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The role of cotyledon cell structure during *in vitro* digestion of starch in navy beans

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ABSTRACT

Studies on the physico-chemical, microstructural characteristics and *in vitro* (under simulated gastric and small intestine conditions) starch digestibility of navy beans were carried out. The microstructure of raw and cooked beans observed through scanning electron microscopy (SEM) showed the presence of hexagonal or angular shaped cotyledon cells (50–100 μm size) containing starch granules with a size ranging between 10 and 50 μm. The extent of starch hydrolysis (%) after 120 min of *in vitro* gastro-intestinal digestion differed between whole navy beans (~60%) and milled bean flour and bean starch (85–90%) after they were cooked under similar conditions. Starch hydrolysis (%) increased significantly when the cotyledon cells in the cooked whole navy beans were disrupted using high pressure treatment (French press). The storage of freshly cooked whole beans resulted in a lower (40–45%) starch hydrolysis whereas re-heating increased the same to 70–80% during *in vitro* small intestinal digestion. The SEM pictures of cooked navy bean digesta after different intervals of *in vitro* gastric and small intestinal digestion showed that the cotyledon cell structure is maintained well throughout the digestion period. However cotyledon cells appear shrunken and developed wrinkles during *in vitro* digestion. Particle size analysis of cooked bean paste taken before and after the *in vitro* gastro-intestinal digestion showed similar particle size distributions.

1. Introduction

Beans (*Phaseolus vulgaris*), a good source of protein and carbohydrates in human diets, are widely grown and consumed in developed as well as developing nations of the world. Apart from proteins (20–38%) and complex carbohydrates (50–60%), beans are rich in minerals, vitamins and polyunsaturated free fatty acids (Rehman & Shah, 2005). The glycemic index of beans is generally low and postprandial glucose response is moderate after ingestion which makes them a preferred source of energy (Jenkins et al., 1981). Furthermore they contain high levels of starch that escapes hydrolysis in the small intestine (resistant starch) and is also known for its prebiotic properties (Rehman et al., 2001; Vargas-Torres et al., 2004). Starch in legumes is naturally situated inside the living cotyledon cells (Hahn, Jones, Akhavan, & Rockland, 1977). Primary cell walls of growing and fleshy tissues have a conserved general composition of cellulose, hemicelluloses and pectin (Chanda, 2005). The non-cellulosic material acts as a "glue" that holds the microfibrils of cellulose together which in turn is responsible for the stability of cell walls (Carpita & Gibeaut, 1993). The starch granules in beans are present in the cotyledon cells and are embedded in the protein matrix of the cellular contents (Daussant, Mosse, & Vaughan, 1983). This situation might restrict the complete swelling of the bean starch during gelatinization due to steric hindrance and other limiting effects including restricted water availability. Hahn et al. (1977) and Kon, Wagner, Becker, Booth, and Robbins (1971) observed birefringence of intracellular starch granules when microscopically examining cooked beans using plain polarized light. Wursch, Delvedovo, and Koellreutter (1986) pointed out that the thick and mechanically resistant nature of the cotyledon cell walls in legumes prevent complete swelling of starch granules during gelatinization which may restrict their interaction with digestive enzymes. Starch degrading enzymes are present in digestive fluids as well as in the brush border of the small intestine (Smith & Morton, 2001). The enzymes present in the human body are difficult to extract or expensive to buy, therefore enzymes from other mammals or from microorganisms are usually used in *in vitro* systems that attempt to simulate the digestive process in the gastro-intestinal tract of human beings. The mammalian enzymes are very similar to human enzymes and are therefore preferred for this work whereas the enzymes from microorganisms may work differently even though they are similarly classified.

α-Amylase inhibitors present in raw beans are known to inhibit the activity of porcine pancreatic amylase, however these inhibitors

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are generally inactivated at or above 100 °C (Singh, Dartois, & Kaur, 2010). Processing leads to an alteration in the food structure and also influence the nutritional characteristics of the food including starch digestibility. The physical characteristics of food; the presence of other food components, such as proteins, lipids and non starch polysaccharides; and the changes and interactions occurring in them during food processing affect the enzymatic digestibility of starch to a considerable extent (Dartois, Singh, Kaur, & Singh, 2010; Kaur, Singh, McCarthy, & Singh, 2007; Singh et al., 2010).Work has been reported on the digestibility of starch in beans in relation to soaking time, microwave and conventional cooking (Oliveira, Queiroz, Helbig, Reis, & Carraro, 2001; Ramirez-Cardenas, Leonel, & Costa, 2008; Salgado et al., 2005). However, the role of cotyledon cell structure during digestion of cooked beans was unclear in the available beans literature.

The aim of this work was to study the influence of cotyledon cell integrity, effect of storage and re-heating on the extent and rate of *in vitro* digestibility of starch in navy beans using a system simulating the human digestive system. Scanning electron microscopy and comparative particle size analysis were used as tools to observe changes that might have occurred to the surfaces and particle size distribution of intact cotyledon cells during the simulated *in vitro* digestion. These insights might further lead to the possibility of changing process and plant structures in order to enhance the positive properties of carbohydrates present in beans.

2. Materials and methods

2.1. Raw material

The raw navy beans (*P. vulgaris*) were obtained from Heinz-Watties Ltd., Hastings, New Zealand. They are the same freshly imported beans that were used by this company to produce canned baked beans in tomato sauce. Pepsin (porcine gastric mucosa; 800–2500 units/mg protein), pancreatin (hog pancreas; 4× USP) and invertase (Invertase, Grade VII from baker's yeast, 401 U/mg solid) were purchased from Sigma–Aldrich Ltd., St. Louis, USA. Amyloglucosidase (3260 U/ml) was supplied by Megazyme International Ireland Ltd., Ireland. The raw beans were stored in an airtight container at 4 °C throughout the study period. The same material lots were used for all experiments.

2.2. Microstructural characteristics of raw and cooked navy beans

Raw as well as cooked (cooking as described in Section 2.6.3) bean cotyledons were sectioned with a razor blade and fixed for 2 h with 2.5% glutaraldehyde in a 0.1 M phosphate buffer at pH 7.2, washed three times in the buffer and post-fixed in 1% (w/v) osmium tetroxide overnight at 4 °C. After buffer washing and dehydration in ethanol series, samples were dried to the critical point (Critical Point Dryer 030- Bal-Tec, Germany), mounted on stubs and then gold sputter coated. The samples were observed under a scanning electron microscope (FEI Quanta 200 FEI Electron Optics, Eindhoven, The Netherlands) at 15 kV.

2.3. Milling of navy beans to flour

A part of the raw beans was milled in two steps. The beans were initially coarsely ground in a plate mill (S100 M, Glen Creston, England) and the resulting particles milled in a high speed centrifugal impact mill that was fitted with a 1 mm sieve the process produced particles ranging from very fine flour to about 1 mm in diameter.

2.4. Starch extraction from navy beans

White navy beans (200 g) were steeped in water (400 ml) containing 0.5% sodium metabisulphite for 18 h at 4 °C. The addition of sodium metabisulphite to the steep water increases the rate of water diffusion into the seeds, assists in breaking down the protein–starch matrix, and controls microbial growth and enzymatic activity in the still living seeds. To extract the starch from the beans they were washed with RO water after draining off the steep water and wet ground in laboratory blender (D-500, Labserv, New Zealand) in 500 ml of RO water. The resulting slurry was filtered through nylon cloth (100 mesh). The material left on the nylon cloth was washed three times with 300 ml of RO water. The filtrate slurry was centrifuged at 1500 × *g* for 15 min. The supernatant was discarded and the solid material re-suspended in 400 ml RO water and again centrifuged at 1500 × *g* for 15 min. This was repeated once before the solid material was transferred to 50 ml centrifuge tubes. The centrifugation and washing procedure was repeated three times before the starch was collected and dried in an oven at 40 °C for at least 16 h. The isolated starch was observed under a light microscope to check the presence of any impurities such as cell wall fragments. The granule size distribution of bean starch was determined using a laser diffraction particle size analyzer (Malvern Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, United Kingdom).

2.5. Microstructural and physico-chemical characteristics of navy bean flour and starch

Electron micrographs of the bean flour and starch were obtained with a scanning electron microscope (FEI Quanta 200 FEI Electron Optics, Eindhoven, The Netherlands). Powdered flour and starch samples were sprinkled on to double-sided adhesive tape placed on an aluminium stub and then sputter coated with gold. The protocol described by Ruiz (2001–2003) was used for the determination of dry matter. A total starch assay kit (K-TSTA (04/2009), Megazyme, Ireland) was used to determine the total starch content of bean flour. The method supplied with the assay kit for samples containing soluble sugars and resistant starch was followed. Exactly weighed samples of bean flour were washed with ethanol and centrifuged. The starch in the bean flour samples was solubilized in potassium hydroxide at 0 °C followed by α-amylase and amyloglucosidase hydrolysis at 50 °C. The glucose released was measured with a glucose oxidase–peroxidase method. The apparent amylose content of bean starch was estimated using the method as described by Singh et al. (2006). The completeness of the starch hydrolysis was verified by adding iodine to the hydrolyzed sample, after taking an aliquot for glucose release measurement. Non hydrolyzed starch binds with iodine to develop a blue–black colour. The starch content of the beans was reported as the starch content of the bean flour on a dry weight basis.

2.6. In vitro digestion

Samples of differently treated bean pastes were prepared according to the protocols described in the next sections. All samples except for the bean flour paste had the same starch concentration of 3.04% (w/w) based on the total starch content of the beans processed (see in Section 2.8).

2.6.1. In vitro digestion model

A 2-stage *in vitro* model of Dartois et al. (2010) representing simulated gastric and intestinal digestion was used. The simulated gastric (SG) and intestinal (SIF) fluids were prepared in accordance with the US Pharmacopeia (2000). The samples were taken for glucose analysis after 0, 15, 30 and 0, 5, 10, 15, 30, 45, 60, 90, 120 min

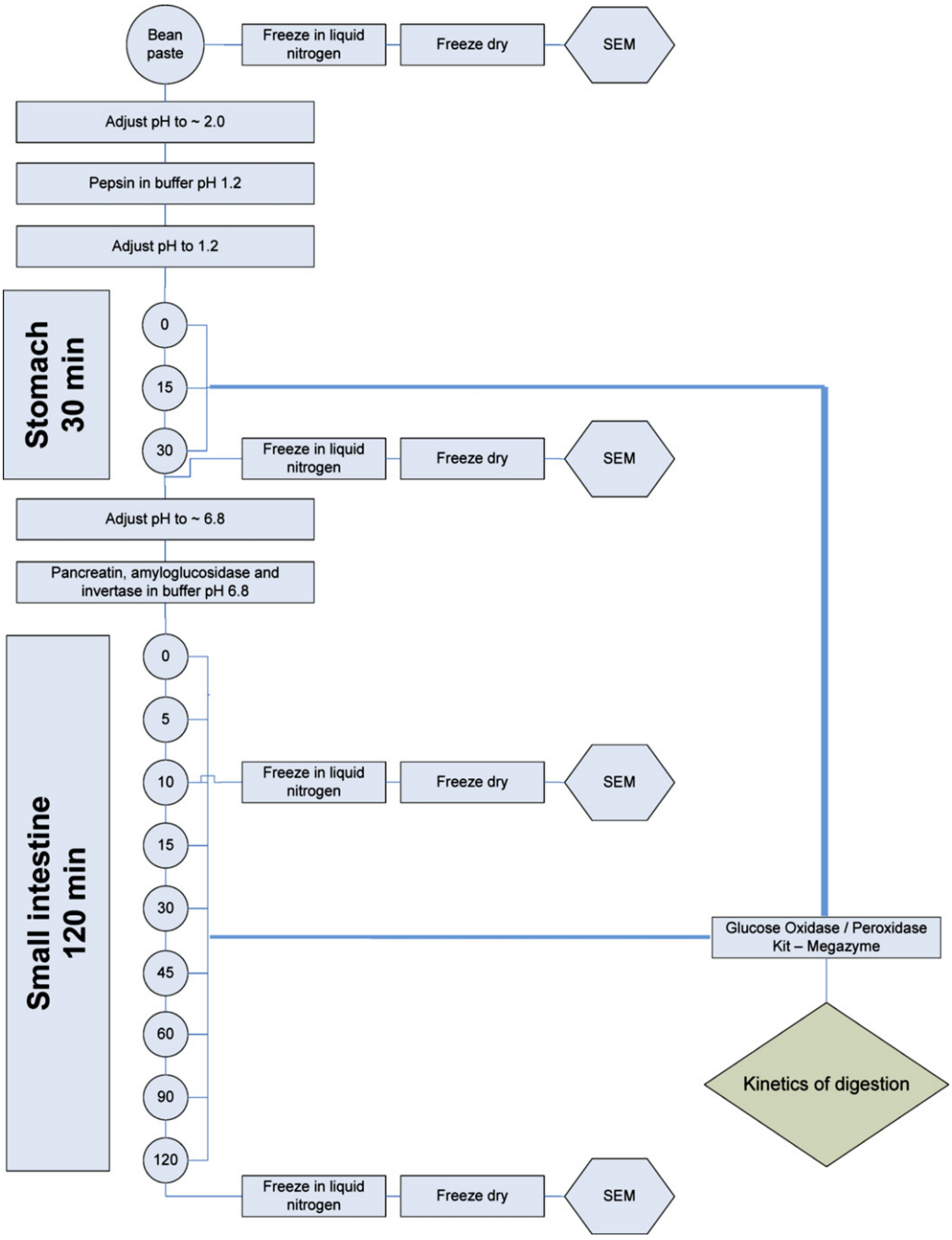

Fig. 1. Flow chart illustrating the *in vitro* digestion of starch in navy beans.

of simulated gastric and intestinal digestion, respectively (Fig. 1). For studying microstructural characteristics through scanning electron microscopy, three individual 1 ml samples were transferred to labelled 2 ml Eppendorf tubes and frozen by immersion in liquid nitrogen and stored at −80 °C before freeze drying. As per the flow chart (Fig. 1), samples were taken from the reactor, immediately before adding the gastric juice, at the end of the *in vitro* gastric digestion, after 10 min and at the end of *in vitro* intestinal digestion.

2.6.2. Measurement of glucose

Glucose release measurement was carried out through a D-glucose assay procedure (GOPOD-FORMAT, K-GLUC 05/2008, Megazyme International Ireland Ltd., Ireland) immediately after the *in vitro* digestion. The ethanol preserved samples were mixed with a test tube shaker (Reax top, Heidolph, Schwabach, Germany) for 10 s each and centrifuged at 1800 × *g* for 10 min. Supernatant (0.2 ml) was secondarily digested with 1 ml amyloglucosidase and invertase in acetate buffer pH 5.2 (0.1 ml amyloglucosidase and 3.125 mg invertase per 10 ml acetate buffer) in a second set of test tubes. The tubes were incubated for 10 min at 37 °C. The concentration of glucose in the tubes was measured according to the instructions of the D-glucose assay and the obtained values were multiplied by a dilution factor of 42 to calculate the concentration of available glucose in the reactor at each point of time (as explained in the assay procedure). Two different kinds of hydrolysis curves were drawn from the results. Curves showing the whole *in vitro* process (stomach and small intestine) include the initially available glucose; the hydrolysis rate (% glucose available) is based on glucose detected in relation to the total glucose available. Curves showing only the small intestinal part are corrected for free glucose

and the hydrolysis rate (% starch hydrolyzed) is based on starch hydrolyzed to glucose in relation to the theoretical total starch content.

2.6.3. Cooking of navy beans for in vitro digestion

The water used in the sample preparation was, unless otherwise stated ultra filtered (Milli Q, Millipore, Billerica, Massachusetts, USA) containing 10 mg $CaCl_2$ per litre according to Van Der Merwe, Osthoff, and Pretorius (2006). Twenty five grams of raw beans and 30 ml of water were weighed in 100 ml glass beaker. After 1 h of soaking, water not taken up by the beans was discarded and the beans were weighed and placed in a 500 ml Schott bottle. Water was added to a total weight of 112.9 g. The bottle was closed and slightly unscrewed to allow pressure balance during autoclaving (3150el- Tuttnauer, Breda, The Netherlands). The temperature in the autoclave was recorded every 5–8 min to assure equality of heat treatment for all the samples processed. The autoclaving procedure comprised heating to 121 °C, holding of temperature for 15 min and rapid cooling to 100 °C. The autoclave was opened upon reaching this temperature and the bottles containing beans and water were put in cold water at 18–20 °C to cool the contents. The bottles were occasionally and carefully shaken during the next 5 min. Two samples were processed at a time. One sample was further processed to a bean paste and the second kept in darkness at room temperature. The whole sample of cooked and cooled beans was placed in a 400 ml Schott bottle to which cold RO water was added to a total weight of 200 g and then blended with a laboratory mixer (D-500, Labserv, New Zealand) for 30 s at a speed of 15,000 rpm and then for 30 s at a speed of 18,000 rpm. The resulting paste was sieved to remove coarse particles and the residue in the sieve (less than 2% of the total weight) washed with 50 ml of RO water and discarded. *In vitro* digestion was carried out on this material as described in the previous section.

2.6.4. Storage and reheating of cooked navy beans for in vitro digestion

Beans were soaked, autoclaved and cooled as described in the previous section and stored in the Schott Bottle in which they were processed. The storage temperature was between 18 °C and 22 °C and the bottles were kept in a dark place. After 1 week of storage the beans were diluted with water and blended to a bean paste in the same way as described above. The content of a Schott Bottle of autoclaved and 1 week stored beans was transferred to a 400 ml laboratory glass beaker. Rinsing the bottle, the total weight of the content was added up to 200 g with RO water. The beaker was covered and heated in a microwave for 60 s at 1100 W. The content was gently stirred and micro-waved for a further 30 s at the same intensity. The reheated beans were cooled at room temperature (20 °C) for 10 min. Blending and sieving was carried out in the same manner as described above for the freshly prepared bean paste followed by *in vitro* digestion as described previously.

2.6.5. High pressure (French press) treatment of cooked navy beans for in vitro digestion

Complete disruption of cells in the cooked and ground bean paste was carried out using a French Press. The French Press comprises a cylinder filled with the material to be processed and a piston that is forced down the cylinder with a hydraulic ram. The material flows out of the cylinder through a needle valve which is used to control the pressure drop during processing. A pressure drop of 38–42 MPa was used to process these samples. In each processing cycle 35 ml of bean paste was processed, a total of 7 cycles was required to process 250 ml of the paste. *In vitro* digestion was carried out in the disrupted material as described previously.

2.7. Microstructural characteristics (light and scanning electron microscopy) of navy bean digesta

Freshly prepared bean pastes were placed on a glass slide and covered with a cover slip before examining them using a transmission light microscope fitted with a camera. Normal light and polarized light was used to illuminate the samples. Scanning electron microscopy was used to examine possible changes on the surface of navy bean cotyledon cells during the *in vitro* digestion process. Preliminary experiments showed that washing step was required to remove soluble hydrolysis products which adhered to the surface of the cells after freeze-drying. The contents of one tube (1 ml) of frozen digesta were washed three times in a 15 ml centrifuge tube by dispersing the frozen digesta in 10 ml of pre-cooled water (0–3 °C) using a test tube shaker. The sample was then centrifuged at 1800 × *g* for 5 min in a pre-cooled centrifuge (1 °C) and the supernatant discarded. The procedure was repeated twice before the residue was transferred to a new, labelled Eppendorf tube, frozen by immersion in liquid nitrogen and freeze dried. The freeze dried powder was then mounted on a stub with double sided adhesive tape, sputter coated with gold (SCD 050, Balzers, Liechtenstein) and examined under a scanning electron microscope (FEI Quanta 200 FEI Electron Optics, Eindhoven, The Netherlands). Micrographs were examined and the general integrity of cells and changes on the cell surface during *in vitro* digestion were noted.

2.8. In vitro digestion of starch in navy bean flour

Bean flour was mixed with water and autoclaved in the same manner as described for whole navy beans. The bean flour paste with a starch content of 3.04% wwb (wet weight basis) resulted in a thick solid paste that was difficult to be transferred to the *in vitro* reactor. The paste was therefore further diluted by adding an equal mass of cold water; this resulted in a viscosity similar to that of the cooked whole bean pastes. To keep the substrate to enzyme ratio comparable with all the digestion experiments, the concentration of enzymes during the *in vitro* digestions of cooked bean flour paste was reduced by 50%. Bean flour (12.3 g) was weighed in a 500 ml Schott bottle and the bottle filled to 200 g with water containing $CaCl_2$ (10 mg/l). The bottle was autoclaved in the same way as the whole bean pastes but differing in that after opening of the autoclave the bottle was vigorously shaken to ensure an even distribution of contents throughout the liquid phase. The contents of the vigorously shaken bottle were sieved and the bottle washed with a total of 50 ml RO water in three steps, each time using the rinse water to wash the sieve residue. *In vitro* digestion was carried out as described previously.

2.9. In vitro digestion of navy bean starch

Bean starch was mixed with water and autoclaved in the same manner as the bean paste. Starch (9.3 g) was weighed in a 500 ml Schott bottle which was then filled to 200 g with water containing $CaCl_2$ (10 mg/l). The bottle was autoclaved in the same way as described in the previous section. The contents of the bottle were transferred to a beaker with 50 ml RO water. Enzymatic hydrolysis of the cooked bean starch was carried out using the *in vitro* digestion method described earlier.

2.10. Particle size distribution of navy bean digesta

The change of particle size of bean paste samples during *in vitro* digestion was determined with a laser diffraction particle size analyzer (Malvern Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, United Kingdom). Bean pastes were measured

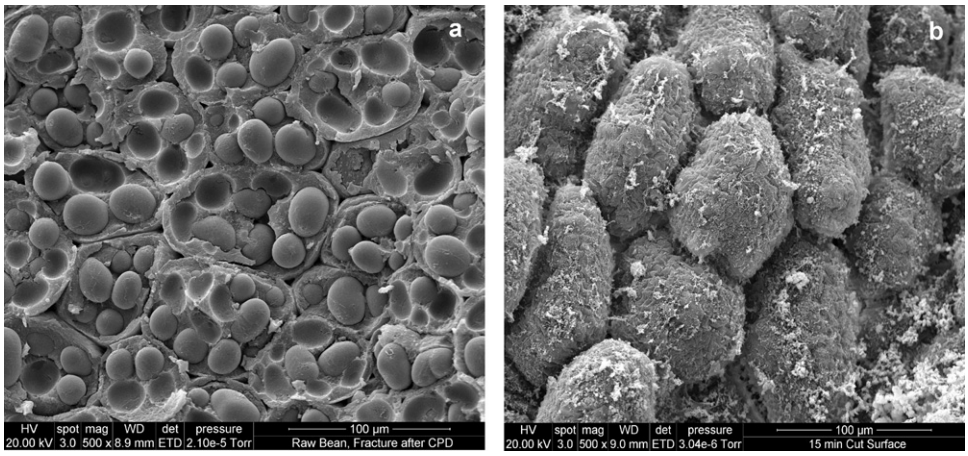

Fig. 2. A and B Scanning electron micrographs showing sections of cotyledon cells. (A) Raw navy beans and (B) cooked navy beans.

before starting the *in vitro* digestion and immediately afterwards. Sample material (~10 ml) was added into the 800 ml water reservoir of the dispersion unit (Hydro 2000 MU) until an obscuration level of 15 ± 2% was obtained. The pump speed was set to 2000 rpm. Refractive indices of 1.50 and 1.33 were used for the bean paste and the water phase respectively. The particle absorbance of the bean paste was set to 0.1.

2.11. Statistical analysis

The *in vitro* digestions were performed in triplicate and the data were analyzed using Microsoft Office Excel 2007 (Microsoft Corporation).

3. Results and discussion

3.1. Microstructural characteristics of navy beans (raw and cooked)

To gain an insight on cotyledon cell morphology and the organization of starch granules in it, the critical point dried, fractured and cut sections of raw and cooked (autoclaved 15 min) beans were observed under the scanning electron microscope. Within the raw bean cotyledon cells, the starch granules were embedded in and surrounded by thick proteinaceous matrix derived from the cell contents (Fig. 2A). A similar morphology for the cellular contents of legumes has been described by Daussant et al. (1983). The size of the bean cotyledon cells ranged between 50 and 100 μm and they were

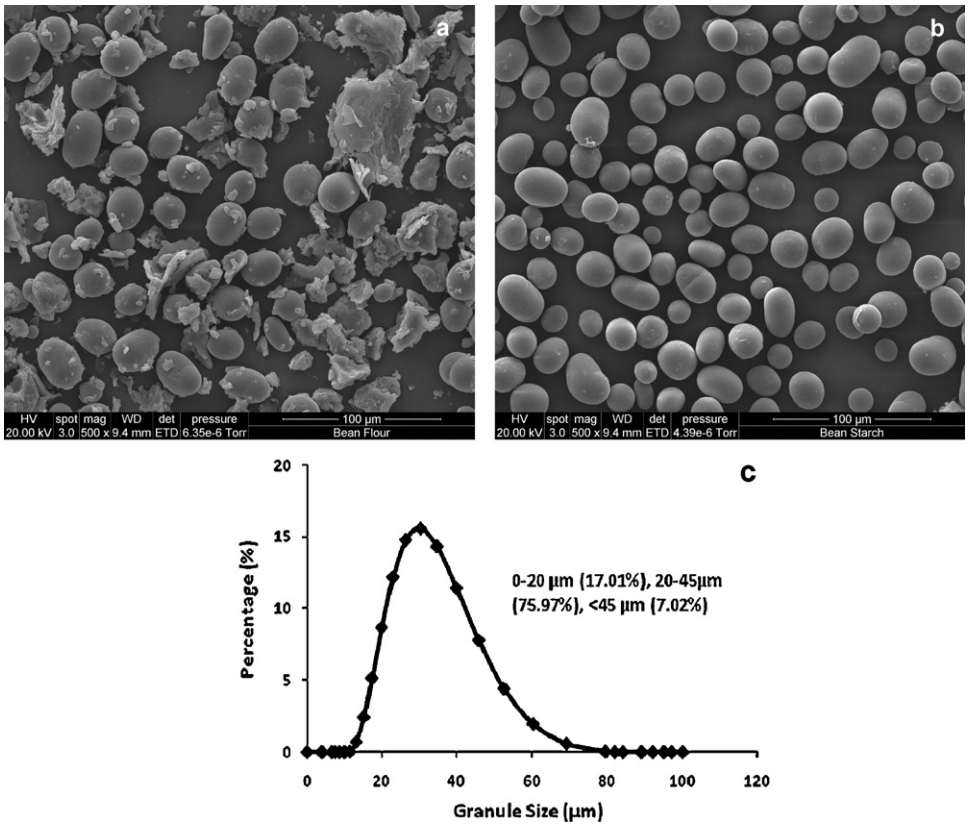

Fig. 3. Scanning electron micrograph of (A) milled navy bean flour, (B) navy bean starch, and (C) granule size distribution of navy bean starch.

hexagonal or angular in shape. The walls of the cotyledon cells were thick robust structures surrounding the starch granules embedded within the proteinaceous cellular contents. The interaction of the cell wall materials such as cellulose and non-cellulosic polysaccharides is responsible for their stability and resistance towards turgor pressure (Carpita & Gibeaut, 1993).

The cut surface of the beans cooked for 15 min showed swollen cotyledon cells due to hydration during cooking (Fig. 2B). The cotyledon cells stayed intact during the cooking process. Strands of dried soluble material were observed on the top surface of cotyledon cell walls. This soluble material might consist of soluble starch containing mainly amylose, soluble sugars and non-starch polysaccharides which oozes out of the cotyledon cells during cooking. Cell wall xyloglucans are composed of β-(1–4) linked D-glucose molecules, an identical primary structure to cellulose but with additional xylosyl units attached to the O-6 position of the glucosyl units. These xyloglucans occur at relatively high levels in legumes, which might be the reason for the extremely high rigidity of cotyledon cells of navy beans when cooked (Carpita & Gibeaut, 1993). In the dry state the cell walls are very brittle and are easily disrupted during milling.

3.2. Microstructural and physicochemical characteristics of navy bean flour and starch

Light and scanning electron microscopy of bean flour revealed that most of the cotyledon cells were disrupted during dry milling of beans. Bean flour comprises free starch granules, protein bodies and cell wall fragments (Fig. 3A). Morphological features captured through SEM are shown in Fig. 3B whereas granule size distribution of the isolated bean starch in terms of percentages of small (0–20 μm), medium (25–45 μm) and large (>45 μm) granules is presented in Fig. 3C. The bean starch granules varied from round for the small granules to oval or irregular for the larger granules. The biochemistry of the chloroplast or amyloplast, as well as the physiology of the plant, mainly dictates the morphology of starch granules (Badenhuizen, 1969; Singh et al., 2002). The membranes and the physical characteristics of the plastids may also be responsible for providing a particular shape or morphology to starch granules during granule development (Jane et al., 1994; Lindeboom, Chang, & Tylera, 2004). The bean starch contained a fairly high percentage (~75%) of medium granules whereas it had a very low percentage (7%) of large and small (17%) granules. The majority of the starch granules ranged between 10 and 20 μm. The granule size distribution of starch has been reported to change during the development of the storage organs of plants (Chojecki, Gale, & Bayliss, 1986). The starch content measured in the beans was 36.1 ± 0.5% on a dry weight basis which is comparable to a value of 36.8% reported by Chung, Liu, Pauls, Fan, and Yada (2008). The moisture content of bean flour was lower than that of intact beans which indicates water evaporation during milling process. Isolation of starch led to a yield of 21.2% of starch based on dry matter of whole beans. A comparable yield of 21% was reported by Hoover and Sosulski (1985). Starch granules in wet extracted starch and in dry milled bean flour had a similar appearance and it is assumed that dry milling did not cause major changes to the granules. The apparent amylose content of isolated bean starch was calculated around 39%. A similar range of amylose content has been reported by Kim, Wiesenborn, Lorenzen, and Berglund (1996) for different Navy bean starches. Legume and bean starches have been reported to have higher amylose content than the other cereal starches (Betancur-Ancona, Guerrero, Matos, & Ortiz, 2001; Gujska, Reinhard, & Khan, 1994). The differences among the amylose content of the starches may be attributed to differences in the activities of the enzymes involved in the biosynthesis of linear and branched components within the starch granules (Krossmann & Lloyd, 2000).

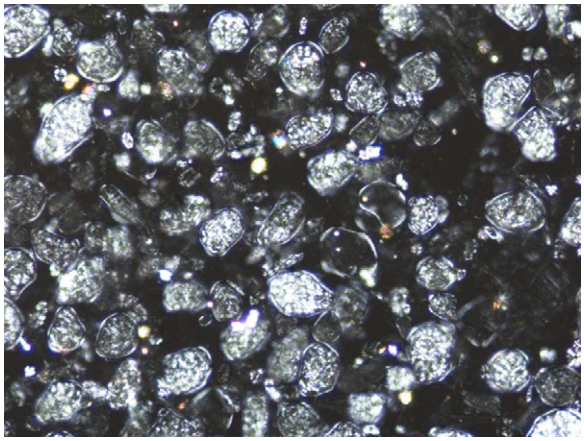

Fig. 4. Light microscopy picture (polarized light) showing cotyledon cells in navy bean paste before *in vitro* digestion.

The amylose content of the starch granules has also been reported to be affected by climatic conditions and soil type during growth, and granule size distribution (Singh, Kaur, & McCarthy, 2007).

3.3. In vitro digestibility

3.3.1. In vitro digestion of starch in cooked navy beans, navy bean flour and navy bean starch

Cells observed under the light microscope in a sample of freshly prepared bean paste were generally intact, with little evidence of disrupted cells. The starch granules present in the cotyledon cells also showed birefringence when viewed in polarized light suggesting incomplete gelatinization in cooked whole navy beans, this is presumably due to restrictions to water uptake imposed by the thick cell walls (Fig. 4). Fig. 5 gives an overview of the kinetics of starch hydrolysis during the *in vitro* digestion process. The first 30 min of hydrolysis represents simulated gastric conditions having low pH whereas the next 120 min represent the simulation of small intestinal conditions at neutral pH. In the gastric step no glucose was released from bean starch whereas in bean flour and autoclaved beans constant levels of glucose in a very low range (2.89 ± 0.54%) were detected. This glucose may be derived from sugars or dextrins initially present in navy beans. In the case of bean starch, all soluble sugars were generally washed away during the purification process. The constant level of glucose throughout the *in vitro* gastric simulation period shows that negligible starch hydrolysis occurred under these conditions.

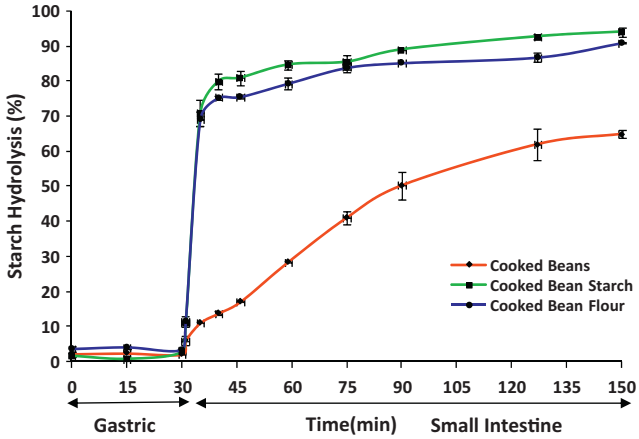

Fig. 5. Starch hydrolysis (%) during *in vitro* digestion of cooked navy beans, navy bean starch and navy bean flour.

Under simulated intestinal conditions, the rate of starch hydrolysis of freshly cooked bean paste, bean flour and starch paste increased progressively over the 120 min period of digestion. However, the percentage of starch hydrolysis rose slowly throughout the digestion of freshly prepared bean paste compared to the rapid rise seen with samples of cooked bean starch and cooked bean flour (Fig. 5). A very low percentage (~20%) of starch hydrolysis was observed during the first 15 min of hydrolysis for the cooked bean paste whereas it was significantly higher (>80%) for cooked bean flour and starch paste.

The starch component of autoclaved bean flour shows a very similar rate of hydrolysis compared to autoclaved bean starch. During autoclaving the starch granules in both cases are completely surrounded by water and unconstrained, allowing them to swell and gelatinize to full extent. Slightly lower levels of hydrolysis of starch in flour compared to extracted starch might be due to the cell clusters which were sieved out in the sample preparation, resulting in an overestimation of theoretical available starch. The presence of proteins and other components in the digestion matrix has been reported to decrease the starch hydrolysis (Singh et al., 2010).

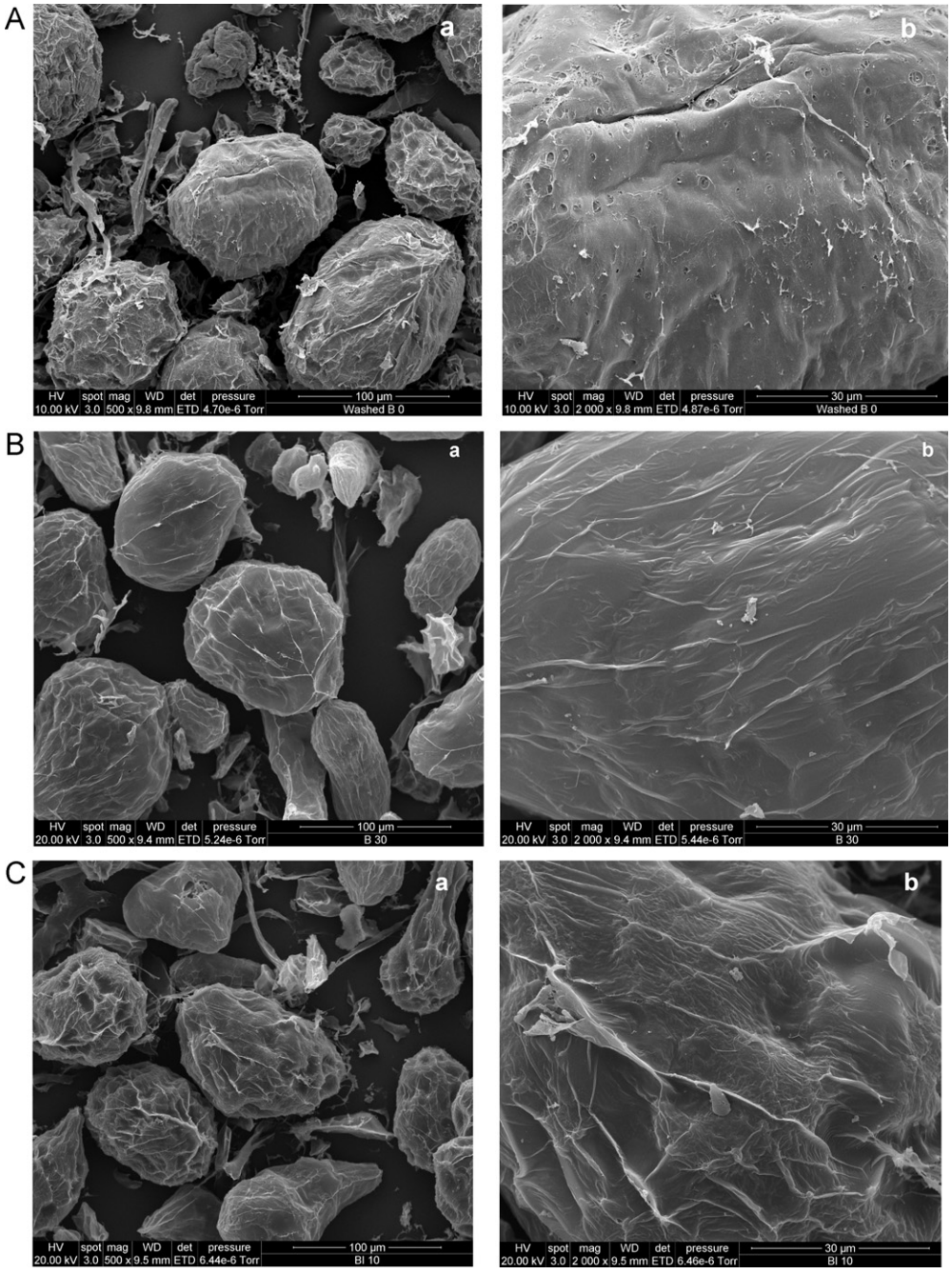

Fig. 6. (A) Scanning electron micrographs collected during *in vitro* gastric and intestinal digestion of starch in cooked navy beans, (a) undigested sample taken at 0 min showing cotyledon cells and (b) magnified view of cotyledon cell wall. (B) Scanning electron micrographs collected during *in vitro* gastric and intestinal digestion of starch in cooked navy beans: Sample taken after 30 min of gastric digestion, (a) cotyledon cells in navy bean digesta and (b) magnified view of cotyledon cell wall. (C) Scanning electron micrographs collected during *in vitro* gastric and intestinal digestion of starch in cooked navy beans: sample taken after 10 min of intestinal digestion, (a) cotyledon cells in navy bean digesta and (b) magnified view of cotyledon cell wall. (D) Scanning electron micrographs collected during *in vitro* gastric and intestinal digestion of starch in cooked navy beans: sample taken after 120 min of intestinal digestion, (a) cotyledon cells in navy bean digesta, (b) magnified view of cotyledon cell wall, and (c) broken cotyledon cells, picture not representative, see text.

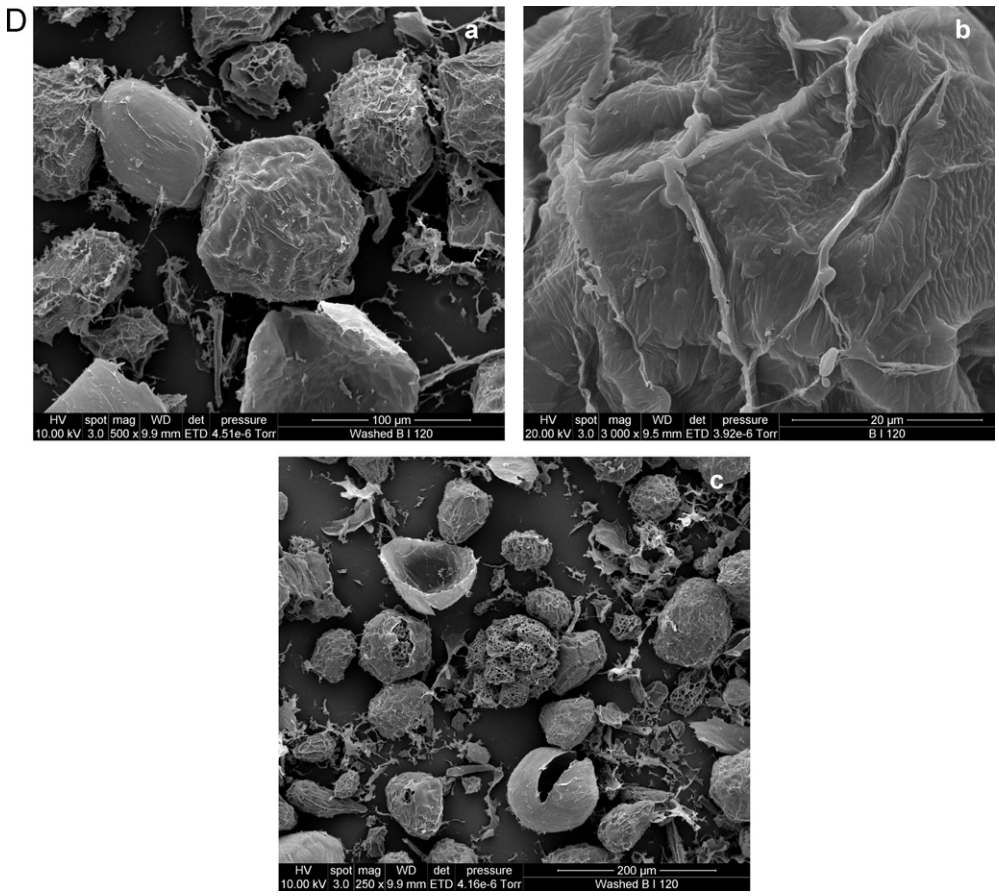

Fig. 6. (*continued*).

Cooking or processing sometimes may reduce the starch digestibility as the conformational changes in proteins may occur that could facilitate the formation of disulphide-linked polymers (Oria, Hamaker, & Shull, 1995). However, the levels of starch hydrolysis for bean flour and pure bean starch were not appreciably different from each other.

The hydrolysis of starch in cooked bean paste at the end of the digestion period was ~65% of the total, while it reached more than 90% for the cooked bean flour and starch paste (Fig. 5). The enzymatic digestibility of different starches is influenced by starch source, granule size, crystallinity, and amylose to amylopectin ratio (Singh et al., 2010). Starches with higher amylose content are more resistant towards digestion (Wolf, Bauer, & Fahey, 1999), however Zhou, Topping, Morell, and Bird (2010) reported that molecular weight of amylose also significantly affects the digestion of bean starch in the human small intestine with high molecular weight amylose preferentially digested.

3.3.2. *Microstructural characteristics of cooked navy bean digesta*

Fig. 6A–D shows freeze-dried samples taken throughout the *in vitro* digestion process of freshly autoclaved beans. The regular cellular structure of cotyledons appears to have been maintained to a good extent during and after cooking by autoclaving. A high level of cell integrity could be observed in all samples. The cotyledon cells appear to have shrunk slightly and showed indentation and wrinkles on their surface. The swelling of starch granules during autoclaving results in the enlargement of cotyledon cells due to absorption of water during cooking. This was evident in the case of undigested samples (Fig. 6A). A magnified view of the cotyledon outer cell wall of the undigested sample showed less wrinkles compared to the samples subjected to intestinal digestion. It might be the case that hydrolysis of starch during *in vitro* digestion and removal of water left a space in which the cell wall could be folded in during freeze drying, causing an even more wrinkled surface and indentation. This phenomenon was especially distinctive in cells that underwent the whole simulated digestive process (120 min). However, the possibility of some artefacts produced due to freeze drying process cannot be ruled out. The development of holes in the cotyledon cell walls during the *in vitro* digestion is nevertheless unlikely because the cell wall is composed of β-(1–4) linked D-glucose molecules and enzymes capable of hydrolysing these linkages are not present in the simulated digestive fluids. The cells

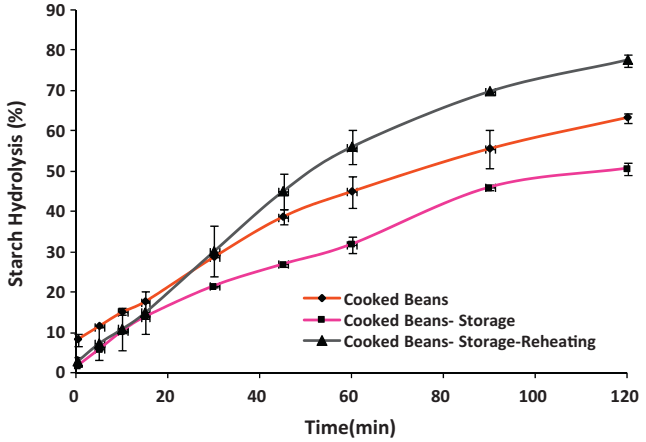

Fig. 7. Starch hydrolysis (%) during *in vitro* intestinal digestion of cooked, cooked–stored, cooked–stored–reheated navy beans.

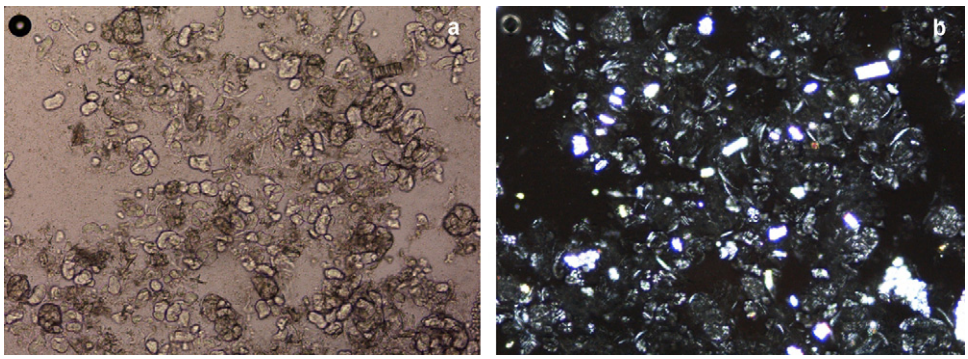

Fig. 8. Light microscopy picture of high pressure (French press) treated navy bean paste showing disrupted cotyledon cells. (A) Normal light (B) polarized light.

stay mainly intact during the enzymatic action. The *in vitro* digestion of starch therefore has to at least partially take place inside the bean cells, implying permeability of the cell wall to digestive enzymes. Fig. 6D(c) is not representative for the sample, but shows a phenomenon that occurred only in a minority of cells that might have been damaged during sample preparation or mixing in the digestion reactor. The cell wall has been completely or partly removed perhaps during preparation, and the cellular contents are exposed (Fig. 9D(d)). The sponge-like open structure has empty cavities from where starch might have been removed during digestion.

3.3.3. *Effects of storage and reheating on in vitro digestion of starch in cooked navy beans*

The influence of storage and reheating on the *in vitro* digestibility of starch in beans is shown in Fig. 7. Starch digestibility significantly decreased during storage possibly due to retrogradation of the cooked starch. Navy bean starch contains high amylose content and it has been reported that the higher amylose content of starch lowers the starch digestibility because of a positive correlation between amylose content and resistant starch formation after cooking (Singh et al., 2010). Even though storage was at room temperature and retrogradation is most pronounced at a low temperature, a clear reduction in final values of hydrolysis was observed. Reheating rendered the starch more susceptible towards enzymatic hydrolysis resulting in final values higher than the ones obtained from freshly autoclaved beans. It is important to note that the direct comparisons between the hydrolysis values obtained for the reheated bean samples and the other samples should be made with care as the conditions of reheating were different from those of the initial heating (see Section 2.6.4). During the reheating of the stored cooked beans, the gelatinization of the partially cooked starch granules may have increased. Another possibility is that the cell structure may have loosened during the initial cooking and subsequent holding period resulting in a more permeable structure after the second heating. A combination of these factors may have led to the increased rates of hydrolysis measured.

3.3.4. *In vitro digestion of starch in high pressure (French press) treated cooked navy beans*

Micrographs of the cooked and high pressure treated bean samples under normal and polarized light are shown in Fig. 8A and B. The French press treatment resulted in disruption of the cotyledon cells which released starch granules. Birefringence in granules was observed under the polarized light which confirmed the partial gelatinization of starch (Fig. 8B). Fig. 9 compares starch hydrolysis of French Press treated beans to that from freshly autoclaved beans. Compared to bean paste samples with intact cotyledon cells, the high pressure treated samples with disrupted cotyledon cells had significantly higher final values of starch hydrolysis. Similar to starch hydrolysis in bean flour and pure bean starch, a sharp increase in the rate of hydrolysis during the first 10 min of *in vitro* simulated small intestinal digestion was observed for French press treated bean paste. However, the overall and end point *in vitro* intestinal digestibility values of French press treated samples were still lower than those obtained for bean starch or bean flour. This lower digestibility may be attributed to the low degree of gelatinization in the cooked bean starch. The French Press treatment disrupted the cells, exposing the partially gelatinized starch granules to the intestinal enzymes. The gelatinized parts of the exposed starch granules were hydrolyzed quickly whereas the non-gelatinized parts were not hydrolyzed completely by the enzymes. The cell walls of bean cotyledons appear to prevent the complete swelling and gelatinization during cooking as indicated by the presence of birefringence remaining in the cooked granules under polarized light (Fig. 4). Kon et al. (1971) reported a similar result. The reduced gelatinization leads to lower starch hydrolysis values as observed in the comparison between French Press treated and only autoclaved beans (Fig. 9).

A second reason for reduced hydrolysis of starch is the intactness of cells throughout the *in vitro* digestion process which has a retarding effect on the enzymatic action. Comparative particle size measurements revealed that the particle size of bean cells does not change during *in vitro* digestion of beans (Fig. 10). Scanning electron microscopy (Fig. 6) revealed a high level of intact cells throughout and after the process. These two findings suggest that the hydrolysis of starch takes place inside the cotyledon cells. The observed intactness of cells presumably leads to a major decrease in the rate of hydrolysis. The most reasonable cause for this observation is that the partly gelatinized starch granules are

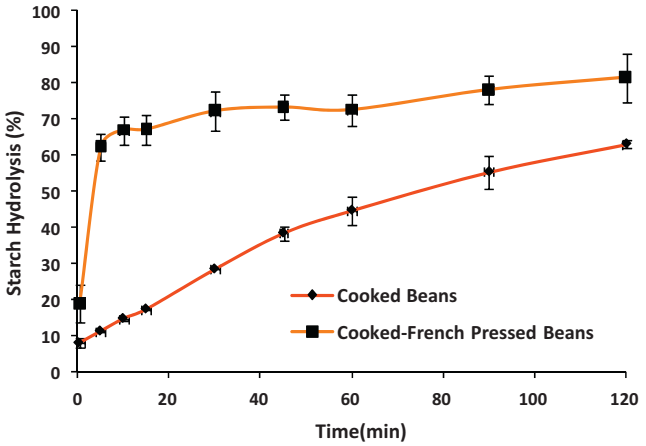

Fig. 9. Starch hydrolysis (%) during *in vitro* intestinal digestion of cooked, cooked-high pressure (French press) treated navy beans.

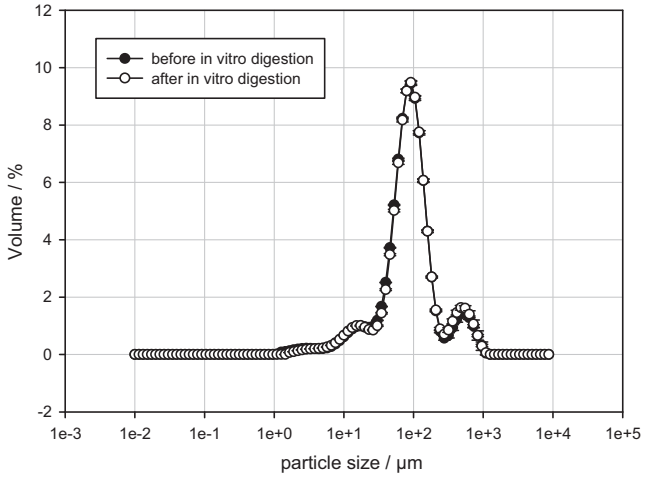

Fig. 10. Particle size distribution of freshly prepared cooked bean paste before and after *in vitro* gastric and intestinal digestion.

tightly packed inside the cotyledon cells. This situation is restricting free access of enzymes to the starch granules and therefore retarding their hydrolysis. In the case of disrupted cells the partly gelatinized starch granules are separated from each other and non-starch components. The free surface area of the starch granules is therefore increased and the enzymatic access is facilitated. Another reason for the increase in the rate of hydrolysis might be that the relatively high pressure and high shear that occurred during the French Press treatment has changed the structure of the partly gelatinized starch granules rendering them easier to hydrolyze. It can furthermore not be ruled out that the cell wall acts as a barrier for the products of starch hydrolysis. In this case the dextrins resulting from the initial hydrolytic action of α-amylase would have to be hydrolyzed to a certain degree, decreasing their size before the resulting oligosaccharides can diffuse out of the cells. Storage and reheating of stored beans clearly affected the final values of hydrolysis but the rate of hydrolysis did not change to a major extent (Fig. 7). Stored and aged starch gels show a characteristic melting endotherm of recrystallized amylopectin around 55–60 °C which is absent in fresh starch gels and breads (Eliasson, 2006). This suggests that reheating of precooked and stored (e.g. canned beans) to low but acceptable temperatures might preserve the lower final value of starch hydrolysis which in turn would lead to a higher content of resistant starch. The stability of cotyledon cell walls in navy beans prevent the complete gelatinization of starch and is therefore responsible for the resistant nature of bean starch and that the intactness of cells throughout the simulated digestive process somehow retards the hydrolysis of starch and thereby the availability of glucose.

3.3.5. Influence of in vitro digestion on particle size of navy bean pastes

The samples taken before and after the *in vitro* digestion for particle size analysis showed almost identical particle size patterns (Fig. 10). It can therefore be concluded that no major changes in the size of particles in bean paste occurred. The peak of the actual particle sizes of ~100 μm is in good accordance with the size of bean cells in scanning electron microscopy pictures as seen in Fig. 9 whereas the peak around 500 μm might represent fragments of the hull of bean cotyledons. Similar results were obtained for all other samples of bean pastes with non disrupted cells. Storage and/or reheating did not affect the particle size (data not shown).

4. Conclusion

Cotyledon cell walls of navy beans impose restrictions on swelling and gelatinization of bean starch during cooking. The incomplete gelatinization of starch granules ultimately reduces the rate and extent of starch hydrolysis measured as glucose release during 120 min of *in vitro* digestion with simulated gastric and small intestinal fluids. The stability of cotyledon cells and the small surface area of the starch granules which are tightly packed inside the cells also appear to restrict the free access of amylolytic enzymes during *in vitro* digestion. Experiments with stored and reheated beans suggest that the extent of gelatinization and availability of water during cooking could be a major factor influencing starch hydrolysis during *in vitro* digestion.

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