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Digestion of isolated legume cells in a stomach-duodenum model: three mechanisms limit starch and protein hydrolysis†

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Retention of intact plant cells to the end of the small intestine leads to transport of entrapped macronutrients such as starch and protein for colonic microbial fermentation, and is a promising mechanism to increase the content of resistant starch in diets. However, the effect of gastro-intestinal bio-mechanical processing on the intactness of plant cells and the subsequent resistance to enzymatic digestion of intracellular starch and protein are not well understood. In this study, intact cells isolated from legume cotyledons are digested in a laboratory model which mimics the mechanical and biochemical conditions of the rat stomach and duodenum. The resulting digesta are characterised in terms of cell (wall) integrity as well as intracellular starch and protein hydrolysis. The cells remained essentially intact in the model with negligible (*ca.* 2–3%) starch or protein digestion; however when the cells were mechanically broken and digested in the model, the hydrolysis was increased to 45–50% suggesting that intact cellular structures could survive the mixing regimes in the model stomach and duodenum sufficiently to prevent digestive enzyme access. Apart from intact cell walls providing effective barrier properties, they also limit digestibility by restricting starch gelatinisation during cooking, and significant non-specific binding of α-amylase is observed to both intact and broken cell wall components, providing a third mechanism hindering starch hydrolysis. The study suggests that the preservation of intactness of plant cells, such as from legumes, could be a viable approach to achieve the targeted delivery of resistant starch to the colon.

1. Introduction

Legumes are known to be nutritionally rich foods in part due to their high protein content[1] as well as slowly digested starch.[2] The rigid cell structure of legumes acts as a physical barrier for diffusion of enzymes inside the cell limiting the hydrolysis of enclosed macronutrients, thus decreasing the glyceamic response under both *in vitro*[3] and *in vivo* condtions.[4,5] Macronutrients which remain unhydrolysed (resistant) at the end of the small intestine pass into the colon, with well-documented health benefits, particularly for resistant starch.[6,7] However, in processed foods, due to possible breakdown of cellular structure by physical force or thermal energy, the enzyme susceptibility of entrapped nutrients could be increased.[8–10]

Apart from physical entrapment limiting the diffusion of enzymes, the intact cellular structure also limits the amount of water inside the cell, thereby restricting the swelling of starch granules during thermal processing.[11] Thus, starch within cooked legume cells or tissue shows a 'maltese cross' birefringent pattern when observed by polarised light microscopy as well as exhibiting an endothermic gelatinisation peak by differential scanning calorimetry[3,11] suggesting that retention of some granular structure could also reduce digestive enzyme susceptibility compared to gelatinised starches.

A few *in vivo* studies of legume food digestion have shown evidence for survival of some legume cells to the end of the small intestine in the digestive tract of rats[4] and humans.[5] Noah *et al.*[5] hypothesized that the mechanical action and acidity in the stomach might lead to the breakdown of cell walls releasing entrapped starch and proteins, however, there are no reports on the behaviour of legume cells in the stomach and duodenum (first section of the small intestine) under

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in vivo conditions. Previous *in vivo* studies used whole or processed legumes representing (after chewing) a mixture of intact cells and broken cells as well as released starch and proteins.

In this study, in order to address the integrity of cells under gastric-duodenal conditions, a Dynamic *In vitro* Rat Stomach Duodenum (DIVRSD) model, that mimics the mechanical and biochemical conditions occurring in the rat stomach and duodenum[12] is used to study the physical integrity as well as the enzymic hydrolysis of entrapped nutrients using isolated legume cells[3] that are devoid of released starch/proteins and broken cells. As the integrity of legume cells is the limiting factor affecting the rate and extent of starch/protein hydrolysis,[3] a dynamic model such as the DIVRSD is appropriate.

It has been previously reported that the DIVRSD model captures the main mechanical and bio-chemical events occurring in the rat stomach and duodenum.[12,13] The rate and extent of food digestion in the DIVRSD model was found to be statistically similar to that found for rats *in vivo*.[13] Whilst the rat model does not represent human dimensions, the mechanical forces and biochemical mechanisms involved in gastro-duodenal digestion in rats and humans are similar.[13] Thus use of the DIVRSD model to study the behaviour of food can be justified as an alternative to static,[14] or dynamic[15] *in vitro* models widely used for such studies.

In addition to physical entrapment, the binding of enzymes to legume cell walls was investigated, as non-specific binding of enzymes to cell wall substrates can reduce the enzyme available for hydrolysis of starch[16] and proteins. The limited enzyme digestibility of starch (and protein) in legume cells is thus described as a combination of at least three mechanisms *viz*. intact cells encapsulating the starch and protein, nonspecific binding of enzymes to cell wall components, and limited swelling of cell wall encapsulated starch during cooking.

2. Materials and methods

Seeds of four legumes, chickpea (CP), pea (P), mung bean (MB) and red kidney bean (RKB) were procured from a local shop in Brisbane, QLD, Australia and used as such for isolation of intact cells, starch and cell wall components.

Purified α-amylase (Sigma A6255 from porcine pancreas), pancreatic amylase (Sigma A-3176 from porcine pancreas), pepsin (Sigma P-6887 from gastric porcine mucosa), pancreatin (Sigma P-1750 from porcine pancreas), porcine bile extract (Sigma B8631), viscozyme® L (Sigma, V2010), trichloroacetic acid (TCA) (Sigma 522082), fluorescein isothiocyanate isomer I (FITC, F7250, Sigma), Calcofluor-white stain (Sigma, 18909), 4-hydroxybenzhydrazide (PAHBAH, Sigma, H9882), azocasein protease substrate (Sigma A2765) and trizma base (Sigma TR1503) were purchased from Sigma-Aldrich. Enzyme glucose reagent (GOPOD) (TR15103) and Pierce BCA protein assay kit (23225) was purchased from Thermo Scientific, Sydney, Australia, and amyloglucosidase (E-AMGDF100) and total starch assay kit (K-TSTA) was obtained from Megazyme, Bray, Ireland.

2.1 Preparation of intact cells, starch–protein fractions and cell wall components

The isolation of cooked intact legume cells, starch and protein fractions as well as cell wall components was carried out as described previously.[3] Briefly, legume seeds were hydrated overnight in ice-chilled water to facilitate the swelling and loosening of outer hulls. Hulls were removed with gentle hand abrasion and then washed in running water. The dehulled legumes were heated at 95 °C for 1 hour, gently mashed using a mortar and pestle and separated with sieves of different size ranges to obtain isolated cooked intact fractions. For the preparation of broken cells, the isolated cooked intact cells were broken by applying a shear mixing force using magnetic stirrer bars that break cell walls and release the entrapped starch and proteins. Briefly, around 15 grams (wet basis) intact cells were mixed with 10 mL Milli-Q water (containing 0.02% sodium azide) overnight at 37 °C using a 12 mm × 4.5 mm micro polytetrafluoroethylene (PTFE) coated magnetic stirrer bar at 1400 rpm.

In order to obtain starch–protein fractions and cell wall components, chickpea seeds after dehulling, were heated at 60 °C for 1 hour, mashed gently and sieved to obtain isolated raw intact structures, which were further broken and allowed to settle in excess of water in a beaker, collected and sieved through different sieve sizes. Material passing through a 150 μM sieve but retained on 32 μM sieve was classified as cell walls, whereas sedimented material that passed through the 32 μM sieve contained starch and protein released from cells. Confocal microscopy was used to confirm the complete breakage of cell wall structure, the purity of cell walls and the separation of starch–protein fractions from cell walls. The moisture, total starch and protein contents were determined as discussed in following section 2.1.1 and the final dry matter content for all the samples was adjusted to 25%. The samples were then incubated at 37 °C in a water bath with very gentle stirring (<100 rpm) for 30 min to assure homogeneity of samples before *in vitro* digestion in the DIVRSD model.

2.1.1 Determination of total starch, total protein and moisture contents. The total starch content was determined using the Megazyme Total Starch Assay Kit (K-TSTA) following the manufacturer instructions. The intact cell structure was damaged overnight by mixing with stirrer bars as described in section 2.1 so as to avoid underestimation of starch content due to intact cellular structure.[3] The protein content for intact legume fractions and starch–protein samples were analysed by combustion (Dumas) protocol using a LECO TruSpec analyser. The moisture content for intact/broken legume fractions, starch–protein sample and cell walls were determined using oven drying at 95 °C overnight under vacuum. All analyses were performed in duplicate and results expressed on percentage dry basis.

2.2 *In vitro* digestion using DIVRSD model

The *in vitro* digestion representing the gastric and small intestinal phases for determination of hydrolysis rate and

extent of starch and protein in isolated cooked legume cells was carried out for both intact and broken cells using the DIVRSD model as described below. The detailed working principle and methodology of the model has been published recently.[12]

Intact or broken legume cells (10 g) adjusted to 25% w/w dry matter (giving a homogeneous pasty consistency) were placed in 50 mL falcon tubes and 1 mL of α-amylase (Sigma A3176) (0.5 U per mg starch) in carbonate buffer (pH 7.0 containing 14.4 mM sodium hydrogen carbonate, 21.1 mM potassium chloride, 1.59 mM calcium chloride and 0.2 mM magnesium chloride) was added. The tubes were then incubated for 30 s at 37 °C and the contents introduced into the stomach region of the DIVRSD model with controlled gastric juice (pepsin 188 U per mg protein in 0.02 M HCL, pH 2) secretion rates as follows:

Time (min)	0–10	10–20	20–40	40–60	60–120
Rate (μL min^{-1})	10	20	40	20	10

Samples were then continuously (~90 μL min^{-1}) passed into the duodenum to undergo simulated intestinal digestion. There, 30 μL per min pancreatin enzyme mix in phosphate buffered saline buffer (pH 7.2, 0.2 M) (containing 2 mg per mL pancreatin, 28 unit of amyloglucosidase and 5 mg per mL bile salt) was continuously added to the digestion process. A constant enzyme to substrate ratio was kept during the whole digestion process. The DIVRSD model system was maintained at 37 °C with compression-rolling extrusion creating 3 contractions per minute in the stomach while a rolling extrusion produced 36 contractions per minute in the duodenum chamber equivalent to a force of *ca.* 3.5 N (ref. 12) in each chamber. The digested aliquot from the duodenal phase was collected in 15 mL falcon tubes at defined intervals up to 120 min (0, 15, 30, 60, 90 and 120 min) and placed into a boiling water bath for 5 min to stop the enzymatic activity. The volume collected was measured, the tube contents gently shaken for homogenous sampling, and immediately analysed for starch and protein hydrolysis as described below.

2.2.1 Determination of starch hydrolysis. Digested aliquots (1.5–2 mL) from duodenal phases (section 2.2) were centrifuged at 6000*g* for 60 s. Since the pancreatin enzyme mix (section 2.2) contained the amyloglucosidase, glucose was the sole end-product. The glucose content in the supernatant was determined using a glucose oxidase colorimetric analysis kit with detection at 505 nm (Pharmacia LKB-Ultrospec III, UK). A factor of 0.9 was used to convert glucose released to calculate % starch hydrolysis.

2.2.2 Determination of protein hydrolysis. The protein content in the supernatant from the duodenal digestion phase (section 2.2) was measured using the Pierce BCA protein assay kit (Product No. 23225) according to the manufacturer's protocol. To separate the small peptides and amino acid residues produced by proteolysis,[17] 100 μl trichloroacetic acid (TCA, 24%) solution was added to an equal volume of digested aliquot making 12% (w/w) TCA in the final mixture. The mixture was then vortexed, incubated for 15 min at room temperature and centrifuged at 15 000*g* for 15 min. Protein in the supernatant was determined using bovine serum albumin as standard. The results were expressed as the percentage protein hydrolysis based on the amount of protein in the supernatant compared with the total protein content.

2.2.3 Determination of amylase and protease activities. For determination of amylase activities in pancreatin and commercial amylase preparations, α-amylase equivalent to approximately 0.02 unit per mg of cooked starch was used to hydrolyse 10 mL of cooked potato starch (1% soluble potato starch cooked at 95 °C for 15 min in PBS buffer pH 7.2, 0.2 M) under mixing conditions. At defined time intervals between 3 and 30 min, 100 μL aliquot was retrieved and mixed with 300 μL of 0.3 M sodium carbonate in ice bath to stop enzyme activity and centrifuged at 6000*g* for 60 s. The maltose concentration in the supernatant was determined using the *para*-hydroxybenzoic acid hydrazide (PAHBAH) assay and the activity expressed as nmol min^{-1} mL^{-1}. For this assay, 1 mL of a freshly prepared mixture (9 : 1) of 0.5 M sodium hydroxide and 5% (w/v) PAHBAH in 0.5 M hydrochloride was added to 100 μL of supernatant. The mixture was then heated in boiling water for 5 min, cooled to room temperature and the absorbance was measured at 410 nm using maltose as standard.

For the determination of protease activity in pancreatin, azocasein digestion assay with some modifications due to Coêlho *et al.*[18] and Benitez, Silva, & Finkelstein[19] was used. Briefly, 100 μL of azocasein (5 mg mL^{-1}) in 100 mM Tris (T1503 Sigma, pH 8.0) was incubated with 100 μL of enzyme solution for 30 min at 37 °C. The reaction was stopped by the addition of 750 μL of 5% trichloroacetic acid (TCA). After centrifugation at 2000*g* for 10 min, the trichloroacetic acid supernatant was added to a 0.5 N NaOH using a 1 : 1 (v/v) ratio, and the optical density was determined at 440 nm. The blank was obtained by mixing the TCA to the substrate prior to the enzyme addition. One proteolytic unit was defined as the amount of the enzyme that released 1 μg of tyrosine per min under 37 °C and pH 8.0. L-Tyrosine standard curve was used to calculate enzyme activity using 10 to 150 mg mL^{-1} tyrosine in 100 mM Tris (pH 8.0).

2.3 *In vitro* starch hydrolysis of starch–protein–cell wall component mixtures

Starch–protein and cell wall components from isolated raw intact chickpea cells were mixed at different ratios (1 : 0.5 to 1 : 2) and monitored for starch and protein hydrolysis using static *in vitro* digestion.[3,20] For amylase and protease digestion of starch–protein–cell wall component mixtures, 15 mg per mL of pancreatin and 80 unit of amyloglucosidase per mg of starch were used. Starch–protein and cell wall components of varying amounts were weighed (100 mg in total) to obtain desired ratios in 50 mL falcon tubes followed by addition of 10 mL of phosphate buffered saline (pH 7.2, 0.2 M containing 0.02% (w/v) sodium azide). The mixture was incubated at 37 °C under constant stirring (100 rpm) with a 3 mm × 6 mm

magnetic stirrer bar. After 0, 5, 10, 15, 30, 45, 60, 90 and 120 min, a 100 μL aliquot was pipetted, and mixed with respective stop solutions for starch and protein determination as described in section 2.2.3.

In a separate experiment, the effect of enzymatic removal of the cell wall from cooked intact chickpea cells on starch hydrolysis was investigated. For this, slight modifications to Guan & Yao's[21] methodology was employed for viscozyme® L treatment of intact chickpea cells. Briefly, 1 g of intact chickpea cells were added to 10 mL of Milli-Q water and mixed to obtain a homogenous dispersion. The pH of the solution was adjusted to 5.0 using 0.5 M HCL and viscozyme® L (50 FBG, 0.5 mL) was added. The activity of viscozyme® L was 100 Fungal Beta-Glucanase Units (FBGU) per mL, in which 1 FBGU is the amount of enzyme required under the standard conditions (30 °C, pH 5.0 and 30 min reaction time) to hydrolyze barley β-glucan to reducing carbohydrates, with a reducing power corresponding to 1 μmol glucose per min. The mixture was then incubated in a water bath at 40 °C for 30 min with continuous stirring at 100 rpm. The samples were centrifuged at 2000*g* for 5 min, supernatant was discarded, the residue mixed with 10 mL of phosphate buffered saline buffer (pH 7.2, 0.2 M containing 0.02% (w/v) sodium azide) and digested with α-amylase (Sigma A6255) as described above. Digestion in a control (without the amylase) was also monitored.

2.4 Observation of cell walls and starch granules

Cell walls and starch granules trapped inside isolated cooked chickpea cells, before and after viscozyme® L treatment, were observed by a double labelling technique employing FITC (specific to starch) and calcofluor-white (specific to cell walls) using a confocal microscope (LSM 700, Carls Zeiss, Germany) at 488 and 405 nm excitation wavelength respectively. Control (no viscozyme® L treatment), viscozyme® L treated and digested samples were stained at room temperature for 1 h using FITC, centrifuged and then rinsed three times with Milli-Q water to remove the excess dye. Confocal microscopic observation was made after treating stained samples with calcofluor-white.

2.5 Amylase–starch–cell wall component interactions

A solution depletion method[16] was used for investigation of binding interactions between amylase and cell wall components under non-hydrolysing conditions at 0 °C. In brief, various ratios of starch to cell wall components (1 : 0.1 to1 : 2) from isolated raw intact chickpea were incubated with 0.5 units of α-amylase (Sigma A6255) in 10 mL of phosphate buffered saline (pH 7.2, 0.2 M) for 30 minutes with intermittent mixing at 0 °C (ice water bath). An aliquot (250 μL) was then transferred into a micro-centrifuge tube, centrifuged at 6000*g* for 60 s and 50 μL of supernatant (containing unbound enzyme) was then used to hydrolyse 10 mL of cooked starch (1% soluble potato starch cooked at 95 °C for 15 min in phosphate buffered saline (pH 7.2, 0.2 M) under mixing). This enzyme volume is equivalent to 0.02 unit of α-amylase per mg of cooked starch, assuming none of the enzyme had bound to cell wall components. At defined time intervals between 3 and 30 min, 100 μL aliquots were mixed with 300 μL of 0.3 M sodium carbonate in an ice bath to stop the enzymatic reaction, and immediately centrifuged at 6000*g* for 60 s. The supernatant was used to determine the reducing sugar content using the PAHBAH assay (described in section 2.2.3) and expressed as % starch hydrolysis using a factorial of 0.95.

2.6 Fluorescent labelling of α-amylase

FITC labelled α-amylase (FITC-AA) was prepared as described previously.[22] Briefly, α-amylase was labelled with fluorescein isothiocyanate isomer I at 10× molar excess in carbonate buffer (0.1 M, pH 9). A desalting column (Sephadex, PD-10) eluted with phosphate buffered saline (pH 7.2, 0.2 M) was used to separate the unbound FITC from the conjugate. Following labelling, the enzyme solution was immediately aliquoted and frozen for storage. The enzyme was defrosted immediately prior to use.

2.7 Visualization of α-amylase bound to starch–protein–cell wall components

The binding of FITC-AA conjugate to starch–protein and starch–cell wall components was monitored at 0 °C following the method described previously.[22] A 1% dispersion of starch–protein and starch–cell wall components (90% starch/protein-10% cell wall component; 10 mL) in phosphate buffered saline (pH 7.2, 0.2 M) in 50 mL falcon tubes was immersed fully in an ice water bath. The dispersion was equilibrated for 15 min with continuous stirring at 100 rpm followed by addition of 50 μL of FITC-AA conjugate and incubated for a further 30 minutes. After 30 min, 100 μL aliquots were transferred to 1.5 mL microcentrifuge tubes and immediately centrifuged at 6000*g* for 60 s. The residue (starch–protein/starch–cell wall components pellet) was recovered after discarding the unbound enzyme in the supernatant. The enzyme bound to starch–protein/starch–cell wall components was observed by confocal microscopy (LSM 700, Carls Zeiss, Germany) using an excitation wavelength of 488 nm for FITC.

2.8 Data fitting to first order kinetics

Progress curves of starch and protein hydrolysis were fitted using first order[23,24] and LOS models[25] and the digestion rate coefficient k determined as described elsewhere.[25,26] As discussed previously,[3] the rate coefficient varies depending on the model used as well as the amount of enzyme and physical conditions of the substrates (*e.g.* intact *vs.* broken). The use of two different models allows this variation to be captured.

3. Results

The digestive behaviour of isolated legume cells under conditions similar to those present in the stomach and small intestine, as well as the mechanisms controlling starch and protein hydrolysis under these conditions were studied using (A): a laboratory model which mimics mechanical and biochemical conditions present in the rat stomach and duode-

num with analysis of starch and protein hydrolysis, and (B): model systems consisting of mixtures of starch and cell wall components with analysis of starch digestion using α-amylase.

3.1 Hydrolysis of starch and protein for intact and broken cells

The chemical composition of different legume fractions and starch–protein samples are presented in Table 1. It is evident in Table 1 that despite differences in total starch and protein contents between chickpea and pea, the ratio of starch to protein (S/P) is similar. Due to relatively higher amount of total protein in mungbean as opposed to red kidney bean, the former showed a lower S/P. Concerning the enzyme activities, it was found that amylase activity from porcine pancreas in Sigma A6255, Sigma A3176 and P-1750 were found to be 9.99, 9.04 and 3.94 nmol min^{-1} mL^{-1} respectively. Likewise, protease activity in pancreatin P-1750 was determined to be 21.28 $mmol^{-1}$ min^{-1} mL^{-1}.

As shown in Fig. 1, starch and protein hydrolysis rates are higher when the cellular structure is physically broken compared to intact cells where very slow hydrolysis is observed. In both cases, the starch and protein digestibility of mung bean cells are higher compared to other legumes studied. Starch hydrolysis (Fig. 1a) extents after digestion for 120 min for broken cells are *ca.* 51, 48, 43 and 32% for mung bean, pea, chickpea and red kidney bean respectively, with corresponding calculated first order rates (k value, min^{-1}) of 0.0056, 0.0052, 0.0044 and 0.0031 (Fig. S2a†), however the corresponding values from LOS plots are 0.0194, 0.0201, 0.0177 and 0.018 respectively (Fig. S2b†). It is to be noted that we exclude the calculation of C∞ from LOS equations as the mechanism of enzymic action is different to that for intact cells. The physical barrier of cell walls is the primary rate limiting step for the intact cells whereas starch structural features and cell wall fragments inhibiting the enzyme action control the rate of hydrolysis in the case of broken cells *i.e.* C∞ should be 100%.

Table 1 Chemical composition of different legume fractions and starch–protein mixture

		Percentage (db)		
Legume fractions	DM	Total starch (S)	Total protein (P)	Ratio (S/P)
Chickpea	21.33 ± 1.04 (18.32 ± 1.09)	57.19 ± 0.48	20.55 ± 0.48	2.78
Pea	22.05 ± 0.25 (20.19 ± 1.06)	64.76 ± 1.76	23.19 ± 0.27	2.79
Mung bean	20.31 ± 1.52 (19.71 ± 1.10)	62.14 ± 0.29	25.50 ± 0.42	2.46
Red kidney bean	19.30 ± 0.59 (20.22 ± 0.65)	57.29 ± 0.85	21.54 ± 0.21	2.66
Starch–protein	20.04 ± 0.82	90.17 ± 1.85	9.60 ± 0.99	9.39
Cell walls	19.51 ± 0.21	—	—	—

DM represents the % dry matter where the values in the brackets represents dry matter contents in the broken cells. The values are the means of duplicate.

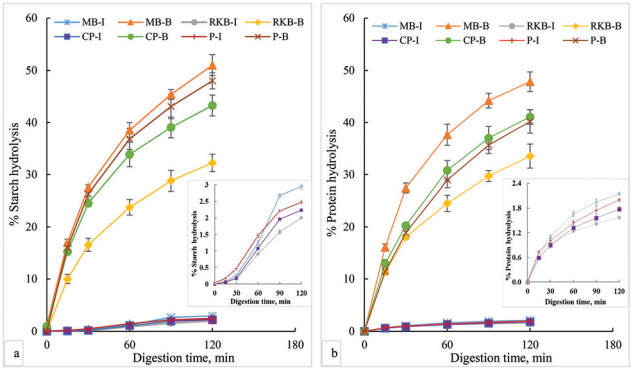

Fig. 1 Progress curves of starch (A) and protein (B) hydrolysis for intact (I) and broken (B) cells in rat stomach duodenum model where MB = mung bean, RKB = red kidney bean, CP = chickpea and P = pea. It is evident that higher starch and protein hydrolysis values were observed for mung bean compared to other legumes both in intact and broken cells. Insets show magnifications of progress curves for intact cells. The corresponding first order and LOS plots are presented in ESI Fig. S1 and S2.†

The same order of susceptibility to 120 min starch hydrolysis of around 3.0, 2.5, 2.2 and 2.0% respectively for intact mung bean, pea, chickpea and red kidney bean was found (Fig. 1a) with corresponding calculated rates of *ca.* 0.0002, 0.0002, 0.0002 and 0.0003 using the first order model (Fig. S1a†). However, the LOS model did not fit well for intact cells (Fig. S2b†) suggesting the overall starch hydrolysis is not truly a first order kinetic process in this system, consistent with the observed hydrolysis being due to a small fraction of damaged 'intact' cells behaving like broken cells.

Protein hydrolysis extent (Fig. 1b) after 120 min digestion was 48, 41, 40 and 33% for broken cells from mung bean, chickpea, pea and red kidney bean respectively. The calculated rate coefficients (k value, min^{-1}) for protein hydrolysis using the first order model (Fig. S2c†) are 0.0052, 0.0043, 0.0042 and 0.0032, while the values obtained using the LOS model (Fig. S2d†) are 0.0224, 0.0175, 0.0181 and 0.016 respectively for these samples. Both models gave comparatively good fits to the protein hydrolysis data for broken cells.

Protein hydrolysis (Fig. 1b) values after120 min digestion are 2.2, 2.0, 1.7 and 1.6% for intact mung bean, pea, chickpea and red kidney bean respectively. In contrast to broken cells, the data for protein hydrolysis in intact cells are not fitted using both the kinetic models. Interestingly, starch and protein are hydrolysed to similar extents within the DIVRSD model.

Although the gastric condition used (pH 2) is more favourable for proteolytic enzymes (pepsin), alpha amylase is still active and retains some amylolytic activity even at pH 2.[20] Kinetic analysis using first order plots (Fig. S1a and c†) and LOS model (Fig. S1b and d†) revealed that starch and protein entrapped inside intact cell walls do not follow general first order kinetics in contrast to broken cells (Fig. S2†), as can be inferred from Fig. 1A for starch.

3.2 Confocal laser scanning micrographs of intact and broken legume cells

Fig. 2 shows confocal laser scanning micrographs of isolated intact mung bean (A, B) and red kidney bean (C, D) cells stained with calcofluor-white before and after 120 min digestion.

It is seen that the isolated cells of both mung bean and red kidney beans appear intact even after 120 min of hydrolysis in the DIVRSD model. This retention of cellular structure clearly hindered the amylase and protease digestion of starch and protein respectively as shown in Fig. 1.

Representative images of physically broken mung bean and kidney bean cells stained with calcofluor-white are shown in Fig. 3. Mung bean cell walls are more fragile and broken to finer particles compared to red kidney bean samples (Fig. 3A) under the mixing conditions used. After being in the DIVRSD model for 120 min, the mung bean cells are further degraded whereas for red kidney bean cells, microscopic images of control and 120 min digesta are similar. This suggest that cell wall fragility is a biological variable that may differ among legume samples. The more fragile nature of the mung bean cells studied here is likely to be one of the reasons behind the higher digestibility of starch and protein for both intact and broken mung bean cells compared to other cells. However as only one sample of each legume was studied, these results cannot be generalised to other samples/varieties of the legumes tested.

3.3 Localization of FITC labelled α-amylase

Isolated legume cells (intact and broken) were incubated with FITC labelled α-amylase. It is observed that in intact cells, the enzymes are predominantly bound to the outer surface of cells (Fig. 4), but in the case of broken cells (Fig. 5) the enzymes are dispersed among the released starch and cell wall fragments suggesting affinity of enzymes towards both catalytic (starch) and non-catalytic (cell wall) substrates.

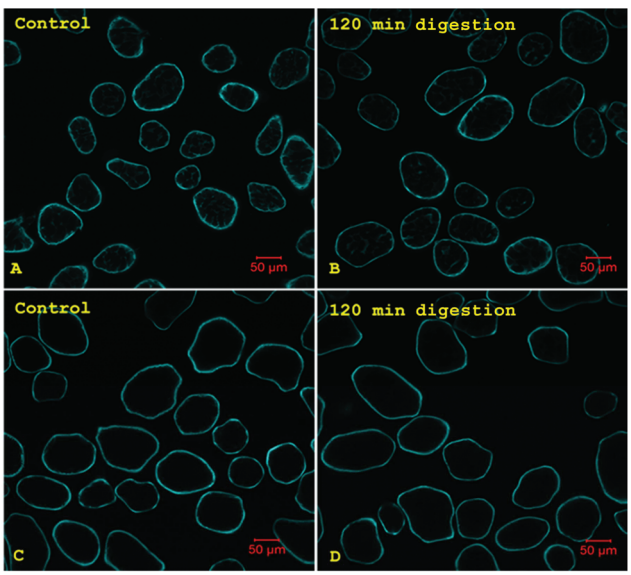

Fig. 2 Confocal laser scanning micrographs of isolated intact mung bean (A, B) and red kidney bean (C, D) cells stained with calcofluor-white. Cell walls labelled with the dye appear intact without visible cracks or breakage.

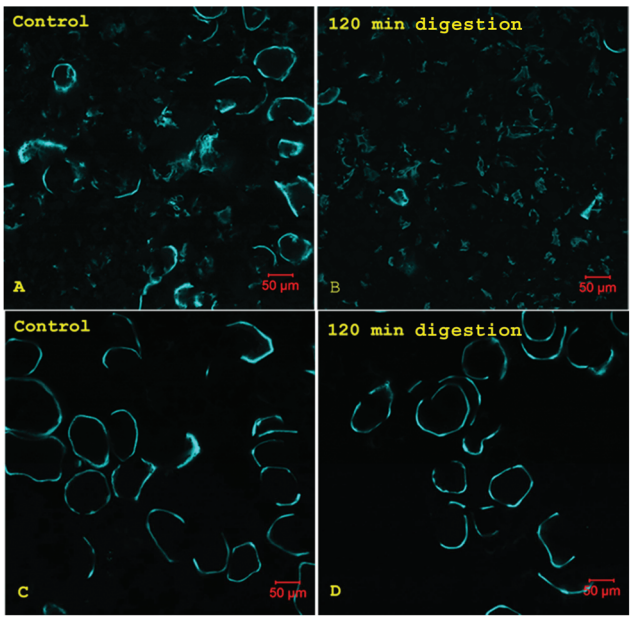

Fig. 3 Confocal laser scanning micrographs of isolated broken mung bean (A, B) and red kidney bean (C, D) cells stained with calcofluor-white.

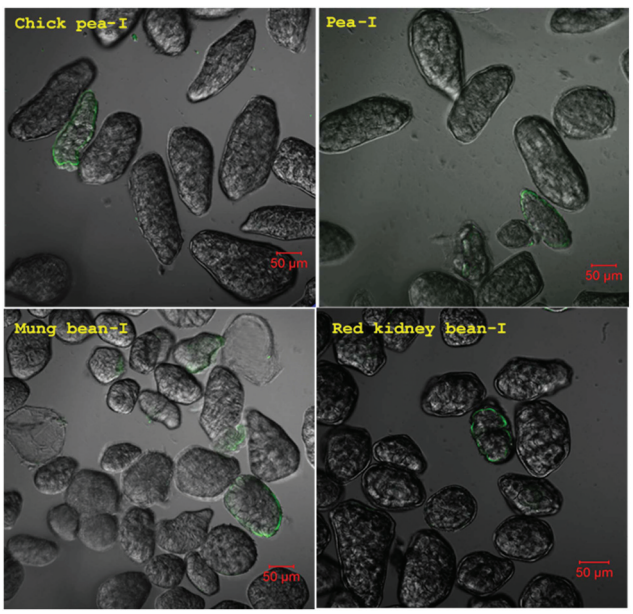

Fig. 4 Confocal laser scanning micrographs: FITC labelled α-amylase (50 μL; 9.99 nmol min^{-1} mL^{-1}) binding to cell walls in intact (I) legume cells (10 mg in 10 mL). As intact cell walls provide a barrier to α-amylase, the enzyme is localized around the cell walls only and could not penetrate inside to hydrolyze internal contents.

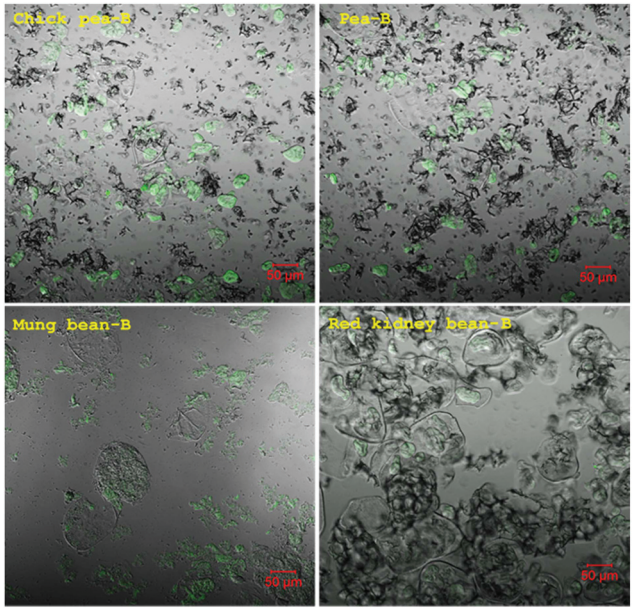

Fig. 5 Confocal laser scanning micrographs: FITC labelled α-amylase binding to both starch and cell wall fragments in broken (B) legume cells. It is evident that α-amylase not only binds to starch granules but also to cell wall components in all legume types.

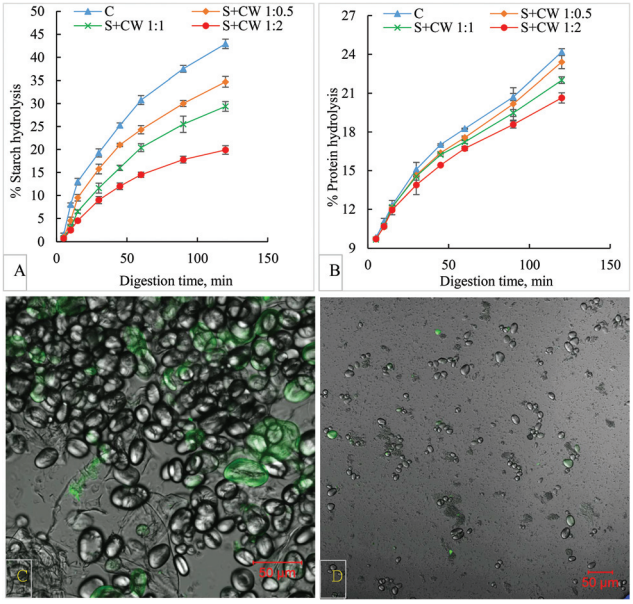

Fig. 6 Progress curves for starch (A) and protein (B) hydrolysis for isolated raw chickpea starch/protein without (control, denoted by C) and with added cell wall components (proportion of 1 : 0.5–2). It is observed that with added cell wall components, starch hydrolysis is decreased markedly (A) whereas protein hydrolysis is only slightly affected (B); (C, D) confocal laser scanning micrographs: FITC labelled α-amylase binding to starch and cell wall components (C) and to starch and proteins (D). The corresponding first-order and LOS plots are presented in ESI Fig. S2a, b and Fig. S3a, b.†

3.4 Non-specific binding of α-amylase to proteins and legume cell walls

It is observed (Fig. 6A) that the addition of cell wall components to isolated legume starch (starch : cell wall components = 1 : 0.5) decreased starch hydrolysis to 35% from 43% after 120 min digestion. Further increase in the amount of cell wall components to 1 or 2 times the amount of starch resulted in further decreases in 120 min starch hydrolysis to *ca.* 30 and 20% respectively. Considering the overall rate coefficient, the addition of cell wall components lowered the rate from an initial value of 0.0058 (control) to a final value of 0.0027 (1 : 2) as revealed using first order kinetic model analysis (Fig. S3a†). A decrease in overall rate from 0.0179 to 0.0146 for these samples was also demonstrated using the LOS model (Fig. S3b†).

Likewise but to a lesser extent, as evident in Fig. 6B, added cell wall components decreased protein hydrolysis from 24% (control) to 20% (1 : 2) with corresponding estimated k values of 0.0015 to 0.0012 (first order, Fig. S4a†) or 0.0198 to 0.0188 (LOS model, Fig. S4b†) respectively, suggesting the higher affinity of α-amylase towards non-specific cell wall binding compared to proteases. This difference in relative properties of digestive enzymes needs further investigation. Fig. 7A shows the activity against cooked soluble potato starch of un-bound α-amylase, retrieved from incubation mixtures consisting of cell wall components and/or starch granules/proteins.

The enzyme activity of residual α-amylase collected from the supernatant was found to be considerably lower than that of control (enzyme in the absence of cell wall components or starch granules/proteins), suggesting binding of α-amylase to cell wall components as well as starch and proteins. With control (α-amylase alone), % residual amylase action was highest (7.6%) compared to starch (6.2%) or cell wall components-starch (4.7–5.5%) added samples (Fig. 7A). The decrease in amylase action was more pronounced (approx. 2.4 times) with higher amounts of cell wall component added as shown in Fig. 7A with corresponding calculated rates (k value, min^{-1}) of 0.1264 (E) and 0.0534 (S + CW 1 : 2) for these samples. Thus, α-amylase not only binds to its preferred substrate-starch, but also shows strong binding affinity to cell wall components, as shown previously for cellulose and wheat bran.[16]

3.5 Effect of removal of cell wall on legume starch hydrolysis

In order to probe the effect of cell wall removal on starch hydrolysis, cooked intact chickpea cells were treated with viscozyme® L for 40 min followed by 120 min amylase digestion. Starch hydrolysis (Fig. 7B) in intact chickpea cells that had been previously incubated with viscozyme® L was found to be considerably greater (*ca.* 45%) than that of control (*ca.* 1%, no viscozyme® L treatment). Whilst kinetic analysis using the LOS model did not fit the experimental data ($R^2 < 0.80$), using the first order model revealed two distinct linear phases with fast and slow rates and corresponding calculated rate coefficients of 0.0107 and 0.0014 respectively for viscozyme® L

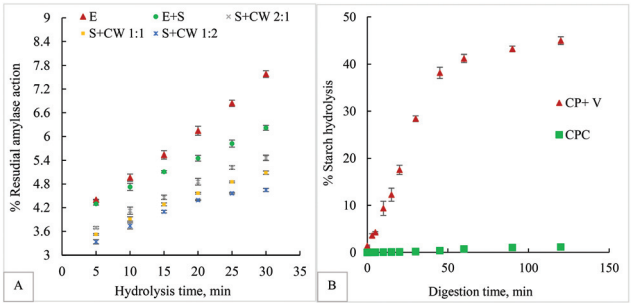

Fig. 7 (A) Activity assay of non-bound α-amylase on cooked soluble potato starch where E = α-amylase, S = starch and CW = cell wall components. The residual amylase action decreased with unbound enzyme retrieved from starch/protein and/or cell wall components added to digestion mixtures compared with control (no added cell wall components or starch/protein). The 50 μL of FITC labelled α-amylase was used for starch–protein–cell wall components in 10 mL buffer where enzyme activity in the labelled enzyme was 9.99 $nmol^{-1}$ min^{-1} mL^{-1}. (B) Progress curve for starch hydrolysis for cooked chickpea (CP) without viscozyme® L treatment (control, CPC) and after viscozyme® L treatment (CP + V). The first order fits of the data are shown in ESI Fig. S3c and d.†

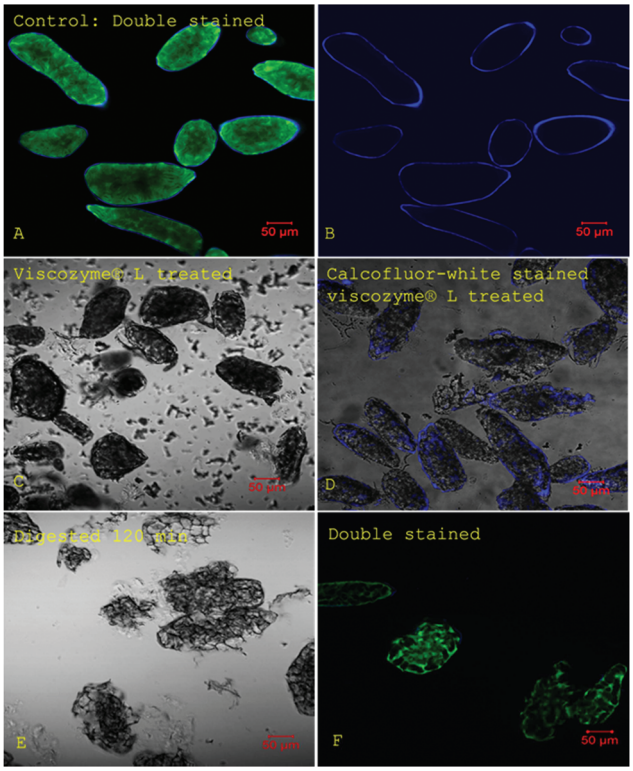

Fig. 8 Confocal laser scanning micrographs of isolated cooked chickpea cells showing viscozyme® L treatment followed by amylase digestion. Intact chickpea cells double stained with calcofluor-white and FITC (A), calcofluor white stain showing intact cell walls (B), viscozyme® L treated chickpea cells (C), calcofluor-white stained viscozyme® L treated cells (D), T-PMT (transmission photomultiplier, *i.e.*, bright field image) view of 120 min amylase digested chickpea cells (E) and calcofluor-white-FITC stained viscozyme® L treated cells (F). It is evident that intact cell walls (A) are removed after viscozyme® L treatment or made more porous (C, D) with the starch is still intact, but after 120 min digestion there is little intact undigested starch (E, F).

treated sample as opposed to a single rate coefficient of *ca.* 0.0001 for control (Fig. S3d†), suggesting effective removal of barriers to access of α-amylase to starch. After viscozyme treatment, the end-point of starch digestion was similar to that for mechanically-broken cells (Fig. 1), but the curve shapes were different showing that enzymic digestion and mechanical breakage of walls have subtly different consequences, possibly due to effects of mechanical but not enzymic treatment on starch/protein interactions.

In order to visualise the effects of viscozyme® L treatment, cell wall integrity and starch location was assessed (Fig. 8). It is seen through calcofluor-white (cell wall stain) and FITC (starch stain) labelling in Fig. 8 that cell walls and the enclosed components, particularly starch are unaltered in control sample (Fig. 8A). After incubation with viscozyme® L for 40 min, the cell walls from most of the cells were either partially or completely hydrolysed while a few were more intact but with visible pores (Fig. 8C and D). It is interesting that cells retain a compact structure of intracellular starch–protein after apparent removal of the cell wall (Fig. 8C). Following 120 min amylase digestion, partial starch depletion is evident (Fig. 8E and F) consistent with the *ca.* 45% digestibility shown in Fig. 7B.

4. Discussion

Isolated legume cells were investigated for their capacity to resist the mechanical conditions encountered in the stomach and duodenum sections in a rat model sufficiently to limit subsequent starch and protein hydrolysis. The present results show that the restricted enzyme digestion characteristic of legumes comes from a combination of factors that include the cell's ability to resist the mechanical force occurring in stomach and duodenum limiting the diffusion of digestive enzymes inside the cells as well as cell wall components binding digestive enzymes.

It is observed from Fig. 1 that, with broken legume cells, starch and protein hydrolysis follows both the general first order and LOS model kinetics (Fig. S2†). Based on the first-order fitting, the mechanism of enzyme action on intact and broken legumes can be differentiated. In intact cells, the barrier provided by the cellular structure controls the diffusion of enzyme inside the cells and this is likely to be the rate limiting step (and would not be expected to follow first-order kinetics). For the case of broken cells, in the absence of a physical barrier, the structural properties of starch and protein are likely to be the rate determinants and in this case, the digestion follows first-order kinetics. The structural basis for amylase digestion kinetics has been recently reviewed.[24] Likewise, in contrast to intact cells (Fig. 1A) where the hydrolysis deviated from first-order kinetics determined using

general first order as well as LOS models (Fig. S1†), but similar to hydrolysis of broken cells (Fig. 1B), legume starch and protein hydrolysis in the presence of externally added cell wall components (Fig. 6) followed first-order kinetics (Fig. S3 and S4†). Whilst starch digestibility in binding experiments was better represented by general first order model (Fig. S3a†), the LOS model also showed a reasonable fit for protein digestion (Fig. S4†).

Similar to broken cells, for the case of isolated starch with added cell wall components, the structural features of starch as well as non-specific binding of enzymes to the fibre components are the limiting steps. Along with binding of amylase to cell wall components, the rate of starch hydrolysis is also affected by non-specific binding of amylase to proteins as has been reported recently for wheat endosperm.[20]

As shown in Fig. 2, the processing of isolated legume cells in the DIVRSD model was unable to rupture the cell wall even after 120 min of digestion. However, using the same model, Wu *et al.*[13] reported reduction in particle size of uncooked rice from 19.38% to 4.42% in large rice (d > 0.85 mm) and to 80.21% from 62.04% in small rice particles (d < 0.60 mm). The disintegration of particles of rice increased the surface area which was proportional to the increased maltose concentration following amylase digestion.[13] However, the intact cells used in this study have typical dimensions of 120 × 50 μm which is an order of magnitude smaller than rice particles. This is consistent with a greater susceptibility to breakage of larger rice particles due to the greater torque imposed by the same mixing conditions, as well as the lack of inter-cellular fault lines in single cells compared with multi-cellular tissues like rice particles.

It is noted that the force (3.5 N) applied in the DIVRSD model[12] is more than what is reported for the human intestine (0.65 N, 11 contractions per min).[27,28] Based on this analogy, we can hypothesise that the retention of plant cell integrity found in this study also applies to humans and other monogastric animals. However, further experiments are needed to test this hypothesis. Whilst the legume cells are apparently intact under confocal microscopy, a slow and steady rise in glucose and soluble peptides, in very minor amounts, is obtained when treated with salivary, gastric and pancreatic juice containing amylolytic and proteolytic enzymes (Fig. 1). Although this may reflect very limited penetration of some cells by enzymes, it is also possible that some starch and protein might have stuck to the outside of legume cells during the isolation process. The overwhelming evidence is that intact cellular structure provides an effective barrier to enzyme hydrolysis of starch and protein under DIVRSD conditions. This is reflected in the hydrolysis curve obtained from broken legumes cell in the DIVRSD model (Fig. 1), where starch and protein hydrolysis increased by almost 20 times when the physical barrier was damaged. In addition, in intact cells, the binding of amylase to cell walls and the presence of protein can protect the starch from hydrolysis. This is evident in Fig. 1 where a lag phase of up to 30 minutes was observed for starch hydrolysis but protein hydrolysis was continuous without any lag phase.

The percentage hydrolysis of starch and protein in completely broken legume cell preparations is still below 50% after 120 min, which is however lower than the previous finding,[3] where starch hydrolysis for broken mung bean, pea, chickpea and red kidney beans were reported to be 74%, 69%, 68% and 64% respectively. The higher hydrolysis in the *in vitro* static system[3] compared to the dynamic (DIVRSD) model is most likely due to the effect of more intense mixing of the contents in the static system (static refers to the lack of transfer of contents to the next digestive stage, not a lack of agitation), as mixing speed can control the rate of enzymic hydrolysis of starch.[29] Hydrolysis rate also depends on the botanical origin; for instance red kidney bean is the most resistant and mung bean the most susceptible of the samples in this study. This might be due to a combined effect of starch properties and cell wall encapsulation. It is seen in Fig. 3 that the highly susceptible mung bean cells are degraded to a greater extent than the less susceptible red kidney bean. The further degradation of cells walls during processing in the DIVRSD model was observed for mung bean but was not clearly evident for red kidney bean. A further explanation for the higher enzyme susceptibility of mung bean starch in broken cells is the degree of residual order of starch cooked inside the cells as monitored by observing the birefringence as well as by the melting enthalpy from differential scanning calorimetry. It was found that the starch molecular order in mung bean isolated cells was comparatively lower than that of red kidney bean even though they were processed similarly.[3] The structure (strength/thickness) and composition of cell wall materials could also affect the diffusion of water leading to destruction of molecular order and requires further investigation.

Apart from the cellular structure of legumes and incompletely gelatinised starch leading to lower enzyme susceptibility, the non-catalytic binding of digestive enzymes to cell wall components could also be important in reducing the rate and extent of starch hydrolysis. As seen in Fig. 6, when FITC labelled alpha amylase was incubated with intact legume cells, the enzyme was predominantly localised on the outer surface of cells *i.e.* on the cell wall components, suggesting the binding of enzyme as recently demonstrated for cellulose and wheat bran.[16] The bound enzyme will thus be unavailable for catalytic activity. When the labelled enzyme was incubated with broken cells, as seen in Fig. 6, the enzymes were found to be adhered to both cell wall fragments and starch, suggesting a competitive behaviour of fibre and starch towards binding of enzymes. This is further illustrated in Fig. 7, where the addition of isolated legume cell wall components (fibres) lowered the hydrolysis of both starch and protein in proportion to the amount of fibre. The inhibition kinetics show that the half maximal inhibitory concentration (IC50-defined as concentration of inhibitor that reduces enzyme activity by 50%) of legumes fibre against amylolysis of legume starch (calculated from data shown in Fig. 6A) is *ca.* 1.94 mg mL^{-1}. This value is lower than the IC_{50} of 10 mg mL^{-1} (ref. 18) for pure cellulose, suggesting that intact cell walls may be more efficient than pure cellulose in binding amylase.

The hydrolysis of entrapped starch and protein in legumes is thus affected by three distinct mechanisms, (i) residual molecular order in starches, (ii) intactness of cells, and (iii) binding of enzymes to cell wall components. In addition, interaction of starch and protein inside legume cells may also inhibit enzyme action. In order to address this complexity, cell wall structures were removed by treating the cells with Viscozyme® L. As seen in Fig. 8, for Viscozyme® L treated cells, even after 120 min of enzyme treatment under mixing condition, the cell contents without cell walls and plasma membrane (as observed through fluorescein diacetate staining) retained an approximately spherical structure and did not disintegrate. This suggests structural interactions between macromolecules, particularly starch and protein, which could affect enzyme susceptibility as reported previously.[20]

5. Conclusions

Using an *in vitro* dynamic model, we observed that isolated legume cells have sufficient mechanical strength to survive mixing conditions in a simulated rat stomach-duodenum. The rate and extent of hydrolysis of starch and protein were greatly increased when the cell wall physical barrier was removed by either mechanical or enzymic processes. Furthermore, evidence for non-specific binding of α-amylase on cell walls, hindering the hydrolysis of starch was observed. Thus, the nutritional benefits from legumes could be enhanced by raw material selection and processing strategies that keep individual cellular structures intact.

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