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A comparison of the kinetics of *in vitro* starch digestion in smooth and wrinkled peas by porcine pancreatic alpha-amylase

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Abstract

This study describes the impact of crop genetics and processing in two pea lines (*Pisum sativum* L.) on starch digestion kinetics. Mutation at the *rugosus* (*r*) locus leads to wrinkled pea seeds, a reduction in starch content and a lower extent of *in vitro* starch digestibility. The Logarithm of Slope (LOS) kinetic model was used to analyse digestion curves obtained using porcine pancreatic α-amylase for a range of particle size fractions. Changes in starch structure induced by the *r* mutation led to clear differences in starch digestion kinetics for purified starches and pea flours. Larger particle size fractions showed slowed starch digestion relative to the purified starch, but significant differences still existed between *r* and wild type pea lines. It is expected that this work will help inform the design of future studies where both starch structure and food structure are important determinants of digestion behavior.

Keywords: starch digestibility; LOS plots, *Pisum sativum* L.*; rugosus* locus; particle size

1. INTRODUCTION:

Starch is the main source of carbohydrate in the diet and accounts for 30-40% of daily energy intakes. The metabolic response to starch is largely dependent on the rate and extent of amylase digestion during gastrointestinal transit, which is in turn influenced by intrinsic starch characteristics as well as food matrix effects (Jenkins, Ghafari et al. 1982, Edwards, Grundy et al. 2015, Lovegrove, Edwards et al. 2017). From a human health perspective, starch sources that are less susceptible to α-amylase action are preferred, as this form of starch has a low impact on postprandial glycaemic responses, and is more likely to enter the colon (i.e., as 'resistant starch'), where its microbial fermentation supports colonic health (Ze, Duncan et al. 2012). Most staple foods tend to contain starch that is highly available and rapidly digested, however, and therefore do not provide such beneficial effects. Thus, there is great interest in increasing dietary provisions of slowly digested and/or resistant starch, yet identifying optimal food sources is challenging due to the complex and interrelated factors (e.g., botanical, structural, processing) that influence starch digestibility in processed foods.

Intrinsic starch characteristics, such as the ratio of amylose:amylopectin, granule size and molecular order, are known to influence the susceptibility of α-glucan chains to amylase hydrolysis, and may thereby contribute to influence the glycaemic response to starch. As intrinsic starch characteristics are defined during biosynthesis (Smith 2001), approaches that control gene expression and enzyme activity during development provide a means of obtaining starch with predictable functional properties (Hedley, Bogracheva et al. 2002). For human nutrition applications, however, it is essential to also consider the context in which starch is normally consumed: Starch tends to be subjected to some form of processing (normally hydrothermal treatment or extrusion) during which major structural changes may occur (e.g., gelatinisation) resulting in a loss of ordered starch granule structure and altered digestion behaviour (Cooke and Gidley 1992, Tahir, Ellis et al. 2011). Starch is also typically incorporated into a more complex food matrix, which may influence processing behaviour and hinder the interaction of starch with

enzymes and digestive fluids during gastrointestinal transit (Grassby, Edwards et al. 2013, Edwards, Grundy et al. 2015, Edwards, Warren et al. 2015). Thus, optimal design of starch sources for use in functional foods requires understanding of both intrinsic (starch granule) and extrinsic (food matrix) factors in order to control the rate and extent of digestion.

In peas (*Pisum sativum* L.), a range of mutants have been identified through breeding and mutagenesis programmes, which affect different steps in the starch synthesis pathway. Wild-type (WT) peas are known to undergo mutation at the R and RB gene loci, resulting in peas with a 'wrinkled' appearance. This study focusses on the WT (*RRRbRb*) genotype pea and a homozygous recessive mutant, '*r*-mutant' (*rrRbRb*), which has a mutation at the *rugosus* (*r*) locus that affects the activity of the starch branching enzyme I (SBEI) during biosynthesis (Smith 1988, Bogracheva, Davydova et al. 1995, Bogracheva, Cairns et al. 1999). SBEI is a transglycosylase involved in amylopectin synthesis (Ratnayake, Hoover et al. 2002), and this mutation causes the amylose content of the *r*-mutant starch to increase to ~70% (Wang, Bogracheva et al. 1998), which is more than double that in the WT pea starch (Bhattacharyya, Martin et al. 1993) Starches in *r*-mutant seeds also have amylopectin with longer chains than in the WT starch granules (Colonna and Mercier 1984). This results in starch with a highly heterogeneous structure and dramatically altered gelatinisation behaviour. Compared to wild type pea starch, the gelatinisation transition of *r*-mutant pea starch is much broader, and the granules have a much less ordered structure, with only indistinct birefringence when viewed by polarised light microscopy (Bogracheva, Morris et al. 1998, Bogracheva, Cairns et al. 1999, Tahir, Ellis et al. 2011).

Previous studies have reported that high amylose starches have a lower digestibility (Panlasigui, Thompson et al. 1991) suggesting that *r* mutant pea starch may also be a useful source of low glycaemic carbohydrate. The form in which the *r*-mutant starch would be delivered for optimal nutritional impact requires further investigation, however, and there are a number of factors to consider. In particular, canned/cooked

peas and indeed other legumes are already known to have a low glycaemic impact, because they contain a high proportion of encapsulated starch post-mastication (Golay, Coulston et al. 1986, Foster-Powell, Holt et al. 2002, Dahl, Foster et al. 2012). In such food systems (i.e., where the starch is encapsulated), the plant cell walls prevent starch-amylase interactions in the upper gastrointestinal tract (Edwards, Grundy et al. 2015). Legume flours on the other hand, consist predominantly of ruptured cells where the starch is highly exposed to digestive enzymes (Edwards, Warren et al. 2014), such that the intrinsic starch characteristics are likely to be major determinants of digestibility and glycaemia. When working with mutant plant tissues, the possibility of pleiotropic effects (e.g. alterations in cell wall structure as a result of mutations affecting starch synthesis) should also be considered (Wang and Hedley 1991).

The aim of this study was to analyse the effects of particle size and botanical origin of pea species on starch gelatinisation behaviour and digestibility. A milling procedure was used to disrupt plant tissue and to systematically expose starch granules entrapped by plant cell wall structures in a controlled manner. We have employed first-order enzyme kinetics and Logarithm of Slope (LOS) analysis to seek new mechanistic insights into the role starch structure, processing and cell wall encapsulation plays in determining starch susceptibility to amyloloysis.

2. MATERIALS & METHODS

2.1 Food Materials

Wild type (*RRrbRb)* and *r*-mutant (*rrRbRb*) pea seeds (*Pisum sativum L.)* were provided as a gift by Dr Claire Domoney (John Innes Centre, Norwich, UK) (*Figure 1*).

2.2 Starch Isolation and Purification

Starch was isolated from these materials based on principles described previously (Edwards, Warren et al. 2014). In brief, hydrated pea seeds were blended with an Ultra-Turrax and washed through sieves (apertures 0.25 and 0.125 mm) to exclude particulates

(e.g., intact cells and seed-coat fragments). The resulting filtrate was then centrifuged (10 min at 3000 x g) to achieve a starch-rich pellet. Purification was achieved by discarding supernatants and discoloured sediment layers, and re-suspending the pellet in 95% ethanol and repeating centrifugation. This step was repeated until the pellet was a uniform white and no layering occurred. Purified starches were then suspended in 99.8% ethanol, transferred to an open container and left at room temperature until dry.

2.3 De-hulling and Milling of Seeds

Pea seeds were manually dehulled following overnight soaking in dH_2O, then dried overnight in an oven (35°C) prior to milling. These materials were then passed through a hammer mill (IKA® MF 10 Basic Microfine Grinder Drive, 6500 rpm) equipped with a 4 mm screen. The milled materials were segregated by size using vertical sieving under gravity with mechanical agitation in a sieve shaker (Endecotts, Ltd., London, UK). Three sieves (1, 0.50 and 0.25 mm aperture) were selected to give a broad spectrum of particle size ranging from 1 mm to 0.25 mm (*Figure 1*). These size fractions were selected to cover a range of particle sizes that occur post-mastication of edible plant tissues (Ranawana, Monro et al. 2010).

2.4 Characterisation of Plant Food Materials

Particles were ground to pass through a 0.5 mm sieve before determination of total starch content using the KOH format of the Total Starch (K-TSTA) AOAC 996.11 procedure (Megazyme International Ireland, Ltd., Wicklow, Ireland). Moisture contents were determined for each particle size by oven-drying at 105°C to a constant weight. All analysis was performed in triplicate.

2.5 Microscopy

Milled particle size fractions of WT and *r*-mutant pea were examined by brightfield and polarised light microscopy using an Olympus BX60F5 (Hamamatsu Photonics,

Hamamatsu, Japan) microscope equipped with a ProgRes® C10plus (Jenoptik Laser, Germany) camera. Samples were suspended in a drop of deioinised water and stained with Potassium iodide for visualisation of starch granules. Birefringence was assessed before and directly after Differential Scanning Calorimetry (DSC) runs.

2.6 Gelatinisation properties

A Multi-Cell DSC (TA Instruments, Elstree, UK) was used to measure starch gelatinisation within milled tissue in excess water under quasi-equilibrium conditions, as described previously (Edwards, Warren et al. 2015). In brief, starch or milled materials were weighed into 1.00 mL Hastelloy® ampoules to which was added 1.00 g of degassed deionised water. The weight of material added was adjusted based on the starch content so that each pan contained 50 mg of starch (i.e. 110 mg or 140 mg of milled WT or r-mutant pea seed, respectively). An ampoule containing 1.00 g deionised, degassed water only was included as a reference. All pans were hermetically sealed, and gently shaken before loading into the instrument. Following an equilibration period (3 h), the samples were heated from 10°C to 120°C at 1 °C.min^{-1}. Measurements were made in triplicate and each repetition was carried out with the pan in a different position in the multi-cell instrument. Peak integration and estimation of gelatinisation parameters were performed using NanoAnalyze software (version 3.6.0, TA Instruments 2015©) Onset, peak and conclusion temperatures (denoted T_o, T_p, and T_c) and gelatinisation enthalpy (ΔH) were obtained from each thermogram as described elsewhere (Bogracheva, Wang et al. 2002).

2.7 *In Vitro* Starch Digestion

Starch digestibility was determined based on principles described previously (Butterworth, Warren et al. 2012, Edwards, Warren et al. 2014). Milled samples and purified starch were weighed into tubes and suspended in 5 mL phosphate buffered saline (PBS, pH 7.4) so that each tube contained 35 mg starch. In this study, we accounted for the lower starch content of the r-mutant pea (compared with the WT), thus enabling the effect of other

material characteristics on starch digestion kinetics to be investigated. The samples were hydrothermally processed in a water bath at 90°C for 10 min with vortex mixing every minute to ensure the complete dispersion and gelatinisation of starch granules. Samples were then allowed to equilibrate at 37°C for 20 min at 30 rpm on a rotary mixer (Stuart rotator SB3). A 'blank' aliquot (100 µL) of each sample was taken into 1.5 mL microfuge tubes containing 100 µL of 0.3 M $NaCO_3$ (pH 9). To start the assay, porcine pancreatic α-amylase (EC 3.2.1.1, supplied in a DFP-treated suspension of 2.9M NaCl containing 2mM CaCl2, A6255, Sigma-Aldrich Co. Ltd, Poole, UK) was added to achieve an activity of 2 U/mL (1U liberates 1 mg maltose from starch in 3 min at pH 6.9, 37°C) in the digestion mixture (i.e. containing 7 mg/mL starch). The tubes were promptly returned to the rotary mixer and incubated at 37°C for the duration of the digestion. After 1, 2, 3, 4, 5, 8, 10, 15, 30, 45 and 60 min, aliquots (100 µL) of the digestion mixture were withdrawn and diluted 1:2 in 0.3 M $NaCO_3$ to stop the reaction. Aliquots were centrifuged at 13,000 x *g* for 5 min to exclude any starch remnants and the supernatants stored at -20°C for subsequent analysis of starch amylolysis products.

The concentration of reducing sugars (i.e. produced from starch amylolysis) in aliquots recovered from various time points were quantified using the PAHBAH assay (Lever 1972). Stored supernatants were appropriately diluted (typically 1:10) in deioinised water, and 20 µL of the diluted sample transferred to a clear plastic flat bottom well 96-well plate, to which was added 200 µL freshly prepared PAHBAH working reagent (250 mg *p*-hydroxybenzoic acid hydrazide dissolved in 4.75 mL of 0.5 M HCL, and made up to 50 mL with 0.5 M NaOH). Standards containing known concentrations of maltose (20-900 µM) were included on each 96-well plate. The plate containing samples and standards was incubated at 100°C for 5 min and subsequently equilibrated for 10 min at room temperature before measuring absorbance (λ =405 nm) in a microplate reader (Bio-Rad Benchmark Plus, Waukegan, Illinois, USA). Reducing sugars were expressed as maltose equivalents by reference to a standard curve.

2.8 Logarithm of Slope Analysis

Starch digestibility data (i.e. concentration of reducing sugars produced over time) for pure starches and milled particles <0.25mm was fitted to a first-order equation (eqn. 1), where C_t represents the concentration of products (i.e. expressed as maltose equivalents) at a given time (t), C_∞ is the concentration of product at the end of the reaction, and k is the digestibility rate constant. The values of C_∞ and k were obtained from Logarithm of Slope (LOS) plots, as described previously (Butterworth, Warren et al. 2012, Edwards, Warren et al. 2014).

$$C_t = C_\infty\left(1 - \mathrm{e}^{-kt}\right) \qquad \text{(eqn. 1)}$$

Data obtained from digestion of larger size-fractions was fitted to the modified first-order equation (eqn. 2) proposed by Edwards et al 2014. The modification accounts for the biphasic nature of starch digestion in heterogeneous particles, e.g., where the starch is encapsulated within intact plant cells. In such materials, LOS plots reveal two or more distinct near-linear phases, each represented by a set of enzyme-kinetic parameters which must be accounted for (as shown in eqn. 2) to describe the overall digestion process.

$$C(t) = \begin{cases} C_{1\infty}\left(1 - e^{-k_1 t}\right), & if\ t \le t_{int} \\ C_{int} + C_{2\infty}\left(1 - e^{-k_2 \times (t - t_{int})}\right), & if\ t \ge t_{int} \end{cases} \qquad (2)$$

Starch amylolysis is represented by two consecutive first order reactions, in which the time identifiers indicate the duration of each reaction phase and t_{int} is the intersections between the two phases. Distinct enzyme kinetic parameters are obtained for each phase (denoted $C_{1\infty}$, $C_{2\infty}$ and k_1, $k_{2)}$ using Logarithm of Slope (LOS) analysis.

LOS plot analysis was performed using data expressed as product concentrations (maltose equivalents), however to aid interpretation, the C_∞ values and digestibility curves are given as a percentage of total starch.

3. RESULTS

3.1 Material Characteristics

The total starch content (mean + SD) of milled fractions of WT and *r*-mutant pea was 50 ± 1.4 and 36 ± 2.6, respectively, expressed on a g per 100 g dry weight basis. Moisture contents were between 6 and 7 % for all size fractions. No significant differences were observed between the starch or moisture content of the different milled fractions within each phenotype. Microscopy observation confirmed that in the smallest fraction, the cells were ruptured and all starch was exposed *(data not shown).*

3.2 Gelatinisation Behaviour

DSC analysis of starch and milled fractions of WT and *r*-mutant peas revealed clear differences in gelatinisation behaviour of the two phenotypes: Starch isolated from WT pea underwent an endothermic transition (gelatinisation) with a peak temperature of 69°C whereas starch isolated from *r*-mutant showed a very broad transition endotherm *(Supplementary Figure 1*), confirming the findings of previous workers for the thermal behaviour of these pea lines (Bogracheva, Cairns et al. 1999). The starch gelatinisation peak observed for the milled fractions of WT samples occurred at a (2-3°C) higher temperature than the purified starch. It should be noted that the starch gelatinisation endotherm overlaps with other endothermic transitions (possibly originating from protein present in these fractions), thus preventing reliable determination of onset and concluding temperatures and gelatinisation enthalpies for the pea flour and milled fractions.

3.3 Starch Digestibility

Digestion progress curves are shown for each particle size of WT and *r*-mutant samples in *Figure 2*. Both particle size and botanical source had an effect on starch digestibility profiles. Intrinsic starch digestibility was 31% lower in isolated starch obtained from the *r*-mutant than from the WT pea. Within each botanical source, the starch and flour fractions had similar digestibility, and the extent of starch digestion was markedly reduced with increasing particle size and therefore degree of cell wall encapsulation *(Figure 3*). In the

largest size fraction (>1 mm), only 24-25% of the starch was digested, and no difference in the extent of digestion was observed between the two botanical sources. This particle-size dependent reduction in the extent of starch digestion is indicative of reduced starch bioaccessibility where the food matrix imposes a physical barrier to enzyme ingress.

3.3 LOS analysis

LOS plots (*Figure 3*) were obtained from digestibility curves to enable identification of first-order rate parameters. Plots obtained from WT and *r*-mutant purified starches and flours (<0.25 mm) were characterised by a single linear phase, denoted by one rate constant. The rate constants were similar for WT and *r*-mutant starch (k = 0.13 and 0.15 .min^{-1}) but a larger proportion of the starch was digested in the WT starch than in the *r*-mutant (C_∞ = 82 vs 57%, respectively). The value of the rate constants were lower for the flour (k = 0.10 min^{-1} for both WT and r-mutant peas) than for the purified starch.

Plots obtained for larger particle size fractions (>0.25 to >1mm) revealed that digestion in these materials occurred by a biphasic reaction (*Figure 3*), characterised by two linear phases of digestion, each with a distinct rate constant (*Table 1).* The value of the rate constants was always higher in the first ('rapid') phase (k_1) than the second phase (k_2). For both WT and *r*-mutant milled fractions, the value of k_1 seemed to increase with increasing particle size. The value of k_2 was always lower in the larger milled particles than the value of rate constants obtained from starch and flour fractions, but from the data obtained k_2 did not appear to be strongly influenced by particle size or botanical source *(Table 1*). As would be expected based on the digestibility curves obtained (*Fig. 2),* the Total C_∞ values (i.e. representing the total extent of starch digestion) were clearly reduced with increasing particle size and were generally lower for milled fractions of r-mutant peas than matched fractions of WT peas.

The total C_∞ values represent the sum of two digestion phases, and thus $C_{1\infty}$ and $C_{2\infty}$ values (*Table 1*) provide insight into the contribution of each reaction phase to total starch digestion; In WT peas, the highest proportion of starch digestion occurred during

the rapid phase, whereas in r-mutant peas, a greater amount of starch is digested in the slow-phase such that, overall, similar proportions of starch were digested in the rapid and slow phase (*Figure 4*). When starch digestibility was expressed as a proportion of the hydrolysable starch fraction (i.e. 57% or 82% for *r*-mutant and WT peas), the digestibility profiles were similar for matched size fractions of WT and r-mutant pea (*Supplementary Fig 2*), suggesting that both materials contained starch that was equally susceptible to amylase hydrolysis, but that r-mutant seeds contain a smaller amount of hydrolysable starch.

4. DISCUSSION

We have used enzyme kinetic analysis and DSC to gain insight into the mechanisms governing starch digestibility in cooked tissues obtained from WT and *r*-mutant peas. Unlike previous digestibility studies which have focussed on extracted starches, we used cooked macro-particles of pea cotyledons to determine the effect of the *r*-mutation on starch digestion kinetics in intact pea tissues. One key finding of this study was that differences in intrinsic susceptibility of the purified starches to α-amylase hydrolysis became less pronounced when the starch was encapsulated within intact plant cells, i.e. as occurs in cooked whole peas and indeed other pulses. There was also no evidence of major differences in apparent cell wall permeability to α-amylase within milled macro particles of *r*-mutant and WT seeds. Furthermore, LOS analysis revealed that the digestible portion of starch in both *r*-mutant and WT pea seeds were hydrolysed by α-amylase at a similar rate, and that the lower extent of starch digestion in the *r*-mutant was attributed to a resistant proportion of starch. These findings have important implications for how *r*-mutant and WT peas are best processed and utilised to achieve desirable postprandial responses (i.e. attenuated glycaemia) in humans.

The differences in intrinsic starch characteristics of the WT and *r*-mutant were reflected in the digestibility curves obtained from the cooked purified starches. As expected, a lower extent of digestion was observed in the *r*-mutant compared with the WT.

Despite the differences in the extent of starch digestion, LOS plots revealed that the hydrolysis of pure starch and flour from both botanical sources occurred through a single first-order reaction represented by one rate constant. For *r*-mutant starch where digestibility was lower, there was no evidence for the contribution of digestion from a different starch fraction, for example as would normally be seen with raw/retrograded starch. Interestingly, the rate constants were similar between both botanical sources. This novel finding suggests that the WT and *r*-mutant pea starch had a similar susceptibility to α-amylase, but that the proportion of starch that could be digested at this rate differed, hence giving rise to different C∞ values. Considering the value of the rate constants in the context of previous studies (Edwards, Warren et al. 2014), it is proposed that the starch that is digested in the first, rapid phase has a more amorphous structure and is situated on the periphery of plant tissue macroparticles. This fraction appears to be present to some extent in both WT and r-mutant peas.

The impact of non-starch components such as protein and fat, which are present in different quantities between WT and r-mutant peas (Bhattacharyya, Martin et al. 1993), is evident from comparison of starch digestibility profiles obtained for pure starch and flours in Figure 2. Although the digestibility profiles were similar, there was a tendency for the flour fraction, which contains protein, lipid and fibre, to be digested more slowly than the purified starch fractions in both WT and r-mutant, although the effect was more marked in the WT pea.

Starch digestibility curves obtained from cooked milled macro-particles (>0.25 to >1 mm) provided evidence that cell wall encapsulation is an important factor that limits the extent of starch digestion within *r*-mutant and WT pea seeds. The reduction in C_∞ with increasing particle size (and thereby proportion of encapsulated starch) was similar to that described previously for milled chickpea fractions (Edwards, Warren et al. 2014), and implies that the cell walls of both pea genotypes are impenetrable to α-amylase. Indeed, although the digestibility of pure, cooked *r*-mutant starch was lower than the WT starch, the differences between the two botanical sources became less evident at larger particle sizes

where a greater proportion of the starch is encapsulated within plant cells. It is also possible that the presence of these cellular structures may play a role in limiting starch gelatinisation during hydrothermal processing (Edwards, Warren et al. 2015), thus limiting the susceptibility of entrapped starch to amylolysis. Overall, this data provides convincing evidence that cell wall encapsulation is a rate limiting factor controlling digestion of encapsulated starch in WT and *r*-mutant peas. This finding is relevant to a number of food systems prepared from cooked peas and indeed other pulses, which have a tendency to cell separate (Waldron, Parker et al. 2003) such that intact, starch-containing cells are present post-mastication (Noah, Guillon et al. 1998). Indeed, this would likely account for a major source of resistant starch (Type 1, 'encapsulated starch') from cooked peas.

The origin of the low digestibility of cooked *r*-mutant pea starch is still the subject of speculation. Previous studies on *r*-mutant pea starch demonstrated that in the native state, *r*-pea starch is hydrolysed at a significantly higher rate than the WT pea starch (Tahir, Ellis et al. 2010), presumably due to the disordered structure of the native granule. Further evidence for this mechanism is supported by (Warren, Royall et al. 2011), who showed that native *r*-pea starch has an unusually high affinity for pancreatic α-amylase. Following hydrothermal treatment, the hydrolysis rate of *r*-mutant starch increases, but its digestibility is still considerably lower than that of hydrothermally processed WT starch (Tahir, Ellis et al. 2010), which was attributed by the authors to the stability of *r*-mutant pea starch to hydrothermal treatment, evidenced here by the broad gelatinisation endotherm and higher melting temperature observed by DSC. Our enzyme-kinetic studies provided further evidence that a fraction of the *r*-mutant pea starch is effectively indigestible following hydrothermal treatment. Speculatively, it may be that the proportion of the *r*-mutant starch which is not fully gelatinised also remains indigestible. Alternatively it has been hypothesised that in high amylose starches an indigestible fraction is formed as a result of α-amylase activity, due to the action of the enzyme releasing linear glucan chains which are able to recrystallize during digestion (Teng, Witt et al. 2016). The data presented in the

present paper do not permit any of these possible mechanisms to be ruled out, and further research is needed into the mechanism of reduced digestibility in *r*-mutant starch.

5. CONCLUSIONS

Enzyme kinetic studies of hydrothermally processed starches and milled fractions (0.25 - >1mm) of WT (*RRRbRb*) and *r* mutant (*rrRbRb*) peas provided new insight into the characteristics responsible for differences in starch digestibility between these pea species. LOS analysis revealed that r-mutant pea starch contains a proportion of starch that is completely resistant to α-amylase hydrolysis. This resistant fraction of the r-mutant pea is thought to occur as a results of resistance to hydrothermal processing, or to be formed during amylolysis of susceptible starch. In materials where this starch was encapsulated within plant cell walls (i.e. as would be expected when eating whole cooked peas) the extent of starch digestion was markedly reduced in both botanical sources, indicating that intact plant cells impose limitations of starch bioaccessibility within both r-mutant and WT peas.

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FIGURE LEGENDS:

Figure 1: Photographs of whole seeds and milled fractions of WT (A) and r-mutant (B) pea seeds. Particle size was defined on the basis of material retention on sieves of known aperture. Note that the scale bar is only applicable to the whole seeds, and that the photographed milled fractions are not to scale. The figure annotations indicate the particle sizes of each milled fraction.

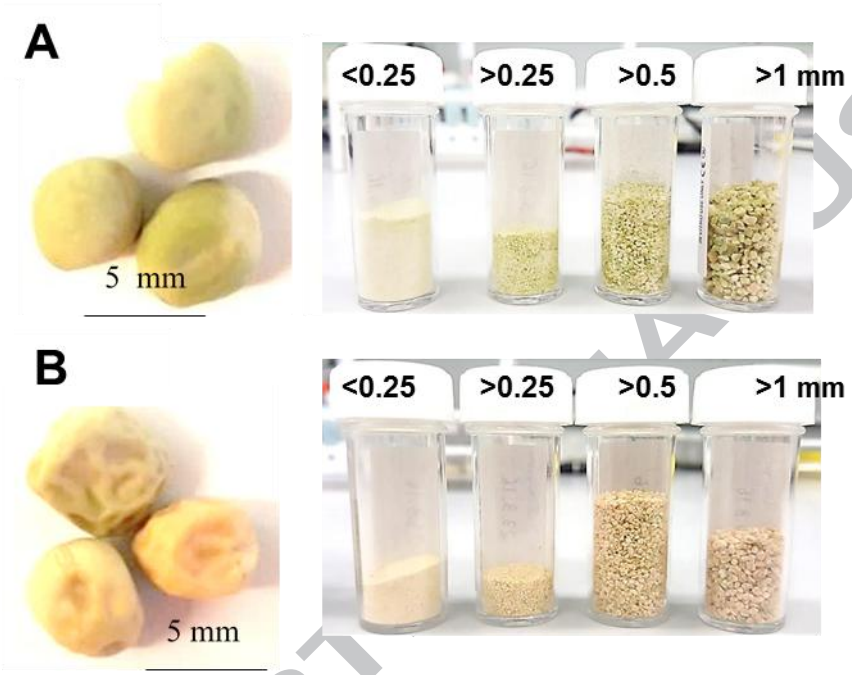


Figure 2: Digestibility curves obtained for milled particle sizes of hydrothermally processed WT (A) and *r*-mutant pea (B) samples digested by porcine pancreatic α-amylase. Values are means of triplicates ± SEM. Curve fits were computed using the parameters obtained from LOS analysis.

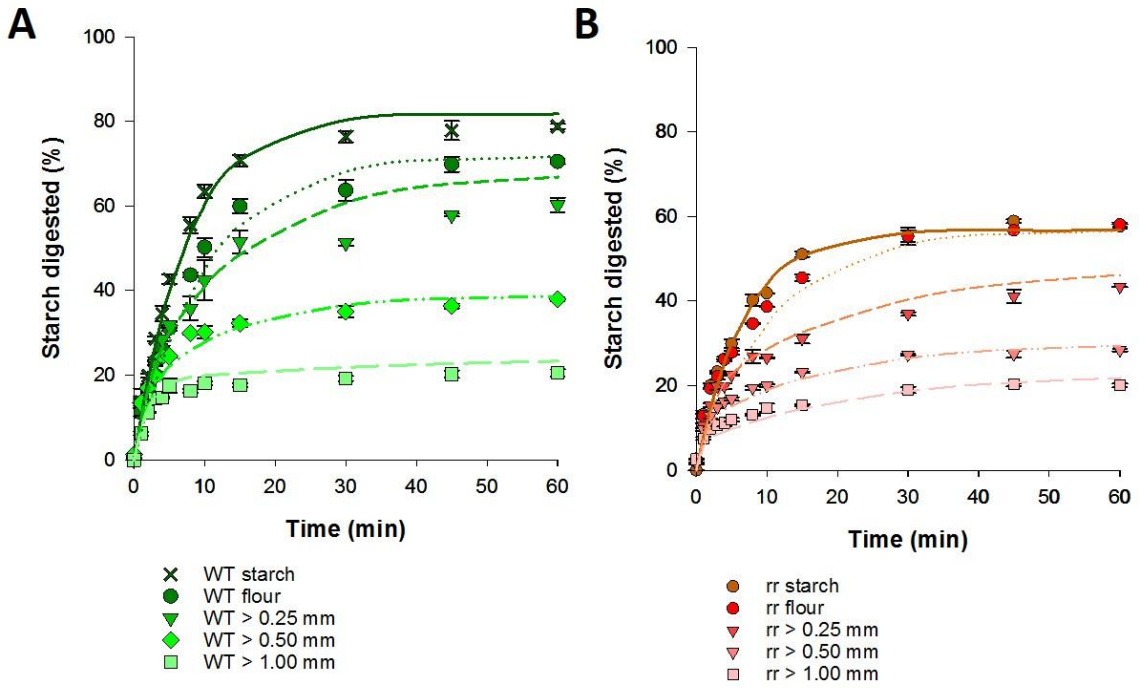


Figure 3: Logarithm of Slope (LOS) plots obtained from digestibility data of WT (A-E) and r-mutant (F- J) hydrothermally processed pea materials. A single linear plot is evident in LOS plots for starches and flours (A,B,F and G), whereas plots obtained for larger particle size fractions are biphasic. Linear equations represent each phase, and provide for determination of enzyme kinetic parameters.

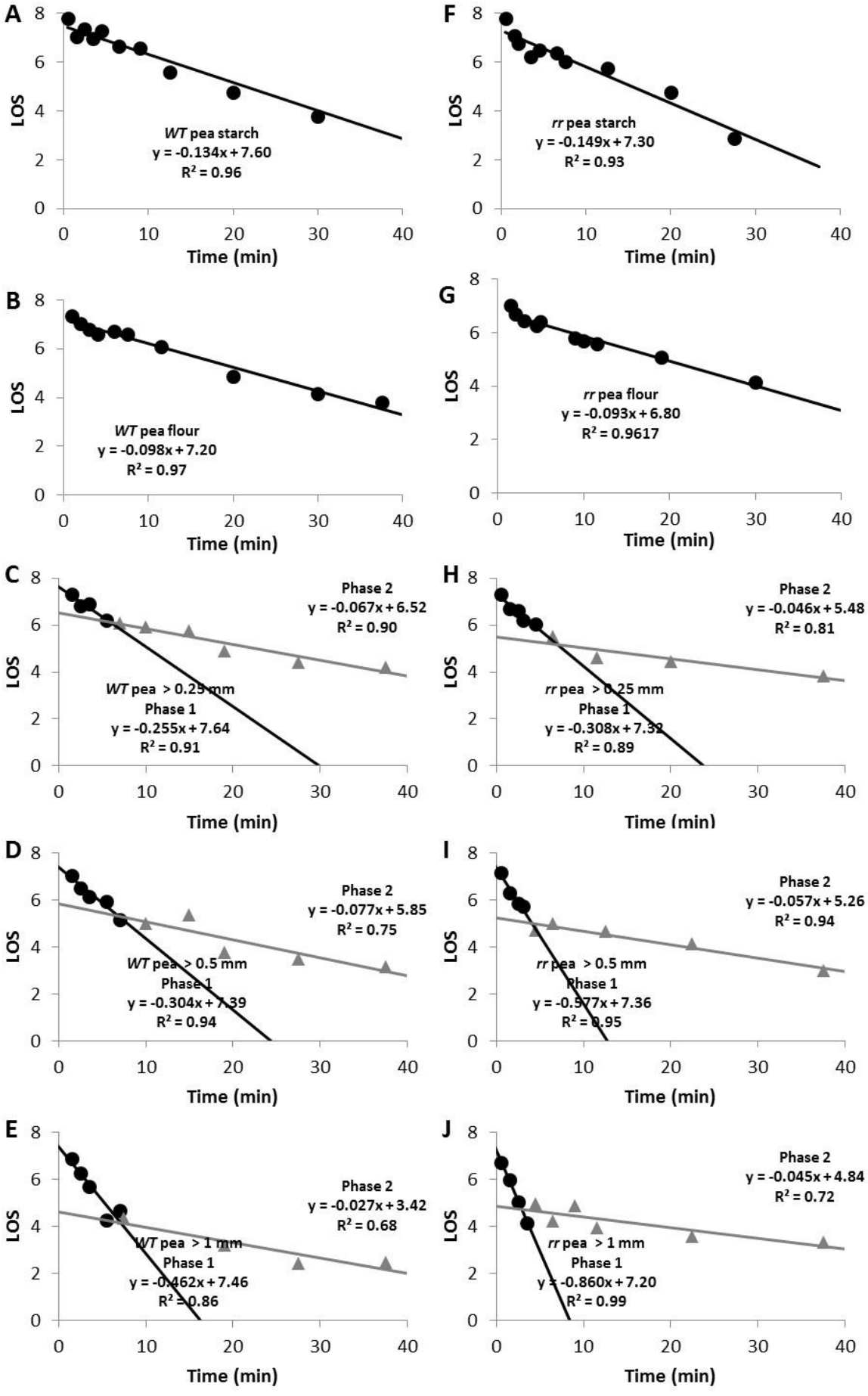
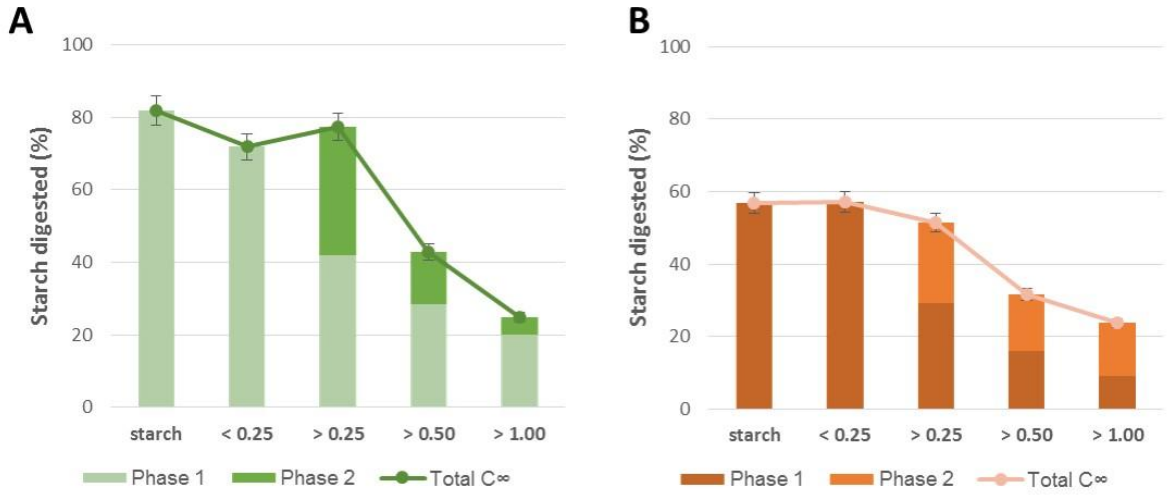
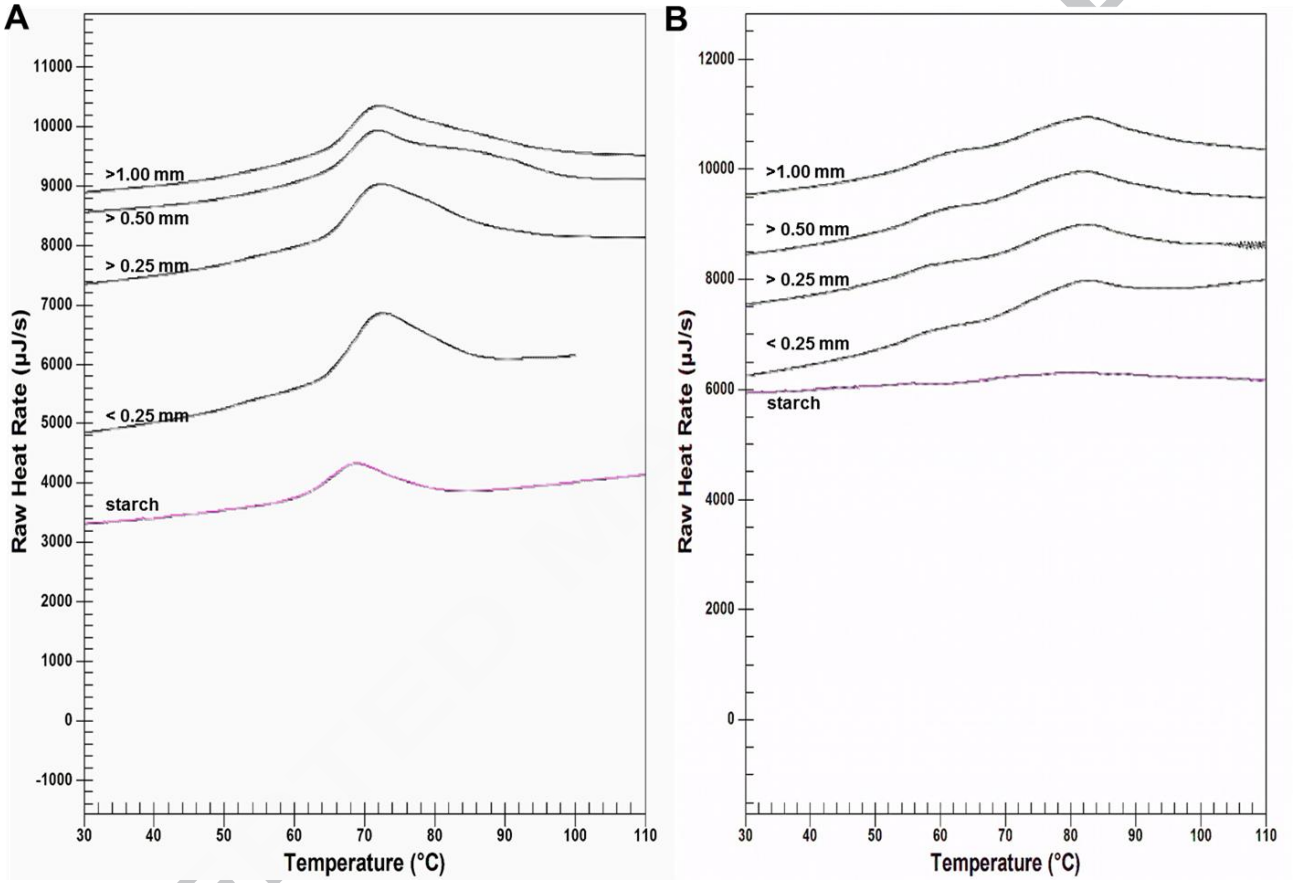

A
WT pea starch
y = -0.134x + 7.60
R² = 0.96
F
rr pea starch
y = -0.149x + 7.30
R² = 0.93
B
WT pea flour
y = -0.098x + 7.20
R² = 0.97
G
rr pea flour
y = -0.093x + 6.80
R² = 0.9617
C
Phase 2
y = -0.067x + 6.52
R² = 0.90
WT pea > 0.25 mm
Phase 1
y = -0.255x + 7.64
R² = 0.91
H
Phase 2
y = -0.046x + 5.48
R² = 0.81
rr pea > 0.25 mm
Phase 1
y = -0.308x + 7.32
R² = 0.89
D
Phase 2
y = -0.077x + 5.85
R² = 0.75
WT pea > 0.5 mm
Phase 1
y = -0.304x + 7.39
R² = 0.94
I
Phase 2
y = -0.057x + 5.26
R² = 0.94
rr pea > 0.5 mm
Phase 1
y = -0.377x + 7.36
R² = 0.95
E
Phase 2
y = -0.027x + 3.42
R² = 0.68
WT pea > 1 mm
Phase 1
y = -0.462x + 7.46
R² = 0.86
J
Phase 2
y = -0.045x + 4.84
R² = 0.72
rr pea > 1 mm
Phase 1
y = -0.860x + 7.20
R² = 0.99
LOS
Time (min)

Figure 4: Contribution of different starch fractions to total extent of starch hydrolysis in WT (A) and *r*-mutant (B) pea fractions. Phase 1 is the rapid digestion phase ($C_{1\infty}$), whereas phase 2 ($C_{2\infty}$), is the slower phase. Starches and flours are represented by a single phase. Error bars are 5% of the Total C_∞ value and the SEM of triplicate measures falls within this range.



Supplementary Figure 1: Representative DSC thermograms obtained from WT (A) and r-mutant (B) pea starches and milled fractions. The endothermic transition associated with starch gelatinisation in milled fractions is thought to overlap with other thermal transitions and therefore gelatinisation parameters could not be reliably obtained.



Supplementary Figure 2: Digestion of hydrolysable starch in milled pea samples. Hydrolysable starch (HS) is defined as the total amount of pure starch that can be hydrolysed by alpha-amylase (82 and 57% of total starch content for WT and r-mutant pea materials, respectively). Similar digestion of HS values between r-mutant and WT pea fractions indicate that the plant matrix is having a similar effect on starch availability in both botanical sources.

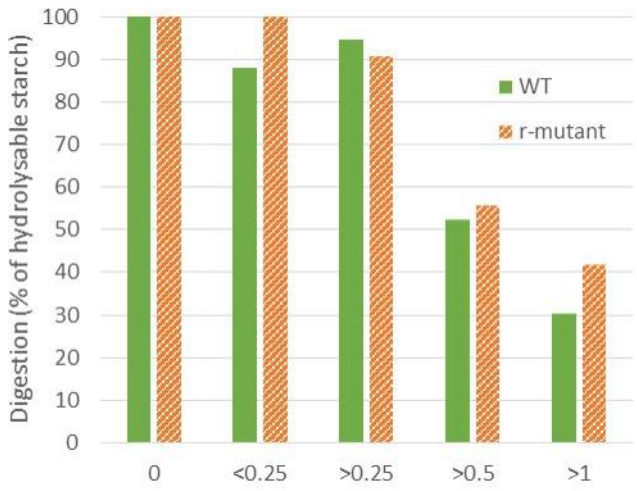


Table 1: Parameters of starch digestibility estimated from the LOS during the rapid phase (C_1) and the slower phase (C_2) of WT and *r*-mutant starches.

	WT					*r*-mutant				
Size (mm)	$C_{1\infty}$	k_1	$C_{2\infty}$	k_2	**Total C_∞**	$C_{1\infty}$	k_1	$C_{2\infty}$	k_2	**Total C_∞**
starch	n/a	n/a	82	0.134	**82**	n/a	n/a	57	0.149	**57**
< 0.25	n/a	n/a	72	0.098	**72**	n/a	n/a	57	0.093	**57**
> 0.25	42	0.255	36	0.067	**77**	29	0.308	22	0.046	**52**
> 0.50	29	0.304	14	0.077	**43**	16	0.577	16	0.057	**32**
> 1.00	20	0.462	5	0.027	**25**	9	0.86	15	0.045	**24**

Highlights

- Wild type and starch branching enzyme mutant pea varieties compared
- LOS analysis revealed effect of mutation on starch digestion kinetics
- Plant cells limit starch bioaccessibility in cooked r-mutant and WT peas
- Particle size following milling influences digestion kinetics of both WT and mutant