83	129.27 $\pm$ 69.05
3. 40% RLB	36	364.14 $\pm$ 72.25*	280.78 $\pm$ 36.65	200.16 $\pm$ 96.40
	44	305.85 $\pm$ 71.85**	245.99 $\pm$ 46.11	177.95 $\pm$ 22.67
4. 60% RLB	36	389.26 $\pm$ 28.00**	282.17 $\pm$ 114.00*	189.02 $\pm$ 45.83
	44	268.54 $\pm$ 20.53**	162.57 $\pm$ 41.97*	143.98 $\pm$ 41.97
5. 89% RLB	36	362.79 $\pm$ 63.45**	209.95 $\pm$ 83.24**	189.95 $\pm$ 62.99
	44	229.34 $\pm$ 67.39**	141.78 $\pm$ 41.73*	129.50 $\pm$ 48.14
6. 100% RLB	36	262.68 $\pm$ 75.63**	206.22 $\pm$ 68.75**	180.32 $\pm$ 56.35
	44	108.40 $\pm$ 26.23**	118.70 $\pm$ 10.84**	157.82 $\pm$ 29.09

Significant difference between the control diet and RLB levels at the same age: * = $P < 0.05$;

** = $P < 0.01$; RLB = Raw lima bean; TAME = Na-p-Toluene sulfony-L-Arginine Methyl Ester; DTEE = N-Benzoyl-L-Tyrosine Ethyl Ester.

against RLB levels (X) gave the following equations:

$$Y_1 = 284.97 - 2.04X + 0.54X^2$$

($P < 0.01$; $R^2 = 0.94$)

$$Y_2 = 135.97 - 1.78X + 0.033X^2$$

($P < 0.05$; $R^2 = 0.67$)

which showed that the relationship between both pancreatic proteinases and RLB levels was negatively quadratic.

Regression of trypsin activity in the small intestine (Y_{si}) and large intestine (Y_{li}) against RLB levels (X) gave the following equations:

$$Y_{si} = 495.58 - 2.85X + 0.0084X^2$$

($P < 0.05$; $R^2 = 0.78$)

$$Y_{li} = 389.22 - 3.14X + 0.0013X^2$$

($P < 0.01$; $R^2 = 0.96$)

which indicated negative quadratic relationship between enzyme activities and RLB in these intestinal areas. Regression of chymotrypsin activity in the corresponding intestinal areas gave the following equations:

$$Y_{is} = 297.42 - 1.72X + 0.0051X^2$$

($P < 0.05$; $R^2 = 0.79$)

$$Y_{li} = 233.52 - 1.88X + 0.008X^2$$

($P < 0.01$; $R^2 = 0.92$)

which implied negative quadratic relation-

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TABLE 3

Effect of Raw Lima Beans on Intestinal Chymotrypsin Activity (Mean + SEM) in the Rat.

CHYMOTRYPSIN (μ mole BTEE MIN ⁻¹ LITRE ⁻¹)				
Diets	Age (Days)	Small	Large	Caecum
1. 0% RLB	36	317.75 $\pm$ 31.80	243.54 $\pm$ 77.85	127.59 $\pm$ 34.69
	44	218.51 $\pm$ 34.51	155.11 $\pm$ 52.85	105.07 $\pm$ 9.90
2. 20% RLB	36	236.88 $\pm$ 87.02	180.14 $\pm$ 52.53	102.58 $\pm$ 17.93
	44	182.62 $\pm$ 31.41*	143.26 $\pm$ 74.29	107.56 $\pm$ 41.43
3. 40% RLB	36	218.48 $\pm$ 43.35*	168.46 $\pm$ 23.19	120.09 $\pm$ 57.84
	44	183.51 $\pm$ 43.11*	147.59 $\pm$ 27.69	106.77 $\pm$ 13.60
4. 60% RLB	36	233.55 $\pm$ 16.79*	169.30 $\pm$ 68.55	113.41 $\pm$ 27.49
	44	125.12 $\pm$ 12.31**	97.54 $\pm$ 29.91**	108.38 $\pm$ 25.18
5. 80% RLB	36	217.67 $\pm$ 38.07*	125.96 $\pm$ 49.90**	125.77 $\pm$ 37.79
	44	137.59 $\pm$ 40.43**	85.06 $\pm$ 25.03**	111.70 $\pm$ 28.69
6. 100% RLB	36	157.60 $\pm$ 45.37**	123.73 $\pm$ 42.25**	111.79 $\pm$ 33.81
	44	131.22 $\pm$ 6.53**	65.04 $\pm$ 15.73**	120.69 $\pm$ 17.45

Significant difference between the control diet and RLB levels at the same age: * = $P < 0.05$;

** = $P < 0.01$; RLB = Raw Lima Bean. TAME = Na-p-Toluene sulfonyl-L-Arginine Methyl Ester; BTEE = N-Benzoyl-L-Tyrosine Ethyl Ester.

ship between chymotrypsin activity and dietary RLB.

Results for experiment two are presented in Tables 4, 5 and 6. Pancreatic chymotrypsin activity (Table 4) was significantly ($P < 0.04$) depressed by a mere 10% replacement of cooked lima bean with the raw bean while pancreatic trypsin was relatively unaffected by up to 15% level of replacement of the cooked bean with the raw.

Trypsin and chymotrypsin activities (Tables 5 and 6) in the small intestine were significantly ($P < 0.05$) depressed (when compared with the control) by 15% replacement of cooked lima bean with the

raw. Trypsin and chymotrypsin activities in both the large intestine and caecum were relatively unaffected by these levels.

DISCUSSION

The present report establishes more firmly that the consumption of raw legume protein alters the levels of some intestinal enzymes by regulating pancreatic enzymes synthesis and/or secretion. Although decreased levels of pancreatic trypsin and chymotrypsin following the ingestion of RLB have similarly been reported for rats and chicks consuming raw soba bean and field bean (Lyman and Lepkovsky, 1957; Salman *et al*, 1968;

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TABLE 4

Effect of Raw Lima Bean on Pancreatic Trypsin and Chymotrypsin Activities (Mean + SEM) in the Rat.

Age Diets	(Days)	TRYPSIN	CHYMOTRYPSIN
		($\times 10$ u mole TAME MIN ⁻¹ LITRE ⁻¹)	($\times 10$ u mole BTEE MIN ⁻¹ LITRE ⁻¹)
1. 0% RLB	36	297.99 $\pm$ 35.93	163.27 $\pm$ 17.68
	44	309.19 $\pm$ 54.39	169.37 $\pm$ 26.75
	36	292.74 $\pm$ 24.08	163.27 $\pm$ 17.69
2. 5% RLB	44	304.42 $\pm$ 53.98	163.84 $\pm$ 21.62
	36	294.68 $\pm$ 39.31	146.29 $\pm$ 24.79*
	44	293.29 $\pm$ 46.37	144.29 $\pm$ 19.40*
3. 10% RLB	36	266.30 $\pm$ 71.56	124.96 $\pm$ 15.93*
	44	284.10 $\pm$ 80.25	138.02 $\pm$ 17.80*
	44		

Significant difference between the control diet and RLB levels: * = $P < 0.05$;

RLB = Raw Lima Bean; TAME = Na-p-Toluene sulfonyl-L-Arginine Methyl Ester;

BTEE = N-Benzoyl-L-Tyrosine Ethyl Ester.

TABLE 5

Effect of Raw Lima Bean on Intestinal Trypsin Activity (Mean + SEM) in the Rat

Diets	Age (Days)	TRYPSIN		
		(u mole TAME MIN ⁻¹ LITRE ⁻¹)	Small	Large
1. 0% RLB	36	265.69 $\pm$ 73.11	177.92 $\pm$ 89.07	186.30 $\pm$ 76.20
	44	248.21 $\pm$ 69.63	166.58 $\pm$ 75.69	175.87 $\pm$ 68.56
	36	261.40 $\pm$ 78.83	176.05 $\pm$ 86.89	188.23 $\pm$ 74.73
2. 5% RLB	44	249.62 $\pm$ 71.53	171.72 $\pm$ 74.82	177.64 $\pm$ 65.47
	36	257.21 $\pm$ 87.64	188.04 $\pm$ 57.37	176.53 $\pm$ 49.18
	44	226.82 $\pm$ 87.93	177.51 $\pm$ 51.88	166.81 $\pm$ 45.32
3. 10% RLB	36	182.16 $\pm$ 19.78*	187.36 $\pm$ 68.32	167.63 $\pm$ 56.61
	44	96.16 $\pm$ 15.95*	171.77 $\pm$ 69.60	163.50 $\pm$ 52.53
	44			

Significant difference between the control diet and RLB levels: * = $P < 0.05$

RLB = Raw Lima Bean; TAME = Na-p-Toluene sulfonyl-L-arginine Methyl Ester

BTEE = N-Benzoyl-L-Tyrosine Ethyl Ester.

TABLE 6

Effect of Raw Lima Bean on Intestinal Chymotrypsin Activity
(Mean + SEM) in the Rat

		CHYMOTRYPSIN				
	Age	(u	mole	BTEE	MIN ⁻¹	LITRE ⁻¹)
Diets	(Days)	Small	Large	Caecum		
1.	0% RLB	36	159.41 ± 43.86	106.75 ± 53.44	111.78 ± 45.72	
		44	148.92 ± 41.76	119.90 ± 45.41	105.52 ± 40.53	
2.	5% RLB	36	156.84 ± 47.29	105.62 ± 52.13	112.93 ± 44.83	
		44	149.70 ± 44.89	103.03 ± 44.89	106.58 ± 39.28	
3.	10% RLB	36	154.32 ± 52.58	112.82 ± 34.42	105.91 ± 29.51	
		44	136.09 ± 52.75	106.51 ± 31.12	100.08 ± 27.19	
4.	15% RLB	36	110.09 ± 11.86*	118.41 ± 40.99	100.57 ± 33.96	
		44	117.69 ± 9.57*	115.06 ± 41.76	98.10 ± 31.52	

Significant difference between the control diet and RLB levels: * = $P < 0.05$;

RLB = Raw Lima Bean; TAME = Na-p-Toluene sulfonyl-L-Arginine Methyl Ester;

BTEE = N-Benzoyl-L-Tyrosin Ethyl Ester

Abbey *et al.*, 1979; Liener, 1980), no attempts were made by these workers to partition the intestinal tract with respect to these enzymes wherein they exhibit differential distribution.

The presence within RLB of potent, heat-labile, proteinase inhibitors is indicated since level as low as 10–20% replacement of cooked bean with the raw significantly depressed pancreatic and intestinal levels of trypsin and chymotrypsin (Tables 1, 2, 3 and 4). The similarity in the nature of quadratic response to dietary RLB by the two proteinases suggests that their inhibition may be mediated via a similar mechanism. It has been suggested that the physiological effect of proteinase inhibitor is to stimulate the pancreas to produce excessive amounts of enzymes which are lost to the animal in the faeces in the form of resistant enzyme-inhibitor complex (Barnes *et al.*, 1965; Khayambashi and Lyman, 1966; Abbey *et al.*, 1979. Since trypsin and chymotrypsin are rich in the sulphur-amino acids (methionine and cystine) [Warsy *et al.*,

1974], the endogenous loss of these enzymes may account for the cystine deficiency noted in animals fed diets containing raw soya bean meal or soya bean trypsin inhibitor. Presumably, therefore, the decreased activities of pancreatic and intestinal trypsin and chymotrypsin in the present study with RLB may be mediated in part via the stimulation of the pancreas to elaborate excessive amounts of these enzymes followed by the formation of resistant enzyme-inhibitor complexes especially in the small intestine. The decreased retention and apparent digestibility of nitrogen reported recently (Aletor and Fetuga, 1984a) in RLB-fed rats may partly be a consequence of depressed activities of these proteinases.

Although the toxic effects of most of the anti-nutritional factors in plant foodstuffs can generally be eliminated by proper heat treatment, conditions do prevail whereby complete destruction may not be achieved especially in mountainous regions where the boiling point of water is reduced. Earlier studies (Korte, 1973)

have shown that in mixtures of ground beans and cereals prepared under field conditions prevailing in Africa, some of the anti-nutritional factors are not completely destroyed, and that such cooked products produce various deleterious physiological effects.

The present study is considered of veterinary importance since farm animals often stray into plots of growing legume seeds. Such a situation could pose nutritional problems where lima bean is a major source of protein.

ACKNOWLEDGEMENT

The authors are grateful to all members of staff of Agronomy Department (Legumes section), International Institute of Tropical Agriculture (IITA) for providing the lima beans and to all members of staff of the pre-clinical rat colony of the College of Medicine, University of Ibadan, for providing the experimental animals.

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