Perspectives into Factors Limiting in Vivo Digestion of Legume Proteins: Antinutritional Compounds or Storage Proteins?

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The in vivo protein digestibility of raw and cooked common bean (*Phaseolus vulgaris* L.) and faba bean (*Vicia faba* L.) and of protein fractions extracted from them was determined with growing rats. Overnight-fasted rats were intubated with a protein suspension or fed the same amount of protein added to a basal diet. The rats were killed 1 h later, the contents of stomach and small intestine were washed out, and their protein contents were measured. The in vivo digestibility of proteins of raw common bean flour was 72.4% and not significantly improved after cooking. In contrast, the digestibility of faba bean proteins was decreased from 86.5 to 60.6% by the thermal treatment. Globulins from either species had similar digestibilities (∼70%). Proteins in the soluble fraction of cooked beans were more digestible than those in the insoluble fraction, which contained the bulk of the proteins. Hemagglutination assay and trypsin inhibitor determination indicated that after the thermal treatment only very low, nonharmful, levels of both lectin and inhibitor remained. Faba bean contained more polyphenols than common bean samples, with most of the polyphenols being bound to globulins. However, protein-bound polyphenols were markedly decreased after cooking. SDS−PAGE characterization of the gastrointestinal digesta of globulins and amino acid analysis of undigested proteins of whole cooked common bean and faba bean suggested that it is mainly the structural properties of the storage proteins and not their binding of polyphenols, which determines the extent of protein aggregation on autoclaving and may therefore be responsible for their low digestibility.

Keywords: *In vivo digestibility; globulins; antinutritional factors; thermal treatment*

INTRODUCTION

Plant seeds represent a major source of dietary proteins, the amount of proteins varying from ∼10% (dry weight) in cereals to ∼40% (dry weight) in certain legumes and oilseeds.

The nutritional value of a protein is determined by its digestibility, its content of essential amino acids, and the availability of individual amino acids (CCVP, 1989). Although plant proteins have been reported to be less susceptible to proteolytic breakdown in vivo than animal proteins (Friedman, 1996), the molecular bases for these differences have yet to be elucidated. In the case of legume proteins the low content of essential sulfur amino acids (methionine and cysteine), the compact structure of the proteins, and the presence of nonprotein compounds (dietary fiber, tannins, phytates) and/or antiphysiological proteins (protease inhibitors, lectins), which can impair digestion and increase endogenous nitrogen excretion, have been proposed to be responsible for the apparent low protein value (Evans and Bauer, 1978; Chang and Satterlee, 1981; Sarwar and Peace, 1986; Jansman et al., 1998).

Evidence from in vitro studies indicates that digestion of native legume seed storage proteins is limited because of the structure and conformation of the proteins (Chang and Satterlee, 1981; Carbonaro et al., 1992). There is a general consensus that antinutritional proteins (protease inhibitors, lectins) can be inactivated by proper heat treatment (Jansman et al., 1998). However, data on the effects of heating on the structural properties that limit the digestion of the native storage protein are conflicting (Chang and Satterlee, 1981; Deshpande and Damodaran, 1989; Nielsen et al., 1988; Carbonaro et al., 1997). Heat denaturation of legume oligomeric proteins leads to the disruption of the multimeric structure followed by the denaturation of the monomers, initiating further rearrangement and/or aggregation (Kinsella et al., 1985). Moreover, severe processing conditions that are often required to inactivate antinutritional factors may promote novel protein−protein interactions or interactions between protein molecules and other endogenous seed components (carbohydrates, polyphenols, phytic acid) that alter the release of amino acids by enzymic degradation (Friedman, 1996). On processing, either soluble or insoluble macrocomplexes can be formed. Although legume proteins become generally less soluble after cooking (McWatters and Holmes, 1979; Carbonaro et al., 1993, 1997), it has to be established how far the thermal aggregation phenomenon may impair digestibility and whether essential amino acids remain trapped inside indigestible protein aggregates. Heating glycoprotein II from *Phaseolus vulgaris* L. has been found to have no effect on either the extent or the rate of proteolysis (Sgarbieri et al., 1982). In a recent in vitro study (Carbonaro et al., 1997) we have shown that, whereas protein digestibility of common bean was

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10.1021/jf991005m CCC: $19.00
Published on Web 02/15/2000

increased upon cooking and that of lentil was unchanged, with faba bean it was significantly decreased. This finding was in agreement with the results on the in vivo digestibility of heat-processed faba bean obtained by other researchers (Fernandez et al., 1993). Undesirable conformational changes leading to impaired susceptibility to proteolysis have been found to occur upon heating pea (*Pisum sativum* L.) vicilin. This was unlike that found with phaseolin, the homologous 7S protein of *P. vulgaris* L. seeds (Deshpande and Damodaran, 1989). However, it is unclear how such structural modifications can influence the in vivo protein and amino acid digestibilities.

Nitrogen balance studies, the most suitable practical method for evaluating in vivo protein digestibility, have been criticized because the pattern of nitrogen excretion is modified by the microflora present in the large intestine (CCVP, 1989). For this reason, protein digestibility is either under- or overestimated, depending on the amino acid or the feedstuff, in comparison to ileal digestibility values (CCVP, 1989). Passage of digesta from the stomach of rats to the cecum takes at least 1.5−2 h (Sgarbieri et al., 1982). Thus, if rats are fed or dosed orally with proteins and killed after 1 h, any undigested protein or peptides will be found in the lumen of the stomach or small intestine. Therefore, in this study protein digestibility was measured in acute (1 h) experiments with growing rats (Rubio et al., 1994). The method also offers the advantage that gastrointestinal digesta is collected and can be therefore characterized. This is a prerequisite for the identification of molecular interactions between various food components that may impair protein digestion and availability.

The digestibility of proteins in raw and cooked common bean (*Phaseolus vulgaris* L.) and faba bean (*Vicia faba* L.) and of protein fractions extracted from them was determined in vivo in 1 h studies with growing rats. A preliminary characterization of the gastrointestinal digesta was also carried out.

EXPERIMENTAL PROCEDURES

Preparation of Legume Samples. Commercial Italian varieties of common bean (*P. vulgaris* L.) and faba bean (*V. faba* L.) were used in the study.

Cooked legumes were prepared after soaking in water for 2 h (1:4 w/v for common bean and 1:5 w/v for faba bean). Seeds, with the soaking water, were autoclaved for 20 min at 120 °C (1 atm) and then freeze-dried. Raw and cooked legumes were ground in a Cyclotec 1093 Tecator (50 μm).

Proteins from raw bean homogenate were extracted in 0.2 M Tris-HCl buffer, pH 8.0 (1:10 w/v), by stirring at room temperature overnight. After centrifugation (45 min, 35000*g*), the supernatant was adjusted to pH 4.5 by the addition of glacial acetic acid and centrifuged again at 12000*g* for 30 min. The precipitate (globulin) was redissolved in distilled water, dialyzed against distilled water, and freeze-dried.

Soluble and insoluble fractions from cooked legumes were obtained by centrifugation (30 min, 4000*g*) of a water suspension of cooked flour (0.02 g/mL) after stirring at room temperature for 2 h. The precipitate (insoluble fraction) and supernatant (soluble fraction) were freeze-dried separately.

Protein content (N × 6.25) was determined in all samples by using the Kjeldahl method (AOAC, 1990).

Trials in Vivo. All management and experimental procedures were carried out in strict accordance with the requirements of the U.K. Animals (Scientific Procedures) Act 1986 by staff licensed under this Act to carry out such procedures.

In vivo digestibility was determined as described by Rubio et al. (1994) in two acute (1 h) experiments with growing rats. Male rats of the Hooded Lister (Rowett) strain (40 days of age), weaned at 19 days of age, were adapted to experimental conditions by feeding them a semisynthetic lactalbumin control diet for 7 days (Grant et al., 1993). Rats (140 ± 1 g) were then housed individually in polypropylene and stainless steel cages and fasted overnight before both experiments.

In the first experiment, rats were intubated with the various samples suspended in phosphate-buffered saline (PBS). In each case the amount administered contained 200 mg of protein (N × 6.25). In the second experiment, 1.0 g of each sample (200−300 mg of protein) was added to 1.0 g of basal diet (maize starch, 500 mg; glucose, 300 mg; glycerol, 200 mg). All animals consumed the meal within 10 min. Control rats were given PBS or protein-free basal diet, respectively, in the same amount that was given to the corresponding treated rats. One hour after either gavage or feeding, the rats were killed by anesthetic (halothane) overdose. A longitudinal incision along the midline of the abdomen was made, and the stomach and small intestine were taken out separately. The stomach and intestinal contents (digesta) were washed out with 10 mL of ice-cold water containing 0.1 mg/mL aprotinin (Sigma Chemical Co., St. Louis, MO), to stop enzyme activity, and centrifuged at 4000*g* for 45 min. The protein content of the supernatants was determined according to the method of Lowry et al. (1951). Endogenous proteins in the stomach and intestine contents were estimated in rats given PBS or protein-free diet. These values were subtracted from the stomach and intestine protein contents determined for the test samples. Using the corrected protein content values, protein digestibility (percent) was calculated by the ratio between protein absorbed in the small intestine and protein ingested, after subtraction of protein in the stomach

$$\mathrm{PD}\ (\%) = \frac{(P_{\mathrm{ing}} - P_{\mathrm{st}}) - P_{\mathrm{int}}}{P_{\mathrm{ing}} - P_{\mathrm{st}}} \times 100$$

where PD is the protein digestibility, P_{ing} = mg of protein ingested, P_{st} = mg of protein in the stomach, and P_{int} = mg of protein in the intestine.

Hemagglutination Assay. Lectin content was determined by using the hemagglutination assay with trypsin-treated rat erythrocytes, as described by Grant et al. (1983). Rat blood was collected into preheparinized tubes, diluted 20-fold with saline (9 g of sodium chloride/L), and centrifuged at 500*g* for 5 min. The cells were washed twice with 0.9% saline and resuspended to their original volume. Trypsin (0.02 mg/10 mL of diluted blood cells) was added, and the mixture was incubated for 40 min at 25 °C. The trypsin-treated cells were centrifuged, washed four times with 0.9% saline, and resuspended in their original diluted volume. Legume samples were extracted with PBS (50 mg of flour/mL of PBS) for 1 h at room temperature with gentle stirring. The extract was centrifuged for 10 min at 25000*g*, and the supernatant was used for the hemagglutination assay.

Twenty-four tubes each containing 200 μL of saline were set up for each sample. Two hundred microliters of sample was added to tube 1, mixed, and serially diluted. Two hundred microliters of blood cells was added to each tube, mixed, and left for 2 h at room temperature. The cells in each dilution were resuspended by gentle agitation, and the degree of clumping was assessed microscopically. Purified phytohemagglutinin from common bean (*P. vulgaris* L.) was included in each assay as standard control. One unit of hemagglutination activity (HU) was defined as the amount of material per milliliter in the last dilution giving 50% agglutination. For comparison, the activity of the samples was expressed as the amount of material [milligrams of dry matter (DM)] containing 1 HU. The limits of experimental accuracy for this technique are ±1 dilution. The results were combined to produce an arithmetic average, but because of the experimental error and for comparison purposes, any value that was not equivalent to one of the sample concentrations used was rounded to the nearest actual value.

Trypsin Inhibitor Determination. Trypsin inhibitor content was assayed according to the method of Van Oort et

Table 1. Protein Content (N × 6.25) and Distribution of Protein of Common Bean (*P. vulgaris* L.) and Faba Bean (*V. faba* L.) Samples[a]

sample	yield (g/kg of dry wt)	protein (g/kg of fraction)	protein (g/kg of meal)
common bean			
raw			
total		266 ± 2.2	266 ± 2.2
globulins	100 ± 15.3	840 ± 5.8	84 ± 0.6
cooked		271 ± 1.6	271 ± 1.6
soluble fraction	160 ± 1.6	225 ± 7.1	36 ± 1.1
insoluble fraction	790 ± 0.7	279 ± 1.3	220 ± 1.7
faba bean			
raw			
total		265 ± 1.8	265 ± 1.8
globulins	120 ± 11.7	920 ± 8.4	110 ± 1.0
cooked		265 ± 1.3	265 ± 1.3
soluble fraction	140 ± 0.6	246 ± 2.2	34 ± 0.3
insoluble fraction	800 ± 0.5	281 ± 1.2	225 ± 1.6

[a] Values are means and standard errors of five replicates.

al. (1989). Samples were extracted with PBS (50 mg of flour/mL of PBS) and centrifuged at 10000*g* for 10 min. Trypsin activity was measured with *N*-α-benzoyl-DL-arginine-*p*-nitroanilide as substrate and trypsin from bovine pancreas (type III, Sigma Chemical, Poole, Dorset, U.K.) as standard. The true enzyme content of trypsin was estimated by active-site titration using *p*-nitrophenyl-*p*′-guanidinobenzoate as substrate (Chase and Shaw, 1967). The reaction was carried out at 30 °C for 30 min and stopped with acetic acid. The absorbance was measured at 405 nm against a blank. Trypsin inhibitor activity was expressed as milligram equivalents of trypsin inhibitor per gram of flour.

Quantification of Total, Free, and Protein-Bound Polyphenols. Polyphenol content was determined after extraction in 0.1 M NaOH (0.02−0.15 g of flour/mL) and centrifugation at 20000*g* for 15 min. The concentration of polyphenols was calculated from the absorption at 328 nm of the supernatant compared to that of a standard tannic acid in 0.1 M NaOH determined at the same wavelength. The contribution of proteins in the 0.1 M NaOH extract to the total absorption was estimated at 328 nm using a bovine serum albumin solution at the same protein concentration as the sample. This value was subtracted from the absorption of the sample. The free polyphenol concentration was determined from the absorption of the supernatant obtained after protein precipitation with 5% trichloroacetic acid and centrifugation. Protein-bound polyphenols were obtained by difference.

SDS−PAGE. SDS−PAGE was carried out according to the method of Laemmli (1970) on a slab gel of 13% polyacrylamide, in the presence of β-mercaptoethanol. Samples and standard proteins were dissolved in sample buffer (0.05 M Tris-HCl, pH 6.8, 3% SDS, 12% glycerol, 2% β-mercaptoethanol, 0.01% bromophenol blue) and heated at 100 °C for 5 min immediately prior to electrophoresis. Thyroglobulin (170.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), pepsin (34.7 kDa), trypsin (23.0 kDa), and cytochrome *c* (12.4 kDa) (Sigma) were used as molecular mass reference proteins. Three hundred micrograms of protein for each legume sample was loaded on the gel. After the electrophoretic run (20 mA, 4 h), the gel was stained with Coomassie brilliant blue R-250.

Amino Acid Analysis. Amino acid composition of cooked common bean and faba bean flour and of their respective small intestine digesta was determined after hydrolysis under vacuum with 6 M HCl at 110 °C for 24 and 72 h. Amino acids were analyzed with a Beckman 118BL amino acid analyzer (Beckman Instruments, Fullerton, CA) and quantified after reaction with ninhydrin (Moore et al., 1958). Cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively, after oxidation with performic acid (Schram et al., 1954). Tryptophan was determined after alkaline hydrolysis (Nielsen and Hurrell, 1985). Values were not corrected for the presence of endogenous proteins.

Statistical Analysis. The results were subjected to analysis of variance. The significance of the differences between means was estimated by a two-side Student *t* test.

RESULTS AND DISCUSSION

About 100 and 120 g/kg were extracted as soluble globulins from common and faba bean seed flours, respectively (Table 1). The salt-soluble protein in globulins represented 35% of the total measured proteins of common and 45% of faba bean, in agreement with previous results (Marquez and Lajolo, 1981). The protein content of globulin extracts was very high and similar to that reported for other globulin preparations (Sathe and Salunkhe, 1981).

After cooking, most of the proteins (80%) of either common or faba bean seeds remained in the insoluble fraction after extraction with water, as a consequence of heat-induced insolubilization (Carbonaro et al., 1993, 1997). In the same extraction conditions, it was previously shown that <20% of protein was not soluble from the raw beans (Carbonaro et al., 1997).

Raw common bean and its globulins had relatively low protein digestibilities in vivo (Table 2). It should be considered that, in the same experimental conditions, digestibility of casein proved to be 98%. Our results are in agreement with the data obtained in digestibility determinations in vitro with similar protein samples (Deshpande et al., 1982; Carbonaro et al., 1997). Cooking did not significantly improve the overall digestibility of common bean proteins ($P > 0.05$). The soluble fraction from cooked beans had a relatively high digestibility. However, the insoluble fraction from cooked beans was very poorly digested. Because the latter fraction comprises the bulk of the proteins in cooked bean (Table 1), the lower than expected digestibility of cooked whole bean was essentially the result of the poor digestibility of the insoluble fraction. Apparently, heat-induced aggregation of *P. vulgaris* L. proteins or particular protein fractions renders them less susceptible to proteolytic breakdown.

Raw faba bean proteins were more digestible than raw common bean proteins (Table 3). Despite this, the digestibility of faba bean globulins was similar to that of globulins from common beans. This may suggest that the factors responsible for the low digestibility of the globulin fraction were the same for both legume species. Digestibility of faba bean proteins was consistent with the value of 85% found by Rubio et al. (1991) for true N digestibility of faba bean meal with rats. However, both biological value and net protein utilization appeared to be very low (Rubio et al., 1991).

Cooking did not improve the digestibility of faba bean proteins, confirming the results previously obtained by

Table 2. Amount of Total Protein (Milligrams) in Stomach and Small Intestine and Protein Digestibility (Percent) of Common Bean in Rats 1 h after Being Given 100 mg of Proteins[a]

			cooked bean		
sample	raw bean	bean globulin	whole	soluble	insoluble
rats fed					
protein in:					
stomach	ND[b]	ND	14.4 ± 0.9[c]	29.1 ± 2.1	9.0 ± 0.9
small intestine	ND	ND	7.1 ± 1.0	2.1 ± 0.4	9.2 ± 0.1
protein digestibility	ND	ND	91.7 ± 1.1a	97.0 ± 1.3b	89.9 ± 0.3a
rats intubated					
protein in:					
stomach	43.0 ± 2.7	54.1 ± 2.5	1.8 ± 1.3	1.3 ± 0.2	9.0 ± 1.6
small intestine	15.7 ± 1.5	13.4 ± 1.1	21.9 ± 3.2	14.3 ± 1.4	34.1 ± 1.5
protein digestibility	72.4 ± 3.1a	70.8 ± 3.8a	77.6 ± 3.3a	85.5 ± 2.7b	62.5 ± 2.2c

[a] Means and standard errors of four replicates. Values in the same row followed by a different letter differ significantly, $P \leq 0.05$. [b] ND, not determined. [c] Lowry basis.

Table 3. Amount of Total Protein (Milligrams) in Stomach and Small Intestine and Protein Digestibility (Percent) of Faba Bean in Rats 1 h after Being Given 100 mg of Proteins[a]

			cooked faba bean		
sample	raw faba bean	faba bean globulin	whole	soluble	insoluble
rats fed					
protein in:					
stomach	ND[b]	ND	22.4 ± 1.4[c]	31.7 ± 2.1	15.0 ± 0.4
small intestine	ND	ND	19.9 ± 1.2	12.4 ± 0.8	24.5 ± 1.2
protein digestibility	ND	ND	74.4 ± 1.6a	81.7 ± 2.1b	71.2 ± 1.6c
rats intubated					
protein in:					
stomach	4.9 ± 0.6	5.4 ± 1.2	4.9 ± 2.8	9.1 ± 1.7	13.9 ± 8.3
small intestine	12.8 ± 0.1	25.6 ± 1.3	37.3 ± 9.9	32.6 ± 1.1	44.4 ± 3.5
protein digestibility	86.5 ± 0.1a	73.0 ± 1.2b	60.6 ± 9.1c	64.1 ± 0.6c	48.4 ± 0.9d

[a] Means and standard errors of four replicates. Values in the same row followed by a different letter differ significantly, $P \leq 0.05$. [b] ND, not determined. [c] Lowry basis.

us in studies in vitro (Carbonaro et al., 1997). Indeed, the values were markedly decreased, especially for the insoluble fraction, which contained a major percentage of total proteins (Table 1).

Proteins of whole flour and soluble fraction of common beans (Table 2) or faba beans (Table 3) after cooking remained longer in the stomach when fed than when the same proteins were intubated. Similarly, when fed, the digestibilities of the proteins appeared to be significantly higher than those gavaged. This was probably the consequence of the higher time of residence of proteins in the stomach given as solid feed and suggests that the gastric phase plays an important role in the overall digestion process.

Raw common bean and common bean globulins contained a high content of lectins (Table 4), in agreement with previous observations (Grant et al., 1983, 1995). Lectins from *P. vulgaris* L. seeds have been found to be deleterious when fed to rats (Pusztai, 1989). Interference with gut function by lectins was therefore likely to be a primary factor responsible for the low digestibility that was measured for these samples (Table 2). The hemagglutination activity in the seed was almost completely abolished upon thermal treatment (Table 4). This may in part account for the slight improvement in protein digestibility found after heat treatment of common bean (Table 2).

In contrast, faba bean contained low amounts of lectins, which were further reduced after cooking (Table 4). Moreover, *V. faba* L. lectin, unlike many other plant lectins, has only limited effects on rat metabolism because it does not bind to or alter the histological

Table 4. Hemagglutinating Activity toward Rat Erythrocytes of Raw and Cooked Common Bean and Faba Bean Samples[a]

	HU/mg of DM[b]	
sample	common bean	faba bean
raw		
total	820	51.3
globulins	1640	6.4
cooked	3.2	6.4
soluble fraction	1.6	25.6
insoluble fraction	1.6	0.8

[a] Values are means of three replicates. The limits of experimental accuracy are ±1 dilution. [b] HU, hemagglutinating units. The amount of material (mg of dry matter, DM) that agglutinated 50% of erythrocytes was defined as containing 1 HU. Accordingly, values are expressed as HU/mg of DM.

structure of rat enterocytes (Pusztai et al., 1990). Therefore, this suggests that neither common bean nor faba bean lectins contribute to any significant extent to the incomplete protein digestion that was found in heat-treated beans.

Trypsin inhibitor content was low in either raw common bean or faba bean and was decreased further to negligible concentrations after cooking (Table 5). Unlike *V. faba* L., a part of the trypsin inhibitor content of *P. vulgaris* L. seed was found in the globulin fraction and this may contribute, together with the activity of lectins, to the low in vivo digestibility of globulins (70.8%) (Table 2). Trypsin inhibitors are thought to exert their antinutritional effect by impairing the efficiency of digestion of dietary proteins in the gut (Gallaher and

Table 5. Trypsin Inhibitor (TI) Activity of Raw and Cooked Common Bean and Faba Bean Samples[a]

	TI mg equivalents/g of flour	
sample	common bean	faba bean
raw		
total	0.385 ± 0.012	0.470 ± 0.025
globulins	0.282 ± 0.008	tr[b]
cooked	0.110 ± 0.005	tr
soluble fraction	0.143 ± 0.002	tr
insoluble fraction	0.051 ± 0.003	tr

[a] Values are means and standard errors of three replicates. [b] tr, traces.

Scheeman, 1986). However, the trypsin inhibitor activity of most legume seeds is generally reduced to nonharmful levels by heat processing (Liener, 1994; Jansman et al., 1998).

Therefore, we investigated the possibility that insoluble complexes between proteins and polyphenols, which are heat-stable bioactive components of many seeds, might have been formed upon heating (Bressani et al., 1988; Carbonaro et al., 1996). Because a high amount of these factors is known to be present in faba bean seeds (Carnovale et al., 1988), such deleterious interaction could account for the decrease in the digestibility of faba bean proteins by cooking (Table 3). Studies about protein−phenolic compound interaction and protein digestibility have demonstrated that the binding between protein and phenolic compounds has an effect on the reduction of the nutritional quality. Indeed, the elimination of phenolic compounds from legume proteins significantly improved the nutritional value [weight gain, protein intake, protein digestibility, net protein ratio, absorbed protein value (Lacroix et al., 1988; Carbonaro et al., 1996)]. Other studies also demonstrated that the structure of the protein is related to the nutritional value. Vegetable proteins are globular proteins and less soluble than animal proteins, and a relationship between protein structure, solubility, and digestibility has been shown (Lacroix et al., 1988; Deshpande et al., 1989; Carbonaro et al., 1997). Vegetable proteins were found to release peptides with higher molecular mass during digestion than animal proteins: best digestibility was observed when peptides released during digestion have a molecular mass ≤3000 Da (Lacroix et al., 1988). Also, an effect of the amount of protein ingested on the digestion time was demonstrated (Rerat et al., 1984).

Quantification of polyphenols indicated that faba bean and faba bean fractions (Figure 1) contained higher amounts of polyphenols than the corresponding common bean samples (Figure 2) with most of the polyphenols being bound to seed proteins (Figures 1 and 3). Because a very high proportion of the polyphenols was protein bound in the globulin fraction (Figures 1 and 3), this suggests they primarly bind to native 7S and 11S storage proteins.

A slight, but significant ($P \leq 0.05$) decrease in the percentage of protein-bound polyphenols was observed after cooking of common bean (Figures 2 and 3). However, protein-bound polyphenols were selectively recovered in the insoluble fraction. The formation of insoluble protein−polyphenol complexes possibly accounts for the lower value of protein digestibility of the insoluble fraction compared to that of the soluble fraction of cooked common bean (Table 2). Nevertheless, the large difference in protein digestibility found between raw and cooked faba bean (Table 3) cannot be

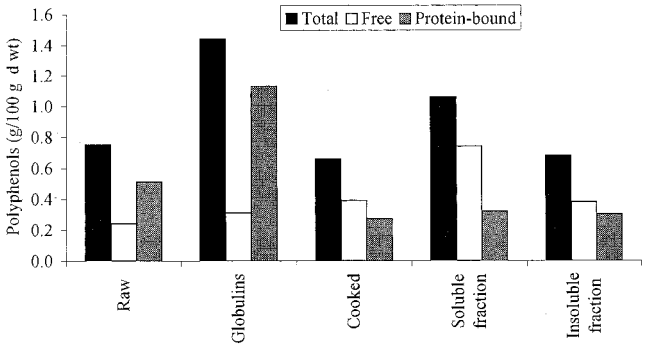

Figure 1. Total, free, and protein-bound polyphenols in raw and cooked faba bean and faba bean fractions (values are means of four determinations with relative standard error <5%).

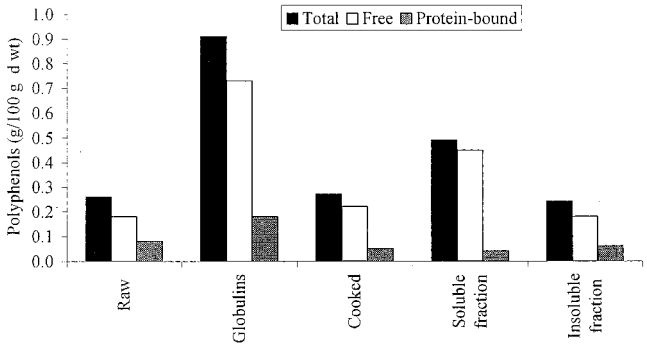

Figure 2. Total, free, and protein-bound polyphenols in raw and cooked common bean and common bean fractions (values are means of four determinations with relative standard error <8%).

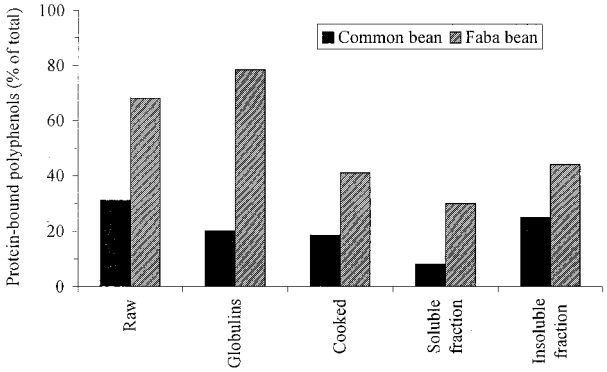

Figure 3. Protein-bound polyphenols (percentage of total polyphenols) in common bean and faba bean samples.

ascribed to the presence of polyphenols, because protein-bound polyphenols were markedly decreased after cooking ($P \leq 0.05$) (Figures 1 and 3). Therefore, the low value of protein digestibility of cooked faba bean cannot be explained by the involvement of antinutritional factors such as lectins, trypsin inhibitors, or the effect of polyphenol binding. It was possible that the different effects of cooking on the in vivo digestibility of common bean and faba bean proteins depend on differences in the specific molecular properties of globulins, the major seed storage proteins.

SDS−PAGE of common bean and faba bean globulins was carried out to point out differences in the proteolytic pattern during gastrointestinal digestion (Figure 4). Globulins extracted from common bean exhibited the three-band pattern at molecular mass (MM) 46−49−50.5 kDa that has been indicated as typical of phaseolin,

Table 6. Amino Acid Composition of Proteins in Cooked Common Bean Flour and in the Small Intestine of Rat after 1 h of Digestion[a]

	cooked common bean flour		small intestinal digest[b]		
amino acid	g/100 g of protein	total mg ingested	g/100 g of protein	total mg recovered	% of ingested
lysine	6.7 ± 0.1	16.9	4.9 ± 0.2	1.06 ± 0.04	6.5
histidine	3.1 ± 0.1	7.9	2.6 ± 0.2	0.57 ± 0.05	7.6
arginine	7.1 ± 0.2	17.9	3.1 ± 0.1	0.67 ± 0.08	3.9
aspartic acid	11.9 ± 0.6	29.9	17.9 ± 0.5	3.87 ± 0.20	13.0
threonine	4.1 ± 0.4	10.4	6.2 ± 0.4	1.33 ± 0.18	12.5
serine	6.0 ± 0.3	15.0	5.6 ± 0.3	1.20 ± 0.07	8.0
glutamic acid	16.5 ± 0.3	41.4	15.6 ± 0.4	3.37 ± 0.15	8.2
proline	3.9 ± 0.4	9.9	4.5 ± 0.5	0.98 ± 0.12	10.1
glycine	4.1 ± 0.2	10.3	12.0 ± 0.3	2.59 ± 0.20	25.2
alanine	4.2 ± 0.2	10.5	3.4 ± 0.3	0.74 ± 0.04	6.7
half-cystine	0.8 ± 0.1	2.1	1.9 ± 0.1	0.41 ± 0.02	19.1
valine	5.6 ± 0.1	14.0	4.6 ± 0.2	0.90 ± 0.05	6.4
methionine	1.3 ± 0.1	3.2	0.9 ± 0.1	0.10 ± 0.02	3.1
isoleucine	4.7 ± 0.2	11.9	3.1 ± 0.2	0.60 ± 0.04	5.0
leucine	8.5 ± 0.2	21.2	6.5 ± 1.0	1.41 ± 0.33	6.6
tyrosine	3.7 ± 0.2	9.3	2.6 ± 0.3	0.56 ± 0.07	6.5
phenylalanine	5.6 ± 0.4	14.0	4.6 ± 0.3	1.04 ± 0.06	7.1
tryptophan	0.9 ± 0.1	2.3	0.7 ± 0.1	0.17 ± 0.03	8.7

[a] Values are means and standard errors of four replicates. [b] Obtained as described under Experimental Procedures.

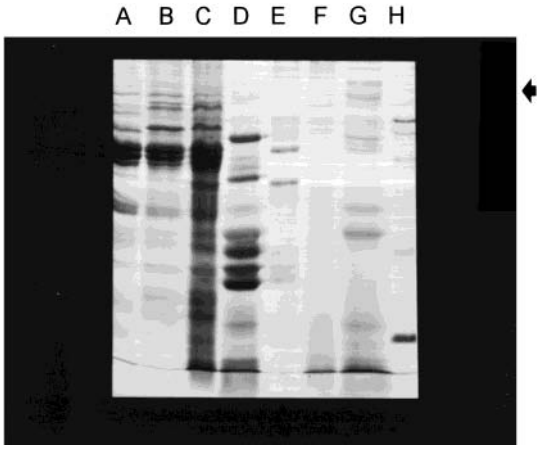

Figure 4. SDS−PAGE of proteins in common bean and faba bean globulin extracts and proteolytic pattern during the in vivo gastrointestinal digestion: (lane A) common bean (*P. vulgaris* L.) standard; (lane B) raw bean globulins; (lane C) stomach content from (B); (lane D) intestinal content from (B); (lane E) raw faba bean globulins; (lane F) stomach content from (E); (lane G) intestinal content from (E); (lane H) molecular mass protein markers [from top to bottom, tyroglobulin (170.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), pepsin (34.7 kDa), trypsin (23.0 kDa), and cytochrome *c* (12.4 kDa)].

the 7S storage protein of *P. vulgaris* L. seed (Pusztai and Stewart, 1980). Also, two other bands at MM 30 and 32 kDa indicated the presence of lectins in the extract, in line with the results of the hemagglutination test (Table 4). Faba bean globulins were resolved into two main bands at MM 41 and 50.5 kDa and three bands of lower mobility corresponding to MM of 24, 27, and 29 kDa. The former components possibly originated from the two different 7S vicilins that have been isolated from *V. faba* L. seeds, whereas the latter species had a molecular mass corresponding to that indicated for basic and acidic subunits of the 11S protein (Derbyshire et al., 1976).

A very high amount of undigested proteins was found in the stomach of rats intubated with common bean globulins, whereas there were no visible protein bands in the case of the stomach digest of rats given faba bean globulins (Figure 4). This result was consistent with the slow rate of stomach emptying that was observed when rats were fed with common bean, but not faba bean, globulins (Tables 2 and 3). Because lectins have been shown to lower the rate of stomach emptying in the rat (Bardocz et al., 1996), this effect can be ascribed to the high lectin content of the common bean globulin preparation (Table 4). In both cases, several bands were found in the small intestinal content, suggesting that only a partial degradation of proteins had occurred during 1 h of digestion. As already reported (Nielsen et al., 1988), the major proteolytic breakdown products of phaseolin were approximately half the molecular mass of the original subunits (23−28 kDa). Main proteolytic fragments from faba bean globulins had a lower mobility and, therefore, a higher molecular mass (35.5 and 38 kDa) compared to that calculated for those arising from common bean globulins (23−28 kDa) (Figure 4). Protein bands at a higher molecular mass than that of the original subunits (>50.5 kDa; see arrow in Figure 4) were also evident in the small intestinal content of rats given faba bean globulins. This suggests a trend toward covalent aggregation of these peptides, as it was previously observed for undigested proteins from faba bean seed (Carbonaro et al., 1997). Examination of the SDS−PAGE patterns of proteins from stomach and gut lumen of overnight-fasted rats (not shown) indicated only a few faint bands that did not match the bands resolved in the electrophoretic profile of gastrointestinal contents of rats intubated with legume globulins. Therefore, a contribution from rat intestinal proteins in the band pattern of Figure 1 could be ruled out.

The amino acid composition of proteins in cooked common bean and faba bean and in their small intestinal digesta was analyzed to determine differences in peptide composition that may account for the lower digestibility of faba bean compared to common bean proteins (Tables 6 and 7). A close similarity in the amino acid composition of both whole seed and small intestinal content of the two legume species was observed. For both legumes, ∼20% of ingested cysteine was still present in the small intestine after 1 h of digestion (Tables 6 and 7). This suggests the involvement of S−S bonds in the stabilization of undigestible peptides or in the formation of novel unavailable species. In addition, in the case of the small intestinal digest of faba bean

Table 7. Amino Acid Composition of Proteins in Cooked Faba Bean Flour and in the Small Intestine of Rat after 1 h of Digestion[a]

	cooked faba bean flour		small intestinal digest[b]		
amino acid	g/100 g of protein	total mg ingested	g/100g of protein	total mg recovered	% of ingested
lysine	6.6 ± 0.2	18.1	4.9 ± 0.3	1.31 ± 0.07	7.2
histidine	2.7 ± 0.1	7.3	1.9 ± 0.2	0.52 ± 0.06	6.9
arginine	8.8 ± 0.1	24.1	3.1 ± 0.3	0.82 ± 0.07	3.3
aspartic acid	11.8 ± 0.2	32.2	15.9 ± 1.1	4.04 ± 0.27	12.4
threonine	4.0 ± 0.1	10.9	5.5 ± 0.4	1.48 ± 0.09	13.8
serine	5.5 ± 0.2	15.0	5.7 ± 0.1	1.52 ± 0.13	10.0
glutamic acid	17.9 ± 0.4	48.8	14.8 ± 0.5	3.54 ± 0.11	7.2
proline	4.3 ± 0.2	11.7	4.3 ± 0.2	1.16 ± 0.04	10.3
glycine	4.6 ± 0.2	12.6	8.0 ± 0.3	2.12 ± 0.17	16.7
alanine	4.5 ± 0.1	12.3	3.9 ± 0.1	1.05 ± 0.05	8.9
half-cystine	1.1 ± 0.1	3.1	2.1 ± 0.1	0.57 ± 0.02	19.4
valine	4.9 ± 0.1	13.5	4.5 ± 0.1	1.20 ± 0.04	8.9
methionine	0.7 ± 0.1	1.8	0.7 ± 0.1	0.20 ± 0.01	11.1
isoleucine	4.2 ± 0.4	11.6	2.8 ± 0.1	0.80 ± 0.04	6.9
leucine	8.3 ± 0.1	22.5	5.2 ± 0.5	1.40 ± 0.12	6.2
tyrosine	3.4 ± 0.1	9.2	3.5 ± 0.2	0.94 ± 0.04	9.8
phenylalanine	4.3 ± 0.3	11.9	4.8 ± 0.5	1.29 ± 0.09	10.9
tryptophan	1.0 ± 0.1	2.6	0.9 ± 0.1	0.23 ± 0.02	7.7

[a] Values are means and standard errors of four replicates. [b] Obtained as described under Experimental Procedures.

(Table 7), the amount (as well as the percentage) of hydrophobic amino acids (alanine, valine, methionine, isoleucine, phenylalanine) and of tyrosine recovered in the gut lumen was higher ($P < 0.05$) than in common bean digest (Table 6). Therefore, complexes of faba bean undigested proteins or peptides were likely to be further stabilized by hydrophobic interactions.

CONCLUSIONS

The results of this study show that protein-bound polyphenols play a minor role in determining the digestibility properties of cooked common bean and faba bean proteins, whereas the plant lectins and trypsin inhibitors do not seem to be involved. However, inherent structural properties of the major storage globulins, 7S for common bean and 11S for faba bean, may be important factors limiting digestion of these proteins during passage through the gastrointestinal tract. The present results confirm our previous findings in in vitro studies showing that digestibility of faba bean proteins can be further impaired by the thermal treatment (Carbonaro et al., 1997).

The low amount of antinutritional factors, such as trypsin inhibitor and lectins, in faba bean seed made likely the possibility that an adverse effect of thermal treatment on protein digestibility could be established. On the other hand, in the case of common bean proteins, the net effect of heating on the digestibility of seed proteins is only slightly positive. Thermal inactivation of the relatively high amount of antinutritional factors present in *P. vulgaris* L. seed may counterbalance the effect of heating on the digestibility of the storage proteins.

Both faba bean and common bean globulins were found to be only partially digested in their native state. The possibility that storage proteins in the two legume species behave quite similarly upon heating cannot be ruled out at this point and seems to be supported by the similarity in the amino acid composition of proteins between the two small intestinal digesta. However, some peculiar features of faba bean proteins may result in a high trend to build up indigestible species upon heating.

Further characterization of peptide complexes that are formed during digestion of cooked common bean and faba bean proteins is currently being performed to determine the molecular basis responsible for the protein digestibility behavior.

ABBREVIATIONS USED

SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; HU, hemagglutinating activity unit.

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Received for review September 7, 1999. Revised manuscript received November 30, 1999. Accepted November 30, 1999. This work is part of the European Concerted Action Program COST 98. We are grateful to the European Commission DGXII, fourth Framework Program, for the Research Training Grant to M. Carbonaro (Contract FAIR-98-5025).

JF991005M