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Factors Affecting Cystine Reactivity in Proteolytic Digests of *Phaseolus vulgaris*

Marina Carbonaro, Luisa Marletta,[†] and Emilia Carnovale*

National Institute of Nutrition, Via Ardeatina 546, 00178 Rome, Italy

Raw/dehulled and cooked/dehulled white and brown beans (*Phaseolus vulgaris*) were tested for in vitro protein digestibility, extent of proteolysis, and cysteine/cystine chemical reactivity. Characterization of the proteolytic digests was carried out by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and ultrafiltration of peptides. The increase in protein digestibility following cooking treatment of both varieties was found to be related to a higher extent of proteolysis, as monitored by SDS–PAGE and recovery of low molecular weight peptides (<30 000) after ultrafiltration of the digests. Dehulling, when it preceded cooking, slightly increased the degree of hydrolysis of the bean proteins. A very low amount of free SH groups was found in the proteolytic digests. The maximum percentage of reactive cystine (cystine susceptible to reduction), detected in the digests after reduction by dithioerythritol, never exceeded 75% of the total cysteine content. Cooking/dehulling had no effect on the cystine reactivity of the white bean (about 65%), while a decrease to 49% was detected in the cooked brown bean and reversed by dehulling. Cystine in all samples was found totally susceptible to reduction after denaturation by urea. The results suggest that the stability of S–S-containing proteins and the presence of tannins are relevant factors for the low cystine reactivity, especially in cooked beans.

INTRODUCTION

Legumes, in spite of their high protein content, contain low amounts of limiting sulfur amino acids. Although much evidence has been produced for the partial bioavailability of sulfur amino acids from legumes (Sarwar and Peace, 1986), the molecular basis of the limited absorption has not yet been clarified. The nutritional value of legume proteins has been reported to be affected by the presence of either heat-labile (mainly protease inhibitors and lectins) or heat-stable (polyphenols) antinutritional factors (Liener, 1979; Fernandez et al., 1982), as well as by the intrinsic resistance of native legume proteins to enzymatic hydrolysis (Nielsen et al., 1988). While changes in the structural stability of phaseolin, the major storage protein in *Phaseolus vulgaris* seeds, which occur upon heating, improve the digestibility of the protein both in vitro and in vivo (Liener and Thompson, 1980; Deshpande and Damodaran, 1989), the same does not always hold true for protease inhibitors, despite the generally reduced inhibitory activity (Bradbear and Boulter, 1984). Since protease inhibitors are stabilized by cysteine in the form of several disulfides, the low digestibility of this protein fraction has been proposed as a major factor for the low availability of legume sulfur amino acids, as most of the other bean proteins are quite poor in these amino acids, especially cysteine (Marquez and Lajolo, 1981; Sgarbieri and Whitaker, 1982).

The essential role of disulfide bonds in stabilizing protein structure and thus lowering the susceptibility to enzymatic hydrolysis has been verified for proteins from different food sources. Breakage of S–S bonds has been shown to increase the in vitro digestibility of soybean and sorghum proteins (Boonvisut and Whitaker, 1976; Hamaker et al., 1987) and of β-lactoglobulin (Reddy et al., 1988).

On the basis of the kinetics of the sulfur amino acid release in small peptides during in vitro proteolysis of raw and cooked beans, Rayas-Duarte et al. (1988) suggest that the location of methionine in a buried hydrophobic region could be the reason for the slow release and, perhaps, also for the limited availability in vivo. On the other hand, cysteine appears to be more accessible than methionine in the protein structure and is completely released after proteolysis of the processed samples, thus not reflecting

* To whom correspondence should be addressed.
[†] Fellowship recipient of Centro Internacional de Agricultura Tropical (CIAT).

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the in vivo situation. Therefore, studying the chemical reactivity of cysteine and cystine would prove to be useful to obtain additional information about the position of this amino acid in legume proteins.

In a previous study (Marletta et al., 1991), aimed at testing the protein quality of different color-coated varieties of *P. vulgaris*, we also examined, by commonly employed chemical methods, the reactivity of sulfur amino acids in the proteolytic digests, as a measure of their availability. We found that while methionine was completely detectable in the whole digests, not more than 60% of cystine appeared to be reactive after addition of dithioerythritol (DTE), a strong reducing agent. Moreover, the percentage of reactive cystine was found to be related to bean color, thus suggesting the involvement of seed coat components in the reactivity of this amino acid. We proceeded here in the evaluation of the factors responsible for the low cysteine availability in legumes by testing raw/dehulled or cooked/dehulled proteolytic digests of white and brown bean varieties with respect to their cysteine/cystine reactivity, in vitro protein digestibility, and extent of proteolysis.

MATERIALS AND METHODS

All chemicals used were of reagent grade.

Two Italian commercial varieties of dry beans (*P. vulgaris*) with white (Cannellini) and brown (Borlotti) seed coats were used in the study.

Dehulled raw beans were obtained by manual removal of the seed coat. Whole or dehulled cooked bean samples were soaked in water (1:3 w/v) at room temperature for 2 h; beans, with soaking water, were autoclaved for 20 min at 1 atm and then freeze-dried. Raw and cooked beans, whole or dehulled, were ground in a Cyclotec 1093 Tecator (50 μm).

Nitrogen content in all bean flours was determined according to the Kjeldahl method (AOAC, 1984).

In Vitro Enzymatic Digestion of Bean Proteins. The protein digestibility of raw and cooked beans, before and after dehulling, was determined according to the multienzyme technique of Bodwell et al. (1980), starting from 10 mL of a 1 mg of N/mL aqueous flour suspension. Immediately after the protein digestibility measure, the enzymatic hydrolysis was stopped by addition of 1.8 M $HClO_4$ (0.2 mL/mL of hydrolysate); the digests were incubated for 30 min and then brought to pH 7.0 by addition of $KHCO_3$. The precipitate was discarded, and the supernatant was assayed for N recovery (AOAC, 1984), which ranged from 80% to 90% of the initial N content in the different samples. The supernatant was used for the subsequent analysis.

SDS-PAGE. SDS-PAGE of the digests was performed according to the method of Fling and Gregerson (1986) on a gradient slab gel of 7.5–20% acrylamide concentration. The molecular weight marker proteins, phosphorylase *b* (97 400), bovine serum albumin (66 200), ovalbumin (42 700), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400) were from Bio-Rad. The gel was stained with a silver stain kit (Sigma).

Ultrafiltration of the Digests. The separation of low molecular weight components present in the proteolytic digests of the different bean samples was carried out by centrifugation with Centriprep 30 or Centriprep 10 (MW membrane cutoff 30 000 and 10 000, respectively) concentrators (Amicon). Ultrafiltration was started with 6 mL of the supernatant obtained after enzymatic hydrolysis and was stopped when the volume of the retentate was approximately 1 mL (total spinning time of 30 min at 3000*g* and room temperature). The N content (milligrams) in the ultrafiltrate and in the retentate was measured according to the Kjeldahl procedure and expressed as a percentage of the total N content of the starting material.

Total Cysteine and Reactive Cystine Assays. Total cysteine content was determined in all bean samples as cysteic acid, after hydrolysis with 6 N HCl (Schram et al., 1954). The determination was carried out on a Beckman 120C amino acid analyzer.

Table I. In Vitro Protein Digestibility (Percent) of the Two Varieties of Raw and Cooked Beans (*P. vulgaris*) before and after Dehulling[a]

	raw bean		cooked bean	
cultivar	whole	dehulled	whole	dehulled
Cannellini (white)	74.0 ± 0.96a	74.2 ± 1.67a	80.5 ± 1.19b	82.6 ± 1.76c
Borlotti (brown)	71.5 ± 1.37d	71.4 ± 1.39d	77.3 ± 1.0e	78.9 ± 1.53f

[a] Mean of 10 determinations ± SD. Values followed by different letter are significantly different ($p < 0.05$).

Reactive cystine (cystine susceptible to reduction) was estimated on the enzymatic hydrolysates according to the chemical method to Zahler and Cleland (1968) after reduction with dithioerythritol (Sigma) and determination of the resulting SH groups with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma) in the presence of arsenite. Reduction of cystine was obtained either by a 30-min incubation at room temperature with DTE at final concentrations of 0.2, 0.6, 1.8, 3.6, and 5.0 mM or by incubation with 0.6 mM DTE for 30, 60, and 120 min. Before reaction with DTNB, the excess of dithiol used as a reducing agent was made unreactive by complexing with a 25-fold molar excess of arsenite. A control without DTE was performed to test the presence of free SH groups in the hydrolysates. Detection of reactive cystine was also carried out after sample denaturation at 38 °C for 1 h with a (final concentration) 8 M urea–3 mM EDTA solution. The chemically reactive cystine content of each sample was corrected for the respective N recovery and expressed as percentage of the total cysteine released after reduction by DTE.

Statistical Analysis. The data were subjected to analysis of variance when necessary. Individual means were compared by Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Protein Digestibility. Table I shows the protein digestibility values detected for the raw/dehulled and cooked/dehulled samples of the white (Cannellini) and brown (Borlotti) varieties of *P. vulgaris*.

The negative effect of tannins on the protein digestibility of cooked beans (Bressani and Elias, 1980) was confirmed by our observation that the white bean, after cooking in the presence of tannic acid (10 mg/100 g of protein), showed a reduction in protein digestibility of about 4%, approximating the value of the brown bean after cooking (77.3%). A similar decrease in protein digestibility values has also been reported following addition of isolated tannins (or tannic acid) to boiled seeds of mung beans (Barroga et al., 1985).

A slight but significant increase was found when removal of the seed coat preceded the cooking treatment of either white or colored beans, while dehulling per se had no effect on the protein digestibility of both varieties, in contrast with previous studies (Deshpande et al., 1982). This might be due to differences in sample preparation, although the influence of polyphenol content and/or reactivity on the protein digestibility of the cultivars investigated cannot be excluded. In this respect it should be considered that the polyphenol content of our bean varieties (Marletta et al., 1991) is low when compared to that of other varieties of *P. vulgaris* (Bressani and Elias, 1980).

Analysis of the Digests. *SDS-PAGE.* The SDS-PAGE pattern of the proteolytic digests of the raw/dehulled and cooked/dehulled bean proteins of the two varieties investigated is shown in Figure 1. No relevant changes in the electrophoretic pattern of the digestion products were observed between the white (Figure 1a–d) and the brown varieties (Figure 1a′–d′), irrespective of cooking and/or dehulling. The raw samples (Figure 1a,a′) still showed the typical band pattern (MW 43 000–51 000)

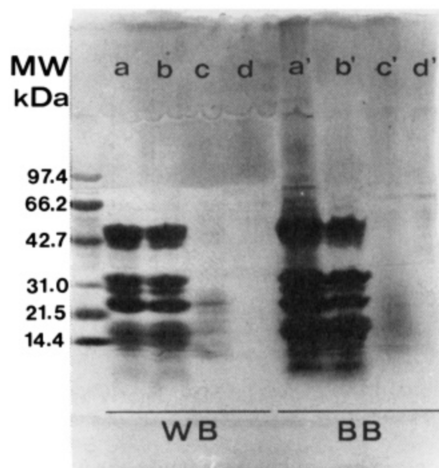

Figure 1. SDS-PAGE of dry bean proteins after enzymatic hydrolysis. WB, white bean; BB, brown bean; a, a′, raw samples; b, b′, dehulled raw samples, c, c′, cooked samples; d, d′, dehulled cooked samples. Molecular weight protein markers (14 400–97 400) are described under Materials and Methods.

described for phaseolin subunits (Bradbear and Boulter, 1984; Deshpande and Nielsen, 1987a,b), together with lower molecular weight bands (10 000–32 000). The 24 000 and 28 000 bands consisted of proteolytic digestion products, since they appeared as faint bands in our raw samples before the enzymatic hydrolysis (not shown). These bands were similar to the well-characterized phaseolin breakdown products, detected in the course of degradation of the purified native protein by different proteases (Liener and Thompson, 1980; Nielsen et al., 1988). The electrophoretic pattern of the raw samples was essentially unchanged by dehulling, except for the disappearance of some components of high molecular weight (likely aggregates) present in the brown variety (Figure 1a′,b′). The major difference in the SDS-PAGE pattern appeared for both varieties after heat treatment and consisted of a conspicuous loss of protein staining bands (Figure 1c,c′), including those corresponding to phaseolin subunits. Only residual amounts of molecular weight proteolytic products (12 000–28 000 MW) were still detectable, but these disappeared after dehulling (Figure 1d,d′). The presence of bands at MW < 10 000, not visible in the pattern of Figure 1, was found difficult to reproduce in these experimental conditions. On the other hand, only a few faint additional bands can be resolved in the hydrolysates of the cooked beans when gels with higher acrylamide concentrations (≥25%) were used. The similarity between our results and those reported in previous works on purified heated phaseolin suggests that the difficulty in the detection of very low molecular weight components is a peculiar feature of the degradation pattern of this protein (Romero and Ryan, 1978; Nielsen et al., 1988). In this respect phaseolin appears different from other seed storage related proteins, like vicilin or glycinin (Nielsen et al., 1988).

The partial loss of protein staining bands observed in the digests of the cooked samples was interpreted in terms of an increase in the degradation rate of the bean proteins by protease. However, the nearly complete absence of protein bands in the cooked dehulled bean hydrolysates cannot be exclusively the result of the increased extent of proteolysis. More probably, some unidentified interactions present in the cooked dehulled beans, resulting in alterations of the solubility of peptides, may be partially responsible for the observed electrophoretic pattern. This aspect is under study.

Ultrafiltration of Low Molecular Weight Digestion Products. The increased proteolytic susceptibility of bean flour proteins subsequent to heat treatment was quantified by the separation of the proteolytic products achieved by ultrafiltration with MW cutoff 30 000 membrane (Figure 2). The extent of proteolysis, measured as the percentage of the total N recovered in the <30 000 fraction, greatly increased in the heat-processed samples of both varieties: 82% and 75% of the total nitrogen was found in the ultrafiltrate for the white and the brown varieties, respectively, while only 20–30% was recovered in the same fraction after proteolysis of the raw samples (Figure 2, WB and BB). This increase in the degree of degradation of the bean proteins promoted by heat treatment is certainly one of the factors responsible for the higher protein digestibility values observed after cooking of beans (Table I) and is consistent with both protein denaturation and inactivation of heat-labile protease inhibitors (Sgarbieri and Whitaker, 1982). A significant correlation was found between the protein digestibility values of the different bean samples and the percentage of the total N recovered in the <30 000 fraction ($r = 0.87$; $p < 0.01$).

Dehulling slightly increased the percentage of N in the <30 000 fraction (from 75% to 85%, Figure 2, BB), only after cooking of the brown variety. However, an increase in the degree of proteolysis in the dehulled and cooked white bean also, compared to the whole cooked bean, cannot be ruled out since the result of the SDS-PAGE showed that modifications in cooked dehulled beans occurred especially in the <30 000 fraction (Figure 1c,d;c′,d′). Actually, when the separation was carried out with a MW cutoff 10 000 membrane, the percentage of low molecular weight products appeared higher after dehulling and cooking than after cooking alone, for the white bean as well (62% and 48%, respectively).

The effect of seed coat removal seems to indicate that the binding of polyphenolic compounds to proteins in the cooked beans may affect the cleavage of peptide bonds by protease. This might be effected either through enzymatic (trypsin) inhibition or by binding to legume proteins (Griffiths and Moseley, 1980; Bressani et al., 1988). In the latter situation the accessibility of susceptible bonds and thus the number, or type, of proteolytic fragments produced might be altered. This effect may also account for the small differences in the SDS-PAGE pattern evidenced between the two varieties after proteolysis of the whole cooked beans (Figure 1c,c′).

Cystine Reactivity. The reduction of cystine by increasing concentration of DTE in the digestion products of the raw/dehulled and cooked/dehulled samples of the two bean varieties is depicted in Figure 3. A very low amount of free thiol groups (<10% of the total cysteine) was determined for all samples before treatment with the reducing agent, indicating that almost all of the cysteine residues in the digestion products were engaged in disulfide bridges. At the lowest concentration of DTE used (0.2–0.6 mM) not more than 40–60% of the disulfide bonds were cleaved in the different bean samples, while a slight increment was observed when the concentration of the reducing agent was increased to 5.0 mM (Figure 3). Similar results were obtained by increasing the time of reduction by DTE up to 120 min at a fixed concentration of 0.6 mM.

The behavior of the disulfides of the digested bean proteins toward DTE was indicative of the presence of groups with different reactivity: 40–60% of the cystine, reduced by lower concentration of DTE, behaved as if freely accessible, while 10–25%, reduced by 1.8–5.0 mM DTE, appeared to be less exposed. In no case were more than 75% of sulfhydryls produced, irrespective of cooking and/or dehulling, in line with our previous data on the low

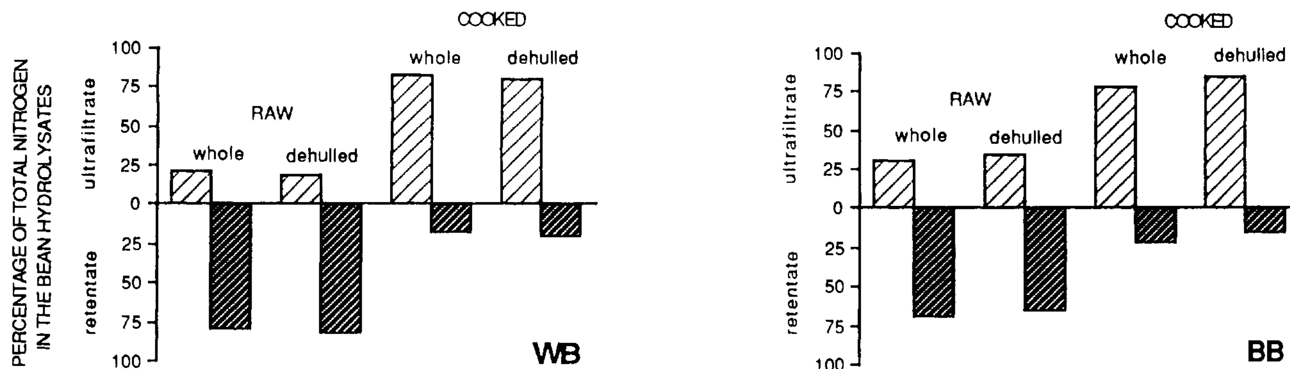

Figure 2. Ultrafiltration of protein hydrolysates: distribution of total nitrogen between the <30 000 MW (lightly hatched) and the >30 000 MW (heavily hatched) fractions. WB, white bean; BB, brown bean.

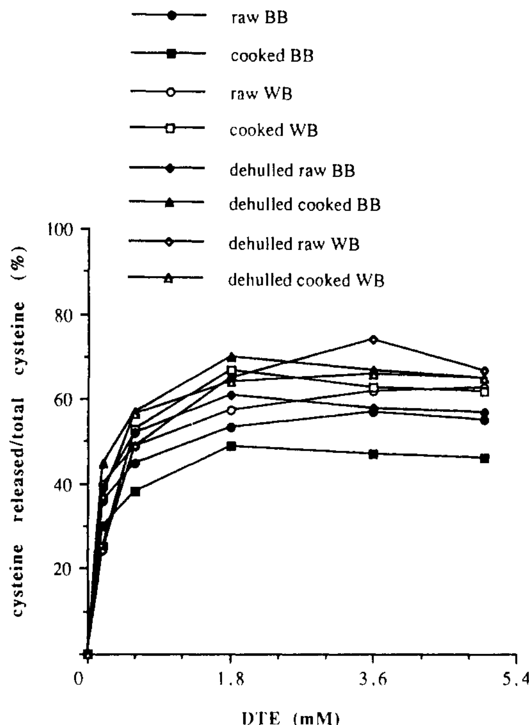

Figure 3. Reduction of cystine in the proteolytic digests of the white and brown dry bean proteins by increasing concentration of DTE. The values are the mean of triplicate determinations (variability coefficient <10%).

cystine reactivity in the digestion products of different bean varieties (Marletta et al., 1991).

The maximum percentages of sulfhydryls produced in the raw bean samples were slightly different (63% and 57% for the white and brown varieties, respectively; Figure 3), but after dehulling, the value for the brown bean rose to that of the colorless raw variety. The latter, after dehulling, showed an unexpected increase to 75% in the S–S reactive groups, for which we have, at this point, no explanation to offer. The cooking treatment had a very different effect on the proportion of disulfide reduced in the two varieties: while in the white bean no significant variations were observed with respect to the raw bean, in the brown bean the value of reactive cystine was found significantly ($p < 0.005$) lower compared to that of the raw sample (49% and 57%, respectively). In addition, dehulling had a marked effect when it preceded cooking of the brown, but not white, bean, raising the value to 70%, so that after dehulling and cooking the percentages of sulfhydryls produced in the two varieties were very close (Figure 3). Moreover, when an excess of tannic acid (1 g/100 g of protein) was added to the hydrolysate of the dehulled cooked brown bean, or when the white bean was cooked in the presence of tannic acid, a decrease to 40% in the maximum percentage of S–S bonds cleaved was achieved (not shown).

These results indicate that tannins are likely to be responsible for the differences observed between the white and the brown varieties in the percentage of cystine reduced by DTE, especially after cooking. A specific effect of tannins on the cystine reactivity has already been suggested by our previous data indicating a higher extent of reduction in similar conditions of cystine from protein hydrolysates of white compared to red or brown beans (Marletta et al., 1991). We propose that tannins can influence not only bean protein digestibility, as already indicated (Barroga et al., 1985; Deshpande et al., 1982), but also the reactivity of cystine. It seems likely that the binding of tannins to the bean proteins, which occurs to a higher extent during the heat treatment but perhaps also in the raw beans, can shield part of the disulfide bonds from the action of the reducing agent. Tannins seem to exert some effect on the degree of proteolysis of the cooked brown bean (Figure 2, BB), but further studies are required to clarify the relationships between the two phenomena. The power of tannins in binding proteins from different food sources stems from several sources. Condensed tannins have been shown to complex with globulin G1 from black bean of *P. vulgaris* over a wide range of pH (Aw and Swanson, 1985). Moreover, the purification of tannin-associated proteins from high-tannin sorghum suggests that, besides prolamines, globulins and albumins are also implicated in the interaction with tannins (Jambunathan and Mertz, 1973; Hagerman and Butler, 1980).

The effect of tannins on cystine reactivity here proposed does not explain why the maximum proportion of free thiol groups detected after reduction by DTE never exceeded 75% of the total cysteine. Thus, if irreversible oxidation processes did not occur, it became necessary to ascertain whether other factors could be involved in burying potentially reactive disulfide bonds away from the action of the reducing agent. When reduction by DTE was performed in the presence of 8 M urea as a denaturing agent, we found that over 90% of the cystine was reduced by 1.8 mM DTE in all samples (Figure 4). No detection of sulfhydryls was obtained when the determination was carried out in presence of 8 M urea but without DTE, indicating that the buried cysteine was in the form of S–S groups. Since the complete reduction of cystine in all samples was accomplished only by the use of urea and DTE, it can be concluded that structural factors intrinsic to the digestion products of legume proteins play a dominant role in the determination of the inaccessibility of part of the disulfides to the reducing agent. It appears

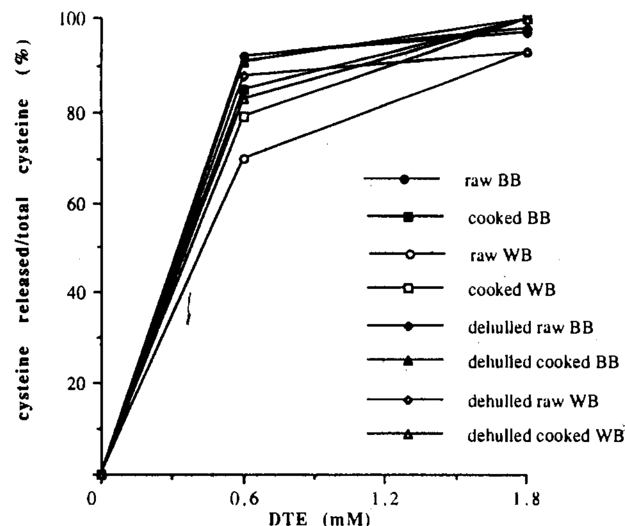

Figure 4. Reduction of cystine in the proteolytic digests of the white and brown dry bean proteins by increasing concentration of DTE after denaturation by 8 M urea. The values are the mean of duplicate determinations (variability coefficient <10%).

that, even after proteolytic digestion, approximately 25–50% of the cystine contained in the bean proteins is still buried by the conformation of the peptides (or proteins), thus turning out to be unreactive unless strong denaturation occurs.

This is not surprising when one considers that most of the cystine in legume proteins is contained in the albumin fraction and mainly in protease inhibitors (Sgarbieri and Whitaker, 1981), which have a high stability conferred by a number of disulfide bonds (Pusztai, 1968; Kowalski et al., 1974). Among bean protein fractions, albumin has been shown to have the lowest protein digestibility and no improvement upon heating (Sgarbieri and Whitaker, 1982). Moreover, proteolysis-resistant fractions in beans seem to consist of heat-stable protease inhibitors (Marquez and Lajolo, 1981).

Our data on cystine reactivity add further support to the idea that the high stability of the S–S-containing proteins (or peptides) plays a major role in the determination of the low availability of cysteine from legumes (and perhaps partly of the low protein digestibility). Tannins are likely to be involved in the additional decrease in cystine reactivity of the colored cooked beans. Data from the literature relative to the specificity of tannin–protein interactions make possible a binding of tannins to protease inhibitors with consequence on cystine reactivity. The preferential binding site of condensed tannins to proteins has been identified in proline residues (Hagerman and Butler, 1981), and the amino acid sequence of protease inhibitors from soybean or beans from *P. vulgaris* shows proline residues to be adjacent or in close proximity to disulfide bridges (Odani and Ikenaka, 1973; Wilson and Laskowski, 1974). The effect of tannins on cystine reactivity could contribute to the lower biological protein quality of the whole colored beans compared to the white bean varieties. Thus, the "reactivity" of cystine might represent a relevant factor in the determination of the release under physiological conditions. As a matter of fact, the cystine fraction easily reduced in our conditions (40–60%) corresponds to the percentage of this amino acid that is reported to be bioavailable from legumes (Sarwar and Peace, 1986).

The commonly employed processing conditions may not always be sufficient to convert all cystine in a bioavailable form. Cysteine in highly stable proteins, like trypsin inhibitors, has been found to be available for rat growth only after extensive denaturation (Kakade et al., 1969). In addition, cooking, in spite of the increase in the degree of proteolysis, could even have a detrimental effect on the cysteine availability of colored beans, as a consequence of tannin–protein interactions, but this effect could be overcome by dehulling. Thus, it is here suggested that the tannin content and the chemical reactivity of cystine need to be evaluated to gain additional knowledge of the nutritional quality of the bean proteins.

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