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PII: S0308-8146(97)00244-6

Food Chemistry, Vol. 63, No. 1, pp. 1–7, 1998
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Printed in Great Britain
0308-8146/98 $19.00+0.00

The inhibitory effect of albumin extracts from white beans (*Phaseolus vulgaris* L.) on *in vitro* iron and zinc dialysability: role of phytic acid

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(Received 19 September 1997; revised version received and accepted 10 November 1997)

The role of phytic acid in determining iron and zinc dialysability in albumin extracts from raw, cooked and cooked/dephytinized white beans, has been investigated. Albumin extract from raw beans was characterized by high iron (19 mg/100 g), zinc (6.9 mg/100 g) and phytic acid (11.5 μmol g^{-1}) contents and a low mineral dialysablity (Fe 0.48%, Zn 2.5%). Cooking did not influence the mineral dialysability from beans but significantly increased the dialysability of iron (1%, $p < 0.001$) and zinc (5.3%, $p < 0.001$) from the albumin extract. Slight modifications in the composition of inositol phosphates after cooking, both in beans and in albumin extract, were observed. The improvement in iron and zinc dialysability from cooked/dephytinized samples was strictly dependent on the residual IP(6+5) (inositol hexa+pentaphosphates) content. Compared to cooked beans, in cooked/dephytinized bean a reduction of IP(6+5) of 49% led to an increase of the iron and zinc dialysability (29% $p < 0.05$ and 42%, $p < 0.001$, respectively). Albumin extract from this sample showed a reduction of 58% in iron and of 45% in zinc content, an almost complete reduction in IP(6+5) content (0.6 μmol g^{-1}) and a strong increase in the iron and zinc dialysability. The albumin digests showed peptides of similar MW profiles but of different amino acid compositions. In particular, in peptides which derived from digestion of albumin extracted from cooked/dephytinized beans, a strong increase in cysteine content was found, indicating that, after the disruption of phytate-mineral-protein complexes cysteine-rich fragments were released. The study indicates that phytic acid is responsible for the low iron and zinc dialysability from the albumin bean fraction and indicates the significance of the amino acid composition of the protein digestion products for the enhancement of mineral dialysability. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Vegetable proteins are largely utilized as ingredients in new formulated food products and are the main protein source in vegetarian diets. They are thought to negatively affect the absorption of iron and zinc (Cook *et al.*, 1981; Hallberg *et al.*, 1982; Derman *et al.*, 1987). However, the actual role of protein in influencing mineral availability has not yet been clarified.

When measuring the mineral availability from food, the influence of specific components, e.g. proteins, is difficult to assess because of the possible interactions with other food constituents. Among these, phytic acid (*myo*-inositol-hexaphosphate) is considered the main factor causing impaired absorption of minerals from cereals and legumes (Hallberg *et al.*, 1987; Sandstrom *et al.*, 1989; Lombardi-Boccia *et al.*, 1991). The ability of phytate to interact with proteins and minerals has long been recognized (O'Dell and de Boland, 1976; Okubo *et al.*, 1976; Honig and Wolf, 1991). These interactions have important nutritional consequences and may result in a reduced bioavailability of minerals as well as in a modification of plant protein properties after intestinal digestion (Lombardi-Boccia *et al.*, 1994).

Studies utilizing isolated protein fractions have been demonstrated to be useful for identifying specific protein interactions affecting mineral availability. The impaired iron absorption from isolated-soy-protein in humans (Hurrell *et al.*, 1992; Lynch *et al.*, 1994) was suggested to be due to both a protein-related moiety and to phytic acid. Previous *in vitro* studies carried out

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on protein fractions of *Phaseolus vulgaris* L. (Lombardi-Boccia *et al.*, 1994, 1996) evidenced a better iron and zinc dialysability from globulins compared to albumin. It is suggested that the low dialysability of iron and zinc from albumin derived mainly from its phytate content.

The present study was undertaken to clarify the role of phytate in albumin extracts from white bean (*Phaseolus vulgaris* L.) on the *in vitro* iron and zinc dialysability. A series of albumin extracts was prepared from raw, cooked and cooked/dephytinized bean flour. The *in vitro* mineral dialysability and inositol phosphates composition were determined. The profiles of the molecular weights of the peptides and their amino acid composition, after *in vitro* gastro-intestinal digestion of the protein extracts, were also determined.

MATERIALS AND METHODS

The study was carried out on white beans (*Phaseolus vulgaris* L.) and albumin extracted from the beans before and after cooking and enzymatic treatments.

Cooking procedure

Beans were soaked for 2 h in deionized water (1:3, w:v ratio) at room temperature, then cooked in the soaking water by autoclaving (125°C, 20 min) and then freeze-dried together with the cooking water.

Total Fe and Zn contents were determined by Atomic Absorption Spectrometry (Varian SpectrAA 400) under standard conditions and following liquid ashing (4 ml HNO_3 + 1 ml H_2O_2). Wholemeal flour (BCR 189, Community Bureau of Reference, Brussels), was analysed as a check on the accuracy of the analysis.

Total protein (N×6.25) content was determined by the AOAC method (AOAC, 1984). Deionized water was used throughout.

Dephytinization

Phytate was removed by enzyme treatment; cooked beans were incubated for 18 h at 37°C under constant shaking in 10 times their weight of deionized water containing 25 mg of wheat phytase (Sigma 0.04 U mg^{-1}) at pH 5.15. Samples were freeze-dried and kept at 20°C until further use.

Phytic acid

Inositol phosphates were analysed by reversed phase HPLC (Sandberg *et al.*, 1991) in duplicate.

Protein extraction

Albumin from samples of white bean (raw, cooked and cooked/dephytinized) was extracted for 1 h under agitation at room temperature from a 20% suspension of the flour in water, filtered and centrifuged at 30 000 g for 25 min. The supernatant was dialyzed for 48 h against deionized water at 4°C (Marquez and Lajolo, 1981). Albumin extracts were freeze-dried before use in the subsequent analysis.

In vitro dialysability

Determination of dialysable iron and zinc from bean was carried out by the *in vitro* method of Miller *et al.* (1981). With the albumin extracts minor modifications of the method were adopted (Lombardi-Boccia *et al.*, 1994). Aliquots of raw, cooked and cooked/dephytinized albumin extracts containing about 1 g of protein were blended in 0.1 N HCl, adjusted to pH 2.0 ± 0.05, and 3 ml of a pepsin solution (16 g pepsin-Porcine stomach, Sigma Chemical Co, St Louis, MO in 100 ml 0.1 N HCl) were 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 of the pepsin digest (20 g), were transferred into 100 ml beakers. Segments of dialysis sac (MW cut-off 6–8000 Spectrapor I, Spectrum Medical Industries Inc., Los Angeles), were pretreated with 1% EDTA solutions and rinsed until the dialysis sacs were free of EDTA. The dialysis sacs were filled with 0.5 N $NaHCO_3$ (in a volume tested in a previous trial to give a pH 7.5) and deionized water to obtain a volume of 20 ml, placed in beakers, and incubated for 30 min. When the pH reached 5.0, 5 ml of a pancreatin-bile solution (0.8 g pancreatin, Porcine pancreas, Sigma Chemical Co, St Louis, MO, and 5 g bile, Porcine, Sigma Chemical Co, St Louis, MO, in 200 ml 0.1 N $NaHCO_3$) were added and the incubation continued for a further 2 h. The dialysates were weighed and iron was determined by bathophenanthroline (Miller *et al.*, 1981). Zinc was determined by Atomic Absorption Spectrometry. Mineral content of each dialysis bag was calculated as a percentage of the total.

SE-high-performance liquid chromatography

Size-exclusion HPLC was performed on dialysates of beans and of the three protein fractions using a Waters instruments equipped with a 510 pump model. Absorbance (210 nm) was measured by an Absorbance detector, model 481, connected to a Millennium 2010. Separation was performed with a Waters Protein PaK 60 column of 300×7.8 mm (MW fractionation range 1000–20 000). The HPLC solvent system was 0.05 mol l^{-1} sodium phosphate-buffer, pH 6.5, containing 0.1 mol l^{-1} NaCl. Flux was 0.5 ml min^{-1}. Samples were filtered through a 0.45 μm fitter (Millipore Corporation, Bedford, MA) prior to injection. All reagents were of analytical grade.

The Standard proteins used were: soy trypsin inhibitor (MW 21 500), ribonuclease A (MW 13 700), cytochrome C (MW 12 300), insulin (MW 6000), baci racin (MW 1450), tryptophan (MW 204).

Amino acid composition

Albumin extracts and the respective dialysates were analysed for amino acid composition after 20 h hydrolysis in 6 N HCl, in vacuum, at 110°C. Hydrolysates were analysed with a Beckman 118BL amino acid analyzer (Beckman Instruments, Fullerton, CA), after reaction with ninhydrin (Moore *et al.*, 1958). Cysteine and methionine were determined as cysteic acid and methionine sulphone, respectively (Schram *et al.*, 1954).

The data were statistically evaluated by one-way analysis of variance (ANOVA) and the differences between means were determined by Duncan's multiple range test (Duncan, 1955).

RESULTS

White beans constitute a rich source of iron, zinc and protein (Table 1). The same is true for its aqueous albumin extract (Table 1) and is in agreement with earlier results (Lombardi-Boccia *et al.*, 1994). Cooking neither affected the mineral nor the protein content of beans. The albumin extracts from cooked bean flour, however, showed a three-fold reduction ($p < 0.001$) in the total protein content with respect to the raw extract (Table 1). This probably is due to lower protein solubility subsequent to the cooking procedure (Gujska and Khan, 1991; Petruccelli and Anión, 1995). Dephytinization of the cooked bean did not significantly change the amounts of minerals or proteins (Table 1). The albumin extract from cooked/dephytinized bean, however, showed a drastic reduction in iron (58.4%, $p < 0.001$), zinc (45%, $p < 0.001$) and protein content (29%, $p < 0.05$) (Table 1) with respect to the sample extracted from cooked beans.

The content of phytic acid in raw white beans was high (12.4 μmol g^{-1}). This is the same for the bean albumin extract (11.5 μmol g^{-1}) (Table 2). Since phytic acid was not dialysable during the extraction of albumin, it was probably complexed with proteins. Also, the high contents of iron and zinc associated with albumin indicate that these minerals might be involved in salt bridges between protein and phytic acid, both negatively charged at neutral pH (Cheryan, 1980). The reduced amount of iron and zinc found in albumin extracts from cooked/dephytinized beans (Table 1) was thus a consequence of the disruption of the ternary complexes subsequent to dephytinization. Dephytinization also lowered the protein content in the albumin extracts (Table 1). This might be due to a release, during albumin extraction, of smaller, dialysable peptides bound to the albumin matrix via phytic acid. Whole white beans, in contrast to the albumin extracts, had an intact cell wall structure, so that neither cooking nor dephytinization could destroy the ternary complex and metals or peptides could not be released.

In cooked white beans a variation in the inositol phosphate composition was observed (Table 2). The inositol hexaphosphate (IP6) content was reduced and the content of IP_5, IP_4 and IP_3 increased correspondingly. In respect of the inhibition of the dialysability of iron and zinc not only the concentration of IP_6 is significant. IP_5 also reduces the bioavailability of both elements (Lonnerdal *et al.*, 1989; Sandberg *et al.*, 1991) while IP4 and IP3 do not (Sandberg *et al.*, 1989). Comparing only the IP6 content of raw and cooked beans, the decrease induced by cooking was 20%; however, in cooked beans, because of the increase in the IP5 content, the amount of IP(6 + 5) was only only 5% less

Table 2. Inositol phosphates content of bean and albumin extracts (dry weight basis)

Samples	Inositol phosphates (μmol g^{-1})[a]				
	IP6	IP5	IP4	IP3	IP(6+5)
Bean					
raw	12.4	1.7	0.7	0.3	14.1
cooked	9.9	3.5	1.0	0.9	13.4
cooked/dephytinized	3.2	3.6	5.0	3.0	6.9
Albumin					
raw	11.5	1.9	0.8	0.3	13.8
cooked	10.9	3.5	1.8	0.6	14.4
cooked/dephytinized	0.6	n.d.	n.d.	0.8	0.6

Abbreviations used: IP, inositol phosphate; IP6, inositol hexaphosphate; IP5, inositol pentaphosphate; IP4, inositol tetraphosphate; IP3, inositol triphosphate.
[a]Determined by reversed phase HPLC (Sandberg *et al.*, 1989)
Mean values ± standard deviations of duplicates.
nd: not detectable.

Table 1. Iron, zinc and protein contents of bean and the respective albumin extracts

	Fe mg/100 g	Zn mg/100 g	Protein g/100 g
Bean			
raw	5.42 ± 0.2	2.86 ± 0.4	24.67 ± 0.2
cooked	5.55 ± 0.1	3.00 ± 0.3	24.35 ± 0.4
cooked/dephytinized	5.69 ± 0.3	2.90 ± 0.5	25.12 ± 0.3
Albumin			
raw	19.00 ± 0.2[a]	6.93 + 0.4[a]	77.79 ± 0.3[a]
cooked	17.58 ± 0.2[a]	6.05 ± 0.6[a]	24.44 ± 0.5[b]
cooked/dephytinized	7.31 ± 0.4[b]	3.30 ± 0.8[b]	17.28 ± 0.4[bc]

Each value represents the Mean ± SD of triplicates.
Values within the same column followed by different letters are significantly different: ([a] vs [b] and [c], $p < 0.001$; [b] vs [c], $p < 0.05$).

than that of the raw sample. Thus the IP6 content alone does not give realistic information on the inhibition of iron and zinc from the different samples. Therefore HPLC analysis of the inositol phosphates is of nutritional importance. A similar trend was found in albumin extracts from raw and cooked beans which did not show differences in the IP(6 + 5) content.

The incubation of cooked bean flour with wheat phytase resulted in a reduction of IP6 content of 68%, and in a parallel increase in IP4 and IP3 contents (Table 2). With respect to cooked beans, the IP(6 + 5) content of the dephytinized beans was reduced by 48%. Albumin extracted from this sample showed a very low amount of inositol phosphates, with IP6 and IP5 almost completely hydrolysed.

It should be emphasized that, in both, raw and cooked samples, IP(6 + 5) constitutes the major fraction of total phosphates. A marked decrease in IP(6 + 5) content was observed only after the enzymatic treatment of bean flour.

IN VITRO MINERAL DIALYSABILITY

Iron and zinc dialysability from beans and albumin (Table 3) was in the range already reported for this species by *in vivo* and *in vitro* studies (Lynch *et al.*, 1984; Sandstrom *et al.*, 1989; Lombardi-Boccia *et al.*, 1995). Albumin showed a small percentage of iron and zinc dialysability, especially when compared with the mineral dialysability of other bean protein fractions (Lombardi-Boccia *et al.*, 1994). In albumin the high amount of minerals and phytic acid and the parallel low iron and zinc dialysability, substantiated the hypothesis of the presence of protein-mineral-phytate complexes.

Iron and zinc dialysability from cooked white bean did not differ with respect to the raw bean. As mentioned above, in this sample a marked variation in inositol phosphates (IPs) composition was observed (Table 2); in particular the increase in IP5 content resulted in an IP(6 + 5) content of 13.4 μmol g^{-1}, with a decrease of only 5% with respect to the IP(6 + 5) content of raw bean. This decrease was too low to cause an increase in mineral dialysability. The finding that IP(6 + 5) had a negative effect on iron and zinc availability is consistent with results deriving from *in vivo* and *in vitro* studies (Sandberg *et al.*, 1989, 1991; Lonnerdal *et al.*, 1989). Albumin deriving from cooked beans showed a significant increase in both iron and zinc dialysability ($p < 0.001$ for both); this finding might be partially explained by the presence of inositols mono- and diphosphates enhancing mineral dialysability.

Compared to cooked beans, the phytase-treated white bean flour showed increases in iron and zinc dialysability of 29% ($p < 0.05$) and 42% ($p < 0.001$), respectively. In this sample the decrease in IP(6 + 5) was 49%, thus, in bean, the increase in mineral dialysability was possible only after a drastic change in IPs composition. Studies have demonstrated that degradation of phytic acid subsequent to food processing leads to a strong increase in iron absorption (Sandberg *et al.*, 1989; Sandberg and Svanberg, 1991). Moreover, it is known that the amount of IP(6 + 5) must decline to a level lower than 0.5 μmol g^{-1} in order to eliminate any inhibitory effect on iron availability (Sandberg and Svanberg, 1991). In the albumin extract from cooked/dephytinized beans, in fact, with an almost complete IP(6 + 5) reduction (0.6 μmol g^{-1}), a five-fold increase in iron dialysability and an about four-fold increase in zinc dialysability ($p < 0.001$ for both) were detected (Table 3).

Table 3. *In vitro* iron and zinc dialysability from white bean and the respective albumin extracts

	Fe %	Zn %
Bean		
raw	3.4 ± 0.3^a	27.5 ± 0.2^a
cooked	3.8 ± 0.2^a	28.9 ± 0.1^a
cooked/dephytinized	4.9 ± 0.7^d	41.0 ± 0.6^b
Albumin		
raw	0.48 ± 0.2^a	2.50 ± 0.3^a
cooked	1.00 ± 0.1^b	5.31 ± 0.6^b
cooked/dephytinized	5.00 ± 0.6^c	23.0 ± 0.6^c

Each value represents the Mean ± SD of triplicates.
Values within the same column followed by different letters are significantly different: (^{a} vs ^{b} and ^{c} $p < 0.001$; ^{a} vs ^{d} $p < 0.05$; ^{b} vs ^{c} $p < 0.001$).

SE-HPLC ANALYSIS OF PAPTIDES IN DIALYSATES

Figure 1 shows the size-exclusion HPLC analysis performed on the peptides released during the *in vitro* gastro-intestinal digestion of cooked and cooked/dephytinized white beans and the respective albumin extracts. The distribution of the molecular weights of the digestion products of cooked and cooked/dephytinized beans showed a range of molecular weights from a maximum of 13 300 to about 200. In dephytinized beans, however, 12 species instead of 10 in the cooked bean were found; the new peptides found in cooked/dephytinized white bean were in the low molecular weight region. In albumin extracted either from cooked beans or from cooked/dephytinized beans, HPLC profiles, showed molecular weights between 12 800 and 190.

In all the samples analysed the peptides with molecular weights lower than the cut-off of the dialysis membrane (MW6-8000) were identical, while the components with molecular weights higher than the cut-off of the dialysis membrane differed among samples. This finding was indicative of protein aggregations occurring in dialysis sacs during digestion and minerals may have been responsible for holding some peptides together.

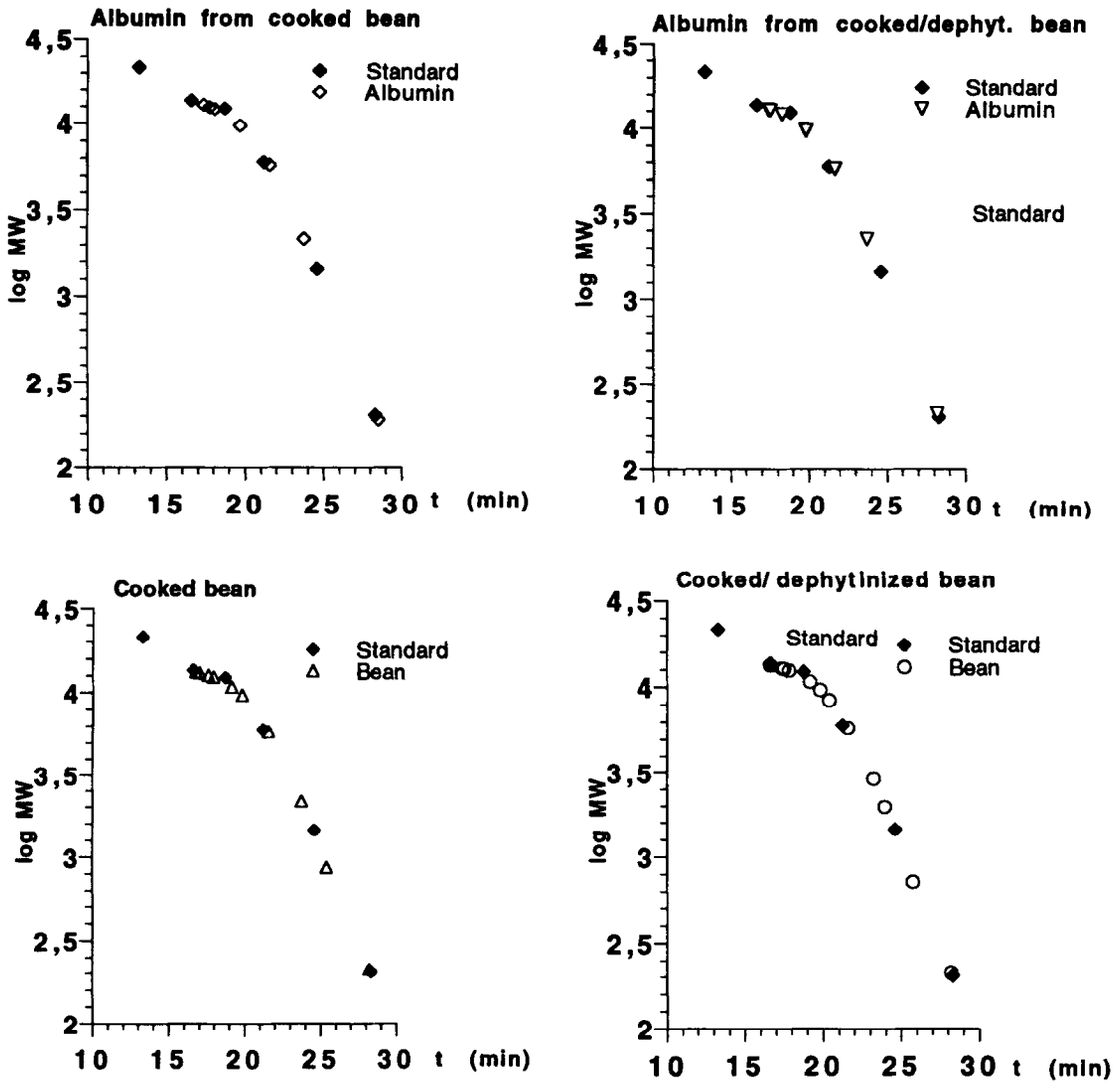

Fig. 1. HPLC separation of peptides dialysed from cooked, cooked/dephytinized bean and from the respective albumin extracts. Separation was performed with a Waters Protein PaK 60 column of 300×7.8 mm (MW fractionation range 1000–20 000). The solvent system was 0.05 mol l^{-1}. Sodium phosphate-buffer, pH6.5, containing 0.1 mol l^{-1} NaCl. Flux at 0.5 ml min^{-1}, absorbance 210 nm. Standards: Soy trypsin inhibitor (MW 21 500), Ribonuclease A (MW 13 700), Cytochrome C (MW 12 300), Insulin (MW 6000), Bacitracin (MW 1450), Tryptophan (MW 204).

AMINO ACID COMPOSITION OF PEPTIDES

Since the products of protein digestion might be responsible for mineral binding and solubilization, the amino acid contents (g/16 g N) of albumin extracts and of peptides deriving from the *in vitro* digestion of albumin samples were analysed (Table 4).

The main differences in amino acid composition among the albumin extracts concerned lysine, histidine, alanine, glutamic acid, glycine, arginine and sulphur amino acids. In particular, the total amounts of sulphur amino acids increased significanly after dephytinization (50%, $p < 0.01$ for methionine; 56, $p < 0.001$% for cysteine). The amino acid composition of the peptides dialysed during the *in vitro* digestion of albumin extracted from cooked bean showed a marked increase in dialysed lysine (47%), glycine (40%), glutamic acid (38%) and alanine (26%) with respect to the raw extract, changes probably consequent to the changes in protein solubility observed upon the cysteine cooking. After dephytinization the only significant change observed concerned the cysteine content: this amion acid, stable in dialysates of both raw and cooked albumin extracts, increased up to 41% ($p < 0.01$). This was probably due to the disruption of the ternary complexes which allowed a higher extraction of cysteine-containing peptides. It is noticeable that the strong increase in total methionine and cysteine content of albumin extracted from cooked/dephytinized bean flour, was actually an increase in dialysed cysteine only. This finding also supports conclusions from an earlier study (Lombardi-Boccia *et al.*, 1994, 1996) showing that globulins, the bean protein fraction with the highest iron dialysability, also had the highest amount of dialysed cysteine.

Table 4. Amino acids in albumin extracts and in peptides dialysed after *in vitro* digestion of albumin extracts (g×16 gN)

	Albumin					
	Raw		Cooked		Cooked/dephytin.	
	Total	Dialysed	Total	Dialysed	Total	Dialysed
Lysine	7.02	5.68	9.85	8.43	8.28	8.61
Histidine	2.57	1.88	3.76	1.94	3.67	2.13
Arginine	5.36	5.61	5.51	6.61	7.96	5.51
Aspartic acid	12.20	9.86	13.27	11.44	12.85	12.13
Threonine	5.29	4.25	6.57	5.05	5.95	6.00
Serine	6.45	5.17	5.57	6.03	4.67	6.05
Glutamic acid	13.65	11.15	17.81	15.38	17.37	15.14
Proline	3.55	2.75	2.80	2.72	2.95	2.42
Glycine	4.86	7.79	6.13	10.94	7.38	11.82
Alanine	5.22	4.97	5.98	6.29	8.10	6.95
Half cystine	1.55	0.77	1.03	0.83	1.61	1.17
Valine	5.33	4.79	4.06	4.34	3.74	4.53
Methionine	1.17	1.29	1.10	1.00	1.49	0.88
Isoleucine	4.41	3.59	2.73	3.30	2.10	3.16
Leucine	8.15	8.42	4.71	6.09	3.64	4.70
Tyrosine	4.00	4.52	2.60	3.27	2.91	3.13
Phenylalanine	5.86	4.33	3.68	3.74	3.27	3.21

Each value represents the mean of three determinations (variability coefficient < 7%).

DISCUSSION

Studies on the nature of the inhibitory effect of vegetable proteins on mineral absorption are still contradictory (Cook *et al.*, 1981; Hallberg *et al.*, 1982; Hurrell *et al.*, 1992). Recent *in vivo* and *in vitro* studies carried out on soy-protein isolates (Lynch *et al.*, 1994) and on bean-protein fractions (Lombardi-Boccia *et al.*, 1994, 1996) regard phytic acid and protein itself as the two major factors responsible for iron and zinc availability.

Present findings strongly suggest that, in albumin, the extent of the interaction between endogenous constituents greatly influences iron and zinc dialysability. Indeed, the contemporary presence, in albumin, of high amounts of phytic acid and minerals — both not dialysable during albumin isolation — indicate that these constituents are bound to protein in phytate-protein or phytate-mineral-protein complexes. Moreover, the lowered ability to extract both mineral and inositol phosphates, together with albumin, after dephytinization of the bean flour, substantiates the presence of ternary complexes in albumin.

Experiments carried out on cooked and cooked/dephytinized samples further suggest a close relationship between inositol phosphates composition and mineral dialysability. Both in beans and in albumin the improvement in mineral dialysability was strictly dependent on the residual IP(6 + 5) content of the samples. The strongest effect was observed in albumin extracted from phytase-treated bean (five-fold increase in iron dialysability and four-fold increase in zinc dialysability) showing an IP(6 + 5) content of 0.6 μmol g^{-1}. This finding is consistent with results of Sandberg and Svanberg (1991) who found a meaningful increase in iron dialysability only after a decrease in IP(6 + 5) below 0.5 μmol g^{-1}.

Even if albumin digests did not show differences in MW profile of the dialysed peptides, the analysis of the amino acids content indicated some differences in the compositions of the protein fragments. In particular, after bean dephytinization, the strong increase in cysteine content found in albumin digests indicated that the disruption of phytate-mineral-protein complexes released cysteine-rich fragments and that cysteine was the amino acid much involved in promoting mineral dialysability. These results are consistent with earlier *in vivo* studies (Layrisse *et al.*, 1984; Taylor *et al.*, 1986), suggesting an involvment of cysteine-containing peptides in enhancing iron absorption.

In conclusion, our findings give evidence that phytic acid in the albumin fraction is the major factor inhibiting the dialysability of iron and zinc. The enhancement of mineral dialysability depends on the amino acid composition of the peptides released during food digestion.

ACKNOWLEDGEMENT

The study was supported by the National Research Council of Italy, Special Project RAISA, Subproject 4, No. 2682.

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