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Susceptibility of phaseolin (*Phaseolus vulgaris*) subunits to trypsinolysis and influence of dietary level of raw phaseolin on protein digestion in the small intestine of rats

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The aim of the present work was (a) to investigate trypsinolysis of denatured purified T phaseolin (*Phaseolus vulgaris*) subunits by MS and (b) to test the effect of raw T phaseolin inclusion level in diets fed chronically to rats on digestion in the small intestine. The diets contained casein as the sole protein source, or casein substituted with 33, 67 and 100 % of purified T phaseolin. Rats were fed for 10 d and then euthanised. Digesta and tissues from the first and second halves of the small intestine were prepared for electrophoresis, immunoblotting and densitometry. α-Phaseolin subunit for the T phaseolin was more resistant to trypsinolysis than β-phaseolin subunit. Nearly intact phaseolin subunits (molecular weight, MW 44–54 kDa) and partially digested phaseolin fragments (MW 17–19 and 20–24 kDa) were identified in small intestinal digesta. The concentration of intact phaseolin and of most undigested phaseolin fragments in digesta increased in the second half of the small intestine with increasing phaseolin intake ($P<0{\cdot}05$–$0{\cdot}01$). The concentration of phaseolin fragments of a MW of 21–22·5 and 23–24·5 kDa in the mucosa increased linearly ($P=0{\cdot}016$–$0{\cdot}084$) when the level of the T phaseolin was increased in the diet. In conclusion, the present work provides evidence that denatured T phaseolin subunits display different trypsinolysis patterns *in vitro*. Moreover, a high intake of raw T phaseolin impacts digestion in the small intestine of rats.

Nutrition: Digestion: *Phaseolus vulgaris*: Phaseolin: Small intestine

Pulses are important sources of protein for human nutrition. The common bean (*Phaseolus vulgaris*) is still a staple food for many people in Latin America and Central Africa and is considered as the 'poor man's meat'[1]. The major components of common bean protein are the globulins. They include the 7S and 11S fractions, which represent 45 and 10 % of the total protein, respectively[2]. The 7S protein phaseolin, like other legume storage proteins, is characterised by a low content in sulphur amino acids. Raw phaseolin is highly resistant to proteolysis[3–5], when compared to the 7S protein of soyabean and pea[6]. The low susceptibility of phaseolin to proteolytic enzymes (digestibility of 20–30 %)[3–5] results from a compact and glycosylated structure, preventing proteases from reaching internal sites of hydrolysis[6,7]. After heat treatment, the susceptibility of phaseolin to proteolysis increases drastically (80–90 %)[3,4].

The α- and β-phaseolin subunits (molecular weight, MW 53, 47 and 43 kDa) are characterised by a high homology[8–10], but differences in amino acid and carbohydrate composition have been shown[11,12]. Such differences may have consequences on subunit structure, thermal stability and surface hydrophobicity as demonstrated for soyabean[13,14]. However, *in vitro* trypsinolysis of phaseolin subunits has not been documented in detail so far.

When raw phaseolin is fed as a single meal, data suggest that its undigested peptide fragments exert a secretagogue activity on the gut[15,16]. After a test meal of raw phaseolin fed 1 d followed by a protein-free diet fed for 3 d, the faecal output of endogenous N increased by 3-fold the day following exposure to phaseolin in rats[15,16]. The faecal excretion of endogenous N was also reported to increase when the amount of raw phaseolin in the test meal was increased[15,16]. By contrast, the faecal excretion of undigested phaseolin did not increase in the same proportions as those observed for endogenous N when the oral dose of phaseolin was increased[15,16]. Chronic feeding of phaseolin, which is more physiological, may provide a different picture following gut adaptation to drastic dietary changes. Indeed, diets containing

Abbreviations: MALDI–QTOF, matrix-assisted laser desorption ionisation–quadrupole time of flight; MW, molecular weight; SI, small intestine.

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a mixture (1:1, w/w) of casein and raw phaseolin and fed to rats for 10 d impacted moderately the gastrointestinal tract, including the architecture and enzyme activities of the small intestine (SI)[17].

The present work aimed to test the hypothesis that (a) patterns of *in vitro* trypsinolysis depend on the molecular origin (α, β) of the T phaseolin subunit, and (b) increasing intake of raw phaseolin affects its digestion in the SI in chronically fed rats. Many common bean varieties consumed nowadays contain phaseolin type T (Tendergreen). As explained previously[17,18], phaseolin type T is made of three subunits, as type S, whereas type I is more rare and contains only two subunits.

Experimental methods

Phaseolin purification

The bean cultivar used in the present study contained phaseolin of the type T. It was provided by the International Centre of Tropical Agriculture (CIAT; Cali, Colombia). Phaseolin was isolated as previously reported[17–19]. Briefly, flour prepared with dehulled beans (200 g/l) 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 at 20 000 ***g*** for 20 min at 4°C. The final precipitate was suspended in 0·5 M-NaCl and dialysed against distilled water (4°C) for 24 h, and then frozen and freeze-dried.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting analysis of purified T phaseolin were conducted as previously described[18]. Briefly, the protein load was 5 μg per well. MW standards (14·4–97·0 kDa; 17-0446-01, Pharmacia, Uppsala, Sweden) were also loaded in a separate well. After electrophoresis, proteins in one gel were stained by Coomassie brilliant blue, while proteins from a similar gel run simultaneously in the same device were electro-transferred to nitrocellulose membranes[18]. Western blotting was conducted using rabbit anti-phaseolin antibodies[18].

In-gel trypsin digestion and MS

In-gel digestion with trypsin was carried out according to the method of Shevchenko *et al.* [20], with a slight modification. The whole procedure has been described in detail elsewhere[18]. Briefly, the intact subunits of purified T phaseolin were excised, washed in acetonitrile – 0·05 M-ammonium bicarbonate (1:1) and dried in a SpeedVac concentrator (Bioblock, Illkirch, France). Proteins in the sliced gels were reduced with dithiothreitol at 60°C for 40 min and alkylated by iodoacetamide for 30 min in the dark. Digestion by trypsin (sequencing grade; Promega, Charbonnières, France) was carried out at 0·5 μg/sample, in 25 μl of 0·05 M-ammonium bicarbonate, at pH 8·0 for 18 h at 37°C. The reaction was stopped by adding 2 μl of 5 % trifluoroacetic acid (v/v) (Pierce, Touzart & Matignon, Vitry sur Seine, France). The supernatant was analysed to determine hydrolysed peptides by means of matrix-assisted laser desorption ionisation–quadrupole time of flight (MALDI–QTOF) MS as previously described[18]. Briefly, collected peptides were deposited onto the MALDI target plate. The plate was introduced into a QTOF MS (Qstar XL; Applied Biosystems, Framingham, MA). oMALDI Xpert2.0 software was used for the MALDI MS and MS–MS experiments. The samples were ionised with a laser beam ($\lambda = 337$ nm) and each spectrum was an average of 250–500 laser shots. The more representative monocharged ions were automatically submitted to fragmentation with an energy of collision of approximately 0·05 eV/Da 20. Typically, the oMALDI Xpert2.0 software treated each sample well individually and generated an MS peak list. This list was submitted for a peptide-mass-fingerprinting search and was used as a 'survey scan' to determine peptide precursors for MS–MS acquisition. All MS and MS–MS data were then used with MASCOT (version 1.9) software for search into several databases such as Swiss Prot or NCBI to identify the proteins present in each gel band.

Animals and diets

The experiment was conducted at the National University of Colombia, in agreement with its guidelines for care and use of laboratory animals[21]. Twenty young adult Wistar female rats with an initial body weight of 190 (SD 20) g were randomly allocated to one of the four dietary treatments and placed in individual metabolic cages (Tecniplast 150–300; Buguggiate, Italy) for the whole experimental period. The control diet contained casein as the sole protein source. In the other diets, casein was replaced by 33, 67 and 100 % of purified T phaseolin[17] (Table 1). The diets were offered to the rats for a period of 10 d, at a daily allowance of 10 g per rat in order to limit food refusals[17].

Preparation and electrophoresis of intestinal digesta and mucosa samples

Intestinal digesta and mucosa samples were collected after the killing of the rats on day 11, 3 h after the last meal[17,18]. Briefly, the digesta from the first and second halves of the SI were collected, immediately frozen and stored at −20°C. Later, they were freeze-dried and ground (1 mm mesh screen). Segments of 3 cm in length of intestinal tissue were also collected from the middle of the proximal and distal SI segments. They were opened longitudinally, washed three times in distilled water (4°C) and immediately frozen in liquid nitrogen. Tissue samples were thawed on ice and homogenised in ice-cold 0·9 % NaCl (40 mg tissue/ml) before analysis.

Soluble protein of digesta was extracted in borate buffer (0·1 M-H_3BO_3, 0·15 M-NaCl, pH 8·0) for 1 h at 4°C (300 mg digesta/ml buffer)[18]. Then, digesta and tissue preparations were centrifuged at 12 000 ***g*** for 10 min at 4°C. The supernatants were fractionated in aliquots and stored at −40°C until electrophoresis and Western blotting analysis, as described previously for purified phaseolin. The protein load was 5 μg for casein and phaseolin, and 30 μg per well for intestinal digesta and mucosa. Soluble protein concentration in digesta and mucosa preparations was measured with the Folin phenol reagent[22].

Table 1. Ingredient and analytical composition of the experimental diets*

	Casein control (g/kg DM)	Phaseolin diets (g/kg DM)		
		P33	P67	P100
Ingredients				
Casein†	118	80	40	0
Phaseolin	0	35	70	106
Starch	582	585	590	594
Sucrose	100	100	100	100
Ground rice hulls	80	80	80	80
Vegetable oil‡	60	60	60	60
Vitamins and trace elements§	10	10	10	10
Sodium chloride	10	10	10	10
Calcium carbonate	15	15	15	15
Calcium phosphate	25	25	25	25
Analysis				
DM (g/kg)	91	91	91	91
Protein (N × 6·25)	109	103	106	102
Diethyl ether extract	65	65	64	66
Ash	71	67	67	64
Neutral-detergent fibre	63	70	70	60
Gross energy (MJ/kg DM)	16·3	16·3	16·4	16·3

* P33, P67 and P100: diets with T phaseolin contributing to 330, 670 and 1000 g/kg of the total dietary protein, respectively.
† Casein was supplemented with 30 g DL-methionine per kg DM casein.
‡ Oil composition (soyabean–sunflower, 1:1).
§ Mineral and vitamin mixture supplied per kg of diet (control and experimental): 7·5 mg vitamin A; 0·2 mg vitamin D_3; 15 mg vitamin E; 6 mg vitamin K; 10 mg vitamin B_2; 35 mg calcium pantothenate; 75 mg niacin; 2·5 mg vitamin B_6; 0·05 mg vitamin B_{12}; 0·05 mg biotin; 200 mg choline; 150 mg Mn; 500 mg Zn; 40 mg Cu, 200 mg Fe; 2 mg I; 0·5 mg Se; 1 mg Co.

Densitometry of stained proteins and phaseolin

Density measurements of stained bands on the gels and membranes were made for each biological sample according to Montoya *et al.*[18]. Briefly, the gels and membranes were scanned using a phosphor imager (Quantum Appligene, version 2.03; Illkirch, France). Staining density was measured vertically for each track and horizontally for each band of interest using image analysis (Image-QuaNT, version 4.2a; Molecular Dynamics, Sunnyvale, CA). The vertical analysis served to generate density profiles according to MW along the track. The horizontal analysis generated density data for each protein band of interest across the tracks that were submitted to statistical analysis (see later). Staining densities are homogenous with concentrations since the same amounts of soluble protein for a given type of sample were deposited at the top of the lanes. The MW of each protein band detected visually was determined by linear regression using the MW standards run simultaneously in the first and last wells of each gel. Densitometry was carried out on both phaseolin polypeptides and endogenous proteins of digesta samples, while it focused only on phaseolin polypeptides in the case of intestinal tissues.

Statistical analysis

An ANOVA was conducted for *in vivo* data using the general linear model procedure of Statistical Analysis Systems statistical software package version 8.0 (SAS Institute Inc., Cary, NC, USA). The effect of the phaseolin level, intestinal site and phaseolin level by intestinal site interaction were tested according to a split-plot design. The effect of the intestinal site was tested against an error within animals, while the effects of the phaseolin level and the interaction were tested against an error between animals. When the F value of the ANOVA was significant ($P<0·05$), the means were compared using Duncan's multiple range test[23]. Furthermore, the influence of the phaseolin level and the interaction was tested for linear, quadratic and cubic variations using orthogonal contrasts.

Results

Trypsin digestion and the MS of the phaseolin subunits

The predicted cleavage sites and the peptides formed after trypsinolysis for each subunit of denatured T phaseolin were identified by MS using the amino acid sequence of α- and β-phaseolin subunits as described by Slightom *et al.*[9] (Fig. 1). The T phaseolin subunits were identified as originated from the α, β- and β-precursors, respectively[18]. In order to distinguish between the two β-subunits (MW 47–50·5 and 44–46·5 kDa), we named them β- and β′-subunits, respectively. The number of identified cleavage sites was twenty-five, thirty and twenty-eight for α-, β- and β′-phaseolin, respectively. Nineteen cleavage sites were common to the three phaseolin subunits. One site was common for the α- and β-subunits only (R_{262}–T_{263} in α and R_{256}–T_{257} in β), three cleavage sites were common for α and β′ only (R_{69}–L_{70}, R_{82}–S_{83} and R_{118}-E_{119}) and six were common for β and β′ only (K_{225}-E_{226}, R_{354}-N_{355}, K_{360}-T_{361}, R_{370}-A_{371}, K_{375}-D_{376} and K_{391}-L_{392}). Only two cleavage sites were specific to the α-subunit alone (R_{62}-F_{63} and K_{277}-E_{278}) and four to the β-subunit alone (R_{28}-E_{29}, D_{116}-R_{117}, K_{237}-S_{238} and K_{241}-Q_{242}).

Food intake and growth in vivo

Food intake did not differ among experimental diets with phaseolin representing 0, 33 and 67 % of the total protein

α 1 MMRARVPLLLLGILFLASLSASFATSLR EEEESQDNPFYFNSDNSWNTLF
β 1 MMRARVPLLLLGILFLASLSASFATSLR↓**EEEESQDNPFYFNSDNSWNTLF**
β′ 1 MMRARVPLLLLGILFLASLSASFATSLR EEEESQDNPFYFNSDNSWNTLF

α 51 K↓**NQYGHIR**↓VLQR↓**FDQQSK**↓**R**↓**LQNLEDYR**↓LVEFR↓**SKPETLLLPQQADAELLL**
β 51 **K**↓**NQYGHIR**↓VLQR FDQQSK↓**R LQNLEDYR**↓LVEFR SKPETLLLPQQADAELLL
β′ 51 K↓**NQYGHIR**↓VLQR FDQQSK↓**R**↓**LQNLEDYR**↓LVEFR↓**SKPETLLLPQQADAELLL**

α 101**VVR**↓**SGSAILVLVKPDD R**↓**R**↓**EYFFLT QGDNPIFSDN QK**↓**IPAGTIFYLVNPDPK**↓
β 101VVR↓**SGSAILVLVKPDD**↓**R**↓**R EYFFLTS DNPIFSD HQK**↓**IPAGTIFYLVNPDPK**↓
β′ 101**VVR**↓**SGSAILVLVKPDD R**↓**R**↓EYFFLTS DNPIFSD HQK↓**IPAGTIFYLVNPDPK**↓

α 152EDLE IIQLAMPVNNPQIHEFFLSSTEAQQSYLQEFSK↓**HILEASFNSK**↓**FEE**
β 151EDL RIIQLAMPVNNPQIHEFFLSSTEAQQSYLQEFSK↓**HILEASFNSK**↓**FEE**
β′ 151EDL RIIQLAMPVNNPQIHEFFLSSTEAQQSYLQEFSK↓**HILEASFNSK**↓**FEE**

α 202**INR**↓VLFEEEGQQEEGQQEGVIVNIDSEQI EELSKHAKSSSRK SH SK QDNTIGNEF
β 201**INR**↓**VLFEEEGQQE G VIVNIDSEQIK**↓ ELSKHAKSSSRK↓**S LSK**↓**QDNTIGNEF**
β′ 201**INR**↓**VLFEEEGQQE G VIVNIDSEQIK**↓ ELSKHAKSSSRK S LSK QDNTIGNEF

α 257GNLTER↓**TDNSLNVLISSIEMK**↓ **EGALFVPHYYSK**↓**AIVILVVNEGEAHVELV**
β 251**GNLTER**↓TDNSLNVLISSIEM EEGALFVPHYYSK↓**AIVILVVNEGEAHVELV**
β′ 251GNLTER TDNSLNVLISSIEM EEGALFVPHYYSK↓**AIVILVVNEGEAHVELV**

α 307**GPK**↓GNK↓**ETLEF ESYR**↓**AELSK**↓**DDVFVIPAAYPVAIK**↓ATSNVNFTGFGINAN
β 301**GPK**↓**GNK**↓**ETLE YESYR**↓**AELSK**↓**DDVFVIPAAYPVAIK**↓**ATSNVNFTGFGINAN**
β′ 301**GPK**↓**GNK**↓**ETLE YESYR**↓**AELSK**↓**DDVFVIPAAYPVAIK**↓**ATSNVNFTGFGINAN**

α 357NNNR NLLAGK TDNVISSIGR ALDGK DVLGLTFSGSGE EVMK LINK↓**QSGSY**
β 351**NNNR**↓NLLAGK↓**TDNVISSIGR**↓**ALDGK**↓**DVLGLTFSGSG DEVMK**↓LINK↓**QSGSY**
β′ 351**NNNR**↓NLLAGK↓**TDNVISSIGR**↓**ALDGK**↓**DVLGLTFSGSG DEVMK**↓LINK↓**QSGSY**

α 407**FVDG HHHQQEQQK**↓GSHQQEQQKGRKGAFVY
β 401**FVD AHHHQQEQQK**↓G RKGAFVY
β′ 401**FVD AHHHQQEQQK**↓G RKGAFVY

Fig. 1. Predicted cleavage sites (arrow) and identified peptides of denatured α- (band b) and β (bands c and d)-phaseolin subunits (Fig. 2) by MS after trypsinolysis using the sequences proposed by Slightom *et al.* [9]. The peptides identified by MS are shown in bold. The differences in identified peptides between β-phaseolin subunits are underlined.

(9·7 ± 0·3, 9·6 ± 0·6 and 8·7 ± 1·8 g/d, respectively). However, it was lower ($P<0·01$) in rats fed with 100 % phaseolin (6·0 ± 1·2 g/d). An ANOVA was made using food intake as the covariate, but it was not significant with the intensities of the protein bands as listed in Tables 2 and 3. The rats that were fed the diets with increasing dietary phaseolin levels lost body weight linearly when compared to the control diet ($P<0·001$; 13, −6, −34 and −44 g for the control, P33, P67 and P100 diets, respectively).

Protein patterns in the digesta of the small intestine of rats

SDS-PAGE. Representative SDS-PAGE gels of soluble proteins extracted from SI digesta and density profiles are shown in Fig. 2(a). Strong bands were observed between 18 and 24 kDa, together with other fainter bands for proximal and especially distal SI digesta of rats fed diets containing phaseolins. However, few faint bands were observed in SI digesta from rats fed the diet containing casein.

The staining intensity of ten out of twelve intestinal digesta protein bands with MW ranging from 15·5 to 54 kDa varied linearly, quadratically or cubically with increasing levels of incorporation of the T phaseolin in the diet ($P<0·001$ to $P=0·019$; Table 2). There was also a significant effect of the intestinal site for half of the bands ($P<0·001$ to $<0·01$). The band intensities were higher in the distal, when compared to the proximal SI for all bands, except one (at a MW of 12·5–13 kDa), whose intensity was lower in the distal SI. Increases in the protein band intensities were much higher for three bands in the MW range of 18–24 kDa than for two bands in the MW range of 44–50·5 kDa. Finally, a significant linear interaction between the T phaseolin level and the intestinal site was observed for two protein bands at a MW comprised between 18 and 21·5 kDa ($P=0·002$). In these cases, the increase in the protein band intensity between the control and the P33 diets was higher in the distal than in the proximal SI. Moreover, the band intensity continued to increase in the distal SI, while reaching a plateau in the proximal SI with increasing dietary phaseolin level. The average densitometry profile (Fig. 2 (a′)) of proteins present in intestinal digesta showed a strong peak at a MW between 19 and 28 kDa in the rats fed the phaseolin-containing diets.

Western blotting. The data obtained for three phaseolin bands ranging in MW between 17 and 54 kDa essentially confirmed the observations made on stained gels (Table 2). The intensity of two immunoreactive phaseolin bands varied (or tended to vary) linearly or quadratically with increasing levels of dietary phaseolin ($P<0·001$ to $P=0·078$). The effect of the intestinal site was significant ($P<0·001$) as the phaseolin band intensities were twice as high in the distal SI, when compared to the proximal SI. Finally, the phaseolin level by site interaction was significant for the three bands ($P=0·003$–$0·024$). Their intensities varied a little across diets in the proximal SI, while they were much higher with

Table 2. Densitometry analysis of protein bands obtained by SDS-PAGE and Western blotting of digesta of the proximal and distal small intestine (SI) of rats fed with different levels of unheated T phaseolin

Protein band* (kDa range)	Proximal SI				Distal SI					*P*		
Diet†	Cas	P33	P67	P100	Cas	P33	P67	P100	RSD	Level	Site	Interaction‡
Arbitrary density units												
SDS-PAGE												
(55–61) a	6·5	7·4	6·9	6·6	10·0	8·9	8·3	5·1	2·1	0·174	0·065	0·081 (L)
(51–54) b	3·9	7·2	7·0	7·1	4·7	8·6	9·2	6·3	2·2	0·007 (Q)	0·172	0·429
(47–50·5) c	4·0	7·1	7·1	7·0	5·2	10·5	9·9	8·0	2·6	0·014 (Q)	0·009	0·564
(44–46·5) d	3·8	6·6	6·9	7·6	4·4	10·6	10·7	9·6	2·7	0·003 (L)	0·004	0·421
(31·5–33) e	4·5	7·6	7·0	6·2	4·9	10·3	8·9	5·6	2·8	0·012 (Q)	0·242	0·634
(29–31) f	3·9	7·8	7·6	6·7	5·0	10·6	9·7	6·5	3·0	0·008 (Q)	0·183	0·768
(26·5–28) g	6·7	12·5	10·1	8·2	7·7	13·9	11·6	8·6	3·2	0·019 (Q)	0·295	0·981
(22–24) h	3·9	19·6	14·4	12·6	4·2	24·3	21·0	24·2	5·1	0·001 (C)	0·001	0·073 (L)
(19·2–21·5) i	$3·4^{d}$	$22·1^{b}$	$15·5^{c}$	$15·5^{c}$	$3·3^{d}$	$26·4^{ab}$	$27·9^{a}$	$30·1^{a}$	5·7	0·001 (C)	0·001	0·002 (L)
(18–19)j	$3·7^{cd}$	$9·6^{bc}$	$10·8^{b}$	$10·8^{b}$	$2·9^{d}$	$24·7^{a}$	$28·2^{a}$	$28·7^{a}$	5·0	0·001 (L)	0·001	0·002 (L)
(15·5–16·5)k	6·1	7·7	7·7	8·4	3·8	7·4	7·1	7·4	2·0	0·018 (L)	0·199	0·816
(12·5–13)l	10·0	12·4	12·5	11·5	8·0	7·4	6·0	5·7	2·7	0·784	0·001	0·406
Western blot												
(44–54)b	$2·8^{d}$	$4·7^{cd}$	$3·5^{d}$	$2·3^{d}$	$3·8^{d}$	$6·8^{bc}$	$8·5^{ab}$	$10·3^{a}$	2·4	0·078 (L)	0·001	0·006 (L)
(20·5–24)h	$1·7^{cd}$	$4·6^{b}$	$4·7^{b}$	$3·4^{bc}$	$1·1^{d}$	$7·5^{a}$	$7·2^{a}$	$7·2^{a}$	1·9	0·001 (Q)	0·001	0·024 (L)
(17–19)j	$3·9^{b}$	$3·1^{b}$	$3·6^{b}$	$2·7^{b}$	$2·6^{b}$	$7·9^{a}$	$9·5^{a}$	$8·1^{a}$	2·6	0·203	0·001	0·003 (Q)

RSD, residual standard deviation; L, linear; Q, quadratic; C, cubic.
[a,b,c] Values with different superscript letters in the same row differ significantly at $P<0·05$.
* The lettering of bands corresponds to that shown in Fig. 1 (a).
† Cas, casein control; P33, P67 and P100, diets with unheated T phaseolin contributing to 330, 670 and 1000 g/kg of the total dietary protein, respectively.
‡ Dietary phaseolin level by intestinal site interaction.

the phaseolin-containing diets when compared to the control in the distal SI. The average densitometry profile (Fig. 2 (b′) for SI digesta of rats fed phaseolin-containing diets showed two important phaseolin peaks, one at a MW of 44–52 kDa (intact molecule) and another one at a MW of 19–25 kDa (fragments resistant to digestion).

Protein patterns associated with the intestinal mucosa

SDS-PAGE. SDS-PAGE of soluble proteins extracted from the intestinal mucosa of rats is presented in Fig. 3 (a). In all tracks, several bands were observed. However, three bands of MW in the range of 19–24·5 kDa were consistently detected in the mucosa of all the rats fed diets containing phaseolin. The level of dietary phaseolin had (or tended to have) a linear effect on the intensity of the two proteins with the higher MW (between 21 and 24·5 kDa; $P=0·016–0·084$; Table 3). The intensity of the intermediate band (at a MW of 21–22·5 kDa) was higher in the distal than in the proximal SI ($P=0·004$). The interaction was never significant. The average densitometry profile (Fig. 3 (a′) of protein bands of the SI mucosa revealed a peak of MW between 18 and 28 kDa (the peak at lower MW is an artifact due to a strong gel non-specific staining with Coomassie blue).

Western blotting. The immunoblotting analysis of the intestinal mucosa revealed consistently across rats three phaseolin bands with MW from 19 to 24·5 kDa (Fig. 3 (b)). Only the phaseolin band with the highest MW (23–24·5 kDa) increased linearly with increasing dietary phaseolin ($P=0·006$). The intensities of phaseolin fragments associated with the mucosa were

Table 3. Densitometry analysis of protein bands obtained by SDS-PAGE and Western blotting in the mucosa of the proximal and distal small intestine (SI) of rats fed with different levels of unheated T phaseolin

Protein band* (kDa range)	Proximal SI				Distal SI					*P*		
Diet†	Cas	P33	P67	P100	Cas	P33	P67	P100	RSD	Level	Site	Interaction‡
Arbitrary density units												
SDS-PAGE												
(23–24·5) h	4·9	6·2	8·6	7·8	5·7	5·9	7·6	8·4	1·7	0·084 (L)	0·912	0·522
(21–22·5) i	3·6	5·4	6·8	6·9	4·6	6·2	8·1	9·3	1·7	0·016 (L)	0·004	0·500
(19–20·5) j	4·2	5·5	7·3	6·6	5·0	6·1	7·4	6·1	1·7	0·179	0·509	0·654
Western blot												
(23–24·5) h	2·0	4·7	7·7	8·7	1·5	21·9	19·2	27·2	8·0	0·006 (L)	0·007	0·280
(21–22) i	3·2	3·1	4·3	4·7	2·8	8·7	11·1	11·2	3·5	0·125	0·002	0·139
(19–20·5) j	$4·7^{b}$	$2·9^{b}$	$2·9^{b}$	$3·8^{b}$	$3·5^{b}$	$10·2^{a}$	$11·8^{a}$	$11·8^{a}$	2·8	0·265	0·001	0·079 (L)

RSD, residual standard deviation; L, linear; Q, quadratic; C, cubic.
[a,b,c] Values with different superscript letters in the same row differ significantly at $P<0·05$.
* The lettering of bands corresponds to that shown in Fig. 2 (a).
† Cas, casein control; P33, P67 and P100, diets with unheated T phaseolin contributing to 330, 670 and 1000 g/kg of the total dietary protein, respectively.
‡ Dietary phaseolin level by intestinal site interaction.

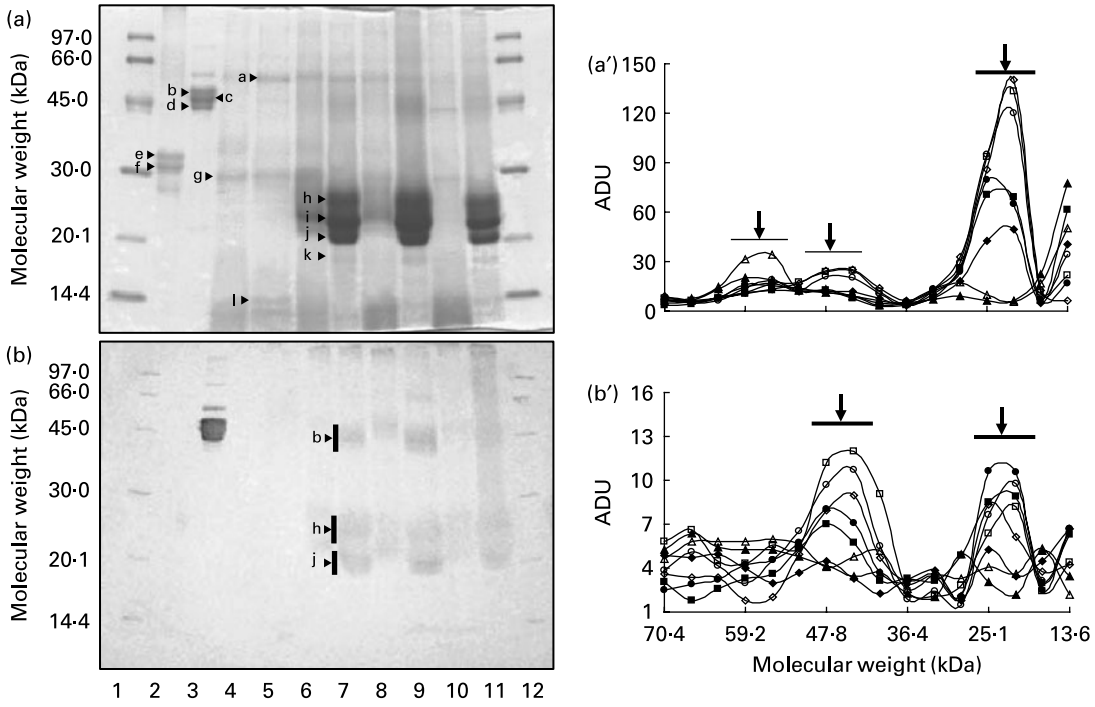

Fig. 2. (a) SDS-PAGE and (b) Western blotting analysis of casein (lane 2), T phaseolin (lane 3) and samples of digesta of the proximal and distal small intestine (SI1, SI2) of rats fed diets containing casein (C) or phaseolin (P) incorporated at 33, 67 or 100 % of the protein in the diet to CSI1 (4), CSI2 (5), P33SI1 (6), P33SI2 (7), P67SI1 (8), P67SI2 (9), P100SI1 (10) and P100SI2 (11). Molecular-weight markers (MW, lanes 1 and 12) are indicated on the left. (a′, b′) Densitometry profiles for the same samples, lines are means for five rats (—▲—, CSI1;—●—, P33SI1;—■—, P67SI1;—◆—, P100SI1;—△—, CSI2;—○—, P33SI2;—□—, P67SI2; —◇—, P100SI2). Letters with (a, b) arrowheads and (a′, b′) arrows for stained bands and densitometry peaks are described in the text.

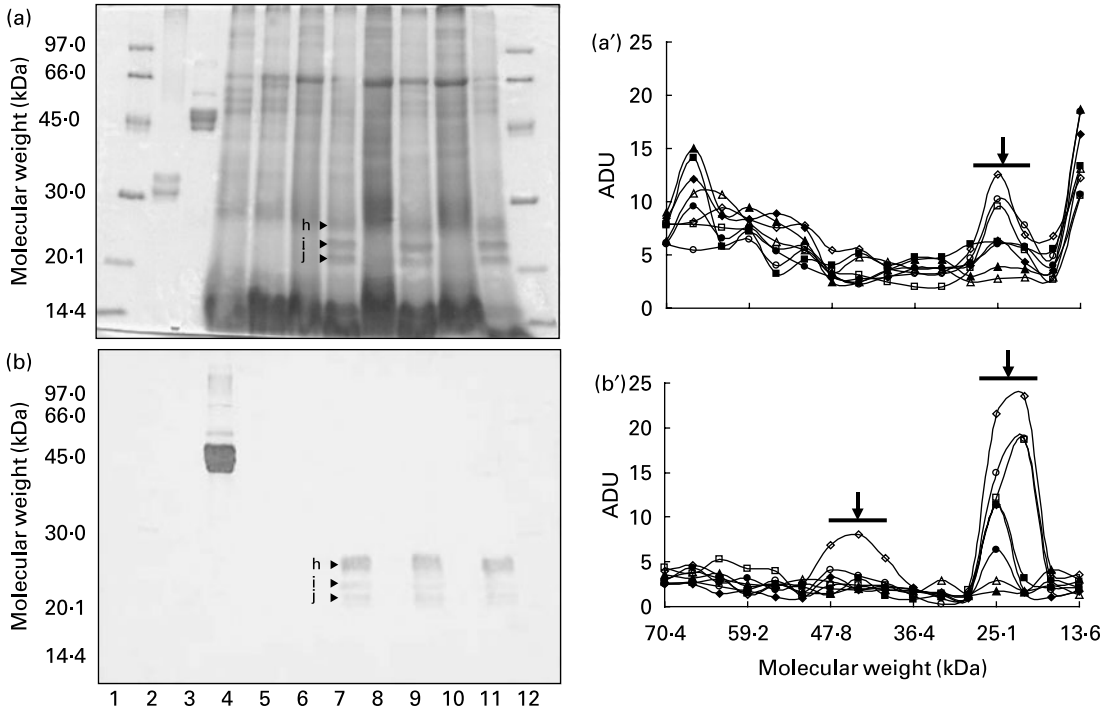

Fig. 3. (a) SDS-PAGE and (b) Western blotting analysis of casein (lane 2), T phaseolin (lane 3) and samples of mucosa of the proximal and distal small intestine (SI1 and SI2) of rats fed diets containing casein (C) or phaseolin (P) incorporated at 33, 67 or 100 % of the protein in the diet to CSI1 (4), CSI2 (5), P33SI1 (6), P33SI2 (7), P67SI1 (8), P67SI2 (9), P100SI1 (10) and P100SI2 (11). Molecular-weight markers (MW, lanes 1 and 12) are indicated on the left. (a′, b′) Densitometry profiles for the same samples, lines are means for five rats (—▲—, CSI1;—●—, P33SI1;—■—, P67SI1;—◆—, P100SI1;—△—, CSI2;—○—, P33SI2;—□—, P67SI2; —◇—, P100SI2). Letters with (a, b) arrowheads and (a′, b′) arrows for stained bands and densitometry peaks are described in the text.

2- to 3-fold higher in the distal than in the proximal SI ($P<0{\cdot}001$ to $P=0{\cdot}007$). The interaction was never significant. The average densitometry profile according to MW (Fig. 3(b′) for the SI mucosa of rats fed diets containing phaseolin showed a marked peak at a MW between 19 and 25 kDa, and another peak at a MW between 44 and 52 kDa in the rats fed the diet with the highest level of the T phaseolin.

Discussion

In the present work, we provide evidence that (a) the T phaseolin subunits display a different susceptibility to trypsinolysis and (b) the raw phaseolin level in the diet impacts protein digestion in the SI of rats.

Characterisation of the phaseolin subunits and of their trypsinolysis

Characterisation of the phaseolin subunits. Phaseolin has a trimeric structure with subunits of MW 53, 47 and 43 kDa[19]. The first subunit originates from the α-precursor and the other two subunits are from the β-precursor, as previously reported[18]. Phaseolin monomers do not differ much in their backbone conformations and α- and β-precursors are known for their high sequence homology[10]. However, some differences were found in amino acid (glutamic acid, glutamine and glycine, mainly) and carbohydrate composition, phosphate binding site and solvent molecule contents[9–12,19]. These factors are responsible for the heterogeneity found among phaseolin subunits[24]. The same phaseolin subunit precursor with different MW was observed in different phaseolin types[18]. The differences in electrophoretic mobility patterns between β-subunits and ascribed to amino acid substitution were observed in soyabean β-conglycinin[13,14]. In spite of the high sequence homology among β-conglycinin subunits, the differences in antibody immunoreactivity were observed due to the differences in amino acid sequence of the recognised epitopes[25].

Trypsinolysis of the phaseolin subunits. The highest number of tryptic fragments, predicted cleavage sites and percentage of sequence coverage of each phaseolin subunit (Fig. 1) show that the β-phaseolin subunit is more susceptible than the α-phaseolin subunit to trypsin hydrolysis. This could be ascribed to the sequence differences between phaseolin precursors and to their manner of reacting to different environmental constraints, as mentioned previously. Indeed, the differences in thermal stability, surface hydrophobicity, solubility and heat-induced association of individual subunits α, α′ and β in soyabean β-conglycinin were observed[26,27].

Analysing peptide sequences after trypsinolysis of native phaseolin revealed that two peptide bonds (R_{236}-K_{237} and K_{241}-Q_{242}) of phaseolin were the most susceptible to this enzyme. Trypsinolysis divides the phaseolin molecule into two structurally identical halves[5]. The cleavage sites in the β-subunit were shown to be R_{69}-L_{70}, R_{77}-L_{78}, K_{187}-H_{188}, K_{197}-F_{198}, R_{203}-;V_{204}, K_{225}-;E_{226}, R_{236}-;K_{237}, K_{241}-;Q_{242}, K_{283}-;A_{284}, K_{303}-;G_{304}, R_{315}-;A_{316}, K_{320}-;D_{321}, R_{354}-;N_{355}, K_{360}-;T_{361} and R_{370}-;A_{371}, as determined by MS after HPLC separation of trypsinolysis fragments[28]. All these cleavage sites (except R_{236}-;K_{237}) were found in the present work. Collectively, these results show the consistency of identification of cleaving sites of phaseolin by trypsin, regardless of analytical approaches.

Subunit v. *phaseolin type susceptibility to proteolysis*. Identification of the subunit precursors of phaseolin types by MS showed the following patterns: α and β for the S phaseolin; α, β and β for the T phaseolin; β and β for the I phaseolin[18]. This indicates that the S, T and I phaseolin types comprise 50, 33 and 0 % of α-phaseolin in the whole molecule, respectively. These differences and those in trypsinolysis among phaseolin subunits could explain the differences in *in vitro* proteolysis observed among heat-treated phaseolin types[29]. The S phaseolin that is richer in α-subunits presented the lowest degree of hydrolysis, when compared to the T and I phaseolins (58 *v*. 71 and 71 %)[29]. Small differences in the protein structure can affect digestion and thermal stability[13,30].

Influence of the phaseolin level on digestion in the small intestine

Phaseolin digestion. Intact phaseolin subunits (MW from 44 to 54 kDa) and also large fragments (18–24 kDa) were detected in the digesta of the SI by Western blotting, as reported previously with different phaseolin types[18]. The band at a MW of 22–24 kDa was identified as originating from the phaseolin α-precursor, and the bands at a MW of 19·2–21·5 and 18–19 kDa from the β-precursor[18]. The bands of phaseolin at a MW of 19–25 kDa were observed in other studies[5–7,16], highlighting the high resistance of these polypeptides to proteolysis.

The differences among diets were found for protein digestion in the SI. First, the identification of many protein bands as originating from phaseolin indicated that it was poorly digested when compared to casein. Similar results were observed in another study with three different phaseolin types[18]. Interestingly, the intensities of intact phaseolin subunits and of various phaseolin fragments in the small intestine responded to the increase in dietary phaseolin. This is at variance with the work by Santoro *et al.* [15,16], where they used a single test meal of phaseolin and reported a level of ileal passage or faecal excretion of phaseolin that was independent of the dietary load. Finally, comparing the proximal to distal SI digesta clearly showed an increase in the concentration of most phaseolin bands in the latter site, due to the accumulation or concentration of undigested material distally. This would suggest an overall resistance of these phaseolin fragments to digestion and a shift of digestion towards the distal intestine.

In pigs fed with toasted soyabean meal, peptides of the α-subunit of β-conglycinin were observed in ileal digesta and identified by peptide sequencing[31]. The data indicated that the α-subunit is more resistant to digestion than the α′- and β-subunits. The major subunits of heated, when compared to raw, pea vicilin appeared more resistant to trypsinolysis, due to a new β-sheet conformation and changes in the tertiary structure following thermal treatment[32]. The opposite is likely to occur with heated phaseolins since their susceptibility to hydrolysis is increased[3,4,17]. This can possibly be ascribed to the changes in the phaseolin structure[7] and a higher accessibility to proteolytic enzymes. Here, we observed between twenty-eight and thirty predicted cleavage sites for the denatured β-phaseolin subunit, whereas Jivotovskaya *et al.* [5] obtained only two of them in the native β-phaseolin subunit.

Endogenous protein. The bands at MW 26·5–28 and 55–61 kDa were identified as anionic trypsin I and pancreatic α-amylase[18]. These bands decreased in intensity when the level of phaseolin increased. First, it could result from the dilution of these endogenous proteins by the undigested phaseolin fractions, which increased with the level of dietary phaseolin. Second, it could be ascribed to a decrease in amino acid availability for gut function due to the high resistance of unheated phaseolin to enzymatic hydrolysis. A protein-free diet fed to rats for 10 d led to a reduction in villous-crypt architecture and digestive enzyme activities of the small intestine[33]. However, Santoro *et al.*[16] assumed that raw phaseolin increases intestinal endogenous protein losses (e.g. cell shedding, digestive enzymes, serum proteins, mucus production) when the level of phaseolin intake increased. Such a discrepancy may come from different experimental approaches, especially acute *v*. chronic feeding experiments. In chronic experiments, one would expect a better adaptation of the gastrointestinal tract to drastic changes in the composition of the diet.

Intestinal mucosa. The presence of fragments of phaseolin with a MW of 20·5–21·5, 22·5–24·5 and 26–27 kDa associated with the SI mucosa of the rats was reported previously[16,18]. These fragments were identified as originating from the α, β- and α-precursors, respectively[18]. Tissue concentrations of phaseolin polypeptides increased with the dietary phaseolin level in the present study, probably reflecting increased luminal concentrations of undigested phaseolin fragments, and possibly the potential adsorption capacity of intestinal tissues.

In conclusion, the present results showed the differences in trypsinolysis among the subunits of the T phaseolin, suggesting that the α-subunit is more resistant than the β-subunit. They also indicated that increasing the level of raw T phaseolin in the diet impacted digestion in rats, increasing the concentrations of intact and partially digested phaseolin in the lumen and associated with the mucosa. The relationships between the molecular precursor origin of subunits and the overall susceptibility of phaseolin to proteolysis should be explored further. This could be carried out with a collection of purified phaseolins displaying highly contrasted subunit profiles and degrees of hydrolysis[34]. Such a new basic knowledge may ultimately help to breed phaseolin varieties with a much higher digestibility after heat treatment that would be of value in improving human food.

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