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Influence of the method of protein extraction on the *in-vitro* evaluation of mineral dialysability from legumes

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The aim of this study was to investigate to what extent the *in-vitro* evaluation of iron, zinc and protein dialysability from legumes is influenced by the method of globulins extraction (G1, G2). According to classical methods, globulins were first extracted into salt solutions containing ascorbic acid. Because of the enhancing effect of ascorbic acid on iron availability, globulins extraction was also carried out in the absence of ascorbic acid. In the presence of ascorbic acid, a parallel reduction in total protein content and in iron and zinc content was detected, suggesting an interaction between minerals and protein components. Ascorbic acid markedly improved protein dialysability and G2 always showed a higher dialysability than G1. In the absence of ascorbic acid, iron dialysability was 3·0% from G1 and 5·5% from G2, while ascorbic acid caused up to a three-fold increase in iron dialysability. Ascorbic acid did not influence zinc dialysability (20% for G1 and 24% for G2). For both globulins, the higher the iron/protein ratio the higher the iron dialysability, indicating that the extent of interaction between iron and protein, as well as influencing protein digestion, likely affects *in-vitro* iron availability.

INTRODUCTION

Several non-nutrients and nutrients in foods have been studied with regard to their effects on mineral availability. Among nutrients, food proteins have been proven to influence iron and zinc availability. Their effect has been found to depend on the source of protein: some proteins, such as those from animal tissue are effective in promoting absorption of both iron and zinc (Cook & Monsen, 1976; Solomons, 1982).

On the other hand, the influence of vegetable proteins on mineral absorption is quite controversial. Vegetable foods contain factors (phytic acid, dietary fibre, tannin), whose influence on mineral availability can hinder the understanding of the actual impact of the protein components (Cook *et al.*, 1981; Hallberg *et al.*, 1987; Sandstrom & Lonnerdal, 1981). Thus, as far as vegetable proteins are concerned, studies performed on protein extracts are very useful for elucidating the potential specific interactions between food components.

In evaluating the nutritional quality of food and diet, attention should be paid to the methodologies adopted in the extraction of the nutrients under study. When investigating the interactions between protein and mineral, the method utilized for protein extraction can greatly affect the subsequent results. Most vegetable proteins are usually fractionated on the basis of their different solubilities in water (albumin fraction) and salt solutions (globulin fractions). In the extraction of globulins from legume seeds, ascorbic acid has been suggested in place of HCl because of its antioxidant properties (Sun & Hall, 1975; Marquez & Lajolo, 1981). In studies on iron availability, however, the presence of ascorbic acid in protein extracts has to be considered, since it has been shown to selectively increase the *in vitro* dialysability/solubility and *in-vivo* availability of iron (Hallberg *et al.*, 1986; Hazell & Johnson, 1987).

The aim of this study was to investigate to what extent the presence of ascorbic acid in the method used for globulins extraction (G1, G2) from *Phaseolus vulgaris* affects *in-vitro* iron and zinc dialysability.

MATERIALS AND METHODS

The study was carried out on globulins (G1, G2) extracted from white bean (*Phaseolus vulgaris* L.). Total protein (N × 6·25) was determined by the method of the AOAC (1984). Total iron and zinc content was determined by Flame Atomic Absorption Spectrometry,

on a Varian SpectrAA 400. Certified standard BCR 189, wholemeal flour (Community Bureau of Reference, Brussels), was analysed as a check on the accuracy of the analysis. Experimental values of iron (69·3 ± 2·3 μg/g) and zinc (56·2 ± 1·5 μg/g) were not statistically different from certified values (68·3 ± 1·9 μg/g and 56·5 ± 1·7 μg/g, respectively). Deionized water was used throughout.

Protein extraction

Globulins (G1, G2) extraction was carried out in a NaCl (0·5 M) solution, as described by Marquez & Lajolo (1981). Two series of extractions were performed: in the presence of ascorbic acid (0·25 M, pH 2·2) (+Asc), as the outline of the method suggested, and in the absence of ascorbic acid (–Asc). In the latter case the pH was adjusted to 2·2 by the addition of HCl. Separation of G1 and G2 was achieved by lowering the ionic strength of NaCl to 0·08 M (Marquez & Lajolo, 1981). Protein fractions were freeze-dried before use in the subsequent analysis.

Dialysability

The *in-vitro* method of Miller *et al.* (1981) was adapted to assess iron and zinc dialysability from globulins (G1, G2). Aliquots of the samples containing about 1 g of protein were blended in 0·1 M HCl, the pH was adjusted to 2·0 ± 0·05 and 3 ml of a pepsin solution (16 g pepsin in 100 ml HCl) was added. The final volume of the homogenates was brought to 70 g by adding deionized water and the samples were incubated at 37°C for 2 h in a shaking water bath. Aliquots (20 g) of the pepsin digest were transferred into 100 ml beakers. Segments of dialysis sac previously treated with EDTA (MW cut-off 6-8000, Spectrapor 1, Spectrum Medical Industries Inc., Los Angeles, CA, USA) containing 0·5 M $NaHCO_3$ (in an amount previously determined in order to titrate a similar sample aliquot to pH 7·5 with 0·5 M KOH) and deionized water to obtain a volume of 20 ml, were placed in each beaker and incubated for 30 min. When the pH reached 5·0, 5 ml of a pancreatin–bile solution (0·8 g pancreatin, 5 g bile in 200 ml 0·1 M $NaHCO_3$) were added and the incubation continued for a further 2 h. The dialysates were weighed and the amount of iron was determined by bathophenanthroline (Miller *et al.*, 1981), while zinc was determined by Atomic Absorption Spectrometry. Mineral content of the dialysis bag was calculated as a percentage of the total. The dialysates were analysed also for protein content (Lowry *et al.*, 1951).

Data were statistically analysed by analysis of variance (ANOVA) and the differences between means were determined by the Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Protein and mineral extraction

Table 1 shows the total protein content and yield of globulin fractions (G1, G2) extracted in the presence and absence of ascorbic acid. When ascorbic acid was included in the fractionation method, a marked reduction in the total protein content of the extract was observed in both globulins. The reduction was higher in G2 than in G1. On the other hand, yield in protein extraction was impaired by ascorbic acid in the G2 fraction only, thus indicating that the decrease in total protein content of G1 was the result of the coextraction of non-protein material. In both the presence and absence of ascorbic acid, the percentage of extracted globulins (G1+G2) was close to about 30% of total protein. This value is lower than those previously reported (Marquez & Lajolo, 1981), possibly because of differences in the solubility properties of globulins among bean varieties. SDS–PAGE analysis (Lombardi-Boccia *et al.*, 1994), however, showed that the present G1 and G2 fractions were quite homogeneous when compared with the same fractions obtained from other varieties of *Phaseolus vulgaris* (Deshpande & Nielsen, 1987). On the basis of the apparent MW obtained from SDS–PAGE (Lombardi-Boccia *et al.*, 1994), the main protein components of G1 and G2 have been found to correspond to phaseolin and lectin, respectively (Chang & Satterlee, 1981; Sgarbieri & Whitaker, 1982).

Table 1. Total protein content and yield of globulins extracted with and without ascorbic acid[a]

	Protein (%)[b]	Yield (% total protein)
G1 (+Asc)	22·7 ± 5·7	24·0
G1 (–Asc)	45·7 ± 6·4	20·7
G2 (+Asc)	13·0 ± 5·0	6·9
G2 (–Asc)	42·1 ± 5·0	12·4

[a]Each value represents the mean ± SD of three determinations.
[b]Referred to extract, dry basis.

Table 2. Mineral's content, yield and mineral/protein ratio in globulins extracted with and without ascorbic acid

	Fe[a] (mg/100 g)	Yield (% tot Fe)	Fe/Protein (μg/g)	Zn[a] (mg/100 g)	Yield (% tot Zn)	Zn/Protein (μg/g)
G1 (+Asc)	3·30 ± 1·0	12·5	146	0·88 ± 0·2	6·0	38·9
G1 (–Asc)	4·68 ± 0·3	8·3	103	2·06 ± 0·4	6·7	45·2
G2 (+Asc)	2·76 ± 0·1	4·9	212	1·83 ± 0·2	5·2	141
G2 (–Asc)	6·78 ± 0·2	6·2	161	2·53 ± 0·6	4·2	60·2

[a]Each value represents the mean ± SD of three determinations.

Ascorbic acid was also found to affect the iron and zinc content of the G1 and G2 extracts (Table 2). The highest values were obtained when extractions were carried out in the absence of ascorbic acid. The similar trend shown by the values of protein and iron/zinc content might reflect an interaction between minerals and protein components. In all extractions, yield values for both iron and zinc indicated that it was possible to extract only a low percentage of these minerals from the bean flour. Such difficulty in extracting iron and zinc may be partly due to the native form (chemical state, complexation with endogenous seed compounds) of the minerals in the seed. Although the interactions between minerals and legume seed components have been only partially clarified, complexation with proteins has been proposed (Yoshida, 1988; Ikeda, 1990).

Ascorbic acid improved the yield of iron extraction from the G1 fraction. Even in the presence of ascorbic acid, however, not more than 12·5% of total iron was recovered (Table 2). It has been shown that ascorbic acid was very effective in mobilizing iron from bean flour. Indeed, some studies (Kojima *et al.*, 1981; Morr & Seo, 1986) reported that ascorbic acid could remove over 50% of the endogenous iron from legumes. Therefore, a higher amount of endogenous iron than that measured at the end of globulins fractionation was probably extracted by the ascorbic acid. The applied method of protein extraction (Marquez & Lajolo, 1981) may have caused a partial loss of the soluble iron complexes. Since the values of both yield and content of protein and iron showed a similar trend, most of the iron recovered in G1 and G2 was probably associated with protein components.

The presence of ascorbic acid resulted in an increase in the iron/protein ratio for both fractions, whereas the zinc/protein ratio was found to have increased in the G2 fraction only.

In-vitro protein and mineral dialysability

Ascorbic acid affected protein dialysability from globulins (Table 3). In the presence of ascorbic acid a two-fold increase in the G1 fraction ($P<0·01$) and a three-fold increase in the G2 fraction ($P<0·025$) were observed. In both the presence and absence of ascorbic acid, G2 showed a significantly higher protein dialysability than G1 ($P<0·01$ and $P<0·005$, respectively). This result is consistent with current findings showing the G1 fraction to be less digestible than the G2 fraction. The G1 fraction has been proven to be mainly constituted of phaseolin (Lombardi-Boccia *et al.*, 1994), a protein whose low digestibility is likely due to its compact structure (Ahn *et al.*, 1991).

Table 3. Protein and mineral dialysability from globulins extracted with and without ascorbic acid[a]

	Protein (%)	Fe (%)	Zn (%)
G1 (+Asc)	40·4 ± 6·0	6·44 ± 1·2	20·2 ± 0·8
G1 (–Asc)	20·4 ± 0·4	3·02 ± 0·7	20·3 ± 1·0
G2 (+Asc)	93·3 ± 6·1	16·7 ± 1·2	23·2 ± 1·0
G2 (–Asc)	31·0 ± 0·4	5·53 ± 0·9	23·9 ± 0·9

[a]Each value represents the mean ± SD of three determinations.

Also, iron dialysability from globulin fractions was strictly dependent on the extraction method and showed the same trend as that reported for protein dialysability: samples containing ascorbic acid showed the highest values of iron dialysability ($P<0·01$ for G1 and $P<0·005$ for G2). This result was expected because the ability of ascorbic acid to enhance non-heme iron availability has long been recognized (Cook & Monsen, 1977; Hallberg *et al.*, 1986). In this study it was found that the presence of ascorbic acid greatly changed the iron and protein contents of the extract, finally resulting in an increase in the iron/protein ratio. Not only did protein and iron dialysability show a similar trend, but the same trend was also followed by the iron/protein ratio of the extracts (Table 2). Indeed, the higher the iron/protein ratio, the higher was the dialysability of both iron and protein. These findings suggest that the extent of the interaction between iron and the protein components influences iron dialysability from globulin fractions. In a previous study (Lombardi-Boccia *et al.*, 1994) it was found that iron solubility from G1 and G2 fractions was less than 50% of the respective total iron content. Furthermore, the solubility of both iron and protein similarly increased during *in-vitro* digestion, suggesting that most of the iron was strictly associated with protein. However, iron dialysability was higher in the G2 than in the G1 fraction, irrespective of the presence of ascorbic acid ($P<0·005$ in both fractions). Thus, differences in the digestibility properties between G1 and G2 likely affect iron dialysability, too.

Zinc dialysability was unaffected by the presence of ascorbic acid. Quite high zinc dialysability values were found in both G1 and G2, only slightly lower than those reported for the whole seed (27%) (Lombardi-Boccia *et al.*, 1994). In spite of a higher Zn/protein ratio in the G2 fraction, in neither the presence or absence of ascorbic acid were significant differences in zinc dialysability evident between the two fractions.

Sandstrom *et al.* (1989) found a relatively high absorption of zinc (24·5%) from white bean. It is generally believed that both type and source of proteins strongly affect zinc absorption (Solomons, 1982). Indeed, zinc absorption from meal based on vegetable protein has been reported to be lower than that from meal based on animal protein. It was suggested that not only the type of protein but also the presence of some vegetable components, such as phytate, can adversely affect zinc absorption (Sandstrom & Lonnerdal, 1989). The isolated bean protein fractions, however, appear to be devoid of those bean constituents interfering with zinc dialysability (Lombardi-Boccia *et al.*, 1994). Unlike iron, high zinc dialysability seems also to be independent of both zinc content and protein availability of the fractions. While even a minor impairment in protein digestion can significantly change iron values, the behaviour of zinc seems not to be affected.

Actually, no difference was found in zinc dialysability between G1 and G2; yet they are known to contain proteins with different digestibilities (Marquez & Lajolo, 1991). Thus, the main determinant of zinc dialysability was probably the amino acid composition of these fractions. Cysteine and histidine have been shown to improve zinc absorption (Snedeker & Greger, 1983; Scholmerich *et al.*, 1987). The histidine residues of glycinine have been found to bind zinc, especially when glycinine is in the denatured state (Appu Rao & Narasinga Rao, 1976; Nosworthy & Caldwell, 1988). This might suggest that zinc interacts more specifically than iron with the protein component.

In conclusion, the methods adopted to investigate the relationship between protein and mineral have been proven to be of importance. The present findings indicate how the evaluation of iron dialysability can be affected by current methods of protein extraction. However, a comparison of the mineral and protein dialysability values obtained when different extraction methods are used might help to understand the role played by specific factors in the interaction between minerals and protein components.

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