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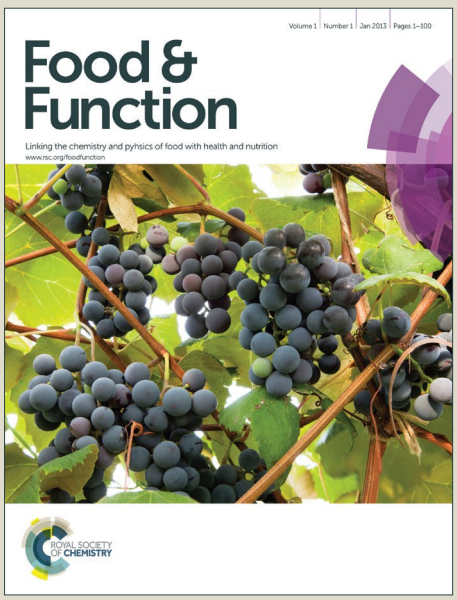

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Intactness of cell wall structure controls the *in-vitro* digestion of starch in legumes

Sushil Dhital, Rewati R Bhattarai, John Gorham, Michael J Gidley[1*]

[1]ARC Centre of Excellence in Plant Cell Walls, Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Qld 4072, Australia.

Abstract

Increasing the level of starch that is not digested by the end of the small intestine and therefore enters the colon ('resistant starch') is a major opportunity for improving the nutritional profile of foods. One mechanism that has been shown to be successful is entrapment of starch within an intact plant tissue structure. However, the level of tissue intactness required for resistance to amylase digestion has not been defined. In this study, intact cells were isolated from a range of legumes after thermal treatment at 60°C (starch not gelatinised) or 95°C (starch gelatinised) followed by hydrolysis using pancreatic alpha amylase. It was found that intact cells, isolated at either temperature, were impervious to amylase. However, application of mechanical force damaged the cell wall and made starch accessible to digestive enzymes. This shows that the access of enzymes to the entrapped swollen starch is the rate limiting step controlling hydrolysis of starch in cooked legumes. The results suggest that a single cell wall could be sufficient to provide an effective delivery of starch to the large intestine with consequent nutritional benefits, provided that mechanical damage during digestion is avoided.

Keywords: Legumes, cell wall, intact cells, cell isolation, *in-vitro* digestion, resistant starch

1 Introduction

Evidences from a number of epidemiological studies as well as from meta-analyses, dietary interventions and metabolic studies strongly support the concept that whole grain foods and legumes have functional properties that protect against metabolic disorders such as Type II diabetes,[1-3] at least in part due to the lower postprandial glycaemic response. Starch is the major glycaemic carbohydrate in both whole grains and legumes. The reduced bio-availability of starch in whole grains and legumes for hydrolytic enzymes has been attributed primarily to the presence of intact tissue/cell structures[1,4-6] that slow down or prevent access/binding of amylase to starch granules enclosed within cell structures. When this access is not limiting, for example as in isolated starch or finely milled grains/legumes, structural features of starch control the rate and extent of amylolysis as reviewed recently.[7] Several studies have shown that physical or thermal breakdown of cellular and granular structure promotes the ingress of digestive enzymes and liberates component structures that are then rapidly digested.[4,8-13] Intact cellular structures encapsulating macro- and micronutrients are recovered from human and animal subjects after consumption of 'whole' foods such as almonds,[14,15] legumes[6,13,16] and carrots.[17]

Similarly, *in vivo* studies have demonstrated the decreases in blood glucose and insulin responses in human subjects fed with whole rice compared to ground rice[18] or whole beans compared to milled beans[4] as well as improved digestibility of nutrients with decrease in particle size of feeds in pigs[19] and chickens.[20] However, these studies did not consider the relative contribution of the particle size of intact cellular structures in controlling digestion. Degradation and digestion of solid particles start from the outer surface (unless they are porous) and there are limits to the capacity of humans or animals to reduce the particle sizes of natural foods during mastication or gastro-intestinal tract transit. [14,17] Thus, larger particles representing clusters of intact cells may provide the physical strength (to protect against disintegration) and a physical barrier for enzyme diffusion and catalysis increasing the probability of survival of all or some of the cellular structure during gastric and small intestinal passage.

The smallest scale of plant tissue structure is an individual cell which is surrounded by an outer, comparatively rigid and hydrophilic, cell wall and an inner lipophilic cell membrane. The outer cell wall comprises a mixture of cellulose, hemicellulose and pectin (in varying proportion depending upon the botanical origins) and provides structural support and protection whereas the cell (plasma) membrane controls the movement of ions or molecules in or out of the cell.[21,22]

The cell membrane, a protein-lipid bi-layer enclosing cellular contents is irreversibly damaged during processing (at ~ 50°C),[23] above this temperature the cell wall can be considered as the primary barrier that limits the diffusion of enzymes inside the cells. The diameter of pores in the normal non-lignified extracellular walls lie in the range 3.5–5.5 nm,[24] which potentially cannot allow the permeation of α-amylase, with a hydrodynamic diameter of ~8 nm.[25] Whilst the exact porosity of cell walls is still debatable as results vary depending upon the measurement techniques,[26] the cell wall may undergo swelling during heating[27] or due to hydration during digestion[15, 17] that may alter the porosity of walls affecting the access of digestive enzymes.

In this study, we characterise the enzyme barrier properties provided by an individual intact plant cell wall taking isolated legumes cells as an exemplar. The mechanism of cell separation and modelling of kinetics of digestion are further discussed. Legumes are chosen as they are known to have relatively thick and strong cell wall structures[28] that resist the cooking process[4,27] allowing the separation of individual cells. The identification of the role of a single cell wall in controlling starch digestion in legumes provides evidence for potential functional food products from controlled legume processing, and also provides inspiration for the development of functional ingredients based on plant cellular encapsulation of starch.

2 Materials and Methods

Legume seeds, chickpea (CP), pea (P), mung bean (MB) and red kidney bean (RKB) were purchased from a local shop and used as such for isolation of cotyledon cells as discussed in the following section.

Alpha amylase (Sigma A6255) was purchased from Sigma-Aldrich, Australia. Amyloglucosidase (E-AMGDF100) and Total Starch Assay Kit (K-TSTA) were obtained from Megazyme, Ireland. The enzyme glucose reagent (GOPOD) (TR15104) was purchased from Thermo Scientific, Australia. The method of preparation of Fluorescent (FITC) labelled alpha-amylase has been described previously.[29]

2.1 Isolation of intact cells

Legume cells were isolated following the method described by Grundy[30] with slight modifications as discussed. Legumes were kept overnight in ice chilled water to swell and loosen the outer hulls whilst minimising *in situ* enzyme activity. Hulls were removed manually with gentle hand abrasion and subsequently washed in running cold water. The de-hulled legumes (300 g) were heated at either 60°C or 95°C (hereafter named as 60°C cells and 95°C

cells) for 1 hour in excess water (1000 mL) with gentle mixing (sufficient to keep the legume particles moving) using a magnetic stirrer bar in a 2L glass beaker. The heated legumes were gently mashed using a mortar and pestle to a paste consistency and separated with sieves as described by Grundy.[30] Starch granules released during the mashing of 60°C cells (hereafter named as 60°C isolated starch (IS)) were isolated according to the method of Hasjim et al.[12] All isolated samples were preserved in 0.02% sodium azide solutions under refrigerated conditions for further use.

2.2 Damaging (breaking) the cell structure

In order to compare the properties of intact cells (95°C-I, 60°C-I) and broken cells (95°C-B, 60°C-B), isolated intact cells were damaged (broken) by applying a shear mixing force. Around 4 grams cells (wet weight) were mixed with 15 ml deionised water (containing 0.02% sodium azide) overnight at room temperature with a 12 mm × 4.5 mm micro polytetrafluoroethylene (PTFE) coated magnetic stirrer bars at 1500 rpm. Complete breakage of cell wall structure was confirmed microscopically. This method was found to be more effective in breaking cells than using a homogeniser, a kitchen blender or ultrasonic treatment.

2.3 Microscopic observations

Confocal microscope (LSM 700, Carls Zeiss, Germany) with and without reflective mode (RM) was used to observe the enzyme-treated and non-treated legume cells using Zen Black 2011 software (Carl Zeiss Version 7.1). For FITC-AA conjugate observation, a FITC filter block with excitation at 488nm was used and emission was collected up to 530 nm. For bright field and polarised pictures, an Olympus BX61 microscope (Olympus Optical Co., Ltd, Tokyo, Japan) equipped with a cross polariser was used.

2.4 Determination of Total Starch

Starch content in isolated cells was determined using the Megazyme Total Starch Assay Kit (K-TSTA) following the manufacturer instructions with modifications in sample preparation. Before treating with enzymes, the intact cell structure was damaged by overnight mixing as described in section 2.2, as underestimation of starch content has been reported for legumes with intact cellular structure.[31]

2.5 Enzymic digestion

Amylase digestion was carried out using 0.5 unit of α-amylase (AA) and 0.28 unit of amyloglucosidase (AMG) per mg equivalent of starch as described by Warren et al.[32] For each

legume variety, five samples were generated, 60°C and 95°C intact cells, 60°C and 95°C broken cells and 60°C isolated starch.

The *in vitro* enzyme hydrolysis was carried out using two different conditions. In one (stationary) condition, 95°C intact and broken cells as well as 60°C isolated starch were hydrolysed without mixing. However, tubes were turned upside down gently at the time of aliquot removal for glucose analysis. This experiment is designed to determine the barrier effect provided by the intact cell structure (as mixing with stirrer bars damages the cells). In a second (mixing) condition, each of the five samples (60°C and 95°C intact cells, 60°C and 95°C broken cells and 60°C isolated starch) were hydrolysed *in vitro* under mixing condition with the use of 4.5 mm × 3 mm micro PTFE coated magnetic stirrer bars at 300 rpm.

The glucose concentration, after enzymic treatment, in the supernatant was determined using the GOPOD assay. The glucose value was multiplied by a factor of 0.9 to convert glucose concentration into starch with results presented as gram of starch hydrolysed per 100 g dry starch.

In a separate experiment, chick pea cells isolated at both 60°C and 95°C were hydrolysed with 0.5 unit fluorescein isothiocyanate (FITC) labelled α-amylase per mg of starch under the same two conditions described previously.

2.6 Fitting of digestion progress curve

The digestion progress curves were fitted with three different models, namely general first order,[33, 34] Goñi[35] and logarithm of the slope (LOS)[36] methods, and digestion rate kinetics (k) from each model were compared in order to elucidate the variability in rate kinetics with respect to the model used.

2.7 Differential scanning calorimetry

Thermal properties were determined using a differential scanning calorimeter (DSC 1, Mettler Toledo, Schwerzenbach, Switzerland) as previously described.[37]

2.8 Statistical analysis

Digestion rate coefficients (k) and thermal parameters, at least in duplicate, were analysed by Analysis of Variance (ANOVA) using Minitab 16 (Minitab Inc., State College, PA, USA). General Linear Model and Tukey's Pairwise Comparisons with confidence level at 95.0% were used in performing the ANOVA.

3.0 Results

3.1 Isolation and microscopic observation of cells

Due to the fragile legume cotyledon structure, attempts to isolate cells directly without any pre-treatment were not successful. As an example, a thin horizontal section of red kidney bean obtained after overnight hydration at 4°C is shown in **Figure 1A i** and **ii**. A vertical cut section from the same cotyledon is shown in **Figure 1A iii**. Although protein bodies are not clearly visible at the magnification used, the compact packing of larger starch granules enclosed within the cell walls is apparent. Sections from other legume samples show that cell shape and size are highly variable among legumes and within a single cotyledon. In general, peas (chick pea and pea) have more ellipsoidal cells whereas beans (mung bean and red kidney bean) have more spherical cells (Figure 1B). In both peas and beans, larger sized and more spherical cells were observed around the central part of the cotyledon whereas smaller and more ellipsoidal cells were observed at the periphery of the cotyledon (data not shown) similar to previous studies on beans and peas.[38]

Reflective and polarised light microscopy images of intact cells isolated at 60°C and 95°C and of starch extracted during isolation of cells at 60°C are shown in **Figure 1B**. Pea and chickpea cells appear to be ellipsoidal in shape whereas cells from mung bean and red kidney bean are more spherical. The separation was clearer in legumes heated at 95°C compared to that of legumes heated at 60°C, where hollow cell wall envelopes and fragments are clearly visible (**Figure 1B**). This is likely to be due to less softening and limited dissolution of middle lamella (the zone defining the boundary between walls from adjacent cells) components at 60°C, resisting the separation of cells. Qualitatively, it was observed that harder grinding was needed to separate cells for legumes treated at 60°C compared to 95°C, and this is likely to have generated some hollow cell envelope or cell fragments due to fracturing during isolation of cells from legumes treated at 60°C.

It should be noted that the isolated cells presented in **Figure 1B** are cells that passed through a 150 µm sieve and were retained on a 53 µm sieve. Thus, it is reasonable to assume that the size and shape as presented in **Figure 1B** are not necessarily representative of all the cells present in raw tissues because they have various shapes and size depending upon the location within the cotyledon (as discussed previously). Furthermore, during heat treatment, the swelling of starch can exert internal pressure on the wall thereby potentially altering the size and morphology of isolated cells.

Under polarised light microscopy, both isolated starch and starch within cells isolated at 60°C are clearly birefringent with distinct Maltese crosses, suggesting the retention of at least some granular structure due to incomplete gelatinisation. Interestingly, the 95°C-isolated cells are also highly birefringent. In order to identify whether the birefringence is due to local organisation of 'gelatinised' starch polymers under the very limited water conditions inside cells or to non-gelatinised (or partially gelatinised starch) granules, the starch inside the cells was extracted by breaking the cells and viewed by polarised light microscopy. As seen in **Figure 1B (**CP-95°C-B-Polarised, RKB-95°C-B-Polarised, RKB-95°C-B-Polarised), the extracted starches are birefringent with the presence of partial Maltese crosses. This illustrates the role of intact cell wall structure on limiting the swelling of granules as well as hindering the melting of starch ordered structures in legumes.

3.2 Thermal properties of cell and isolated starch

The thermal properties of starch-containing cells as well as of isolated starches are shown in **Table 1** and representative thermograms are shown in **Figure 2.** In spite of the presence of birefringent starches in 95°C isolated cells, suggesting the presence of ordered structure, the melting enthalpy was almost negligible. A minor endotherm (equivalent to $\Delta H < 1$ J/gm, values not shown in **Table 1**) was observed in thermograms of cooked 95°C cells (**Figure 2**). Significant melting enthalpy was observed for 60°C cells and 60°C isolated starches. Despite the fact that both 60°C cells and isolated starches are obtained from the same experiment, the melting enthalpy (ΔH) of isolated starch is significantly higher than that of 60°C isolated cells (Table 1). In all cases, the onset of gelatinisation temperature (T_o) of 60°C intact cells was higher than that of 60°C isolated starches and the difference was significant for pea, mung bean and red kidney bean.

3.3 Amylase digestion under non-stirring (non-mixing) conditions

In order to investigate the effect of intactness of cells on amylase susceptibility, 95°C isolated intact cells as well as broken cells and 60°C isolated starch were treated with amylase and amyloglucosidase under non-stirring (stationary) conditions. Typical resulting hydrolysis curves are shown in **Figure 3A.** It is clearly seen that the rate of hydrolysis of intact cells is very low (less than 5% in 120 minutes), whereas significant hydrolysis of starch was obtained for broken cells isolated at 95°C and starch isolated at 60°C. The extent of hydrolysis of starch from broken cells (95°C) is higher than that of starch isolated at 60°C, consistent with greater retention of granular structure compared with broken cells treated at 95°C.

3.4 Amylase digestion under stirring (mixing) conditions

The amylase susceptibility (measured for up to 300 mins in order to capture progressive changes due to stirring/mixing conditions) of 60°C and 95°C intact and broken cells as well as 60°C isolated starch are presented in **Figure 3B.** The digestion progress curve is fitted to a first order equation **(Figure 4)** with rate coefficients determined using three different models[33,35,36] and compared in order to evaluate the consistency between models. Amylase susceptibility of legume starch was found to be dependent upon the intactness of the cell structure rather than the botanical source i.e. the intactness of cell wall structure controls the in vitro digestion of starch in legumes. This is evident from the lower susceptibility of both 60°C and 95°C intact cells compared to broken cells as seen in **Figure 3B** suggesting that intact cell walls provide a barrier to the diffusion of amylase inside the cells. Similar information is also obtained from the digestion rate coefficients (**Table 2**) obtained from fitting first order models. However the apparent rates obtained for intact cells isolated at both 60°C and 95°C are probably not the true starch hydrolysis rate as they are likely determined by cell breakage under the mixing conditions used during the assay rather than the direct hydrolysis of starch. The first order plots of chick pea under different processing conditions (95°C intact and broken, 60°C intact and broken, and 60°C isolated starch) are shown in **Figure 4** as an exemplar. It is noted that the experiments shown in **Figure 3B** were performed under mixing conditions, using a magnetic stirrer bar rotating at 300 rpm in order to keep cells in suspension. The slow and steady rise in starch digestibility of 'intact cells' is most likely due to the damage of cell walls by the physical force of the rotating stirrer bar. The breakage of cells during the experiment was confirmed microscopically (figures not shown).

The location of enzymes during the hydrolysis of 60°C and 95°C chick pea intact cells under mixing condition is shown in **Figure 5.** After 30 min hydrolysis time**,** it is clearly seen that enzymes are concentrated at the outer periphery of intact cells whereas they are inside the broken cells. Even after 4 hours of hydrolysis, the majority of 95°C isolated chick pea cells are shown to be resistant to enzymic hydrolysis whereas starch in 60°C cells is more degraded due to greater access of enzymes inside the broken or damaged cells.

First order rate coefficients vary depending upon the model selected for calculation. In general, models proposed by Al-Rabadi et al[33] and Goñi et al[35] give close values (**Table 2**) and can be fitted to a wide range of digestibility values compared to the LOS model proposed by Butterworth et al.[36] However, **Figure 4** shows that the LOS model is able to discriminate between those systems where there is no barrier to access of starch (isolated starches and

previously broken cells) and which therefore are expected to show first order kinetics in contrast to the 'intact' cell samples where the rate-determining factor is proposed to be the progressive rupture of cells under the stirring conditions of the assay, a process that is not expected to necessarily follow first order kinetics. The LOS model is therefore better able to diagnose first order kinetics more precisely compared to the other two models which are more generic and can be fitted to all digestograms.

4 Discussion

4.1 Isolation of cotyledon cells

The structure of plant cell wall components and their molecular organisation is complex, but in general, non-lignified primary cells of e.g. legume cotyledons are fused together by a middle lamella (**Figure 1A)** that is rich in pectin. The primary wall is more structurally robust, consisting of a complex interacting network of mainly cellulose, hemicellulose and pectin. In principle, the efficient separation of intact cells requires the ability to loosen /dissolve the middle lamella whilst minimising damage to the underlying primary cell wall that encapsulates the micro- and macro nutrients.A schematic representation of cell isolation from legume cotyledons is shown in **Figure 6**, based on light microscopy images (**Figure 1A**). When soaked in excess water, the legume seed coat is softened, and cell wall polymers are hydrated with particular plasticisation of the middle lamella. This enables softening of the cotyledon as well as loosening of the adhesion between cells. The soaking process can be accelerated by inclusion of salts such as sodium chloride, sodium tripolyphosphate, sodium carbonate and bicarbonate.[39-41] Increasing the pH of the soaking solution or use of chelating agents such as EDTA or CDTA speeds up the soaking and separation process by removal of divalent cations, particularly calcium and magnesium from their cross links with pectin thereby softening the middle lamella.[27, 30,42,43] Cells can be isolated from potatoes and legumes without gelatinizing starches by successive treatments with the mild acid and alkali for prolonged periods,[44,454] however the effect of acid and alkali on cell wall permeability and solubilisation of proteins has not been reported. To avoid potential complications from the use of additional reagents, the current method involves just water as a medium.

Grinding of cotyledons after hydration at either room or refrigerated temperature causes extensive rupture of cell walls making it almost impossible to isolate intact cells directly (data not shown). One of the underlying causes is the turgor pressure which acts outwards from each cell onto the cell membrane and wall and rigidifies the tissue. However, application of heat

causes melting of the lipid bilayer of the cell membrane at around 50°C[23] and effectively removes turgor pressure by allowing the movement of water through the tissue structure. For legume cotyledons, this process softens the tissue allowing subsequent separation of cells at a temperature below that required for full gelatinisation of starch. Further application of heat (to e.g. 95°C) partially depolymerises and solubilises the pectin in the middle lamella reducing the adhesion between the cell clusters and facilitating the release of intact cells under mechanical stress (e.g. grinding with mortar and pestle or squeezing between glass slides). Furthermore, swelling of starch granules within the cells can also impart mechanical stress within the tissue, easing the cell separation process.[27,46] The facilitation of the separation process at higher temperature is evident from **Figure 1B**, where more clearly discrete 'clean' cells were isolated from legumes heated at 95°C compared to 60°C.

4.2 Enzymic susceptibility of intact cells and isolated starches

Intact cells isolated from cotyledons heated at both 95°C and 60°C, are impervious to alpha amylase under non-stirred conditions, as evident from the negligible glucose released after 2 hours of enzyme treatment (**Figure 3A**) and also evident from Figure **5**, where fluorescently labelled enzymes accumulate at the outer boundary of intact cells. This accumulation is probably related to the previously demonstrated binding of α-amylase to cellulosic materials.[47] However, if the cells are damaged or broken by external force, the susceptibility to amylase increases as seen in digestion progress curves (**Figure 3B**) and apparent digestion rate coefficients (**Table 2**). This is in line with previous reports where cooked and homogenised as well as milled and cooked legumes had markedly increased *in vitro* starch digestibility compared to non-homogenised and non-milled counterparts.[5,31,48] Although, these reports hypothesised that the cell structure was the key determinant of the digestion rate of starch in legumes, in this study using isolated cells we, for the first time, have explicitly demonstrated that amylolytic enzymes are unable to penetrate intact legume cells that are extracted at both non-gelatinisation (60°C) and cooking (95°C) temperatures. Thus we can conclude that intactness of cell wall structure controls the in vitro digestion of starch in legumes. The natural porosity of non-lignified cells allowing free passage of digestive enzymes is still debatable due to the difference in methods applied for the measurement of porosity of cell walls as well as the size of digestive enzymes.[26] Whilst it is known that the cell wall hydrates and swells during both intestinal digestion and thermal processing[15,17,27] and the permeability of the cell membrane is increased with increase in temperature, the present results show that legume cells isolated at both 60°C and 95°C are impervious to digestive amylases.

Starch and proteins are the two major components of cotyledon cells. During heating, starch undergoes swelling whereas protein denatures. The thermal parameters monitored for intact cells isolated at 95°C and 60°C cells as well as 60°C isolated starches provide some interesting information. In spite of the presence of birefringence and partial Maltese crosses in all four types of legume cells isolated at 95°C (**Figure 1B**), the melting enthalpy as measured by DSC for those cells is low compared with that for isolated starches, e.g. the melting enthalpy of 60°C isolated starch is twice the melting enthalpy of 60°C intact cells (**Table 1**). This is most likely due to the swelling of starch entrapped in intact cells being impeded by the presence of strong cell walls even under pressure in sealed DSC pans. As seen in **Figure 1A-B**, the starch granules in legumes cells are densely packed within the boundary of the cell wall. We propose that during heating in excess water (as in cooking or a DSC experiment), swelling of starch granules is limited due to the space constraints and leached molecules from limited swollen granules further restrict swelling as well as impede water penetration within the intact cells thus decreasing the porosity of the isolated cells. This is thus different from the melting of helices in low water condition generating a high temperature 'tail' in the DSC plot, named as the M1 endotherm by Donovan.[49]

4.3 Apparent digestion rate coefficients

Recently the mechanisms of starch digestion by alpha amylase have been reviewed.[7] The kinetics of starch digestion, unless in extremely high or low enzyme to substrate ratio[32] generally show simple decay curves with apparent first-order behaviour (**Figure 3**) This pattern can often be described by a single exponential decay equation $C = C\infty (1 - e^{-kt})$.[35] The decay equation suggests that the rate of reaction decreases with time due to substrate depletion, such that a semi logarithmic plot of starch digested against time shows a linear relation (**Figure 4**) with a slope of –k, where C represents the starch hydrolysis at time t and $C\infty$ is the total concentration digested at the end point or the maximum extent of hydrolysis. The k values reflect the susceptibility of starch or starchy foods towards hydrolysis by amylase, and typically range from 10^{-5} to 10^{-3} min^{-1} but are dependent on enzyme concentration.[32, 36] Although, the fraction of the starch remaining after the digestion plateau is reached has been described as enzyme resistant,[50] provided the starch is not chemically modified, all starches can be hydrolysed completely by a combination of endo- and exo-enzymes given enough time and enzyme activity, so absolutely resistant starch without chemical modification may not be possible.[7] Expressing the digestion as a kinetic parameter can give a straightforward description of relative rates of digestion of starch or starchy foods. The digestion rate

coefficient, a single parameter that can describe the whole digestion curve, is however dependent on the mathematical model used to determine the parameters as shown in **Table 2** and **Figure 4**. It is noted that numerous mathematical models, beyond the three used in the present experiment, have been developed and used to describe the hydrolysis rate of starch and starchy foods.[51] The description (merits and demerits) of each method is out of scope of the present paper. However, in view of the current ambiguity in the kinetic rate parameters obtained from different models, the time has come to have a broad agreement on a relevant mathematical model and standardised *in vitro* conditions so as to achieve a harmonised description of potential nutritional value from starch and starchy foods.

Conclusions

We have shown explicitly that the intactness of cell wall structure is the limiting factor that controls the rate and extent of hydrolysis of starch trapped inside legume cotyledon cells isolated after treatment at temperatures either below (60°C) or above (95°C) the temperature range of (isolated) starch gelatinisation. This shows that cooking of fine milled legume flour will not have the equivalent slow digestion property as cooked whole legumes because the former lacks an intact cell wall structure. Thus physical processes that have less damage to cell structure are desirable to achieve low and slow digestion of starch in legumes. Studies of the micro-mechanical properties of cell walls of isolated legumes and the role of intactness of cell structure in *in vitro* digestion of cereals are ongoing, and will be reported separately.

In addition to direct relevance to the nutritional functionality of legumes, the present results demonstrate the principle that a single plant cell can act as an efficient barrier to digestive enzyme action, providing a potential route to controlled delivery of functional nutrients to the large intestine where fermentation by the resident microbiota will allow the release of cell contents. However, the relative fragility of isolated cells demonstrated in the current study suggests that some strengthening of the wall structure may be needed to guarantee that intact cells survive to the large intestine.

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Author Information

Corresponding Author

Email: m.gidley@uq.edu.au (M. J. Gidley). Phone: +61 7 3365 2145; Fax: +61 7 3365 1177.

Notes

The authors declare no competing financial interest.

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Table 1: Thermal properties of isolated legume cells and starches[1]

Legumes	Treatments	ΔH (J/gm)	T_o (°C)	T_p(°C)	T_c(°C)
Chick pea	60°C-IS	11.14^{a}	72.86^{cd}	75.32e	78.67^{e}
	60°C-I	4.10b^{c}	73.65^{cd}	77.00^{de}	81.77^{cd}
Pea	60°C-IS	4.85b^{c}	75.79^{bc}	81.47^{bc}	85.69^{b}
	60°C-I	3.60^{c}	80.38^{a}	84.95^{a}	90.35^{a}
Mung bean	60°C-IS	6.06^{b}	73.84^{cd}	75.24^{e}	83.66^{bc}
	60°C-I	3.26^{c}	77.94^{ab}	82.14^{b}	85.26^{b}
Red Kidney bean	60°C-IS	9.89^{a}	70.30^{d}	75.34^{e}	80.89^{de}
	60°C-I	2.65^{c}	75.15^{bc}	79.39^{cd}	83.83^{bc}

[1] ΔH = Melting enthalpy, T_o= Onset temperature, T_p= Peak temperature, T_c= Conclusion temperature. Values with similar superscripted letters in each row are not significantly different (P>0.05)

Table 2: Apparent digestion rate coefficients calculated using different models[1]

Legumes	Treatments	Kinetic Constant (Al-Rabadi model, $\times 10^{-3}$ min^{-1})	Kinetic Constant (Goñi model, $\times 10^{-3}$ min^{-1})	Kinetic Constant (LOS model, $\times 10^{-3}$ min^{-1})
Chick Pea (CP)	95°C-B	$8.15^{BCD\ b}$	$16.00^{ABCD\ a}$	$14.40^{ABC\ a}$
	95°C-I	$2.40^{F\ b}$	$3.15^{J\ a}$	-
	60°C-B	$5.25^{DEF\ b}$	$9.80^{EFGH\ a}$	$11.60^{ABC\ a}$
	60°C-I	$2.60^{F\ a}$	$3.95^{IJ\ a}$	-
	60°C-IS	$4.90^{DEF\ b}$	$7.30^{GHIJ\ a}$	-
Pea (P)	95°C-B	$7.50^{CD\ b}$	$12.40^{BCDEFG\ a}$	$12.20^{ABC\ a}$
	95°C-I	$3.25^{EF\ a}$	$5.75^{HIJ\ a}$	-
	60°C-B	$10.4^{ABC\ b}$	$17.45^{AB\ a}$	$15.30^{AB\ a}$
	60°C-I	$6.85^{CDE\ b}$	$11.50^{CDEFGH\ a}$	$10.20^{BC\ a}$
	60°C-IS	$11.95^{\ AB\ b}$	$21.00^{A\ a}$	-
Mung Bean (MB)	95°C-B	$9.90^{ABC\ b}$	$14.70^{BCDEF\ a}$	$12.85^{ABC\ a}$
	95°C-I	$5.35^{DEF\ b}$	$9.25^{FGHI\ ab}$	$9.70^{BC\ a}$
	60°C-B	$11.85^{AB\ a}$	$15.88^{ABCD\ a}$	$14.25^{ABC\ a}$
	60°C-I	$7.70^{CD\ a}$	$15.20^{ABCDE\ a}$	$15.20^{AB\ a}$
	60°C-IS	$12.70^{\ A\ a}$	$17.2^{ABC\ a}$	$17.40^{A\ a}$
Red Kidney Bean (RKB)	95°C-B	$10.05^{ABC\ a}$	$10.70^{DEFGH\ a}$	$10.15^{BC\ a}$
	95°C-I	$4.90^{DEF\ a}$	$6.10^{HIJ\ a}$	-
	60°C-B	$8.25^{BCD\ a}$	$12.30^{BCDEFG\ a}$	$12.65^{ABC\ a}$
	60°C-I	$2.05^{F\ a}$	$3.95^{IJ\ a}$	-
	60°C-IS	$7.00^{CDE\ a}$	$8.25^{GHIJ\ a}$	$7.65^{C\ a}$

[1]Abbreviations used in the table are as follows: chick pea (CP), pea (P), mung bean (MB), red kidney bean (RKB), isolated intact cells extracted at 95°C (95ºC-I) and broken by mixing with stirrer bar (95ºC-B), isolated intact cells extracted at 60°C (60°C-I) and broken by mixing with stirrer bar (60°C-B), starch isolated at 60°C (60°C-IS). In order to show differences between legumes, values with the same superscripted uppercase letter in each column for each legume type are not significantly different (P>0.05). Similarly, in order to identify differences between different models, kinetic constant values with the same superscripted lowercase letter in each row are not significantly different (P>0.05)

Figure Captions

Figure 1A: Microscopic observation of thin sections of hydrated legume (red kidney bean) cotyledon. i: Thin slice showing empty and starch-filled cells; ii: Transverse section showing the starch entrapped in cell matrices; iii: Vertical section showing the packing of starch within the cells.

Figure 1B: Microscopic observation of legume cells isolated at 60°C and 95ºC. Abbreviations used in figure are as follows: chick pea (CP), pea (P), mung bean (MB), red kidney bean (RKB), Reflective mode image taken from confocal microscope (RM), broken cells (B), bright field image (Bright field), BF image taken with cross polarisation (Polarised).

Figure 2: Thermograms of legume cells isolated at 60°C and 95ºC. Abbreviations in figure legends are as follows: chick pea (CP), pea (P), mung bean (MB), red kidney bean (RKB), starch isolated at 60°C (60°C-IS), intact cells isolated at 60°C (60°C-I), intact cells isolated at 95°C (95ºC-I). Data have been offset for clarity.

Figure 3A: Amylase digestion (up to 120 mins) under non-stirring (mixing) conditions of intact (I) and broken (B) chick pea (CP), pea (P), mung bean (MB) and red kidney bean (RKB) cells isolated at 95°C and starch (IS) isolated at 60°C.

Figure 3B: Amylase digestion of intact and broken legume cells (up to 500 mins) under stirring (mixing) conditions isolated at 60°C and 95ºC. Abbreviations in figure legends are as follows: chick pea (CP), pea (P), mung bean (MB), red kidney bean (RKB), isolated intact cells extracted at 95°C (95ºC-I) and broken by mixing with stirrer bar (95ºC-B), isolated intact cells extracted at 60°C (60°C-I) and broken by mixing with stirrer bar (60°C-B)

Figure 4: First order fits of data from **Figure 3A** (chick pea) with three mathematical models: General first order (Al-Rabadi et al.,[33] Dhital et al.,[34]) abbreviated as First Order, Goñi et al model (Goñi et al.,[35]) abbreviated as Goñi and logarithm of the slope (LOS) (Butterworth et al., [36]) model abbreviated as LOS. Other abbreviations in figure legends are as follows: Chick pea (CP), isolated intact cells extracted at 95°C (C-I) and broken by mixing with stirrer bar (C-B), isolated intact cells extracted at 60°C (60°C-I) and broken by mixing with stirrer bar (60°C-B)

Figure 5: Confocal and reflective mode (RM) images of chick pea (CP) cells isolated at 95°C and 60°C hydrolysed with FITC labelled alpha amylase for 30 min and 4 h. The location of enzyme is shown by red arrows. Scale bar = 100 μm

Figure 6: Schematic diagram showing physical changes during separation of legume cells

Figure 1A

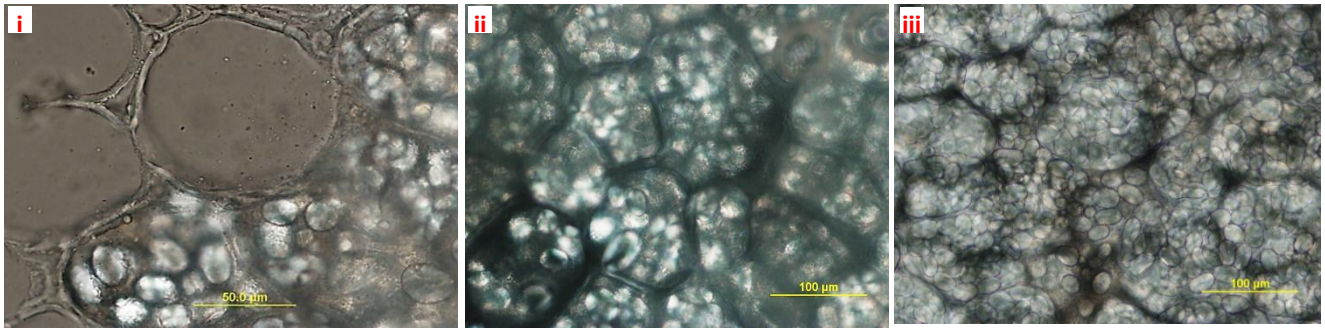
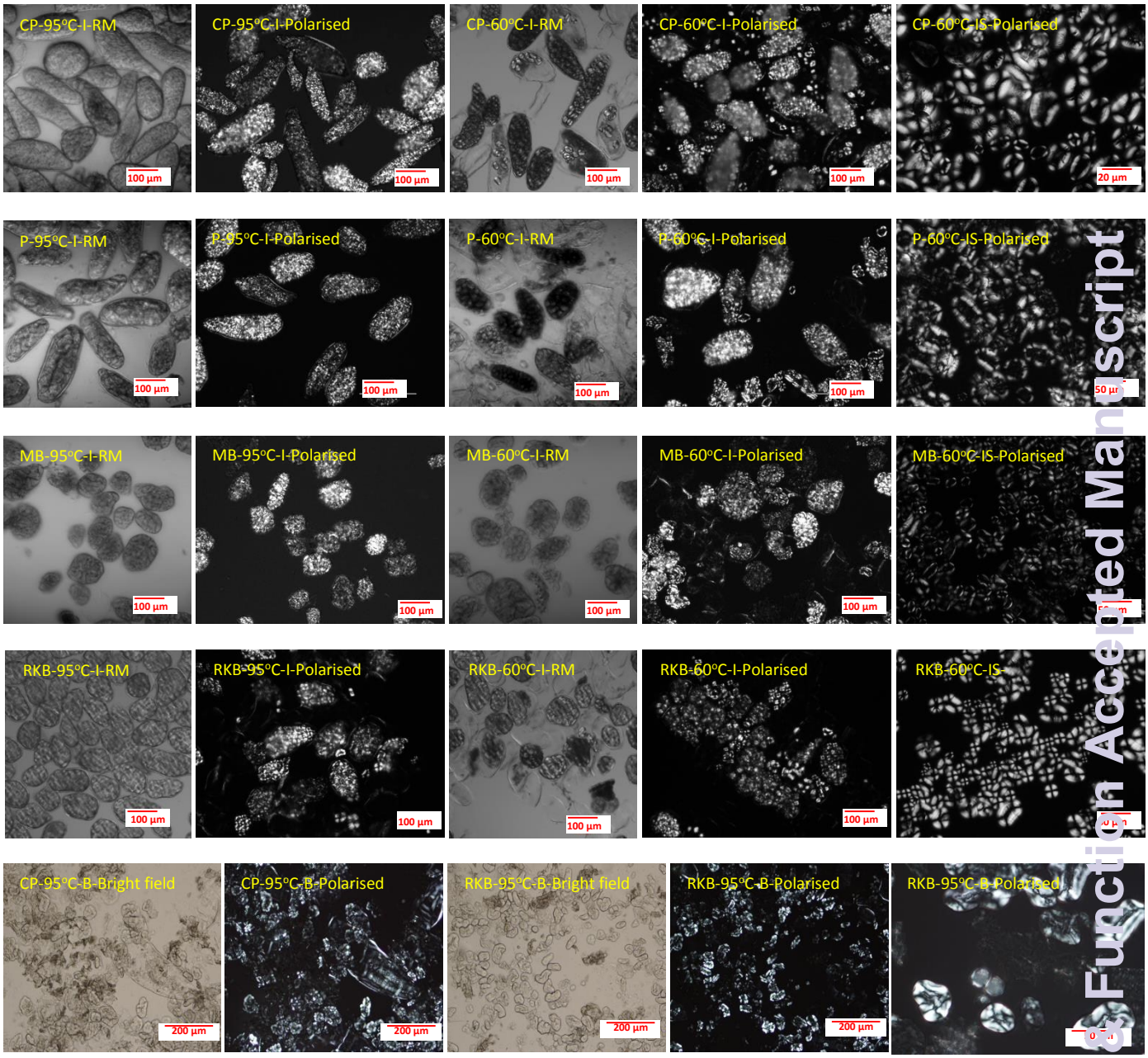


Figure 1B



565 **Figure 2**

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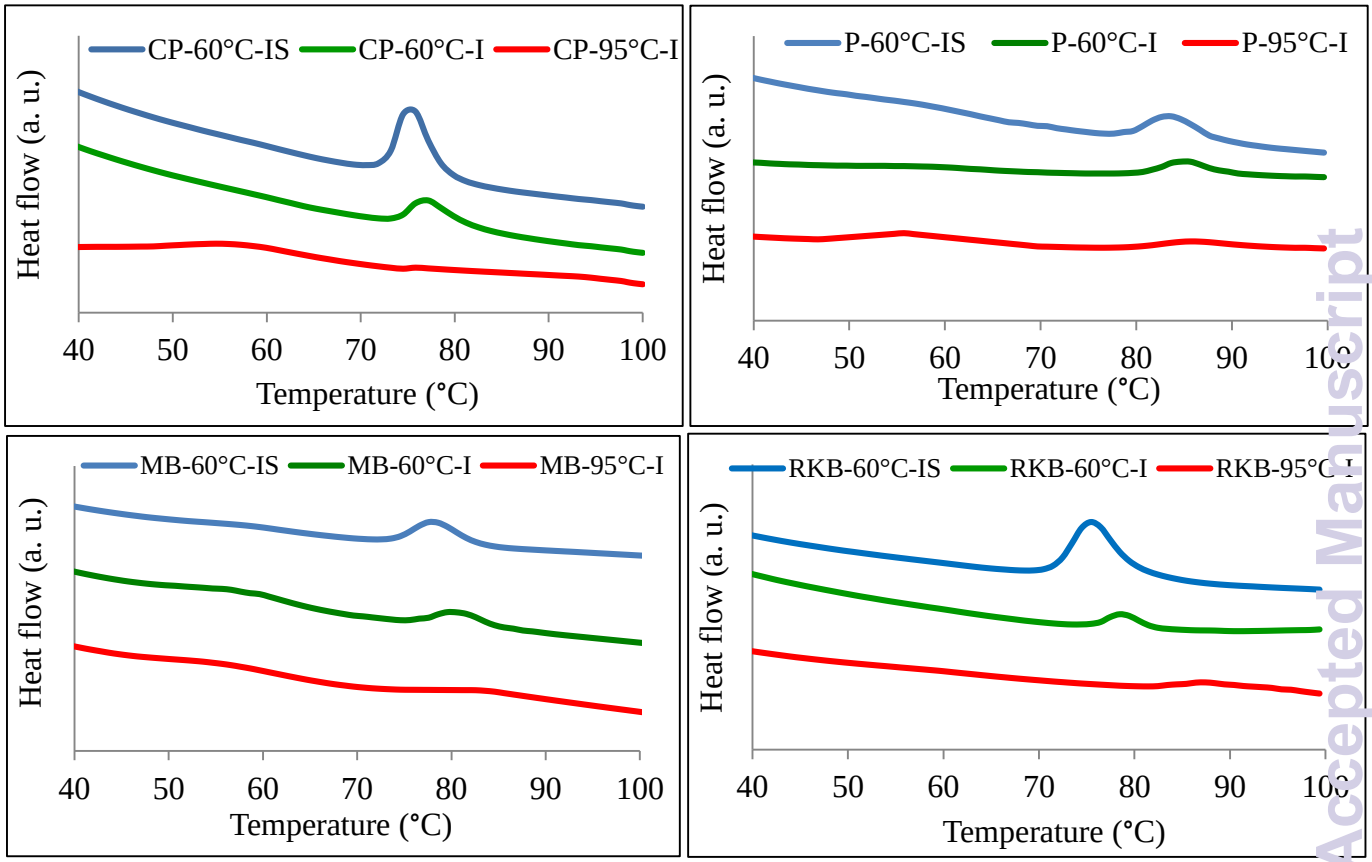

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Figure 3A

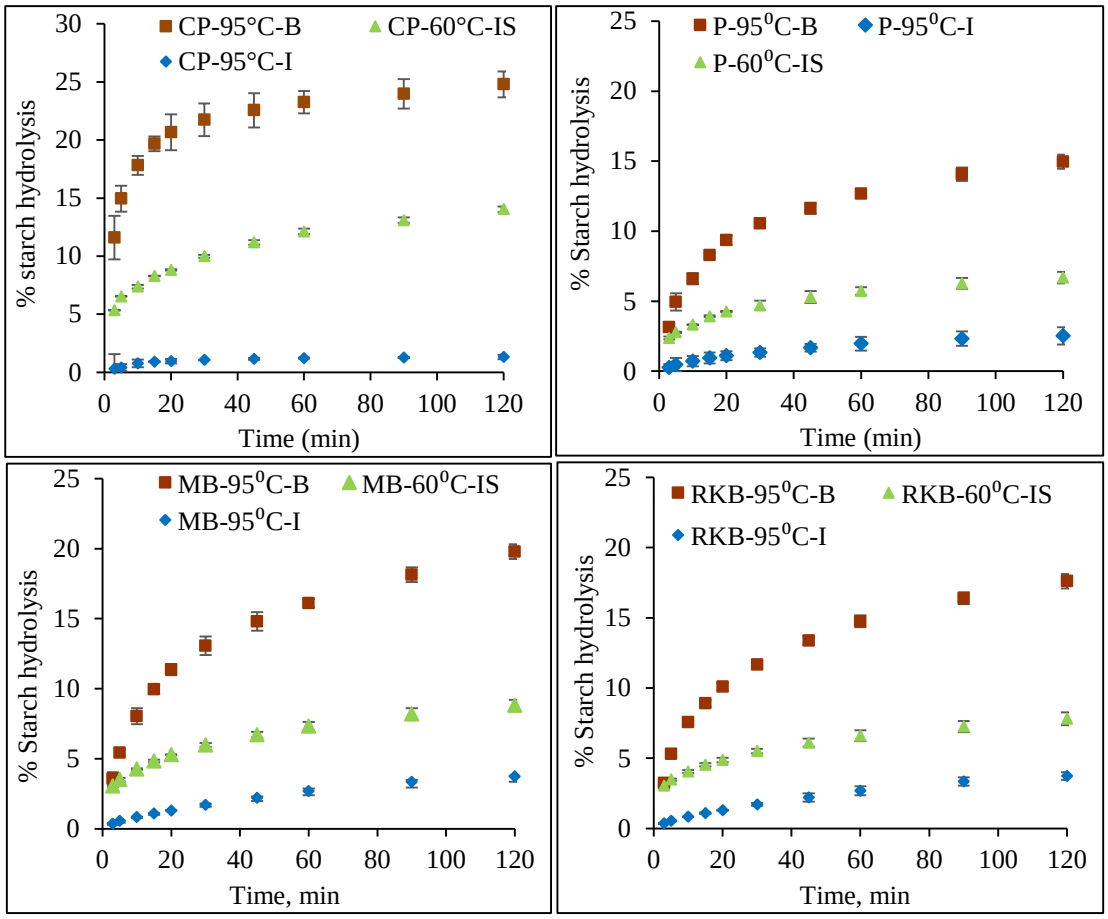
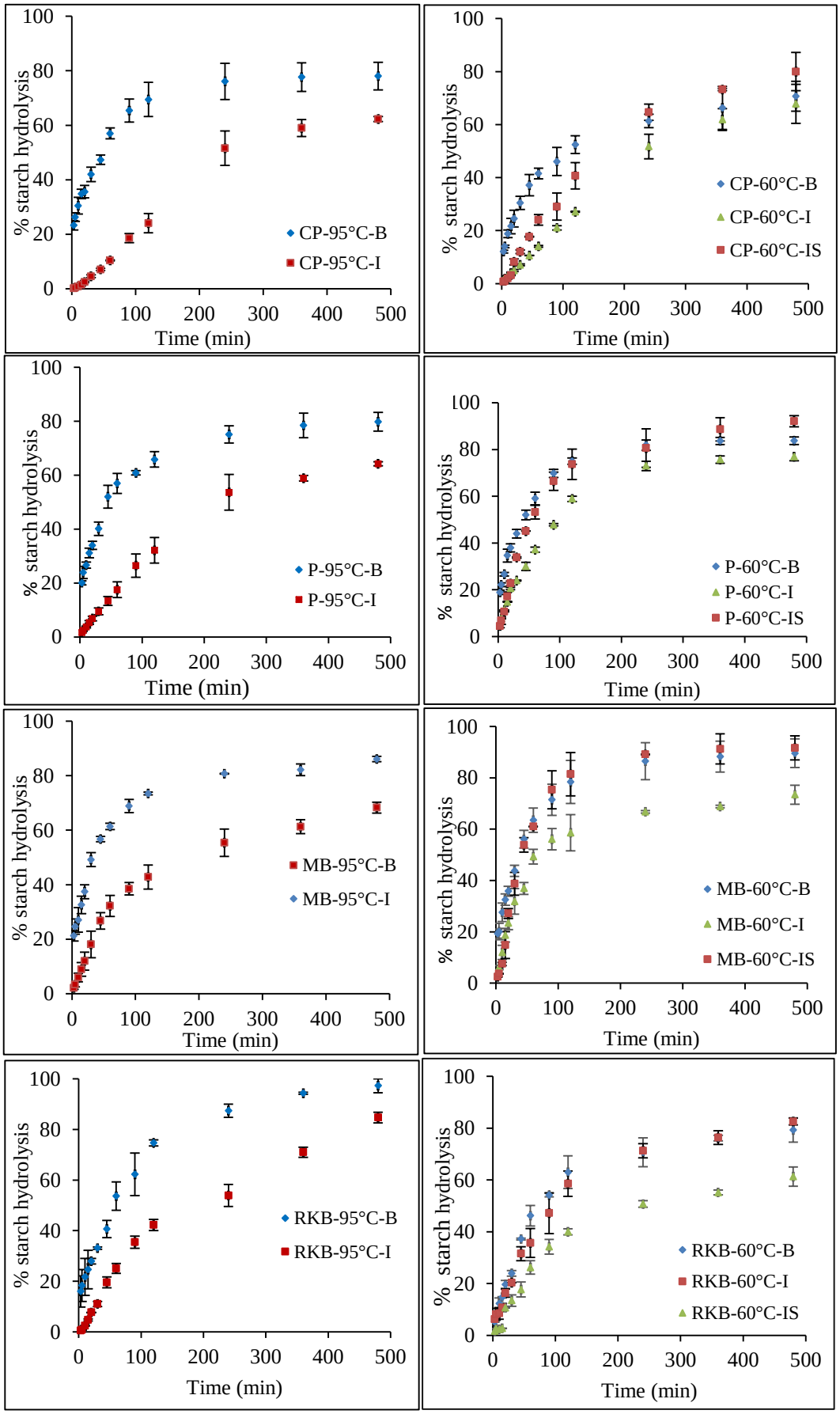
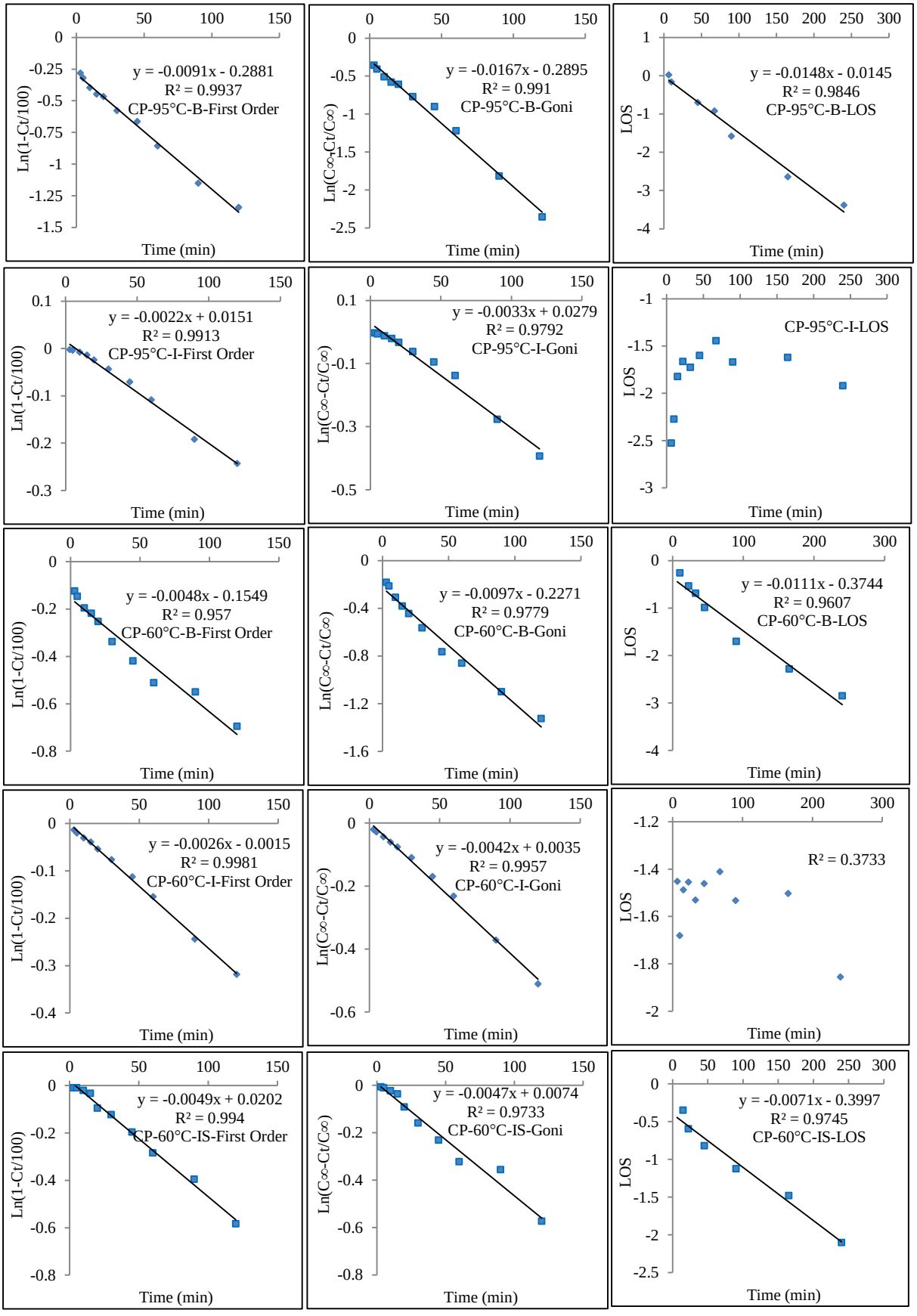


Figure 3B

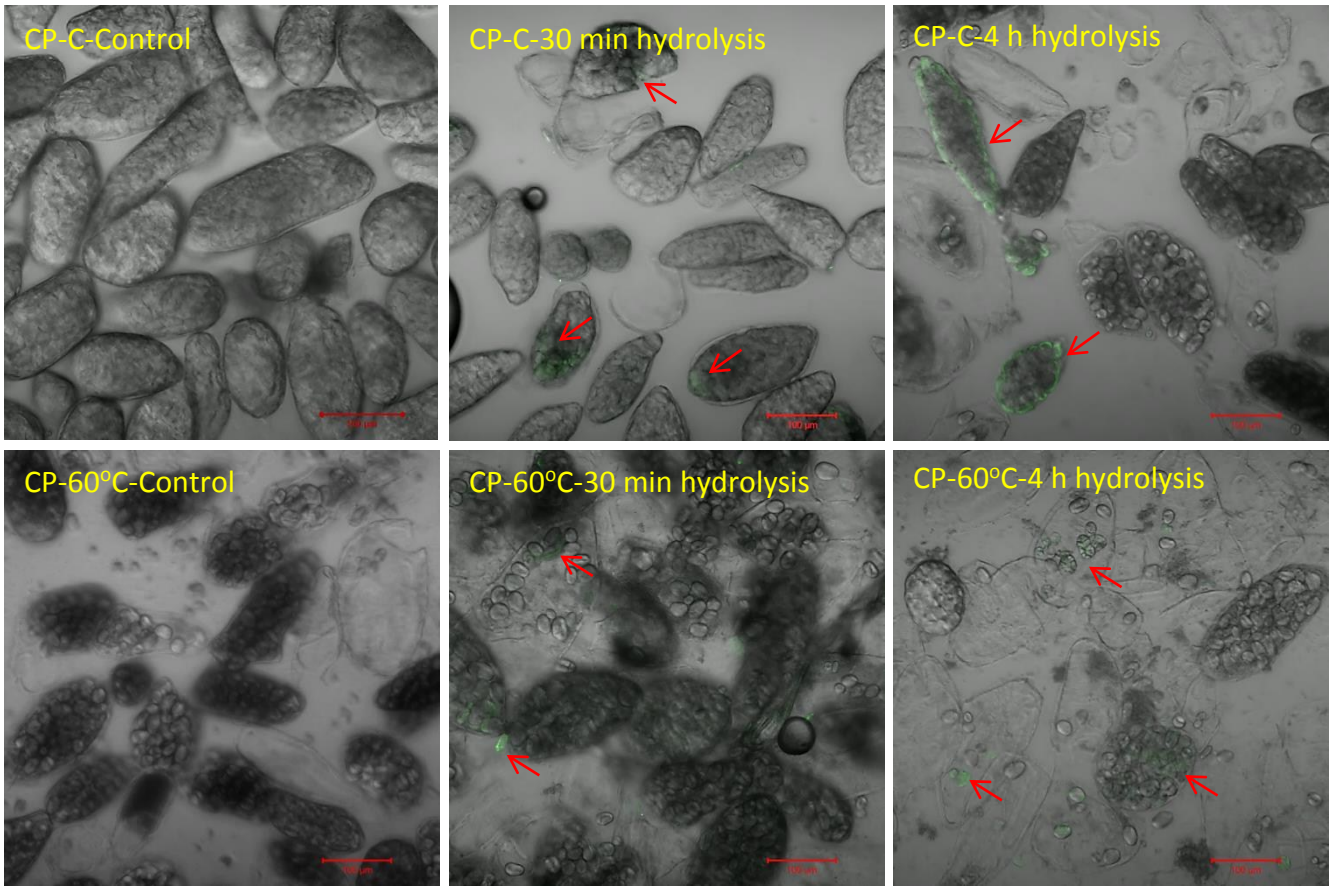


611 **Figure 4**



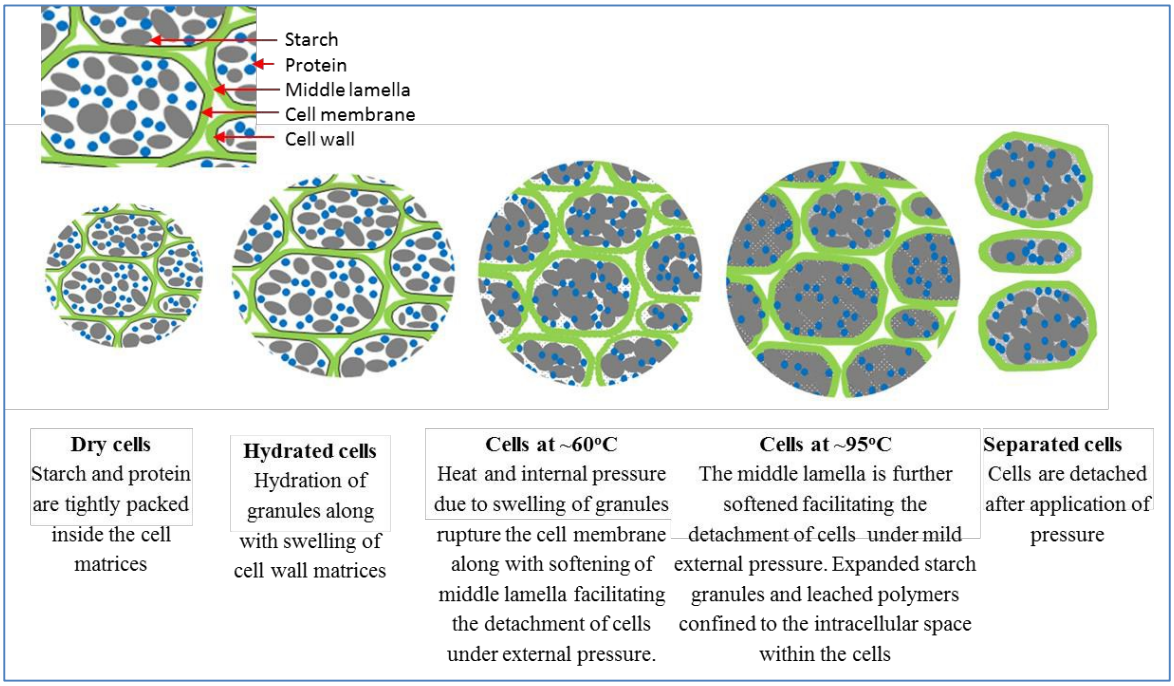
612

Figure 5



628 **Figure 6**

629



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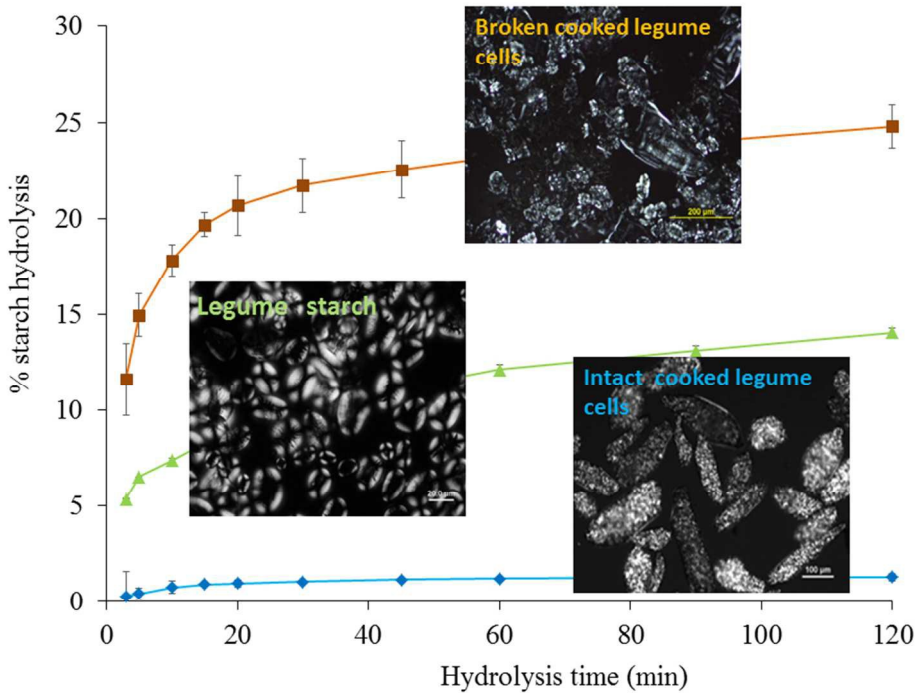

Graphical Abstract
254x190mm (96 x 96 DPI)