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Sulphur γ-glutamyl peptides in mature seeds of common beans (*Phaseolus vulgaris* L.)

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The non-protein fractions of five common bean varieties (*Phaseolus vulgaris L.*) have been studied in order to determine their total contents of γ-glutamyl-S-methyl-L-cysteine (γ-Glu-Cys(S-Me)) and of free S-methyl-L-cysteine. After isolation by extraction with 70% ethanol and purification by ion-exchange chromatography on cationic and anionic supports, the identification of these compounds was achieved by amino acid analysis after HCl hydrolysis and by mass spectrometry. γ-Glu-Cys(S-Me) was present in high levels in all the varieties studied: the average content was 11 μmol g^{-1} seed weight, accounting for approx. 50% of the bean methionine content determined by ion-exchange chromatography. Free S-methyl-L-cysteine constituted up to 20% of the total amount of S-methyl-L-cysteine found. The presence of γ-glutamyl-L-methionine did not seem to interfere in the total methionine content since its concentration in the non-protein bean fractions studied was negligible. These results suggest (1) that the presence of γ-glutamyl-S-methyl-L-cysteine in whole beans may overestimate the methionine content up to 50%, when this is determined by the traditional method of cyanogen bromide cleavage followed by gas chromatographic analysis of the resulting methyl thiocyanate; (2) a routine quantification method for these sulphur compounds in beans on a laboratory scale is now possible.

INTRODUCTION

Approximately 20 species of edible dry legumes are cultivated and recognized as important sources of food supply, especially the seeds from the common bean, *Phaseolus vulgaris*. The 500 varieties known differ in their colour, size, shape and flavour characteristics. Human taste is so well adapted to them that other cheap protein sources are unable to reduce their high consumption around the world. These beans are very well known in terms of their protein quality, nutritive value, and a few undesirable attributes (Bressani, 1993).

Most nutritional problems related to bean seeds are associated with their low protein digestibility (Nielsen, 1988, 1991), reduced content and bioavailability of sulphur amino acids (Sarwar *et al.*, 1989; Eggum *et al.*, 1989; McDonough *et al.*, 1989), presence of antinutritional factors such as proteolytic enzyme inhibitors and lectins (Van der Poel, 1993), occurrence of natural products such as polyphenolic compounds or phytates (Aw and Swanson, 1985) and diminution of cookability and protein utilization due to storage (Garcia and Lajolo, 1994; Reyes-Moreno and Paredes-López, 1993; Sotelo *et al.*, 1987; Antunes and Sgarbieri, 1979). Some other aspects of these seeds, however, call for more studies of their nutritional significance.

It has been reported that the bean seed non-protein fraction (NPN) contains a significant amount of free amino acids and γ-glutamyl peptides. γ-Glu-Cys(S-Me) and γ-glutamyl methionine (γ-Glu-Met) are examples of those peptides. In the plant kingdom these compounds are restricted to the *Leguminosae, Alliaceae* and mushrooms and they are found in variable quantities (Kasai and Larsen, 1980).

The presence of γ-Glu-Cys(S-Me) is reported to interfere with the determination of the total seed methionine content when the cleavage is performed by cyanogen bromide and the subsequent quantification of methyl thiocyanate (MeSCN) is done by gas chromatography (Duncan *et al.*, 1984). As a consequence, the methionine content might be overestimated leading to its wrong quantification. The extent of this interference, however, has not been determined.

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Despite some results to the contrary (Evans and Bandemer, 1967; Eyre *et al.*, 1983), some antinutritional effects of the free S-methyl-L-cysteine on laboratory animals have been reported (Benevenga, 1974; Evans and Boulter, 1975; Benevenga *et al.*, 1976). Mice growth depression has been observed when these animals received a diet composed of a mixture of synthetic amino acids supplemented with 75% Cys(S-Me) and 25% methionine in molar proportions (Friedman and Gumbmann, 1984; Friedman, 1994). Compared to a control diet containing 100% methionine (1.17 g $100\,g^{-1}$ diet), the experimental group presented a weight gain of only 13%. The most widely accepted explanation is that Cys(S-Me) and methionine compete in a common metabolic pathway for the amino acid transport and utilization (Finkelstein and Benevenga, 1986), so these authors postulated a transamination pathway producing some intermediate toxic products. Because of the presence of Cys(S-Me) in its structure, the same kind of toxic effect might also be expected for the dipeptide γ-Glu-Cys(S-Me).

The first studies related to the detection and quantification of the γ-glutamyl peptides in legumes were performed between 1950 and 1970 (Thompson and Morris, 1956; Rinderknecht, 1957; Zacharius *et al.*, 1958, 1959; Morris *et al.*, 1963; Zacharius, 1970). Most of them were focused on the investigation of their NPN fraction constituents. Kidney beans (*P. vulgaris*) and *Vigna radiata* seeds have been reported to contain considerable amounts of γ-Glu-Cys(S-Me) (Evans and Boulter 1975; Otoul *et al.*, 1975); *Vigna mungo* has high levels of γ-glutamyl-methionine, but its γ-Glu-Cys(S-Me) total content is very low (Kasai *et al.*, 1986). *Glycine max* contains γ-glutamyl-phenylalanine and γ-glutamyl-tyrosine (Morris and Thompson, 1962). Evans and Boulter (1975) studied the distribution of sulphur-containing peptides in 14 genus and species of legumes and found the highest amount of γ-Glu-Cys(S-Me) in kidney beans (0.87% in protein). Although the peptide pattern seems to be typical for each species no additional work has been done in order to detect their presence in other *P. vulgaris* bean seeds. Moreover, no routine quantification method for these compounds in legumes has yet been reported.

Based on these data and on the elevated consumption of *P. vulgaris* by humans, we identified and quantified the total amount of free Cys(S-Me) and its γ-glutamic acid dipeptide present in the NPN fractions of five *P. vulgaris* bean varieties. Also, we estimated the percentage interference of these sulphur compounds on the determination of the available methionine content in beans. Here we report the results of these investigations and propose a routine quantitation method on a laboratory scale.

MATERIALS AND METHODS

The amino acids and ion-exchange resins were from Sigma Chemical Company, St Louis, MO.

The *P. vulgaris* seeds were field grown at the Agronomic Institutes of Campinas (SP) and Paraná (PR), except for the Carioca variety that were bought in a local market. All seeds were stored at 4°C and powdered in a Polymix (Kinematica, Luzern) mill to 60 mesh size for analysis. The moisture content was measured in an oven at 80° for 24 h being expressed on a percentage basis. The crude protein was determined by the micro-Kjeldahl method (AOAC, 1995). The seeds showed, at the time of analysis, a mean 11.6% humidity and a protein content of 22.0% ($N \times 6.25$).

Preparation of crude extracts

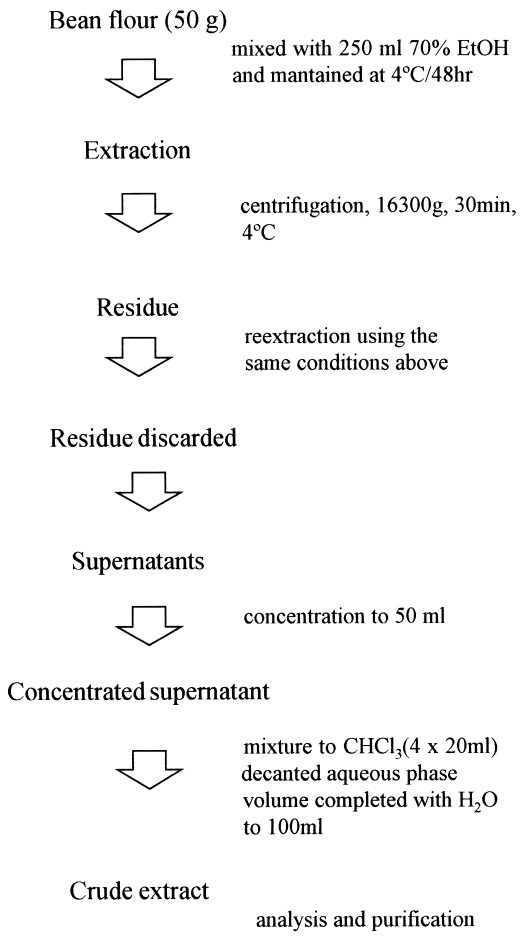
The whole bean flour samples were extracted with 70% EtOH during 96 h, ensuring the inactivation of enzymes and allowing the extraction of amino acids and γ-glutamyl peptides according to the general procedure described by Kasai *et al.* (1986). This procedure adapted to a 50 g sample scale is outlined in Scheme 1.

Preliminary identification and purification of the crude extracts by ion-exchange chromatography

The non-protein crude extract from the Carioca variety was analysed by TLC on silica-plates using *n*-butanol:acetic acid:water (4:1:1) as the mobile phase.

The crude extract was submitted to purification to first remove the neutral compounds and later the acidic amino acids that coelute with the peptide.

A 20 ml sample of the crude extract, representing 10 g of seed weight, was applied to an Amberlite IR 120 column (1.5×30 cm, 32.5 meq, H^+) previously washed with 300 ml of water for removing the neutral compounds (mainly carbohydrates). The elution of nitrogenated compounds was performed with 350 ml of 2N NH_4OH (50 ml fractions). All fractions were neutralized with 2N acetic acid and lyophilized. The separation of the acidic components was performed by ion-exchange chromatography on a basic anion exchange resin in the acetate form using acetic acid as eluant. Fractions 2, 3 and 4 were applied to Dowex 1 X 4 (25 meq, AcO^-). The column was thoroughly washed with distilled water to remove all the basic amino acids and most of the neutral ones. The elution with 350 ml 2N acetic acid produced seven fractions of acidic amino acids and of γ-glutamyl peptide. The fractions 3–6 were combined, adjusted to pH 4.0 with diluted ammonia and applied again to a Dowex 1 X 4 column in order to separate the individual acid amino acids by elution with increasing concentrations of acetic acid from 0.2 to 2.0 N. For each concentration, five fractions of 25 ml were collected, adjusted to pH 4.0 with dilute ammonia and lyophilized. Column flow rates were 100 ml h^{-1}. The chromatographic procedures were performed at 4°C. The elution with 0.2 N acetic acid removed the last basic and neutral amino acids still present in the extract. At an acetic acid con-



Scheme 1. Extraction of gamma-glutamyl peptides from bean flour.

centration of 0.5 N, glutamic acid was eluted and the elution of aspartic acid began. At 1.0 N acetic acid the peptide was partially recovered together with aspartic acid. At 2.0 N acetic acid, the peptide could be recovered without contamination by any other amino acid.

Fractionation by ion-exchange chromatography showed the appearance of γ-Glu-Cys(S-Me) sulphoxide, probably a by-product produced from the Cys(S-Me) oxidation.

Identification and quantification by amino acid analysis

Amino acid analyses were carried out by ion-exchange chromatography under the experimental conditions recommended for protein hydrolysates. Samples containing free amino acids and peptides (5 mg protein equivalents) were acid-hydrolyzed with 1 ml 6 N HCl in vacuum-sealed vials at 110°C for 3 h. The dried residues were dissolved in citrate buffer, pH 2.2 (Na-S, Beckman Instr. Inc., CA) and aliquots were analysed in a Beckman automated amino acid analyzer, model 7300, equipped with a 200 mm column ion-exchange Na^+ resin.

Each sample analysed corresponded to three hydrolysates, so the mean values were taken.

Identification by mass spectrometry

The method of plasma desorption mass spectrometry (PDMS) is a well known technique for the mass determination of nonvolatile biomolecules (MacFarlane and Torgerson, 1974; Chait *et al.*, 1981). The spontaneous fission radionuclide ^{252}Cf is the principal ingredient of this technique. Each fission event gives rise to a pair of energetic heavy ions moving in opposite directions. One of them hits a detector ('start detector') triggering a high precision electronic chronometer, while the other strikes the sample desorbing and ionizing molecules from the surface. The secondary ions emitted from the sample surface are accelerated electrostatically towards a grid and travel through a field-free region with constant velocity before striking a second detector ('stop detector'). The time interval between the start and stop signals (which can be measured quite accurately with appropriate circuitry) is proportional to the square root of the mass to charge ratio of the analyzed ion. The time of flight (TOF) information can be converted into mass of the secondary ions by a calibration procedure.

A compact time of flight mass spectrometer (25 cm of flight length) developed at 'Laboratório de Instrumentação e Partículas', Physics Institute, University of São Paulo (LIP-IFUSP) was used in the present analysis (Macchione *et al.*, 1994). The spectrometer was operated at a pressure of 2.10^{-5} Torr, and acceleration potential of +6 kV and the TOF information about secondary ions was accumulated for 2. 10^6 start events.

For preparation of PDMS sample, 10 μl of solution (14 mg of purified peptide dissolved in 1.5 ml of 0.1% TFA) were deposited on 1.5 μm thick aluminized molar foil and dried.

RESULTS AND DISCUSSION

Isolation and identification procedures

The ethanol:water extracts from the five bean varieties studied contained between 12 and 23% of the total bean nitrogen. The co-extracted proteins were eliminated by precipitation in chloroform. The resulting crude extracts contained on average 5.3 ± 0.4% of total bean nitrogen (constituted mainly by free amino acids and small peptides). These values are in agreement with the data available in the literature. Zacharius (1970) found 7.54% NPN in beans; Ma and Bliss (1978) reported that, in common beans, the free amino acid pool accounts for 5–9% of total seed nitrogen in common beans and Sgarbieri and Galleazzi (1990) described the isolation of non-protein nitrogenous substances varying from 2.73 to 7.94% of the total nitrogen from 60 *P. vulgaris* varieties after protein precipitation with 10% TCA.

The TLC analysis of these extracts revealed that they contained mostly an unknown substance of R_F 0.24 which could be visualized as a large spot by both

reaction with ninhydrin and with iodoplatinate. The fact that it reacted with the last indicated a reduced sulphur compound such as methionine or Cys(S-Me) (Toennies and Kolb, 1951). Its hydrolysis yielded glutamic acid and Cys(S-Me), which appeared in the aminogram in nearly equal amounts.

The crude NPN extracts also had a very high carbohydrate content which made it difficult to lyophilize them (due to their elevated hygroscopicity). The elimination of these neutral compounds was achieved by ion-exchange chromatography, which removed 90–100% of the carbohydrates.

The constituent amino acids of the γ-glutamic peptide purified from the Carioca variety were identified after acid hydrolysis. The experimental conditions used for classic acid hydrolysis (6N HCl at 110°C during 22 h) were shown to be very drastic for peptides containing a γ-linkage, so the hydrolysis time was previously optimized. The results showed that the maximum release of Cys(S-Me) occurred at 3 h. Longer hydrolysis caused increasing loss of this amino acid: up to 50% after 7 h hydrolysis (results not presented). Under the same experimental conditions, glutamic acid is completely stable. In conclusion, 3 h was the most suitable hydrolysis time for the routine quantitation of the of γ-Glu-Cys(S-Me). Glutamic acid and S-methyl-L-cysteine were found in molar proportions of 1:1 causing the disappearance of the original material.

During the purification procedures of the crude extracts, the presence of an unknown by-product of retention time 2.8 min was detected. We supposed that this could be γ-Glu-Cys(S-Me) sulfoxide. This was fully confirmed by comparing it with a standard produced by intentional oxidation of the isolated peptide with H_2O_2. This oxidation was performed by addition of 100 μl H_2O_2 to 100 μl of aqueous solution containing 0.2 μ mol of the reduced peptide, maintenance of the mixture at room temperature for two hours and lyophilization. The hydrolysis of the oxidized peptide was performed as described in Materials and Methods.

Figure 1 shows the aminograms of the purified γ-glutamyl peptide before (a) and after (b) acid hydrolysis, respectively. Its identity was also confirmed by plasma desorption mass spectrometry (PDMS). Figure 2 shows peaks corresponding to cationized molecular ions $[M+H]^+$, $[M+Na]^+$ and $[M+2Na\text{-}H]+$. The molecular weight of the peptide was deduced to be M = (264.3 ± 0.2) Daltons which is in very good agreement with the expected value.

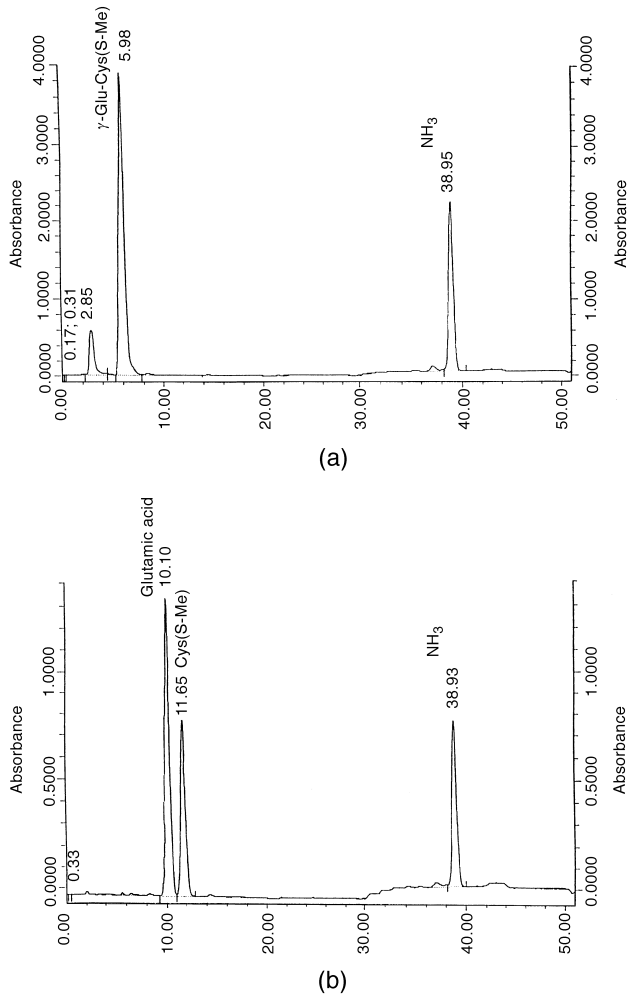


Fig. 1. Amino acid composition of the purified γ-glutamyl-S-methyl-cysteine before (a) and after (b) acid hydrolysis.

Estimated level of γ-glutamyl-S-methyl-L-cysteine

The aminograms of the five crude extracts studied were similar, revealing a complex pattern containing all the protein amino acids. Figure 3(a) illustrates the aminogram from the Carioca variety before hydrolysis. Aspartic acid, glutamic acid, threonine, valine, histidine, tryptophan and arginine were present in high concentrations, but the major component was attributed to the intact peptide γ-Glu-Cys(S-Me), which was eluted

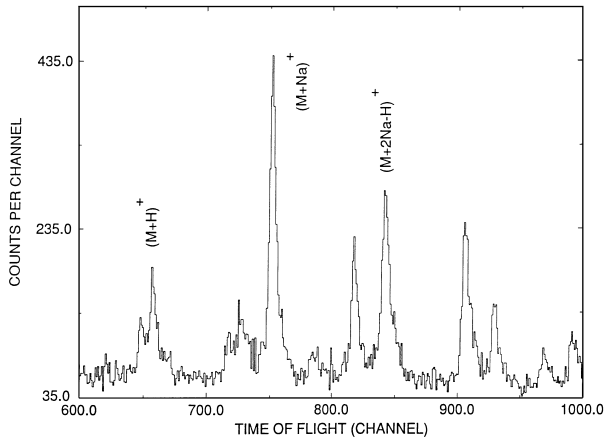


Fig. 2. PDMS time of flight spectrum (molecular region) of positive ions from γ-Glu-Cys(S-Me) peptide sample extracted from bean seeds. The peaks corresponding to the cationized ions of the peptide molecule were identified and assigned as $[M+H]^+$, $[M+Na]^+$ and $[M+2Na\text{-}H]^+$ (note suppressed zero in the vertical scale).

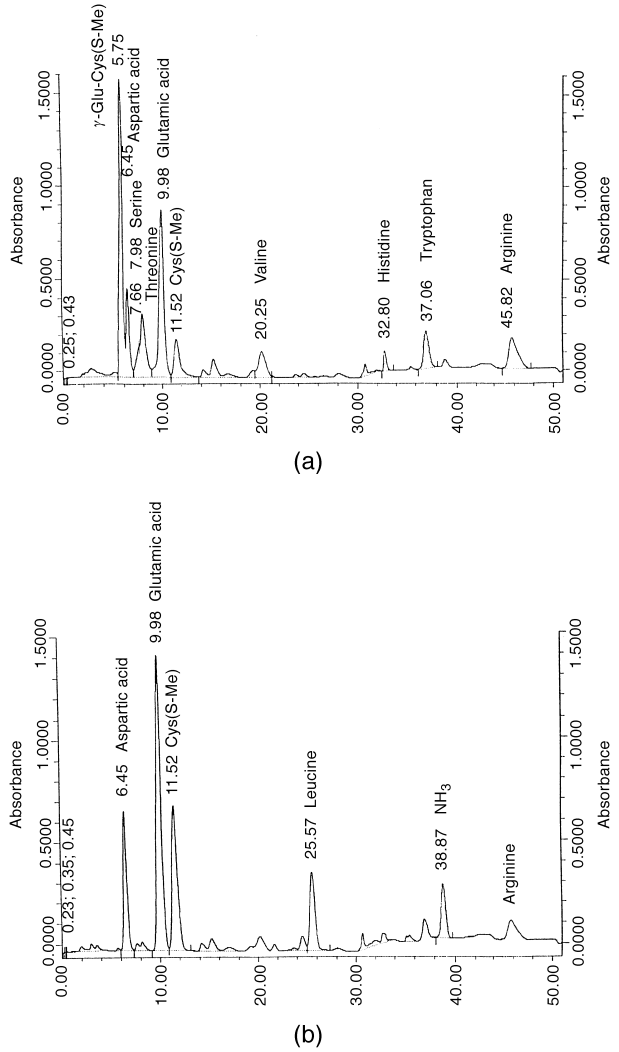


Fig. 3. Amino acid composition of the Carioca bean variety crude extract before (a) and after (b) acid hydrolysis.

immediately before aspartic acid (retention time of 5.75 min). Free Cys(S-Me) coeluted with proline at 11.5 min. This could be identified by comparison with an authentic standard. The differentiation from proline became possible by comparison of the peak area ratios obtained at 570 and 440 nm. The proline content in the crude extract at 440 nm was negligible.

Figure 3(b) presents the aminogram obtained after acid hydrolysis of the same crude extract. As expected, the peak corresponding to γ-Glu-Cys(S-Me) disappeared completely while those of glutamic acid and Cys(S-Me) appeared. These results suggested that this dipeptide was the most abundant source of the overall amino acids present in the NPN bean extracts.

The measurement of γ-Glu-Cys(S-Me) and free Cys(S-Me) of five varieties of *P. vulgaris* beans revealed that the levels of this sulphur peptide in the bean extracts are high with a mean content of 10.9 ± 1.0 μmol of the peptide per gram bean seed (Table 1). Data about *P. vulgaris* in the literature are scarce and each of the previously published studies uses only one sample as the object of a single analysis. Zacharius (1970) reported 11.5 μmol of this peptide per gram of bush bean seed measured as total Cys(S-Me) after a 22 h acid hydrolysis. Evans and Boulter (1975) estimated a total of Cys(S-Me) in protein, corresponding to 14.1 μmol g^{-1} seed of kidney bean with a protein content of 22%. This was the highest level found among the legumes studied.

Table 1. Gamma-glutamyl-S-methyl-L-cysteine and free S-methyl-L-cysteine in *Phaseolus vulgaris* bean varieties[a]

Bean variety	Total Cys(S-Me)[b] μmol g^{-1} seed	Free Cys(S-Me)[c]
Carioca	12.2 ± 1.0	1.8 ± 0.2
Carioca-IAC	11.7 ± 0.8	2.3 ± 0.1
Safira	8.4 ± 0.4	1.7 ± 0.1
Iapar-14	10.3 ± 1.8	2.8 ± 0.3
Iapar-57	12.0 ± 1.1	2.3 ± 0.6
Mean	**10.9 ± 1.0**	**2.2 ± 0.3**

[a]Differences between total and free S-methyl-L-cysteine contents represent the gamma-glutamyl-S-methyl-L-cysteine. Three extractions per sample were carried out and results expressed as μmol g^{-1} seed ± SD.
[b,c]Measured after and before HCl hydrolysis, respectively.

Our results show high levels of γ-Glu-Cys(S-Me) in five varieties of common beans which strongly suggest that this could be a genotypic characteristic of this legume species. This hypothesis might be confirmed by performing a systematic trial in which geographic, soil and climatic influences are considered.

The free form of Cys(S-Me) was present at a low level (2.2 μmol g^{-1} bean seeds (Table 1). After hydrolysis, this level rose to 10.9 μmol g^{-1} seed, indicating that 80% of the Cys(S-Me) found came from γ-Glu-Cys(S-Me).

Possible changes in amino acid composition of the crude extracts were investigated by analysing their aminograms before and after hydrolysis. Table 2 shows the amino acids undergoing major changes. The amounts of glutamic acid, S-methylcysteine, leucine, methionine and lysine increased after hydrolysis, whereas the amounts of the other amino acids decreased or were not affected by the hydrolysis procedure.

Acid hydrolysis released approx. 8.7 μmol of Cys(S-Me) and 13.4 μmol of glutamic acid per gram bean. The liberation of ~35% more glutamic acid than Cys(S-Me) may suggest the presence of other peptides containing glutamic acid.

γ-Glu-Leu and γ-Glu-Met have been detected in blackgram (Otoul *et al.*, 1975), in kidney bean seeds (Morris *et al.*, 1963) and in *Phaseolus lunatus* (Rinderknecht *et al.*, 1958). The increment observed by us in the amount of leucine determined after hydrolysis corresponds to 1.3 μmol g^{-1} seed. The amount of methionine showed only a very slight increment, indicating a negligible contribution of the γ-Glu-Met peptide to the overall composition of the NPN fraction, confirming previous results of Zacharius (1970) who reported a 4% contribution of γ-Glu-Met in relation to

Table 2. Changes in selected amino acid levels in the crude extracts of five bean varieties after acid hydrolysis

Bean variety	Glu	Cys(S-Me)	Asp	Leu	Met	Lys
			μmol g^{-1} seed[a]			
Carioca	16.1	10.4	6.5	1.2	0.4	0.5
Carioca IAC	14.0	9.4	4.1	0.9	0.7	0.3
Safira	10.7	6.7	3.5	1.1	0.5	0.5
Iapar-14	11.8	7.5	2.0	1.6	0.8	0.4
Iapar-57	14.5	9.7	4.4	1.5	0.5	0.5
Mean	**13.4 ± 2.1**	**8.7 ± 1.6**	**4.1 ± 1.7**	**1.23 ± 0.3**	**0.56 ± 0.2**	**0.5 ± 0.1**

[a]Values refer to the differences between the levels measured before and after acid hydrolysis. Data are expressed on a 11.6% moisture weight basis.

γ-Glu-Cys(S-Me). The possibility of the presence of other peptides like γ-Glu-Glu, previously isolated from *Acacia georginae* (Ito and Fowden, 1972), and γ-Glu-γ-Glu-Cys(S-Me), detected in *V. radiata* seeds by Kasai *et al.* (1986), could not be neglected. Finally, the increase in the amounts of aspartic acid and ammonia after hydrolysis seems more likely due to the presence of the asparagine moiety. For these reasons, the quantitation of γ-Glu-Cys(S-Me), by measuring the release of glutamic acid after hydrolysis, does not seem to be the best procedure to use.

Interference of S-methyl-L-cysteine with the methionine quantitation

The high levels of the sulphur dipeptide in common beans may have analytical and nutritional implications. The free Cys(S-Me) does not release MeSCN by reaction with BrCN, but the predominant dipeptide γ-Glu-Cys(S-Me) does. This may cause an interference with the determination of methionine by analytical methods (Ellinger and Duncan, 1976).

The cleavage of methionine by cyanogen bromide and subsequent gas chromatographic quantification of methyl thiocyanate (MeSCN) involves the participation of the S-CH_3 group, also present in γ-Glu-Cys(S-Me) (Duncan *et al.*, 1984). This method is frequently employed to estimate the intact methionine considered nutritionally available, as distinct from methionine sulphoxide (Ellinger and Duncan, 1976; McIntosh and Ellinger, 1976; Duncan *et al.*, 1984; Kohnhorst *et al.*, 1990).

The mean methionine content in beans is approx. 1.4 g per 100 g protein (Rayas-Duarte *et al.*, 1988; Koehler and Burke, 1988; Peace *et al.*, 1988; Marletta *et al.*, 1992) which corresponds to 20.6 μmol g^{-1} seed if the protein content is 22%. According to our results, the difference between γ-Glu-Cys(S-Me) and free Cys(S-Me) contents varied from 7.0 to 10.4 μmol g^{-1} seed, so it accounts for an interference of up to 50% of the methionine content estimated by the reaction with BrCN.

There are some contradictory results on BrCN methodology applied to legumes reported in the literature. For instance, Mackenzie (1977) described a procedure for methionine quantification in some vegetables that does not refer to the possible interference of γ-Glu-Cys(S-Me). Apostolatos and Hoff (1981) reported that the contribution of this dipeptide to the amount of methionine is negligible. Some investigations on *P. vulgaris* seeds report total methionine contents lower than those obtained by measuring MeSCN from BrCN cleavage, suggesting an overestimation caused by the presence of the γ-glutamic peptides containing a sulphur amino acid (Evans and Boulter, 1975; Duncan *et al.*, 1984; Durigan *et al.*, 1987; Lanfer Marquez *et al.*, 1996). The isolated bean protein fractions, devoid of this peptide, were not affected by this kind of interference, giving more reliable methionine values after reaction with BrCN (Lanfer Marquez *et al.*, 1996).

In conclusion, the considerable interference of this peptide in the analysis of methionine by MeSCN determination, suggests the method should not be used for legumes containing γ-Glu-Cys(S-Me). The extent of γ-Glu-Cys(S-Me) distribution and levels among edible legumes will need to be determined. For that, initial experiments are been undertaken in our laboratory.

In view of the high content of γ-Glu-Cys(S-Me) in common beans, more information about its metabolic pathways and possible antinutritional or toxic properties are needed.

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