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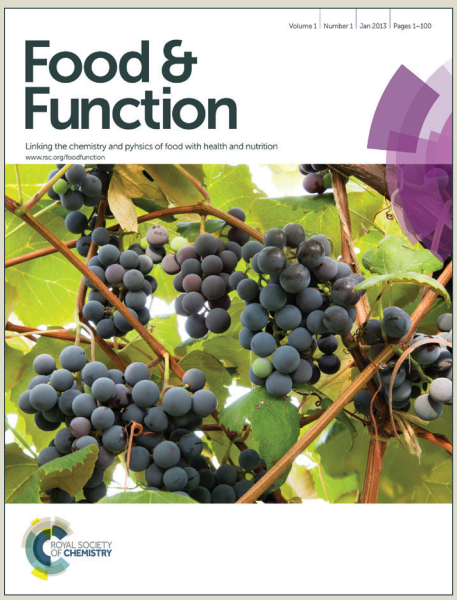

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Impact of dehulling on the physico-chemical properties and *in vitro* protein digestion of common beans (*Phaseolus vulgaris* L.)

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The objective of this study was to study the effect of dehulling on the microstructural, physico-chemical characteristics, and *in vitro* protein digestion of common bean flours with particular regard to differences between adult and infant human beings. The microstructure of flour samples from undehulled (WB) and manually dehulled (SB) beans, observed through scanning electron microscopy, showed that WB starch granules appeared surrounded by an integral matrix, while the SB starch granule structure was still visible although covered by protein clusters. The starch granules were oval and spherical, with heterogeneous sizes ranging from 19 to 30 μm in diameter. Particle size analysis determined with a laser diffraction particle size analyzer showed similar bimodal particle size distributions of small (1–25 μm) and large (>100 μm) granules, though the particle size of WB was obviously higher than SB. Color and other physico-chemical analyses showed that dehulling had significant ($P < 0.05$) influence on all investigated characteristics. The *in vitro* gastric and duodenal digestion experiments carried out under physiological conditions showed that the SB samples are more prone to be digested by infants. From our results, it is possible to conclude that the dehulling process improves bean flour protein digestion which could be utilized in various food applications.

Introduction

Leguminous seeds are an important source of nutrient compounds such as proteins, starch, dietary fibers and minerals.[1] In particular, aside from being an excellent source of some vitamins and minerals, the white beans (*Phaseolus vulgaris* L.) are rich in nutrients and have significant amounts of proteins (20–38%) and complex carbohydrates (50–60%), besides to be rich in unsaturated fatty acids (linoleic acid) and dietary fibres, some of which are particularly soluble.[2,3] Hence, representing a good source of proteins in human diets, they are widely grown and consumed in developed as well as developing nations of the world.[4] The glycemic index of beans is generally low and postprandial glucose response is moderate after ingestion which makes them a preferred source of energy.[5,6] The inclusion of beans in the daily diet has many beneficial physiological effects in controlling and preventing various metabolic diseases such as diabetes mellitus, coronary heart, colon cancer.[7] On the other hands, bean seeds are also rich in non-nutrient components,[8,9] such as inhibitors of proteases, lectins, anti-vitamins, saponins, tannins, allergens, phytic acid and toxins.[10] In particular, the bean seed coat has a greater phenolics content than has the cotyledon.[11] Among phenolics, the coat is a very rich source of condensed tannins that occur mainly in the brown and black varieties of seeds than in white beans.[1,9] Condensed tannins (i.e. proanthocyanidins) are flavan-3-ol-based biopolymers that, at high temperature in alcohol solutions of strong mineral acids, release anthocyanidins and catechins as end groups. Although several studies have reported on the antioxidant and antiradical activity of tannins,[1] these molecules are able to interfere with digestibility of

bean proteins by decreasing their hydrolysis significantly. In fact, they have the ability to bind the proteins via hydrogen bonds thus preventing the hydrolysis by proteases.[12] In this work we have investigated the impact of manual dehulling on the microstructure and on some physico-chemical characteristics (such as color, pH, moisture, water holding capacity and bulk density) of common bean flour. Moreover, the influence of coat on infant and adult digestion was also studied with the aim to use dry bean flours to create functional foods addressed to different kinds of consumers, such as athletes but also patients affected by diabetes or coeliac disease.

Materials and Methods

Materials

White beans (*Phaseolus vulgaris L.*) were provided by Select (Agria, San Giuseppe Vesuviano, Italy) and stored at 4 °C until processed. Chemicals for electrophoresis were from Bio-Rad (Segrate, Milano, Italy). α-amylase (product A1031), Pancreatin from porcine pancreas (containing trypsin, chymotrypsin and pancreatic amylase) (product P3292), pepsin from porcine gastric mucosa (product P6887), soybean Bowmann-Birk trypsin-chymotrypsin inhibitor, bile salts, and all other reagents were purchased from Sigma Chemical Company (Pool, Dorset, UK). Chemicals were of analytical grade, unless specified.

Bean flour preparation

White dried beans (*Phaseolus vulgaris L.*) were divided into two portions which were treated as undehulled (or whole beans, WB) and manually dehulled (or shelled beans, SB) respectively.

Manual dehulling was done by soaking the seeds in cold water for 5 h, followed by vigorous hand-rubbing to detach the seed coats. The dehulled seeds were next dried in an oven at 65 °C for 24 h.

Each bean samples (WB and SB) were ground using a variable speed laboratory blender (LB20ES, Waring Commercial, Torrington, Connecticut, USA), so that the flour would pass through a 425 µm stainless steel sieve (Octagon Digital Endecotts Limited, Lombard Road, London, UK). The flour samples were collected and stored in polyethylene bags at 4 °C until used for analysis.

Microstructural analysis

Samples were dried at the critical point and coated with gold particles. Microstructure of samples was examined by means of Scanning Electron Microscopy (SEM) (LEO EVO 40, Zeiss, Germany) with a 20 kV acceleration voltage and a magnification of × 2000.

2.4. Particle size analysis

Particle size distributions of the flour samples were measured by light scattering (Mastersizer 3000, Malvern Instruments, UK) in ethanol. The measurement range of the equipment was 0.01–3500 µm. The Fraunhofer diffraction model, assuming a standardized spherical shape, was used to analyse all samples. The results obtained were diameters of equivalent spheres expressed in volume. Each average value represents the mean of 3-7 independent measurements.

2.5. Determination of sample properties

Colour of flour samples was measured with a tristimulus colorimeter (Minolta Chroma Meter model CR 300, Milan, Italy) with a circular measurement area (D = 8 mm). The colorimeter was calibrated using a white standard plate (L = 100) at the beginning of each session. Chromatic coordinates L* (brightness), a* (+a red; -a green) and b* (+b yellow; -b blue) were reported as the average of six measurements on each sample.

From the parameters determined, chroma and total colour difference (ΔE) were calculated by the equations:

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$$

$$\text{Chroma} = (a^{*2} + b^{*2})^{½}$$

The moisture content, in triplicate for each sample, was determined by the AACC method[13] (number 44-15.02, 1999). The results were calculated as percentage of water per sample weight (%).

The pH of samples was measured by using a digital pHmeter (MP220, Mettler, Toledo) according to the AACC method[13] (number 02-52.01).

Bulk density of flour samples was determined according to the method of Okaka and Potter.[14] A 50-g sample was filled into a 100-mL graduated measuring cylinder. The cylinder was tapped gently several times on a laboratory bench to a constant volume. The results for bulk density were reported as g/mL.

Water holding capacity (WHC) was determined by the method of D'Apollonia[15] with some modifications. Five grams of the samples were weighed into a 50 mL centrifuge tube to which 30 mL water was added. The slurry was stirred for 5 min and then allowed to stand for 30 min at ambient conditions. The flour was then centrifuged at 4500 rpm for 25 min and the weight of free liquid measured. WHC was calculated as:

$$\text{WHC} = \frac{\textit{weight of wet sample - weight of dry sample}}{\textit{weight of dry sample (d. b.)}} \times 100$$

Results were expressed as means and standard deviation of at least three independent experiments.

Protein determination

Protein determination was carried out by the Bio-Rad Protein Assay (Bio-Rad), using bovine serum albumin as standard.[16]

***In vitro* adult digestion model**

The oral phase

To simulate oral phase digestion, both whole and shelled flour (5.2 mg) boiled for 15 minutes, were incubated for 2 min at 170 rpm and 37°C with α-amylase dissolved (150 units/mL) in 4 mL of Simulated Salivary Fluid (SSF) (0.15M NaCl, 3 mM $CO(NH_2)_2$, pH 6.9.

The gastric phase

The simulation of human digestion was done according to Giosafatto et al.[17] and Minekus et al.[18] with modifications. Aliquots (100 µL) of Simulated Gastric Fluid (SGF, 0.15 M NaCl, pH 2.5) were placed in 1.5 mL microcentrifuge tubes and incubated at 37° C. 100 µL of oral samples, the pH of which was adjusted to 2.5 with 6 M HCl, were added to each of the SGF vials to start the digestion reaction. The ratio of pepsin to test protein was 20:1 (w/w). At intervals of 1, 2, 5, 10, 20, 40, 60 min 40 µL of 0.5 M ammonium bicarbonate (NH_4HCO_3) were added to each vial to stop the pepsin reaction. The control was set up by incubating the sample for 60 min without the protease.

The small intestine phase

The small intestine digestion was performed by using, as the starting material 60 min gastric digests, adjusted to pH 6.5 with 0.5 M Bis-Tris HCl, pH 6.5. Bile salts (sodium taurocholate and sodium glycodeoxycholate) dissolved in Simulated Duodenal Fluid (SDF, 0.15 M NaCl pH 6.5) were added to a final concentration of 10 mM. After preheating at 37 °C for 10 min, pancreatin-containing enzymes (added in sufficient quantity to provide 100 U/mL of intestine phase, based on trypsin activity), dissolved in SDF, were added to the duodenal mix. Aliquots were removed over the 60 min digestion time course and proteolysis stopped by addition of a two-fold excess of soybean Bowmann-Birk trypsyn-chymotrypsin inhibitor above

that calculated to inhibit trypsin and chymotrypsin in the digestion mix. The control was carried out by incubating the sample without the proteases for 60 min. The samples were then analyzed using the SDS–PAGE procedure described below.

***In vitro* infant digestion model**

The infant digestion model was applied to the same samples of bean flour using the same protocol described by Mandalari et al.[19] and Dupont et al.[20] with some modifications. In particular:

1) pH of the gastric digestion mix was adjusted at 3.0 instead of 2.5;

2) the pepsin concentration in the gastric digestion mix was decreased by a factor of 8;

3) the duodenal digestion mix was altered by reducing the bile salt concentration by a factor of 4, whilst pancreatin concentration was reduced by a factor of 10.

As a consequence of the reduced levels of pancreatin a 10-fold lower concentration of Bowmann-Birk inhibitor was added to terminate simulated duodenal proteolysis.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

5 µL of sample buffer (15 mM Tris-HCl, pH 6.8, containing 0.5% w/v SDS, 2.5% v/v glycerol, 200 mM β mercaptoethanol, and 0.003% w/v bromophenol blue) were added to aliquots of 20 µL of each proteolysed sample and analyzed by 12% SDS-PAGE, as described by Laemmli.[21] Electrophoresis was performed at constant voltage (80 V for 2-3 h), and the proteins were stained with Coomassie Brilliant Blue R250. Bio-Rad Precision Protein Standards were used as molecular weight markers.

The image analysis was carried out using Image Lab software (Bio-Rad, version 5.2.1) following the procedure described in Giosafatto et al.[17]

Statistical analysis

All experimental results are reported as means and standard deviation of at least three independent experiments. Statistical differences between two flour samples (WB and SB) were evaluated by using a t- Student test where $P < 0.05$ was considered as statistical significant. The statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Microstructural and particle size analyses

In order to study differences between flour samples processed from WB and SB at microstructural level, the characterisation of the microstructure by means of SEM and of light scattering was performed. SEM image and particle size distribution of flour samples, WB and SB, are shown in Figure 1. Morphological features captured through SEM of WB and SB samples showed globular structures reported as starch granules, spherical protein and lipid bodies (Fig. 1. Panel A). In general, the size of starch granules is larger than that of lipid and protein bodies,[22,23] thus, the larger globular structures found in the SEM images (Fig. 1. Panel A) could possibly be starch granules, varying in shape from ovoid to spherical, with heterogeneous sizes ranging from 19 to 30 µm. The milling of beans resulted in extensive exposure of cotyledon cells. The discernible globular or irregular particles attached to or located between the starch granules were the protein bodies or fragments of protein matrix disrupted during milling. Particles might also have included mineral and fiber components, as reported in different legumes by other authors.[24,25] In particular, WB samples showed

starch granules with a smoother surface compared to the ones observed in SB samples, where starch granule structure was still visible although covered by protein clusters (Fig. 1. Panel A).

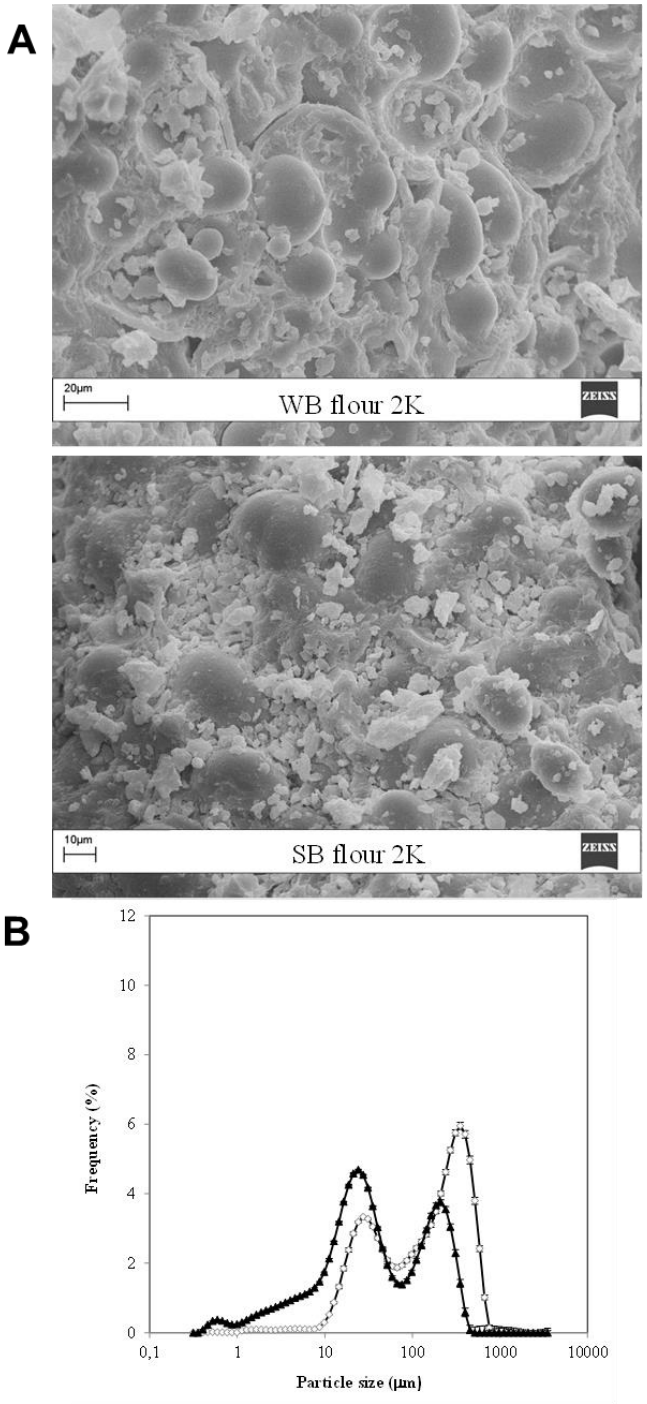

Fig. 1 Microstructural and particle size analyses of WB and SB flour samples. Panel A: SEM images of WB and SB samples. Panel B: particle size distribution of WB (◊) and SB (▲) samples.

The size distributions of WB and SB samples (Fig. 1. Panel B) exhibited a similar bimodal distribution of small (1–25 µm) and large (>100 µm) granules. As expected, the particle size of WB was higher than SB samples. Both flour samples showed a first population around 20 µm diameter for the starch granule fraction, while the second population was around 310 µm and 185 µm, respectively for WB and SB samples. Smallest particles less than 10 µm that were obviously more relevant in SB samples, consist essentially of interstitial proteins and small starch fragments. WB samples were characterized by a substantial presence of large particles with sizes greater than 300 µm that are probably starch - protein aggregates and kernel tissues, while the curve of SB flour exhibited a large peak ranged from 100 to 200 µm for cell particles containing protein matrix holding starch granule. These observations agree with the microstructural study of Berg et al.[26] on navy bean flour and starch.

Physico-chemical properties

As physico-chemical properties, color and other properties were investigated. The color parameters recorded for WB and SB are shown in Table 1.

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Table 1

Colour values L*, a*, b* and ΔE and chroma of flour samples, expressed as means ± s.d.

Colour values	WB flour	SB flour
Lightness (L*)	86.77± 0.95a	91.91 ± 0.18b
Redness (a*)	0.79 ± 0.20b	0.14 ± 0.10a
Yellowness (b*)	11.65 ± 0.36b	8.91 ± 0.22a
ΔE	17.66 ± 0.91b	12.06 ± 0.16a
Chroma	11.68 ± 0.34b	8.91 ± 0.22a

[ab] Means within the same row with different letters differ significantly at $P < 0.05$

The colour analysis of WB and SB showed significant differences ($P < 0.05$) between flour samples with regard to all colour values probably due to higher bran contents of WB resulting in greater amounts of pigment and thus increase in the hue red (a*) and yellow (b*) values, the color grade values (ΔE and chroma), decrease in lightness (L*) value. In fact, a*, b*, ΔE and chroma of WB were significantly higher ($P < 0.05$) than SB, while 'L*' had the lowest (86.8) values, indicating a lower degree of lightness of WB.

Table 2 reports the main properties of the flour samples (mean ± SD).

Table 2

Properties of WB and SB bean flour, expressed as means ± s.d.

Samples	pH	moisture (%)	WHC (g/g flour)	Bulk density (g/cm^3)
WB flour	4.55 ± 0.15a	11.25 ± 0.30b	177.5 ± 15.1b	0.75 ± 0.02b
SB flour	5.60 ± 0.26b	9.83 ± 0.34a	133.1 ± 9.6a	0.66 ± 0.02a

[ab] Means within the same column with different letters differ significantly at $P < 0.05$

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The pH values of both WB and SB samples were measured and equal to 4.6 and 5.6, respectively. The pH of the WB was observed to be significantly lower ($P < 0.05$) than SB, due to the fact that this sample has higher content of bioactive compounds, such as antioxidants, tannins, phytic acid, phenols and γ-aminobutyric acid (GABA)[27] that are released in solution. Significant differences ($P < 0.05$) were detected also for moisture content of samples. The moisture content of the WB containing shell bean with coarser particle size was significantly higher ($P < 0.05$) than the moisture content of SB with fine particle size and without seed coats. Similar results have been obtained previously by several authors that have studied how the higher moisture content of coarser particles influence the quality of bakery products made of wheat bran flour,[28] whole barley,[29] and pulse (legumes) flours.[30] Moisture content of WB and SB samples reflect results obtained for WHC that is significantly higher ($P < 0.05$) in WB compared to SB (Table 2). This variation in water absorption is mainly related to differences in both chemical composition and mean particle size of samples. In fact, it is well known that protein content, starch damage degree and larger bran particle size generally tend to increase flour water sorption properties.[31,32] Thus, it is not surprising that the bulk density results (Table 2) were significantly different ($P < 0.05$). In fact, bulk density is depended upon the particle size of the samples and it is a measure of heaviness of a flour sample. In particular, the bulk density values for SB were significantly lower ($P <0.05$) than WB values. SB results were somewhat similar both to those of dehulled bean flours (*Phaseolus vulgaris L.*)[33] and of all-purpose wheat flour.[34]

In vitro protein digestion

Protein digestibility is a nutritional parameter that evaluates the use of a protein source. This is influenced by several factors, for example, phenolic compounds, inhibitors of protein, and heat treatment.[35] In particular, *in vitro* methods simulating digestion processes are widely used to study the gastro-intestinal behaviour of food or pharmaceuticals. Although human nutritional studies are still being considered the "gold standard" for addressing diet related questions, *in vitro* methods have the advantage of being more rapid, less expensive, less labour intensive, and do not have ethical restrictions.[18] In particular, in this work we have WB and SB digestibility after assessing that both samples possess a similar protein content with no significant differences ($P < 0.05$) and equal to around 22%±0.5%. Thus, gastric and duodenal digestion experiments were set up under physiological conditions following both the adult[17,18] and infant model[19,20], in order to study the digestion of the flour sample proteins by the human gut. As it possible to note from Figure 2, the flour was promptly digested by the adult gastric environment already after 1 min incubation with pepsin.

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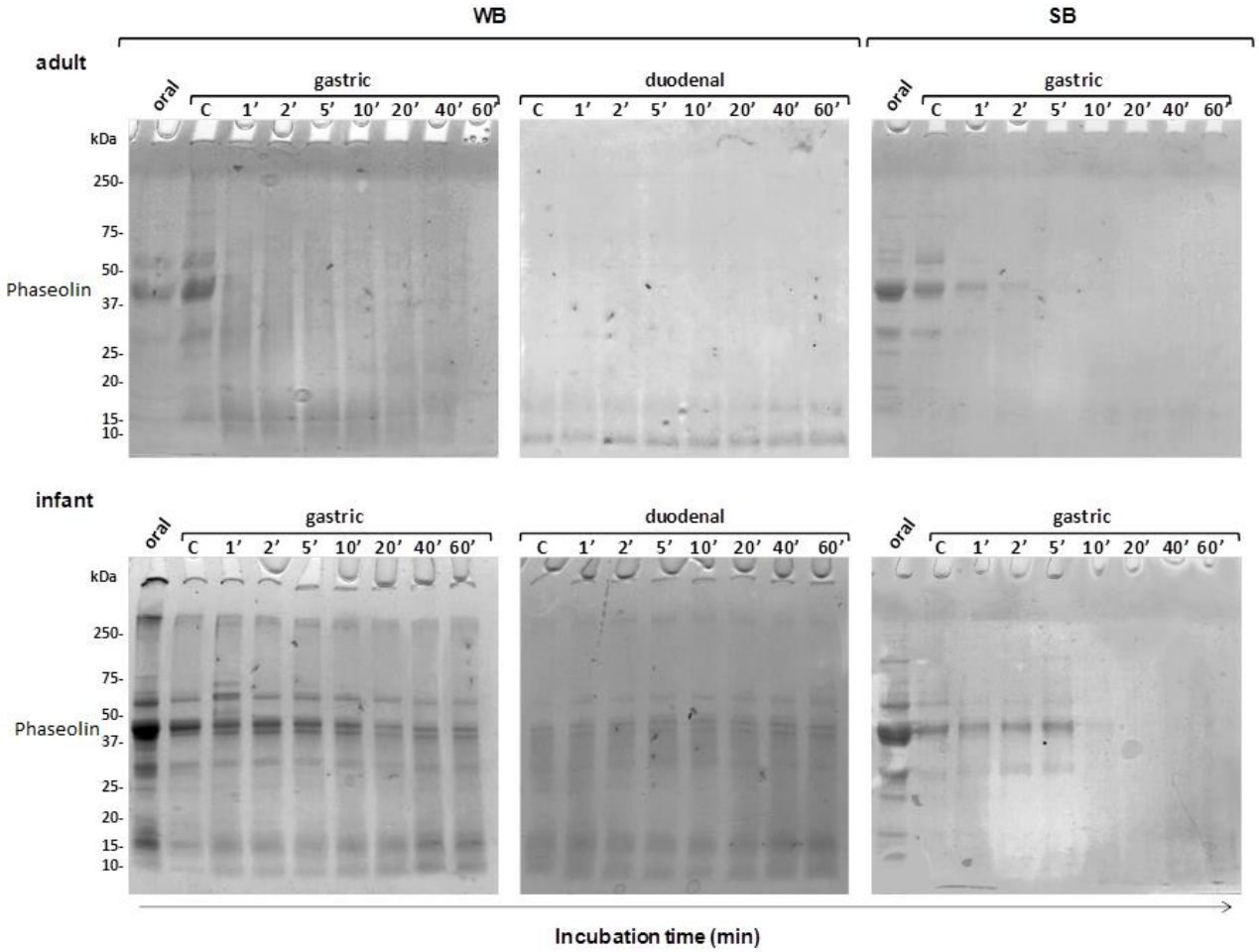

Fig. 2 SDS-PAGE analysis of WB flour and flour from SB subjected to oral, gastric and duodenal *in vitro* digestion following the adult and infant model. Lane c corresponds to the samples before the digestion. Further experimental details are given in the text. St, molecular weights standards, Bio-Rad.

The samples obtained after 60 min of simulated gastric digestion were further subjected to a process mimicking duodenal digestion with pancreatin. As expected, the results showed that following subsequent incubation with the duodenal enzymes, the flour proteins were totally degraded. It must be said that a slightly difference in digestion was revealed between WB and SB samples regarding the adult model. In particular, the gastric digestion of WB samples provokes the formation of peptide fragments of low molecular weights that are completely absence in gastric digestion of SB samples already after 5 min. Regarding infant model, a markable difference in digestion of WB and SB samples is observed. Starting from gastric digestion, the WB samples seem to be quite resistant to

pepsin treatment since the presence of intact proteins is still detectable after 60 min of incubation. In fact, taking into account the main protein band, corresponding to phaseolin protein, (having a molecular mass between 50 and 37 kDa), densitometry analysis showed that the relative density of the 60 min sample is still 52% in comparison to the control (C). Moreover, the proteins present in the WB flour during the pancreatin digestion appear still stable and they were only gradually digested upon incubation to release low molecular weight fragments, suggesting that certain amount of proteins persisted through duodenal digestion, since 40% of phaseolin was still present after 60 min duodenal digestion (Fig. 2) in comparison to the control (C). On the other hand, gastric digestion of SB samples is quite rapid as demonstrated by densitometry analysis of residual intact phaseolin in the SDS-PAGE, that indicated that only 64% of protein was observed still intact already after 5 min of incubation with pepsin. After 10 min of incubation, no protein bands were detected, demonstrating that the removal of the coat makes common bean more digestible in infants (Figure 2). The decreased digestibility of WB flour samples by using conditions found in the infant gut might be explained by the fact that the condensed tannins, anthocyanins and flavonols are mostly found in seed coats[36,37] ,while the phenolic acids are concentrated mainly in the cotyledons. Recent results[9] have demonstrated that tannins are able to interfere with digestibility of bean proteins by decreasing the hydrolysis of phaseolin significantly. While the potential of the bean proteins is high, it is associated with antinutritional factors and other substances that are harmful to health,[38,1] such as inhibitors of proteases, lectins, anti-vitamins, saponins, tannins, allergens, phytic acid and toxins.[10] In particular, studied carried out by Beninger et al.[39] and by Petri et al.[40] have demonstrated that phytate is primarily localized in the cotyledon where it is likely the major inhibitor of Fe availability[39,40] ;whereas the tannins found especially in the coat, are highly chemically active and they can form complexes with starch and proteins influencing the digestibility and bioavailability of essential aminoacids. Furthermore, tannins are attributed with other harmful effects such as off-flavor food with a decreased palatability due to astringency.[41]

Conclusions

Structure characterisation of flour samples showed that WB starch granules processed from whole beans appeared surrounded by an integral matrix, while the SB starch granule structure was still visible although covered by protein clusters. The starch granules of bean flour samples were oval and spherical, with heterogeneous sizes ranging from 19 to 30 μm in diameter. The size distributions of WB and SB exhibited a similar bimodal distribution of small (1–25 μm) and large (>100 μm) granules. In particular the particle size for WB samples made from whole beans was higher than SB from dehulling process. The main physico-chemical properties of both WB and SB were significantly different ($P < 0.05$). These different functional properties could be due to variation in chemical compositions and mean particle size of samples.

Moreover, after simulation of human digestion it is possible to conclude that the presence of the coat influences dramatically the digestion of bean proteins by the infants both in the gastric and duodenal environment. Possible applications of the dehulled beans can be determined from their functional properties and protein digestibility. Indeed, our results suggest a promising future for bean flour prepared after the removal of coat for the production of bean-based food addressed to infant consumption. Moreover, due to the high nutritional value of their proteins, bean flours could be used as wheat flour substitute in foods for coeliac disease affected patients.

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