ELSEVIER

PII: S0308-8146(97)00040-X

Food Chemistry, Vol. 61, No. 1/2, pp. 167–172, 1998

0308-8146/98 $19.00 + 0.00

Dialysable, soluble and fermentable calcium from beans (*Phaseolus vulgaris* L.) as model for *in vitro* assessment of the potential calcium availability

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(Received 29 November 1996; accepted 9 December 1996)

Calcium potential availability from mottled and white bean was studied by using an *in vitro* model which simulates the conditions existing in the small intestine and the fermentation process in the colon. Beans contained high amounts of calcium which distribution in the seed varied greatly (hull 70%, cotyledon 30%). The percentage of calcium dialysability in both bean varieties was in the range of 14%. Dehulling greatly improved calcium dialysability; when compared to whole seed, increases of up to 97% ($p < 0.01$) were found. Calcium dialysability from the hull was very low (4–8%). Dialysable calcium and soluble calcium were not affected by cooking. Results indicated that phytate was not the main inhibitor of calcium dialysability from beans. The overall calcium dialysability from beans was mainly influenced by the calcium status in the hull. Calcium released from retentate (from 24 to 72%), during *in vitro* fermentation experiments indicated a substantial degradation of the undialysable High Molecular Weight (HMW) calcium complexes within the 14 h of the fermentation process.

INTRODUCTION

Beans contain high amounts of calcium and could substantially contribute to an adequate calcium intake in our diets. However, the presence of certain components of vegetable foods which can hinder the absorption of calcium is a matter of concern. Only two plant components, oxalate and phytate, have so far been regarded as the main complexing agents interfering with calcium absorption from beans (Weaver *et al.*, 1993; Heaney *et al.*, 1991). Some studies have shown that calcium absorption was greater from low-oxalate food, such as kale, than from high-oxalate food, such as spinach or broccoli (Weaver *et al.*, 1987; Heaney and Weaver, 1990; Heaney *et al.*, 1993). Phytate has also been shown to interfere with the absorption of calcium (Lonnerdal *et al.*, 1989; Heaney *et al.*, 1991), and calcium itself increases the severity of phytate impact by forming insoluble complexes with other minerals (Ellis *et al.*, 1987; Hallberg *et al.*, 1991).

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On the other hand, the impact of dietary fibre on calcium bioavailability is still debated. Results from *in vivo* studies on the effect exerted by high fibre diets on calcium absorption are not consistent (Kelsay *et al.*, 1979; Wisker *et al.*, 1991). James *et al.* (1978) reported that calcium binding by dietary fibre was highly correlated to the uronic acid residues of pectins and there is little evidence of the binding of calcium to other fibre constituents (Behall *et al.*, 1987; Rossander, 1992).

The contribution of microbial fermentation to the overall calcium absorption is a variable, and this has to be considered when studying calcium availability. Studies have shown that the ideal balance does not reflect the total mineral turnover and that the caecal–colon region takes part in calcium absorption (James *et al.*, 1978; Larsen and Sandstrom, 1983; Sandstrom *et al.*, 1986; Younes *et al.*, 1996).

Few data are available on calcium absorption from legumes, and these data usually result from *in vivo* studies. A more complex task is studying, *in vitro*, the mechanisms which affect calcium availability. There is at present, no *in vitro* model which accounts both for the calcium absorbable in the small intestine and the

calcium fraction which appears after fermentation in the colon. Trinidad *et al.* (1996) recently proposed an *in vitro* model which simulates the conditions existing in the small intestine and the colon in order to estimate calcium availability from a basal diet enriched with available and unavailable carbohydrates.

This study aimed to determine the *in vitro* potential availability of calcium from bean (*Phaseolus vulgaris*), and to assess the influence of cooking on calcium availability. *In vitro* dialysability and solubility of calcium from bean were used as indicators of potential calcium availability. In order to investigate the influence of calcium distribution in the seed structure on calcium potential availability, the evaluations were carried out both on whole seeds and seed fractions (cotyledon, hull).

In order to achieve further information on the overall *in vitro* calcium availability, the digests (only the retentates) were subject to a simulated process of colon fermentation after the gastro-intestinal digestion, and the amount of calcium released from digests was then evaluated.

MATERIALS AND METHODS

The study was carried out on samples of two varieties of *Phaseolus vulgaris* (mottled and white bean). Experiments on raw beans were conducted on whole seed and seed fractions (cotyledon, hull). Experiments on cooked beans were conducted on whole seed and cotyledon only.

All reagents were analytical grade and deionized water was used throughout. Glassware was acid washed in concentrated HCI and rinsed with deionized water.

Cooking procedure: The samples were soaked for 6 h in deionized water (1:3, w:v ratio) at room temperature, then cooked by autoclaving (125°C, 20 min) and freeze-dried together with their cooking water.

Calcium analysis: This was performed by Atomic Absorption Spectrometry on a Varian SpectrAA 400. A nitrous oxide-acetylene flame was used in order to avoid the formation of refractory compounds, and 0.2% caesium chloride was added to samples to suppress ionization. The certified Standard NBS 1567a, wheat flour (National Bureau of Standards, Gaterborough, MD 20899) was analysed as a check on the accuracy of the analysis. Experimental values were not statistically different from certified values (19.2 ± 0.05 mg $100\,g^{-1}$; 19.1 ± 0.4 mg $100\,g^{-1}$, respectively).

Phytic acid: This was determined by the method of Harland and Oberleas (1986).

Dialysable calcium: This was assessed by using the *in vitro* method of Miller *et al.* (1981). Aliquots of each sample (containing about 10 mg of calcium) were blended with 0.1 N HCL, the pH was adjusted to 2.0 ± 0.05, and 5 ml of pepsin solution (10 g pepsin in 100 ml 0.1 N HCl) were added. The final volume of the homogenates was brought to 100 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 digests were transferred into 100 ml beakers. Segments of dialysis sac (MW cut-off 6–8000 Spectrapor 1, Spectrum Medical Industries Inc., Los Angeles), containing 0.5 N $NaHC0_3$ (an amount previously determined to titrate a same sample aliquot to pH 7.5 with 0.5 M KOH) and sufficient deionized water to obtain the 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 N $NaHCO_3$) were added and the incubation continued for a further 2 h. The dialysates were weighed and the amount of calcium in the dialysis bag was determined by Atomic Absorption Spectrometry. Retentates were frozen for later use.

Soluble calcium: This was determined in the retentates deriving from the gastro-intestinal digestion. At the end of the simulated gastro-intestinal digestion the retentates were centrifuged at $3\,500 \times \boldsymbol{g}$ for 20 min. Supernatants were weighed and analysed for calcium content by Atomic Absorption Spectrometry. Soluble calcium was expressed as a percentage of total calcium.

In vitro fermentation: Retenates deriving from the *in vitro* gastro-intestinal digestion of white bean were thawed and fermented *in vitro* by using human fecal inoculum. Fecal samples were from a healthy male whose diet had been rich in vegetables. The subject was not affected by gastro-intestinal diseases, and had received no drug therapy in the two months preceding the study. *In vitro* fermentation experiments were carried out in complete anaerobiosis in order to reproduce the anoxic conditions of the large bowel. All manipulations were performed inside an anaerobic glove cabinet (Forma Scientific mod. 1025, Marietta, OH), in an atmosphere of 85% N2, 10% H2 and 5% CO2 at very low O2 concentration (O2 < 10 ppm) (Brusa and Ferrari, 1985). All employed solutions were pre-reduced in the same cabinet for 48 h until use. Stool specimens were collected in sterile contaners, introduced in the anaerobic cabinet no later than 1 h after sampling, suspended (10%) in dilution blank (Holdeman *et al.*, 1977) and homogenized with glass balls. Fecal suspensions were inoculated at 10% rate in Erlenmeyer flasks containing 30 ml of retentates, which were then placed in airtight Gas-Paks (Oxoid, Unipath Ltd, Basingstoke, UK) and closed in the anaerobic cabinet. The gas-Paks were incubated at 37°C on an alternative agitator. Blanks, whitout substrate, were incubated under the same conditions. Fermentation lasted 14 h and samples were taken after 4, 8 and 14 h of incubation. At the end of each incubation time, the pH was registered and samples were centrifuged at $3500 \times \boldsymbol{g}$ for 20 min at 4°C. Supernatants were weighed and analysed for calcium content by Atomic Absorption Spectrometry. The percentage of calcium released was calculated by dividing the calcium content in supernatant by the calcium in the sample $\times 100$.

Table 1. Total dialysable and soluble calcium from raw and cooked beans and seed functions

		Total Ca (mg 100 $^{-1}$)	Raw		Cooked	
			Dialysable (%)	Soluble (%)	Dialysable (%)	Soluble (%)
Mottled bean	Whole seed	133 ± 0.9	13.8 ± 1.9a	13.7 ± 2.1a	15.2 ± 0.9a	14.1 ± 1.2a
	Cotyledon	44.5 ± 0.6	24.2 ± 1.6b	41.1 ± 2.5b	27.8 ± 0.9b	37.9 ± 1.9b
	Hull	808 ± 1.8	8.7 ± 2.6c	14.5 ± 2.6a	nd	nd
White bean	Whole seed	90.5 ± 0.9	14.3 ± 1.4a	13.1 ± 2.0a	14.8 ± 0.6a	14.3 ± 1.8a
	Cotyledon	26.3 ± 0.7	28.2 ± 1.4b	37.3 ± 2.6b	33.1 ± 1.2b	40.2 ± 1.1c
	Hull	967 ± 1.7	4.3 ± 2.9c	13.3 ± 2.5a	nd	nd

Each value represents the Mean ± SD of five determinations. nd = not determined. For each variety, values within the same column with different letters are significantly different (a vs b, $p < 0.01$; a vs c and b vs c, $p < 0.001$).

Data were subject to analysis of variance (ANOVA). The differences between means were determined by the Duncan's Multiple and range test (Duncan, 1955).

RESULTS AND DISCUSSION

Mottled and white bean differed for total calcium content which was higher in the mottled variety (Table 1); as concerns calcium distribution between the seed fractions, the hull was particularly rich in calcium, accounting for about 70% of the total seed calcium content.

The percentage of dialysable calcium in both bean varieties was about 14% (Table 1). Dehulling greatly improved calcium dialysability: when compared to whole seed values, increases of up to 97% ($p < 0.01$) were found in cotyledon of both bean varieties. Calcium dialysability from the hull was very low, white bean hull showing the lowest value.

In whole seeds of both bean varieties, the percentages of dialysable and soluble calcium were similar. In both bean varieties, dehulling also increased the amount of soluble calcium: when compared to whole seed, a threefold increase ($p < 0.01$) was found in the retentates deriving from the *in vitro* digestion of cotyledon.

Cooking did not affect dialysable or soluble calcium (Table 1). Showing a similar pattern to that found in raw beans, calcium dialysability and solubility were significantly higher in cotyledon than in whole seed ($p < 0.01$ and $p < 0.001$, respectively). The similarity in behaviour of calcium dialysability in white and coloured beans indicated that tannins (which were present only in the coloured bean) did not bind calcium in insoluble and unavailable complexes, as reported also by Proulx *et al.* (1993).

Phytic acid in bean is regarded as the major inhibitor of calcium absorption (Lonnerdal *et al.*, 1989; Heaney *et al.*, 1991; Proulx *et al.*, 1993). The effect of phytate on calcium was examined in our previous study demonstrating that dephytinization significantly increased calcium dialysability from beans (Carnovale *et al.*, 1996). Bean had a high phytate content which was mainly located in the cotyledon (Table 2). Small reductions in total phytate content were detected after cooking (up to 22% in whole bean, up to 7% in cotyledon). It must be taken into account that the method employed for phytate determination does not discriminate between phytic acid and the inositol phosphates with a lower number of phosphate groups which might originate upon cooking. Some of these may positively influence calcium availability (Lonnerdal *et al.*, 1989). A previous study (Lombardi-Boccia *et al.*, 1995) reported a similar decrease in total phytic acid content upon cooking. However, as far as inositol phosphates are concerned (Lombardi-Boccia *et al.*, submitted), cooking induced a decrease in inositol hexaphosphate and an increase in the other IPs. In particular, because of the increase in IP5, the reduction in IP(6 + 5) was rather small and did not allow an increase in mineral dialysability. These findings could explain why no significant increases in calcium dialysability upon cooking were detected in the present study.

When comparing calcium dialysability between the hull and cotyledon, the latter seed fraction contained almost all the phytate content of the bean, which showed that it was not phytate which exerted the main inhibitory action on calcium dialysability. Our results are consisitent with those reported by Weaver *et al.* (1993), who suggested the presence of an inhibitor different from phytate, presumably located in the coat of common bean. Calcium dialysability from whole seed was thus largely determined by the hull fraction which contains endogenous constituents such as oxalic acid and dietary fibre components, both known to adversely affect calcium absorption (Weaver *et al.*, 1987; Persson *et al.*, 1991).

The higher calcium dialysability and solubility showed by the cotyledon in comparison to whole seed might thus depend on the removal, together with the hull, of firmly bound calcium not easily available in the upper intestine. The great difference in calcium dialysability between the seed fractions suggests that calcium localization in the seed did affect the overall calcium availability from the seed. This may thus be predictive of calcium potential availability.

Interactions between calcium ions and pectins in cell walls play a key role in stabilizing the wall structure

Table 2. Phytic acid content of raw and cooked beans and seed fractions (%)

		Phytic acid	
		Raw	Cooked
Mottled bean	Whole seed	1.10	0.86
	Cotyledon	1.18	1.10
	Hull	0.06	—
White bean	Whole seed	0.81	0.69
	Cotyledon	0.83	0.81
	Hull	0.04	—

Each value represents the Mean ± SD of five determinations.

Table 3. Percentage of calcium released after *in vitro* fermentation of retentate deriving from *in-vitro* digestion of white bean

	4 h	8 h	14 h
White bean	31 (24.2–39.9)	53 (44–75.4)	61·9 (54.8–72.3)

Ten replicates for each time of fermentation.

(Demarty *et al.*, 1984; Selvendran *et al.*, 1987). Differences in calcium dialysability between the hull and cotyledon could find an explanation in the different structures of the cell walls of the two fractions. Cotyledons consist of thin-walled parenchyma tissue in which pectins are the main component. Pectin chains are linked by calcium ionic bonds through the non-methylate free carboxylic acid groups of uronic acids (James *et al.*, 1978; Selvendran *et al.*, 1987). Cotyledons are surrounded by hulls which are specialized cell layers, the pericarp and the testa, which can contain thickened, lignified and suberised cell walls. Calcium is bound in this structure by ionic bonds between pectin chains and by covalent bonds to ferulic acid (McDougall *et al.*, 1996). The mild conditions used in the cooking process did not lead to pectin degradation (Lintas *et al.*, 1995). Depolymeration of pectin with solubilization and loss of calcium occurs only after drastic processing conditions (Amadò, 1994; Carnovale and Lintas, 1995).

The presence in the seed fractions of a greater amount of soluble calcium, in proportion to dialysable calcium, indicated that not all soluble complexes containing calcium were dialysable under the digestion conditions employed in the present study. A portion of soluble calcium was likely bound to substances of MW > 8000, which are unable to cross the dialysis membrane. HMW calcium complexes could thus be degraded in the colon and calcium could become available for absorption.

In vivo studies (Favus *et al.*, 1980; Nyman & Asp, 1988) reported that calcium bound to indigestible components was released into the large intestine, where the fibre was degraded by colonic bacteria. Both dietary fibre components and other substances, such as phytate, which are largely indigestible in the stomach and small intestine, might be degraded in the colon and a portion of the bound calcium might still be absorbable.

Results from *in vitro* fermentation experiments are shown in Table 3. As fermentation was protracted, pH decreased from 6 to 4 corresponding to a production of short-chain fatty acids and the amount of released calcium increased from a minimum value of 24.2% to a maximum of about 72.3% of the total calcium. Since no significant differences were observed in the results obtained after 8 and 14 h, most of the bound calcium in the digests was likely released within 8 h of the beginning of the fermentation process.

Few literature data are available on *in vitro* measurements of calcium in bean. An *in vivo* study (Heaney *et al.*, 1991) reported a value of 21.9% for calcium absorption from soybean. Weaver *et al.* (1993) found (for three varieties of bean) a fractional calcium absorption ranging from 19.3 to 23.1%. Absorbable calcium in several plant foods, calculated on the calcium load of a typical serving size (Weaver and Plawecki, 1994), is estimated as 17% fractional absorption for beans. When studying the *in vitro* fermentation of a basal diet enriched with some fibre sources, Trinidad *et al.* (1996) found a 17–44% calcium release, depending on the different carbohydrate fermentabilities. Sandstrom *et al.* (1986) instilled ^{47}Ca solution into the colon, and estimated a 14.1% mean calcium absorption.

Although the *in vitro* fermentation could not provide a quantitative assessment of colonic calcium absorption, the high amount of calcium which was released from the retentates in the present study indicated that a substantial digestion of fibre components occurred during *in vitro* fermentation of bean. Thus the calcium bound in complexes unabsorbable in the upper intestine (e.g. Ca-pectate, Ca-phytate) shifts the release of calcium, and hence its potential absorption, to the colon.

Most of the insoluble complexes containing calcium were thus broken down during the *in vitro* fermentation, and potentially absorbable calcium was released. Microbial fermentation thus most significantly contributes to calcium solubilization, even if the overall calcium absorption largely depends on the colon absorptive capacity.

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