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In vitro and *in vivo* protein hydrolysis of beans (*Phaseolus vulgaris*) genetically modified to express different phaseolin types

Carlos A. Montoya [a,b], Arturo S. Gomez [a], Jean-Paul Lallès [b], Wolfgang B. Souffrant [c], Stephen Beebe [d], Pascal Leterme [a,e,*]

[a] *Universidad Nacional de Colombia, Departamento de Producción Animal, Carrera 32 Chapinero, Palmira (Valle), Colombia*
[b] *INRA, UMR1079 SENAH, F-35590 Saint-Gilles, France*
[c] *FBN, Department of Nutritional Physiology, Oskar Kellner, Wilhelm-Stahl-Allee 2, 18196 16 Dummerstorf, Germany*
[d] *Centro Internacional de Agricultura Tropical, AA 6713 Cali, Colombia*
[e] *Prairie Swine Centre, P.O. Box 21057, 2105 8th Street East, Saskatoon, SK, Canada S7H 5N9*

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Abstract

Experiments were conducted to study whether phaseolin type could influence proteolysis susceptibility and nutritional value of total bean protein. The DOR-390 bean cultivar was genetically modified to express different phaseolin types (S, T or I). Beans were soaked and autoclaved. A sequential hydrolysis was carried out *in vitro* with pepsin and pancreatin. Differences in the degree of protein hydrolysis among bean lines started at 30 min and remained until 240 min, with the S bean proteins presenting lower values ($P < 0.05$). Subsequently, rats were fed with diets containing beans expressing different phaseolin types as the only source of protein for N digestibility and nutritional value determination. No differences ($P > 0.05$) in ileal protein digestibility and rat growth were observed. In conclusion, the differences in *in vitro* hydrolysis between bean lines expressing different phaseolin types had no consequences on growth and N retention in rats.

Keywords: *Phaseolus vulgaris*; Phaseolin; Nutritional value; *In vitro* hydrolysis

1. Introduction

The common bean (*Phaseolus vulgaris*) is a staple food for many people of Latin America and Central Africa. It is rich in protein with high lysine content as well as in minerals, starch and dietary fibre (Leterme & Muñoz, 2002).

Phaseolin, its major storage protein represents 40–50% of the total seed protein (Chagas & Santoro, 1997). Its nutritive value is limited by low sulphur-containing amino acid and a high resistance of raw phaseolin to proteolysis. Thermal treatment drastically improves phaseolin digestion (Montoya et al., 2006).

More than 40 phaseolin types have been classified by electrophoresis according to their subunit composition. The S (Sanilac) and T (Tendergreen) phaseolin are the two major phaseolins found in cultivated beans. They typically have three subunits with a MW ranging from 45.6 to 54.4 kDa (Chagas & Santoro, 1997; Salmanowicz, 2001). They differ in subunit composition: the S type has 2α, 2β and 2γ subunits which appear as three main bands in SDS-PAGE (Brown, Ma, Bliss, & Hall, 1981). However, the S phaseolin may appear as two main bands when less resolution equipment or conditions are used (Gepts, 1988; Koening, Singh, & Gepts, 1990; Montoya et al., 2006). The T phaseolin has only 1α, 1β and 1γ subunits, appearing as three main bands in SDS-PAGE (Brown et al., 1981). Finally, the I (Inca) type is unique among the phaseolin family in that it lacks the largest polypeptide

* Corresponding author. Address: Prairie Swine Centre, P.O. Box 21057, 2105 8th Street East, Saskatoon, SK, Canada S7H 5N9. Tel.: +1 306 667 7445; fax: +1 306 955 2510.
E-mail address: pascal.leterme@usask.ca (P. Leterme).

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(52 kDa; Koening et al., 1990). Thus it appears with two bands only in SDS-PAGE.

Plant breeders would like to improve the nutritional quality of beans. They screen bean accessions for possible differences between phaseolin types in methionine content or digestibility. However, phaseolin type will be integrated in breeding programmes only if evidence is made that genetic modification will result in an improvement of the nutritional value of whole cooked beans. The susceptibility to hydrolysis *in vitro* varies considerably among phaseolin types (Montoya, unpublished data). These differences could be due to differences in protein subunit composition (Fukuda et al., 2005) or in amino acid composition.

Differences between phaseolin types have been reported for *in vivo* digestibility (S and T phaseolins in whole bean; Begbie & Ross, 1993) and *in vitro* hydrolysis (S, T and I purified phaseolins; Montoya, Lallès, Beebe, Souffrant, & Leterme, in press). However, it is difficult to compare the value of different phaseolin types in whole beans, since the latter vary in size, colour and composition. In order to overcome that problem, the DOR-390 bean variety with small black seeds and naturally expressing the S phaseolin type, has been genetically modified to express either the T or the I phaseolin types.

The present work aimed to study the *in vitro* and *in vivo* proteolysis of cooked beans differing in their phaseolin types only and the possible consequences on the nutritional value of beans in rats.

2. Materials and methods

2.1. Production of bean lines expressing different types of phaseolins

The initial bean was the DOR-390 cultivar, a bean with small black seeds, which normally expresses the S phaseolin type. Through backcrossing, the T and I phaseolin types were introduced in the DOR-390 bean line. The source of the T phaseolin was G19892 of the CIAT gene bank, a wild bean accession from Argentina. The source of I phaseolin was G23585, a wild bean from Ecuador. The F1 plants of the backcross-2 generation were analyzed by electrophoresis to identify the heterozygotes carrying the T or I phaseolin. The beans were self-pollinated and the individual F2 plants were analyzed by electrophoresis. Lines with T and I phaseolin in the homozygous state were selected. Then, the lines with the three different phaseolin types were grown in order to produce enough material for the study.

2.2. Preparation of the beans and the purified phaseolins

The beans were soaked in distilled water (1:3, w:v) overnight at room temperature. The following day, the water was changed and the beans were washed, autoclaved in distilled water (1:3, w:v) at 121 °C (15 psi) for 20 min, frozen and freeze-dried. The beans were then ground (0.5 mm-mesh) and kept at −20 °C until use.

The phaseolins were isolated as described by Montoya et al. (2006). Briefly, flour prepared with dehulled beans (1 g/20 ml) was extracted with 0.5 M NaCl in 0.025 M HCl at pH 2.0. Then, it was centrifuged at 20,000*g* for 20 min. The supernatant fraction was mixed with five volumes of distilled water (1:5) at 4 °C and centrifuged for 20 min at 4 °C and 20,000*g*. The final precipitate was suspended in 0.5 M NaCl and dialyzed against distilled water (4 °C) for 24 h, and then frozen and freeze-dried.

2.3. In vitro hydrolysis experiment

2.3.1. Enzymatic hydrolysis protocol

Porcine pepsin (Merck No. 107197) and pancreatin (a mixture of pancreatic enzymes, Sigma No. P1750), were used to study the kinetics of *in vitro* protein hydrolysis of the beans. Casein was used as a reference protein. Samples were mixed with HCl 0.1 N (pH 2.0; 9.4 mg protein/ml HCl) and pre-incubated for 30 min at 39 °C in a water-bath under continuous stirring. Then, pepsin was added to the medium with an enzyme to protein ratio of 1:67 (w:w). Aliquots were taken after 0, 30 and 120 min of pepsin hydrolysis. Phosphate buffer saline (0.2 M, pH 8.0) was then mixed (1:1) with the remaining incubation medium and pancreatin was added with an enzyme to protein ratio of 1:30 (w:w). Aliquots were taken 20, 120 and 240 min after pancreatin addition (i.e. at times 140, 240 and 360 min after pepsin addition).

Aliquots were immediately added with trichloroacetic acid [TCA, 7.5% (w:v) final concentration] for precipitating protein. After centrifugation at 21,000 *g* for 10 min, the supernatants were collected for TCA-soluble N determination by the Kjeldahl method.

2.4. Sample treatment and SDS-PAGE electrophoresis

Other aliquots collected as above were immediately mixed with SDS 20% with a final concentration of 7% (v:v). They were incubated at 100 °C for 3 min and centrifuged at 21,000 *g* for 5 min. The supernatants were frozen at −20 °C.

Electrophoresis was conducted in 62.5 mM Tris–HCl buffer with 3.4 mM SDS for 3 h under a current of 70 V, as previously described (Salgado et al., 2003), except that we used concentration and separation gels with 4.8% and 15% polyacrilamide, respectively. Molecular weight (MW) standards (14.2–66.0 kDa; MW-SDS-70L, Sigma) were added to specific wells in each gel. The MW of each protein band detected visually after enzymatic hydrolysis was determined using a linear regression obtained from MW standards migration (Salgado et al., 2002).

2.5. In vivo experiments

2.5.1. Diet preparation

The diets of Experiment 1 (digestibility trial) were formulated to contain 100 g protein/kg dry matter (DM). In

Experiment 2 (growth and N retention trial), diets were formulated to contain 90 g digestible protein/kg DM, based on results of Experiment 1. In both experiments, the beans provided all the protein, except in a reference diet based on casein and in a protein-free diet that were also formulated (Table 1). The reference diet here was the same as in our previous work with purified phaseolin (Montoya et al., 2006). The protein-free diet aimed at measuring the basal endogenous protein losses of the rats. Chromium oxide (Cr_2O_3) was added as an indigestible marker for estimation of the ileal digestibility.

Table 1
Characteristics and nutrient composition of bean lines expressing different phaseolin type (S, T or I)

	Bean lines		
	S	T	I
Bean characteristics[a]			
Weight of 100 seeds (g)	19	18	17
Cotyledon to testa ratio	7.4	7.5	7.4
Bean analysis[a] *(g/kg DM)*			
Protein (N × 6.25)	209	243	236
Ether extract	21	15	19
Ash	46	45	46
Starch	332	320	319
NDF[b]	246	237	260
ADF[c]	91	90	89
Lignin	10	12	8
Gross energy (MJ/kg)	15.2	15.7	15.6

	Experiment 1[d]				Experiment 2[d]				
	C	S	T	I	C	S	T	I	PF[e]
Diet ingredients (g/kg DM)									
S-bean[f]	0	454	0	0	0	565	0	0	0
T-bean[f]	0	0	391	0	0	0	476	0	0
I-bean[f]	0	0	0	406	0	0	0	492	0
Casein[g]	120	0	0	0	115	0	0	0	0
Starch	599	265	328	313	604	154	243	227	719
Others[h]	281	281	281	281	281	281	281	281	281
Diet analysis (g/kg DM)									
Protein (N × 6.25)	111	103	102	100	101	126	120	122	10
Digestible protein	108	73	74	72	104	90	87	88	
Ether extract	65	72	69	76	69	76	74	70	65
Ash	45	63	61	61	45	67	62	65	44
NDF[b]	65	187	163	176	67	220	185	201	59
Gross energy (MJ/kg)	18.7	18.7	18.7	18.6	19.1	18.7	18.9	18.8	19.1

Composition and nutrient content of the experimental diets used in the *in vivo* experiments (1 and 2).

[a] S, T or I beans containing S, T or I phaseolin, respectively.Values are means of four repetitions.

[b] NDF: neutral detergent fibre.

[c] ADF: acid detergent fibre.

[d] Diets containing 10% total protein in Experiment 1, and 9% digestible protein in Experiment 2.

[e] PF: protein-free diet.

[f] DOR-390 bean lines expressing S, T or I phaseolin. Beans were thermally treated at 121 °C for 20 min before incorporation into diets.

[g] Casein was supplemented with 30 g DL-methionine per kg DM casein.

[h] g/kg: sucrose 100; ground rice hulls 80; vegetal oil 60; $CaPO_4$ 14; $CaCO_3$ 3; NaCl 2; KCl 7; $MgSO_4$ 2; Cr_2O_3 3; vitamin-trace elements 10 (mg/kg of diet: vitamin A 7.5; vitamin D_3 0.2; vitamin E 15; vitamin K 6; vitamin B2 10; calcium pantothenate 35; niacin 75; vitamin B6 2.5; vitamin B12 0.05; biotin 0.05; choline 200; Mn 150; Zn 500; Cu 40; Fe 200; I 2; Se 05; Co 1).

2.6. Animals and experimental design

The experiments were conducted under the guidelines of the National University of Colombia for care and use of laboratory animals. The rats (male, Wistar) provided by the Animal Laboratory Facility of the National University of Colombia (Bogota), had initial bodyweights (BW) of 100 ± 7 and 50 ± 5 g in Experiments 1 and 2, respectively. They were kept in individual metabolic cages (Tecniplast 150–300; Buguggiate, Italy) for the whole experimental period.

A completely randomized design comprising five treatments ($n = 6$ per treatment) were used for both experiments. The treatments were diets containing S, T or I beans or casein and a protein-free diet. In Experiment 1, the rats were adapted to the diets for 5 days. The faeces were collected totally for 5 days and kept at −15 °C until analysis for digestibility determination. At the end of the period, the rats were fasted for the rest of the day. They received a single meal the following day 3 h before being euthanized with an injection of Ketamin and Rompun® (1:1, v:v). The abdomen of the rats was immediately opened, the small intestine was unrolled and the content of the last 20 cm (ileum) was collected.

In Experiment 2, the animals were adapted to the diets for 5 days and then fed with the experimental diets for 28 days for growth trial. Every 5 days, the rats were weighed and the supply of food adjusted to 10% of BW. The last 10 days, faeces and urine were collected and kept at −15 °C until analysis of N. At the end of the trial, the rats were fasted overnight and then refed a meal 3 h before being euthanized as mentioned above. They were weighed, their abdomen was immediately opened and the weight of the gastrointestinal segments was measured (Montoya et al., 2006).

2.7. Analysis

The diets, faeces and digesta were analyzed for DM and N. The diets were also analyzed for ash, ether extract, neutral and acid detergent fibres (NDF and ADF) and gross energy as previously reported (Montoya et al., 2006). The chromium concentration in the diets and ileal digesta was determined colorimetrically after nitro-perchloric acid hydrolysis (Furukawa & Tsukahara, 1966). The amino acids in bean flour, purified phaseolin, diets and ileal digesta were analyzed in duplicate, by ion exchange chromatography using a Biochrome 20 analyzer (Pharmacia Biotech Ltd., Cambridge, UK). Methionine and cysteine were determined by the same method after oxidation with performic acid before hydrolysis. Tryptophan was also

determined using the same method, after alkaline hydrolysis of protein with 4 N BaOH.

3. Calculations

3.1. In vitro hydrolysis

The degree of hydrolysis of N was calculated according to the following equation:

$$\text{Degree of hydrolysis}_N = ([Ns_{(TX)}] - [Ns_{(T0)}]) \times 100/([N_{(total)}] - [Ns_{(T0)}])$$

where $Ns_{(TX)}$ is the soluble N in TCA at time X of the kinetics; $Ns_{(T0)}$ is soluble N at time 0; and $N_{(total)}$ is the total N of the sample.

3.1.1. Digestibility trial (Experiment 1)

Apparent and true faecal digestibility coefficients of DM, N and energy were calculated as previously reported (Montoya et al., 2006).

The ileal digestibilities were calculated using the following equations:

$$\text{Apparent ileal digestibility} = (100 - [(Ni/Nf) \times (Crf/Cri)]) \times 100$$

$$\text{Endogenous N losses(ENL)} = [Ni \times (Crf/Cri)]$$

$$\text{True ileal digestibility} = (Nd - [Ne - ENL])/Nd$$

where Ni is the N or AA_x content of ileal digesta; Nf is the N or AA_x content of feed; ENL is the ileal flow of endogenous N or AA_x determined with the free-protein diet; Crf is the chromium content of feed; Cri is the chromium content of ileal digesta; Nd is the amount of N or AA_x ingested; and Ne is the ileal flow of N or AA_x.

3.1.2. Growth and N retention trial (Experiment 2)

For the growth and N retention experiment, the following equations were used:

$$\text{Protein efficiency ratio (PER)} = WG/Pi$$

$$\text{Adjusted PER} = \text{PER test protein/PER casein control.}$$

$$\text{Net protein ratio} = (WG + WL)/Pi$$

$$\text{N balance} = Ni - (Nf + Nu)$$

$$\text{Biological value} = Ni - [(Nf - Nfe) + (Nu - Nue)]/[Ni - (Nf - Nfe)]$$

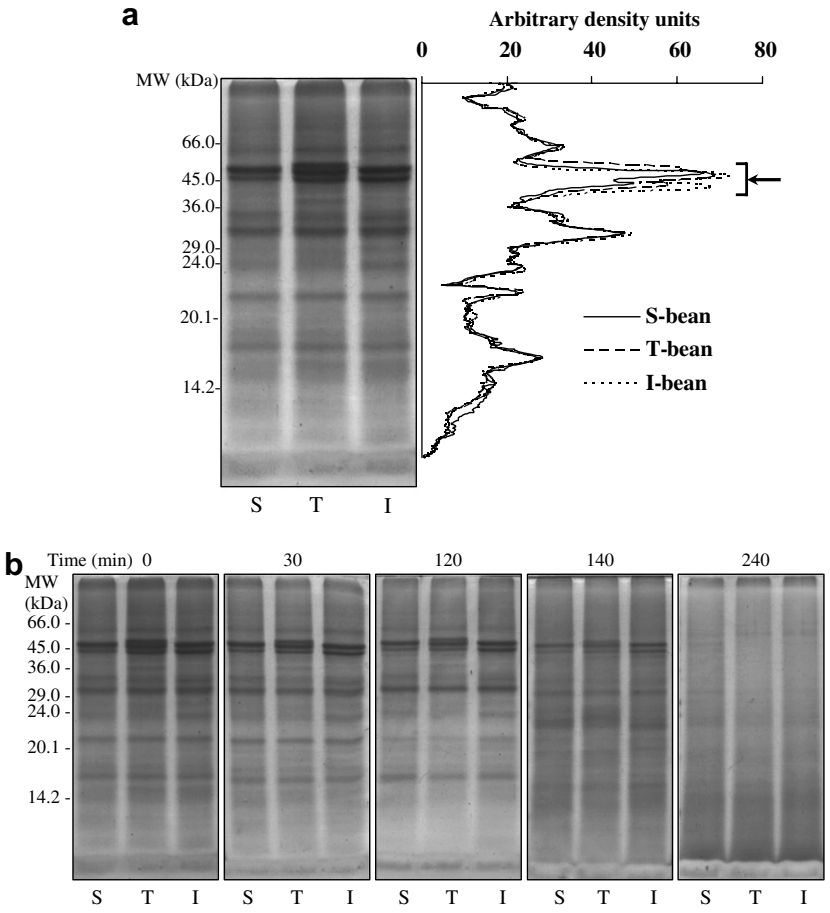

Fig. 1. (a) SDS-PAGE pattern of soluble protein and densitometry profile in DOR-390 bean expressing S, T or I phaseolin ($n = 2$ per bean type). The arrow shows the major differences in protein bands between bean lines. (b) Soluble protein from beans at different times points during sequential hydrolysis by pepsin (0–120 min) and pancreatin (120–240 min). Arrow heads indicate protein bands not hydrolysed after 240 min. Molecular weight markers (MW) are indicated on the left.

Net protein utilization = [Ni − (Nf + Nu)] × 100/Ni.

where WG is the weight gain of rats fed test diet; Pi is the protein intake from test diet; WL is the weight loss of rats fed the protein-free diet; Ni is N intake; Nf is N in faeces; Nu is N in urine; Nfe and Nue are faecal and urine endogenous N output measured with the protein-free diet.

3.2. Statistical analysis

All statistical analyses were performed using the General Linear Model procedure of SAS (SAS, 1999). An analysis of variance of the data obtained in the *in vitro* sequential hydrolysis and growth changes of rats in Experiment 2 was conducted in order to test the effects of beans, time and beans by time interaction (PROC MIXED procedure of SAS). An analysis was made to test the effect of bean at each time point (PROC GLM procedure of SAS). In Experiment 1 and for the variables obtained at the end of Experiment 2, analyses of variance of the data were conducted in order to test the effect of the diet. When the *F*-value of the analysis of variance was significant ($P < 0.05$), the means were compared using the Duncan's multiple range tests. The distance of χ^2 was calculated between two proteins on the basis of their AA composition profile according to the method of Guilloteau, Sauvant, and Patureau (1983).

4. Results and discussion

4.1. Composition of the beans

The SDS-PAGE and densitometry profiles of the soluble proteins of the beans confirmed that differences among bean lines were limited, except for the bands corresponding to the phaseolin subunits, between MW 43.1 and 51.5 kDa (Fig. 1a). Here we used a high percentage of polyacrylamide (15%) in our gels for seeing the maximum of proteins from the beans which reduces the resolution of bands for phaseolin. In these conditions, T and I phaseolins appeared with three and two subunits respectively. However, S phaseolin appeared with only two visible bands, in agreement with figures published by Gepts (1988) and Koening et al. (1990) and our previous work (Montoya et al., 2006).

The protein content was higher in the T and I lines as compared to the S bean (Table 1). According to Gepts (1988), the T phaseolin is associated with an increased seed size and protein content as compared to S phaseolin. This could explain the lower protein content of the S bean here, although the size of the beans was kept constant.

Differences in AA composition between the purified phaseolins were observed (χ^2, S/T = 10.4; S/I = 16.1; T/I = 6.5) (Table 2). However, these differences did not have any effect on AA composition between the bean lines expressing different phaseolin types (χ^2, S/T = 3.9; S/I =

Table 2
Amino acid composition (g/16 g N) of purified S, T and I phaseolin types and DOR-390 bean lines expressing the same phaseolin types

	Purified phaseolin types				Bean lines				
	S	T	I	AAS[a]	S	T	I	AAS[a]	PDCASS[b]
N (g/kg)	154	155	155		33.4	38.9	37.8		
Essential									
Arginine	4.6	4.9	4.7		4.6	5.3	4.9		
Histidine	3.0	3.2	2.9	1.6 ± 0.08	3.0	3.0	3.0	1.6 ± 0.01	106.9 ± 2.2
Isoleucine	4.0	4.0	3.8	1.4 ± 0.06	3.7	3.7	3.8	1.3 ± 0.01	89.9 ± 1.7
Leucine	7.2	6.8	6.5	1.0 ± 0.05	6.2	6.2	6.2	0.9 ± 0.01	63.8 ± 0.9
Lysine	6.2	6.0	5.6	1.0 ± 0.04	5.7	5.7	5.8	1.0 ± 0.01	67.3 ± 1.2
Methionine	0.7	0.7	0.7	0.4 ± 0.01	1.0	0.9	1.0	0.9 ± 0.03	58.2 ± 1.2
Phenylalanine	5.4	5.6	5.2	1.3 ± 0.02	6.2	6.1	6.2	1.1 ± 0.02	75.1 ± 2.1
Threonine	2.4	3.1	2.9	0.8 ± 0.11	4.0	3.9	4.1	1.2 ± 0.02	79.6 ± 1.5
Tryptophan	nd[c]	nd	Nd	nd	0.8	0.6	0.6	0.6 ± 0.10	39.5 ± 6.5
Valine	4.3	4.4	4.1	1.2 ± 0.05	4.5	4.4	4.5	1.3 ± 0.02	86.5 ± 0.8
No essential									
Alanine	3.1	3.4	3.5	4.6	4.5	4.5			
Aspartic acid	10.6	9.6	9.3	9.3	9.1	9.4			
Cysteine	0.3	0.3	0.2	1.2	1.2	1.2			
Glutamic acid	13.6	13.4	11.2	11.1	10.9	11.1			
Glycine	3.3	3.2	3.0	3.5	3.4	3.5			
Proline	3.6	3.3	3.1	4.4	4.4	4.5			
Serine	5.2	4.7	4.5	4.6	4.5	4.6			
Tyrosine	2.7	2.7	2.9	2.0	2.2	2.2			
Total AA	80.3	79.4	74.1	77.8	77.7	78.6			

[a] Amino acid score = amino acid × (g/16 g N) in test protein/requirements amino acid × (g/16 g N) for a children of 2 to 5 years old.
[b] Protein digestibility corrected amino acid score = AAS × true ileal digestibility.
[c] Not determined.

2.1; T/I = 1.2). In the other hand, differences were observed between the purified phaseolins and the bean lines ($\chi^2 = 41 - 68$). Collectively, these data suggest a relative stability of bean AA composition with phaseolin and non-phaseolin proteins having different AA composition profiles.

The AA score showed Met + Cys (0.4 ± 0.01) and Trp (0.6 ± 0.1) as the limiting amino acids for purified phaseolins and bean lines, respectively. The calculation of the PDCAAS confirmed that Trp ($39.5 \pm 6.5\%$) and S-containing ($58.2 \pm 1.2\%$) AA are the main limiting AA (Table 2).

4.2. In vitro hydrolysis

The kinetics of hydrolysis of the bean proteins and casein are shown in Fig. 2a. The proteins of the T beans were less hydrolyzed than those of the I beans after 30 min of pepsin hydrolysis ($P < 0.05$). After 140 and 240 min of hydrolysis, the T and I bean proteins were hydrolyzed better than those of the S bean (55, 61 and 47%, respectively). This was confirmed by SDS-PAGE (Fig. 1b). At 240 min, the S bean presented protein bands in the MW ranging from 43.1 to 51.5 kDa, which corresponds to intact phaseolin subunits. Between 240 and 360 min, the situation did not change anymore (data not shown).

The higher protein hydrolysis observed for the I beans compared with S beans could result from the higher hydrolysis during the pepsin step. Banerjee et al. (1996) suggested that slight differences in the tertiary structure of a protein cause distinct quaternary structures and maybe differences in susceptibility to enzyme attack. In a previous *in vitro* study with purified and heat-treated S, T and I phaseolins (Montoya et al., in press) and in similar experimental conditions, we also observed the lowest degree of hydrolysis for the S phaseolin. This result suggests that the main differences in the degree of hydrolysis between bean lines could be explained by differences in the susceptibility of phaseolin to hydrolysis. *In vitro* investigations carried out in our laboratory with more than forty purified phaseolins (C. Montoya et al., unpublished) should provide more insights into the relationships between compositional and/or structural changes in phaseolin types and their susceptibility to hydrolysis *in vitro*.

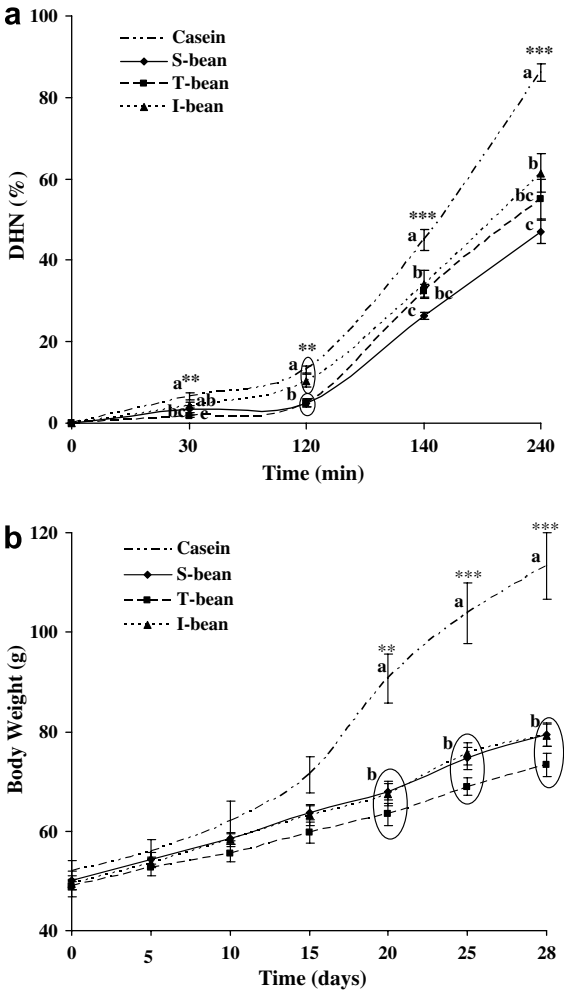

Fig. 2. (a) Kinetics of protein hydrolysis of bean lines expressing S, T or I phaseolin and casein, expressed as the percentage of N soluble in TCA 7.5% (DH_N%). Values are means and SEM for five sets of kinetics per source of protein. (b) Changes with time in BW of rats fed diets containing bean lines expressing S, T or I phaseolin and casein. Values are means and SEM for six rats. Values with different letters for a given time point differ ($P^* < 0.05$; $P^{**} < 0.01$; $P^{***} < 0.001$).

Table 3
Apparent and true faecal and ileal digestibilities of bean lines expressing S, T or I phaseolin (Experiment 1)

	Diet[A]				RSD[B]	P[C]
	C	S	T	I		
Faecal digestibility (%)						
DM	88.7a	80.0b	80.1b	80.0b	1.9	0.001
Apparent N	91.6a	64.9b	66.2b	65.9b	2.5	0.001
True N	97.3a	71.1b	72.5b	72.4b	2.5	0.001
Energy	90.8a	80.9b	81.8b	82.2b	2.3	0.001
Ileal digestibility (%)						
DM	80.4	74.2	72.8	73.2	5.3	0.105
Apparent N	80.0a	62.8b	64.5b	63.4b	5.6	0.001
True N	84.1a	67.1b	68.9b	67.9b	5.6	0.001
Essential AA						
Arginine	82.2a	71.7b	77.8ab	75.9ab	6.1	0.050
Histidine	84.7a	64.7b	67.5b	67.7b	5.1	0.001
Isoleucine	75.1	68.9	70.9	70.3	5.0	0.300
Leucine	85.9a	71.4b	73.4b	71.6b	4.3	0.001
Lysine	89.8a	68.6c	76.7b	74.4bc	7.1	0.044
Methionine	96.1a	79.6b	84.8b	71.8c	6.0	0.001
Phenylalanine	91.0a	68.5b	76.3b	74.4b	6.3	0.001
Threonine	76.2a	61.1bc	55.6c	67.7ab	7.4	0.001
Valine	77.1a	66.3b	68.3b	64.3b	5.6	0.006

[A] Diets based on reference diet C based on casein and thermal-treated bean lines expressing S, T or I phaseolin.
[B] RSD: residual standard deviation.
[C] Values with different letters in the same row differ significantly at $P < 0.05$.

4.3. Digestibility trial (Experiment 1)

No difference in feed intake was observed between diets (data not shown). Faecal and ileal digestibilities for N were not different between bean types ($P > 0.05$). However, differences were observed between bean types for some AA (arginine, lysine, methionine and threonine, Table 3). The bean-based diets had lower digestibilities than the casein diet ($P < 0.01$), with the exception of some AA (isoleucine, arginine, Table 3).

The low faecal and ileal digestibilities of N found for bean protein cannot be ascribed to heat-labile antinutritional factors such as lectin and protease inhibitors. Indeed, soaking and cooking beans reduce trypsin inhibitors (by 98%), tannin content (by 85%) and phytic acid (by 32%), resulting in improved protein digestibility (Antunes & Sgarbieri, 1980; Barampama & Simard, 1994; Wu et al., 1996). We observed a high faecal digestibility (92% on average) for heated phaseolins (S, T and I) in rats (Montoya et al., 2006). The differences in protein digestibility between the present study and the one with pure phaseolin (Montoya et al., 2006) can be ascribed to the cell walls of the whole seeds, the presence of low digestible albumin (Genovese & Lajolo, 1996) and phaseolin interaction with tannins (Del Pino & Lajolo, 1996). Additionally, it has been shown that the consumption of toasted beans induce the excretion of large amounts of endogenous N in piglets (Huisman et al., 1992) and in rats (Marquez & Lajolo, 1991; Oliveira & Sgarbieri, 1986), which results in lower apparent protein digestibility.

4.3.1. Growth and N retention trial (Experiment 2)

No difference in growth rate was observed between rats fed different bean types ($P > 0.05$) (Table 4 and Fig. 2b) in agreement with the lack of differences in AA composition of the bean lines. Rat BW started to be higher ($P < 0.01$) with the casein diet as compared to the other diets after 20 days of treatment. The BW gain of rats fed the casein diet was twice as high as of the rats fed with the bean diets (Table 4). This was also observed by Oliveira and Sgarbieri (1986) and can be ascribed to: (1) the lower digestibility of the bean proteins, (2) their imbalance in essential amino

Table 4
Food intake, growth, N retention and gastro-intestinal tract characteristics in rats fed diets containing bean lines expressing S, T or I phaseolin (Experiment 2)

	Diet[A]				RSD[B]	P[C]
	C	S	T	I		
Initial BW[D] (g)	52.0	50.1	49.4	49.6	4.9	0.795
Final BW (g)	110.1a	83.1b	71.1b	79.3b	12.3	0.001
Food intake (g/day)	6.9	6.3	6.0	6.3	0.6	0.146
N intake[E] (mg/day)	118	126	118	124	9.8	0.470
Growth (g/day)	1.8a	1.0b	0.9b	1.0b	0.3	0.001
Protein efficiency ratio	2.5a	1.3b	1.2b	1.3b	0.3	0.001
Adjusted PER[F]	1.0a	0.5b	0.5b	0.5b	0.1	0.001
Net protein ratio	3.0a	1.7b	1.7b	1.7b	0.3	0.001
Conversion index	4.0b	6.3a	7.1a	7.0a	1.2	0.002
N intake[G] (mg/day)	149	137	126	135	11.6	0.086
N balance	0.8a	0.6b	0.5b	0.5b	0.1	0.001
Apparent NPU[H]	54.6a	40.7b	41.1b	41.3b	4.3	0.001
True NPU	68.1a	54.8b	57.6b	55.8b	3.6	0.001
Weight of fresh contents (g/100 g BW)						
Total	10.7	10.9	11.2	12.1	2.5	0.653
Stomach	7.9	4.4	5.6	6.7	1.5	0.052
Small intestine	2.0b	3.6a	2.7b	2.2b	0.5	0.005
Caecum	0.4b	2.0a	2.0a	2.0a	0.5	0.001
Colon	0.5b	1.0a	1.1a	1.2a	0.3	0.001
Weight of tissues (g/100 g BW)						
Total	5.0b	6.6a	6.6a	6.8a	0.5	0.001
Stomach	1.2	1.2	1.3	1.2	0.3	0.822
Small intestine	2.8b	3.5a	3.6a	3.9a	0.4	0.002
Caecum	0.4b	1.0a	1.0a	1.0a	0.2	0.001
Colon	0.5b	0.9a	0.7a	0.8a	0.1	0.001

[A] Diets based on reference diet C based on casein and thermal-treated bean lines expressing S, T or I phaseolin.
[B] RSD: residual standard deviation.
[C] Values with different letters in the same row differ significantly at $P < 0.05$.
[D] BW: body-weight.
[E] Adjusted PER by casein diet (Wu et al., 1996).
[F] N intake during the growth trial.
[G] N intake during N retention measurement.
[H] NPU: net protein utilization.

acids and (3) the higher endogenous losses generated by bean intake. Endogenous losses markedly reduce the bioavailability of the amino acids, because dietary amino acids are used for the synthesis of digestive enzymes, mucus and epithelial cells, at the expense of protein accretion (Hess, Sève, Langer, & Duc, 2000).

4.3.2. Weight characteristics of the gastrointestinal tract (GIT) (Experiment 2)

The characteristics of GIT segments and their digesta contents are shown in Table 4. Stomach contents tended ($P = 0.052$) to be lighter and small intestine digesta heavier ($P < 0.05$) with the diet containing S beans, as compared to T and I bean diets. The length of the gut segments and the pH of digesta were not affected ($P > 0.05$) by diet treatment (data not shown).

The tissue weights of the rat small intestine, caecum and colon were not influenced by the bean type in the diet. They were higher ($P < 0.05$) in the rats fed the bean-based diets than in those receiving the casein diet. These observations are in agreement with previous experiments on purified phaseolins in rats, which induced limited alterations in the weight and architecture of the GIT tissue (Montoya et al., 2006). As expected, the casein diet with lower fibre content and higher digestibility generated GIT tissue lighter than with bean diets (Johnson & Gee, 1986).

In conclusion, the present work on sequential hydrolysis *in vitro* provides evidence that phaseolin type influences the susceptibility of total bean protein to hydrolysis. Such an effect was not observed in vivo, probably because phaseolin represents only 40–50% of total bean protein. The possible effects of other bean proteins or bean components on endogenous protein losses by the rat could mask the differences found in the *in vitro* hydrolysis among phaseolin types. Therefore, the S, T and I phaseolins do not appear as suitable marker proteins for breeding programs aimed at nutritional value improvement. Finally, the present work highlights the added value of combining *in vitro* and *in vivo* approaches in studying plant protein digestion.

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