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Juscelino Tovar

Alicia de Francisco

Inger Bjorck

Nils-Georg Asp

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RELATIONSHIP BETWEEN MICROSTRUCTURE AND *IN VITRO* DIGESTIBILITY OF STARCH IN PRECOOKED LEGUMINOUS SEED FLOURS

Juscelino Tovar[1], Alicia de Francisco[2], Inger Björck[1] and Nils-Georg Asp[1]

[1]Department of Applied Nutrition and Food Chemistry, Chemical Center
University of Lund, P.O. Box 124, S-221 00 Lund, Sweden
[2]Carlsberg Research Center, Gamle Carlsberg Vej 10,
DK-2500 Valby, Denmark

Abstract

Precooked flours (PCFs) were prepared by milling boiled and freeze-dried red kidney beans, white beans and lentils. As demonstrated by scanning electron microscopy, PCFs were rich in relatively large particles which contained cell structures filled with starch. In contrast, flours from raw seeds contained a large number of free starch granules. The *in vitro* α-amylolysis rate of PCFs was remarkably low, but increased after physical and chemical treatments of the flours. Homogenization resulted in the largest increase of hydrolysis rate. The susceptibility to enzymatic hydrolysis was also enhanced when PCFs were preincubated with pepsin or submitted to additional boiling. These treatments promoted evident alterations in the microscopic appearance of the cotyledon cell walls of the PCFs, changes that ranged from an apparently thinner surface (pepsin effect), to an almost complete disruption (homogenization effect). A flour prepared from boiled and vacuum-dried red beans showed less structural integrity and greater rate of amylolysis than the corresponding PCF, indicating that the drying procedure may influence the microstructural and digestibility features of precooked leguminous materials. Neither cell walls nor starch granules were observed after suspending PCFs in 2N KOH, giving support to the use of alkaline pre-treatment for the evaluation of total starch content of PCFs by enzymatic procedures. The present results suggest the persistence of starch granules enclosed in cotyledon cells as a primary reason for the limited enzymatic availability of starch in PCFs.

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Direct inquiries to J. Tovar
Telephone number: 46 46 109267
Telefax number: 46 46 104532

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Introduction

Dried legume seeds have interesting nutritional properties. Among them, generally low postprandial glycaemic responses to pulses have been reported repeatedly (Jenkins et al., 1980; Shaheen and Fleming, 1987). Legumes, therefore, constitute a suitable food item for dietary management of diabetics (Jenkins et al., 1984). However, industrial use of legume flours has a number of restrictions. Apart from the need for control of the antinutrients, such as, proteinase inhibitors, lectins, and polyphenols (Jaffé, 1980; Liener, 1979; Rascón et al., 1985), the "slow carbohydrate" feature of legumes is sensitive to extensive processing (Golay et al., 1986; Jenkins et al., 1982; Traianedes and O'Dea, 1986; Wong et al., 1985).

The importance of plant cell wall matrix as a physical barrier for digestion and nutrient absorption is recognized (Schneeman, 1990). Würsch et al. (1986) identified the rigidity of cotyledon cell walls as an important determinant of the low availability of starch in cooked common beans. Two different procedures have been reported for the preparation of processed legume seed products. Instant flakes were obtained from white beans (Golay et al., 1986; Tappy et al., 1986) and precooked flours were prepared from lentils and several bean varieties (Tovar et al., 1990a, b). Both preparations are rich in starch granules entrapped within cell walls, resulting in a notably low rate of α-amylolysis *in vitro*. The aim of this study was to investigate the microstructure of precooked flours from red beans, white beans and lentils, and the changes related to various chemical and physical treatments that lead to a higher starch availability.

Materials and Methods

Seeds

Green coat lentils (*Lens culinaris* Medik) and red and white common beans (*Phaseolus vulgaris* L.) were obtained from the local market. Cooking properties and starch digestibility of these seed lots were studied before (Tovar et al., 1990b).

Processing of Legumes

Seeds were soaked in twice their weight of water for 20 minutes and drained. The soaked seeds were cooked by boiling for 70 minutes in water, employing a constant seed to water ratio of 1:3 (w/v). This procedure rendered lentils and red kidney beans soft ("cooked" seeds), whereas white beans remained slightly undercooked (Tovar et al., 1990b).

The softened seeds, along with cooking water were freeze-dried and ground to pass a 1 mm screen in a Cyclotec 1093 mill (Tecator AB, Höganäs). The resulting flour was kept in a desiccator until used. This preparation is referred to as precooked flour (PCF) (Tovar et al., 1990a, b).

In some experiments directed to study the influence of the drying procedure on the microstructure and susceptibility to enzymatic hydrolysis of precooked red beans, the seed and cooking water mixture was dried in a vacuum chamber for 48 hours at room temperature. The dried material was then milled as described before, and a precooked and vacuum-dried flour was obtained. For comparative microscopic examination, raw legumes were ground to a flour using the same mill.

Additional Processing of Legume Flours

In order to evaluate the impact of additional processing of the PCFs on the microstructural features and on enzymic susceptibility of starch, various treatments were applied before the starch availability assay (Tovar et al., 1990a):

a) Homogenization - The PCFs (500 mg, dmb) were suspended in 20 ml distilled water and submitted to five one-minute pulses at maximal strength with a Polytron blender (Kinematica GmbH, Luzern).

b) Additional boiling - A water suspension of the PCF (1-1.5 g/20ml, dmb) was boiled for 20 minutes in a water bath.

c) Pepsin incubation - One hundred milligrams of pepsin (2000 FIP-U/g; Merck, Darmstadt) were added to a suspension of PCF (500 mg/20 ml, dmb) adjusted to pH 1.5 with 5N HCl. The mixture was incubated for 1 hour at 37°C, and neutralized with 5N NaOH for enzyme inactivation.

The effect of an alkaline treatment on the microstructure of PCFs was also studied: the PCFs (500 mg, fresh basis) were suspended in 10 ml of water and an equal volume of freshly prepared 4N KOH solution was added. The mixture was kept for 30 minutes at room temperature and neutralized (pH 6.5 - 7) with 5N HCl. This procedure is used for assessment of total starch content in PCFs (Tovar et al., 1990a, b).

Treated PCFs were either used directly for determination of starch content and digestibility, or freeze-dried for scanning electron microscopy (SEM).

Scanning Electron Microscopy

Flours were mounted on aluminium specimen stubs with double-sticky tape, sputter-coated with gold in a Balzers SCD-030 sputtering device, and examined in a Cambridge Stereoscan 100 SEM, operated at 10 kV. The SEM photomicrographs were taken on Polaroid 55, negative/positive film, using back scattering to avoid charging (Cambridge Instruments, 1982).

Starch Determination

Starch was analyzed according to Holm et al. (1986). In this method, starch is gelatinized and enzymatically hydrolyzed after sequential incubation with a thermostable amylase, Termamyl (Novo BioLabs, Bagsvaerd, Denmark), at boiling temperature and with amyloglucosidase from *Aspergillus niger* (Boehringer Mannheim, Germany) at 60°C. Finally, free glucose is measured using the combined glucose oxidase/peroxidase colorimetric assay.

In Vitro Starch Digestibility

The *in vitro* digestibility of starch in PCFs and in precooked vacuum-dried red bean was determined as described by Holm et al. (1985), using 200 units of porcine pancreatic α-amylase (Sigma Chemical Co., St Louis) per gram of starch. The hydrolysis index values obtained after 15 minutes of incubation gave a measure of the initial rate of digestion, whereas the 60 minute values were taken as indicators of the final degree of hydrolysis. The digestibility of wheat starch suspensions boiled for 20 minutes was assayed as a reference. Starch content in legume samples was calculated on the basis of values obtained after the homogenization treatment, i.e. the "potentially available starch" content (Tovar et al., 1990b).

Statistics

Means were compared by one-way analysis of variance followed by the Duncan multiple comparison test, using the SPSS/PC$^+$ program.

Results

Scanning Electron Microscopy

The flour obtained from cooked and freeze dried red kidney beans (PCF) was rich in large particles (50-110 μm) corresponding to rather intact cell structures, presumably entrapping gelatinized starch granules (Figure 1A). On the other hand, mainly free starch granules (10-30 μm) and protein bodies (2-4 μm) were observed in the flour obtained from raw seeds (Fig. 1B). A similar pattern was observed for corresponding PCFs and raw flours from white beans and lentils (Figures 2 and 3). The precooked and vacuum-dried red bean powder contained smaller particles (25-50 μm) than the PCF (Figure 4), suggesting a higher degree of disruption of the starch-containing cells.

All PCFs were similarly affected by each physical or chemical treatment. Additional boiling of the flours resulted in microstructural changes. Exposure of starch by disruption of cell structures, as well as a "melted" appearance of the surface of some cells were observed. This is illustrated for red beans in Figure 5.

After a prolonged homogenization step, extensive cell disruption was appreciable in PCFs. Broken cells and aggregated starch granules were mainly noticed (Figure 6).

Table 1. *Im vitro* digestibility of starch in variously treated precooked legume flours.

Treatment	*Degree of Hydrolysis(%)*[1]					
	Legume					
	Red Beans		White Beans		Lentils	
	15 minutes	60 minutes	15 minutes	60 minutes	15 minutes	60 minutes
None	8(1.5)[a]	19(1.5)[e]	10(2.2)[a]	30(4.4)[c]	11(3.0)[a]	31(3.7)[c]
Homogenization	50(1.0)[b]	54(0.6)[f]	41(1.0)[b]	67(1.0)[d]	55(3.1)[b]	73(2.7)[d]
Boiling	28(2.0)[c]	44(2.0)[b]	34(2.0)[c]	64(1.0)[d]	46(2.8)[b]	76(3.4)[d]
Pepsin	36(0.0)[d]	52(2.0)[b]	21(2.0)[d]	45(1.5)[d]	31(1.2)[c]	67(2.3)[d]

[1]Calculated after 15 and 60 minutes of incubation as:

maltose equivalents (mg) X 0.95 X 100 ÷ starch submitted to hydrolysis (mg)

Values are the mean of a minimum of four assays. SD is indicated in parentheses.
Means from same legume without common superscript letters are significantly different ($p < 0.05$).

Treatment of PCFs with pepsin resulted in a thinner particle surface. In some cases the cell wall was missing completely, leaving naked clumps of starch (Figure 7).

Neither defined cell structures nor starch granules were identified in PCFs following solubilization in 2N KOH (Figure 8).

Starch Availability In Vitro

Figure 9 depicts the α-amylolysis curves of the precooked and vacuum-dried powder and the PCF from red kidney beans. Both flours were hydrolyzed at a lower rate than the boiled wheat starch used as a reference, though the PCF was less susceptible to amylolysis than the vacuum-dried bean flour.

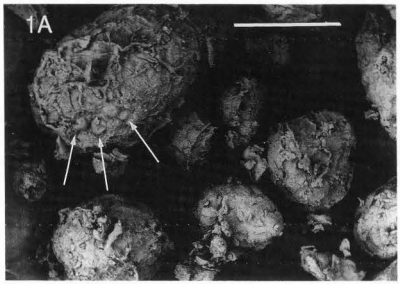
As summarized in Table 1, the digestibility of PCFs by pancreatic amylase was remarkably low, though the red kidney bean preparation was less efficiently hydrolyzed than PCFs from lentils and white beans (60 minutes, $p < 0.05$). All of the treatments applied to PCFs resulted in a significant increase in the rate of α-amylolysis (Table 1). The largest increment in availability was observed after prolonged homogenization.

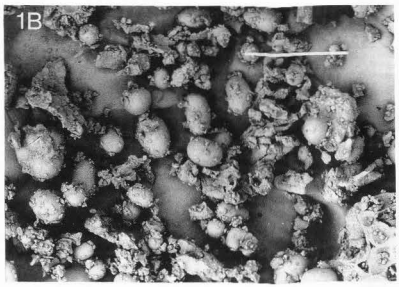
Discussion

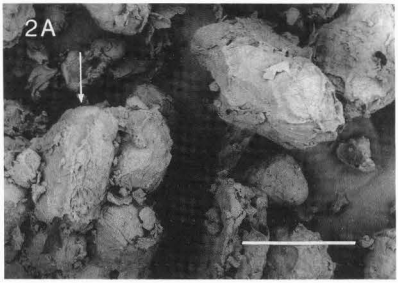
We have recently reported the preparation of PCFs from cooked and freeze-dried common beans and lentils, which showed a limited enzymic starch availability *in vitro* (Tovar et al., 1990a, b). Besides their potential use as ingredients of processed food items for diabetics, PCFs represent a valuable system to study the mechanisms governing the low rate of digestion of starch in legume seeds. The limited availability of starch in the PCFs was attributed to the physical insulation of starch granules within cell walls, as observed by light microscopy. A similar phenomenon was described by Würsch et al. (1986) as determinant for the low rate of hydrolysis shown by cooked and gently mashed white beans. The SEM observations reported in this paper stress the importance of the insulation of starch granules by cell walls as a primary reason for the slow digestibility of starch in PCFs.

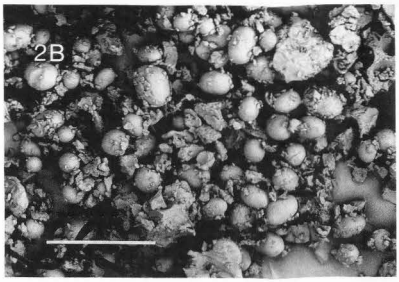
After milling of raw seeds, particles of two different sizes were observed. Those ranging between 10 and 30 μm were identified as free starch granules, whereas the smaller ones, 2-4 μm, probably represent protein bodies (Hughes and Swanson, 1989). The size of the particles in PCFs was larger, 50-110μm (Figures 1-3), resembling the dimensions of cotyledon cells in gently processed white and red kidney beans (Fleming et al., 1988; Golay et al., 1986). Enzymic availability of starch in the PCF from red kidney beans is limited, resulting in incomplete hydrolysis even after extensive incubation with microbial amylases (Tovar et al., 1990a). Present observations suggest that the differences in susceptibility to enzymatic attack observed between PCFs and the corresponding raw flours (Tovar et al., 1990a, b), are mainly due to the release of starch granules during milling of uncooked seeds, whereas starch-containing cotyledon cells remain in the PCFs.

The drying procedure applied to the cooked legumes may influence the properties of the final product. The precooked and vacuum-dried flour from red kidney beans contained fewer intact cells and was more rapidly hydrolyzed than the freeze-dried one (PCF) (Figures 4 and 9). Interestingly, flours prepared after autoclaving and warm air-drying of common beans, do not contain intact cells (Hughes and Swanson, 1989). Thus, the mild conditions prevailing during boiling and lyophilization should be preferred for cooking and dehydration, in









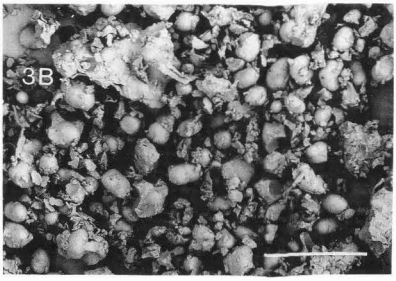
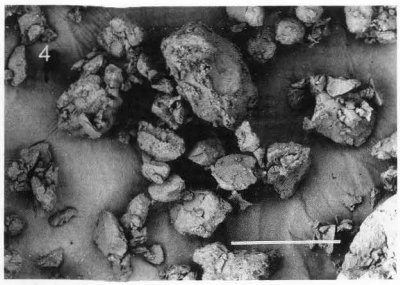


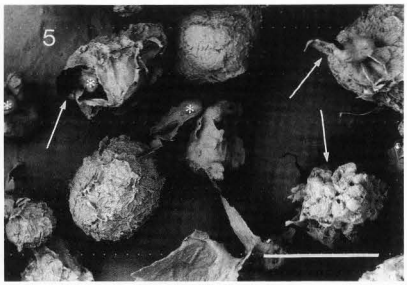
Figure 1. SEM micrographs of red kidney bean PCF (A) and raw flour (B). Arrows indicate outlines of starch granules entrapped within cells in (A). Free starch granules and adhering protein bodies are evident in (B). Bar = 100 μm.

Figure 2. SEM micrographs of white bean PCF (A) and raw flour (B). Agglomerates of cotyledon cells enclosing starch granules (arrow) are evident in (A). Abundant free starch granules and protein bodies are observed in B). Bar = 100 μm.

Figure 3. SEM micrographs of lentil PCF (A) and raw flour (B). Starch granules (arrow) are contained within rather intact cells in (A). Free starch granules and protein bodies predominate in (B). Bar = 100 μm.

Figure 4 (on the facing page). Flour from cooked and vacuum-dried red kidney beans. The predominant particle size is smaller than in PCF (Figure 1A), indicating disruption of cotyledon cells. Bar = 100 μm.





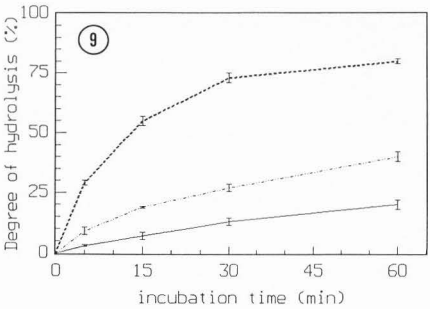


Figure 5. Red kidey bean PCF after additional heat treatment (reboiling), showing both a "melted" appearance of the cell surface (arrows) ad broken cells with exposed starch granules (*). Bar = 100 μm.

Figure 6. Homognized PCF, with extensive cell disruption and starch release. Bar = 100 μm.

Figure 7. Pepsin-reated PCF. Changes in cell surface ranged from thinning to complete removal of cell walls. The starch contents of most cells and even naked clumps of starch granules are clearly observed. Bar = 100 μm.

Figure 8. PCF after alkaline (2N KOH) treatment. No recognizable structures were detected. Bar = 100 μm.

Figure 9. Hydrolsis of starch in pre-cooked red kidney bean flours by pancreatic α-amylase *in vitro*. ——, flour from cooked and freeze-dried seeds; -·-·-, flour from cooked and vacuum-dried seeds; ----, boiled wheat starch reference. Bars indicate the standad deviation of the mean, n = 4.

order to prepare slowly-digestible precooked legume flours.

As previously reported for precooked powders from red kidney bean (Tovar et al., 1990a), the hydrolytic behavior of PCFs was partially altered by additional heat treatment. Starch granules showed a melted appearance of the tubular structures (Figure 5). The later change suggests cracking as a consequence of cell wall disruption/maceration, and interpretation supported by the release of a gelatinous and iodine-stainable layer over the particulate sediment in freeze-boiled PCF samples (results not shown). These observations are in agreement with the 2-fold increase in the rate of amylolysis recorded for boiled flours (Table 1). Sensitivity to additional treatment must be considered when PCFs are incorporated into foods (Tovar et al., 1990a, b).

The results presented herein to explain why a prolonged homogenization step is necessary for the measurement of the potentially available starch content in cooked beans and PCF (Tovar et al., 1990a, b). The mechanical treatment produced extensive cell disruption (Figure 6), leading to starch release. Furthermore, it promoted the large increase in the rate of amylolysis (Table 1), suggesting complete exposition of the gelatinized starch granules to enzymatic attack.

Acidic incubation of CIs with pepsin, which simulates the gastric phase of digestion, resulted in a greater rate of hydrolysis (Table 1). Although the occurrence of protein/starch interactions decreasing the digestibility of the carbohydrate (Yong et al., 1985; Tovar et al., 1989) cannot be ruled out, a thinned, or even missing, cell surface seems to be the main consequence of the treatment (Figure 7) and this weakened insulating barrier favoured the amylolytic process. Incubation under acidic conditions, in the absence of pepsin, results in only minor changes of the *in vitro* digestibility of starch in gently blended cooked beans (Würsch et al., 1986). Therefore, the microstructural alteration and higher amylolytic rate observed in CIs following incubation with pepsin, may be regarded as an enzymatic rather than a merely pH dependent effect.

Although leading to inaccurate estimates of the starch content, the presence of cell-entrapped starch does not affect the enzymatic-gravimetric determination of dietary fiber in red bean PCF (Tovar et al., 1990a), a procedure that includes a digestion step with pepsin (Asp et al., 1983). Hence, the alteration of the cell surface by pepsin might contribute to an efficient *in vivo* digestion of starch in beans and other seeds. The total *in vivo* digestibility of starch in intact cell-containing leguminous materials will be further investigated.

Because of their relatively high amylose content (Hoover and Sosulski, 1985; Eliasson, 1988), wet heating of legume starches may produce appreciable amounts of amylase resistant starch (Tovar et al., 1990b). Resistant starch is available to enzymatic hydrolysis after solubilization in dimethylsulphoxide or concentrated KOH (Englyst and Cummings, 1984; Siljeström et al., 1988). The initial solubilization in 2N KOH used to determine total starch in PCFs by enzymic methods (Tovar et al., 1990a, b) may be considered appropriate since, in addition to the chemical modification of resistant starch, the treatment led to profound deterioration of cellular structures and left no evidence of enclosed starch granules (Figure 8).

The cooking procedure used, boiling for 70 minutes, resulted in softened red kidney beans and lentils, whereas undercooked white beans were obtained (Tovar et al., 1990b). However, SEM comparison of PCFs obtained from these seeds did not reveal significant microstructural differences. Thus, the presence of cells filled with starch is characteristic of PCFs, both after complete and incomplete cooking. The effect of prolonged cooking times on the cell wall integrity of PCFs should be evaluated.

Factors, other than the starch insulation phenomenon, may also affect the digestibility of legume starches. Despite the similar microstructure, an unequal degree of starch hydrolysis was recorded for the various PCFs after 60 minutes of digestion (Table 1). These differences may be due to thermostable factors, like polyphenolic compounds and dietary fiber (Björck and Nyman, 1987; Socorro et al., 1989), as well as to the intrinsic properties of the starch and its degree of gelatinization (Hoover and Sosulski, 1985; Socorro et al., 1989; Würsch et al., 1986), which are considered elsewhere (Tovar et al., 1990b).

Conclusion

As a general characteristic, precooked legume flours (PCFs) contained a high proportion of starch granules enclosed by intact cotyledon cell walls, which contrasted with the predominance of free granules in the flour from raw seeds. Cell-enclosed starch is less easily attacked by amylolytic enzymes, which results in a limited availability *in vitro*.

The present SEM study showed that physical and chemical treatments of PCFs may affect the integrity of cell walls and increase the exposure of starch, facilitating the amylolytic process. The alteration of the cell surface caused by incubation of PCFs with pepsin may be of importance in a nutritional context, since it results in a more available starch preparation. The deterioration of cell integrity and increased digestibility associated with mechanical and thermal manipulation of the flours is relevant from the technological point of view.

Acknowledgements

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Discussion with Reviewers

B.G. Swanson: Were starch granules identified strictly by SEM observation?

Authors: No, light microscopy of iodine-stained samples was also used to visualize free and cell-enclosed starch granules.

F.R. Dintzis: How soon after being cooked were the seeds freeze-dried? Would the *in vitro* digestibility properties be affected by the time the cooked seeds were

allowed to remain at room temperature prior to freeze-drying?
Authors: The cooked seeds were allowed to cool down to room temperature, for about 1 hour, frozen at -20°C overnight and freeze-dried. The influence of the length of the cooling and resting steps on the cell integrity and *in vitro* digestibility of starch in PCFs has not been studied. However, they probably are less critical than the heating, freezing and drying periods.

R.S. Bhatty: Much of the visible material may be artifacts or cell debris produced on various treatments and grinding of the seeds.
Authors: Our conclusions were not drawn from the observation of artifacts. The microphotographs shown are representative pictures of each flour. They were selected after thorough observation of two independently prepared samples.

R.S. Bhatty: Figure 8 seems to be unique in not containing anything visible in other figures (e.g., Figures 4 and 7). Could you explain this?
Authors: Figure 8 represents a PCF treated with 2N KOH. The strongly alkaline conditions resulted in extensive disruption, and perhaps dissolution, of cell walls plus dispersion of the resistant starch fraction. Therefore, neither intact cell structures nor starch granules were present in the sample.

R.S. Bhatty: In Figure 9 hydrolysis of red bean starch is compared with isolated wheat starch. Why wheat starch? Is it surprising then that hydrolysis of isolated wheat starch was about three folds higher than that of bean?
Authors: Gelatinized wheat starch is rapidly digested *in vitro* and promotes high glycemic responses in normal and diabetic individuals. Wheat starch, either isolated or in bread, is therefore frequently used as "rapid starch" reference in both *in vitro* and *in vivo* studies (Jenkins et al., 1982; Socorro et al., 1989). The 3-fold lower hydrolysis rate of starch in red bean PCF compared to boiled wheat starch is not surprising, it provides additional evidence of the retained "slow" properties of the powdered precooked legumes.

D.J. Gallant: You said that differences in susceptibility to enzymatic attack between PCFs and the corresponding raw flours are mainly due to the release of starch granules during milling of uncooked seeds. They may also be due to the nature of cell walls; did you study this aspect? Do you know how the cell walls disintegrated under the different kinds of treatments?
Authors: We did not study cell wall properties in the PCFs, but they are expected to be different than in raw whole seeds. Cell wall changes during cooking and drying probably contribute to increased resistance to disruption during milling, which is critical to obtain a slowly hydrolyzed powder. Cytochemical studies would be a way to investigate the effect of the different treatments.

D.J. Gallant: In your paper, Tovar et al. 1990b, soaked seeds were cooked by boiling in water until soft as felt between fingers. Why did not you determine the exact cooking time from textural measurements, for instance from the Instron penetrometer curves, and choose the same softness for both red kidney beans and white beans?
Authors: In this investigation we decided to compare the microstructure of beans and lentils submitted to the same cooking procedure. A previous work (Tovar et al., 1990b) dealt with the digestibility features of "undercooked" and "cooked" white beans and included Instron measurements of textural properties. The influence of cooking length and conditions on the cell integrity in PCFs will be investigated.

D.J. Gallant: Behavior of foodstuffs in cooking may be different according to the quality of water. For example, lentils, when cooked in distilled water lost about 80% of the iron they contained and much more (98%) when cooked with water and salt according to domestic uses (Gallant, unpublished data). Could you give details of the kind of water you used for soaking and boiling the lentils and beans?
Authors: Tap water was used for both soaking and boiling. A typical analysis indicates Ca^{++} + Mg^{++} content of 82 mg/l and conductivity of 49 mho.

D.J. Gallant: During soaking time (which may be several hours, depending on seed moisture and storage conditions), components such as water soluble antinutritional factors (protease inhibitors, phytohemagglutinins, phytic acid and oligosaccharides) are leached out and can be recovered in the soaking water. In your technique, which could be a process for industrial use, PCFs are prepared by soaking 20 minutes and then cooking for 70 minutes in water, freeze-drying and grinding the seeds. Do you know what is the behavior of antinutritional components during your process? Are you certain that all the protease inhibitors have been removed or denatured?
Authors: The main reason for the short soaking time employed was to avoid starch losses by leaching (Tovar et al., 1990b). We did not analyze the soaking water, but no important losses of macromolecules would be expected in 20 minutes. In spite of the relatively short soaking and boiling treatments, less than 5% of the trypsin inhibitor activity remained in the PCFs.