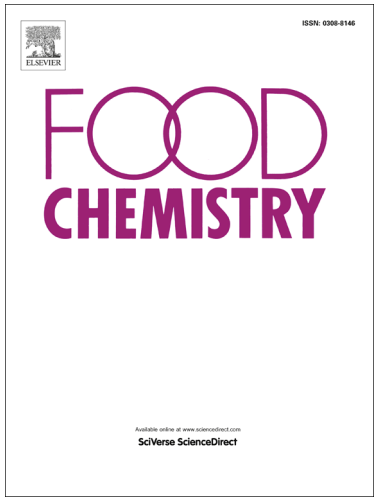

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Combined techniques for characterising pasta structure reveals how the gluten network slows enzymic digestion rate

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Abstract

The aim of the present study is to characterise the influence of gluten structure on the kinetics of starch hydrolysis in pasta. Spaghetti and powdered pasta were prepared from three different cultivars of durum semolina, and starch was also purified from each cultivar. Digestion kinetic parameters were obtained through logarithm-of-slope analysis, allowing identification of sequential digestion steps. Purified starch and semolina were digested following a single first-order rate constant, while pasta and powdered pasta followed two sequential first-order rate constants. Rate coefficients were altered by pepsin hydrolysis. Confocal microscopy revealed that, following cooking, starch granules were completely swollen for starch, semolina and pasta powder samples. In pasta, they were completely swollen in the external regions, partially swollen in the intermediate region and almost intact in the pasta strand centre. Gluten entrapment accounts for sequential kinetic steps in starch digestion of pasta; the compact microstructure of pasta also reduces digestion rates.

Keywords:

Pasta structure; Gluten entrapment; Starch Digestion Rate

1. Introduction

Pasta is a widely consumed carbohydrate-based food with a relatively low glycemic index (GI). Consumption of foods with a low glycemic index may help to reduce the risk of metabolic diseases, such as type 2 diabetes, cardiovascular disease and obesity (Bonora & Muggeo, 2001; Ludwig, 2002; Morris & Zemel, 1999), although there are significant problems with the statistical validity of GI measurements (DeVries, 2007; Miller-Jones, 2008; Whelan, Hollar, Agatston, Dodson, & Tahal, 2010). It has been confirmed by many *in vitro* (Colonna, Barry, Cloarec, Bornet, Gouilloud, & Galmiche, 1990; Fardet, Hoebler, Baldwin, Bouchet, Gallant, & Barry, 1998; Riccardi, Clemente, & Giacco, 2003) and *in vivo* (Berti, Riso, Monti, & Porrini, 2004; Granfeldt, Björck, & Hagander, 1991; Jenkins, et al., 1981; Karinthi, 1995; Monge, Cortassa, Fiocchi, Mussino, & Carta, 1990) experimental results that starch digestion in pasta proceeds more slowly than in other comparable starchy foods. The mechanism by which pasta exhibits a lower GI can be generally attributed to two aspects of its structure. The first is its compact and relatively dense local microstructure, which limits the surface area where the digestive enzymes can access available starch (Jenkins, Wolever, Jenkins, Lee, Wong, & Josse, 1983), and which prevents starch granules from being hydrothermally swollen during cooking (Heneen & Brismar, 2003; Sissons, Aravind, & Fellows, 2010). The second is the presence of a continuous gluten matrix, which entraps starch granules and reduces the accessibility of α-amylase to starch entrapped by the gluten network, as has been demonstrated by a number of workers (Colonna, et al., 1990; Cunin, Handschin, Walther, & Escher, 1995; Dexter, Dronzek, & Matsuo, 1978; Favier, Samson,

Aubled, Morel, & Abecassis, 1996; Singh & MacRitchie, 2004; Sissons, et al., 2010).

The effect of pasta structure on starch digestion rates can be characterised by measuring the percentage of starch digested as a function of time. One of the most widely used methods is the Englyst classification system, in which the acronyms RDS, SDS and RS were created for rapidly and slowly digestible starch, and resistant starch, respectively (Englyst, Englyst, Hudson, Cole, & Cummings, 1999). The starch digestion rate can be further characterised by utilising digestion curves to calculate hydrolysis indices (HI), equal to the area under digestibility curves (AUC) between the starting time and a selected completion time (Aravind, Sissons, & Fellows, 2011; Butterworth, Warren, Grassby, Patel, & Ellis, 2012; Edwards, Warren, Milligan, Butterworth, & Ellis, 2014). However, these methods are limited in the degree to which they allow for a rigorous, quantitative comparison of the rates and extents of starch digestion, and cannot be used to detect changes in the digestion rate constant quantifiably as starch digestion proceeds. One means of overcoming this limitation is an empirical, modified first-order kinetic model (Goñi, Garcia-Alonso, & Saura-Calixto, 1997) and the accompanying logarithm of the slope (LOS) plot (Poulsen, Ruiter, Visser, & Iversen, 2003), which allows for sequential first-order steps, previously applied to characterise the reaction rate of starch amylolysis (Butterworth, et al., 2012; Patel, Day, Butterworth, & Ellis, 2014). The LOS plot can reveal whether the reaction rate constant remains unchanged throughout the whole reaction, by demonstrating if there is a linear relationship between the time (t) and logarithmic form of digestion data (ln(dC/dt)) (Poulsen, et al., 2003). The LOS plot can reveal two or more first-order kinetic steps, in which each slope can provide

the rate coefficient. A food matrix, such as cooked pasta, may contain starch fractions in different structural environments. These could be starch that is totally gelatinised in the external region (the outer ring of the cylindrical section of cooked pasta) or dispersed into solution; partially swollen and gelatinised in the intermediate region (transition area between the outer and inner sphere of the cylindrical section of cooked pasta); and intact in the central region (the inner part of the cylindrical section of cooked pasta) (Heneen, et al., 2003). LOS plots assist in understanding how structural differences may affect starch digestion rates. Another advantage of this method is that it enables the product concentration to be predicted at the end of the reaction, thus avoiding the need to carry out prolonged digestions that may result in unacceptable errors because of end-product inhibition or enzyme inactivation (Edwards, et al., 2014).

The aim of this study is to explore the roles that the gluten network and pasta microstructure play in the kinetics of starch digestion in pasta. Therefore, in this study, the modified first-order kinetic model and LOS plot are used to obtain the instantaneous reaction rate of *in vitro* starch digestion during the progress of pasta digestion. The aim is to observe differences in digestion rate constants that may arise as a result of the digestion of different starch structures of cooked pasta. Meanwhile, in order to characterise the effect gluten entrapment plays in reducing starch digestion rates, pepsin hydrolysis is employed to hydrolyze the gluten network, mimicking the effect of stomach digestion *in vivo*, and changes in the starch digestion rate as a result of the destruction of the gluten network are observed.

An essential complement to kinetic characterisation is also used: morphological characterisation. This is implemented using confocal laser microscopy, which visualizes the nature of the starch and protein components during the whole digestion process.

2. Materials and methods

2.1. Materials

Three commercial durum wheat varieties (Jandaroi, Caparoi and Yawa) were sourced from a large field trial grown at the Wagga Wagga Agricultural Research Station (NSW, Australia) in the 2011 season. Semolina was obtained from the grain of these varieties using a laboratory scale Buhler MLU202 mill according to procedures described elsewhere (Sissons & Hare, 2002). Pepsin (Sigma P-6887, from gastric porcine mucosa), α-amylase (Sigma P-6255, from porcine pancreas, 1173 U/mg, one unit liberates 1.0 mg of maltose from soluble starch in 3 min at pH 7.0 at 37 °C) and amyloglucosidase (Megazyme E-AMGDF, 0.16 U/mg, one unit liberates 1.0 mmol of maltose from soluble starch in 1 min at pH 7.0 at 37 °C) were used. All other chemicals were of analytical grade.

2.2. Preparation of purified starch, pasta and pasta powder

Purified starches were obtained using a slight modification of Vansteelandt & Delcour 1999, as described. Semolina (140 mg) was steeped in 50 ml 0.2 (*w/v*) % sodium bisulfite at room temperature. The samples were centrifuged, the supernatants decanted and the pellet resuspended in 80 (w/v) % ethanol, before being centrifuged to remove ethanol and left to dry at 50 °C for two days prior to storage. The semolina was processed into pasta using a small-scale extruder as

described elsewhere (Sissons, Gianibelli, & Batey, 2002). Pasta was dried at 65°C at 70% RH for 45 min, then for 13 h at 50°C and 80-70% RH followed by cooling to 25°C at 55% RH for 4 h. The pasta was kept at room temperature for a minimum of one week to stabilise moisture movement before further analysis. (Sissons, Gianibelli, & Batey, 2002), and the diameter can be seen in Table S1. Aravind, Sissons, Fellows, Blazek, & Gilbert, 2012 presented X-ray scattering data for pasta prepared using this technique, indicating that starch in the uncooked pasta is still in its native state. The spaghetti strands were ground using a coffee grinder at room temperature for 30 seconds into a powder (see particle size distribution in Figure S1).

2.3. Composition of durum wheat semolina

The starch content of durum wheat semolina and the ground pasta powder was measured using a megazyme total starch (AA/AMG) assay kit. Briefly, 100 mg of sample was weighed into a 15 ml centrifuge tube, and 0.2 ml of aqueous ethanol (80% v/v) was added to disperse the sample. A blank was also prepared without sample addition. To this, 2 ml of DMSO was added, and the tube was mixed using a vortex mixer for 30 s, before being placed into a boiling water bath for 5 min. Thermostable α-amylase solution was prepared by diluting 1 ml of the solution provided in the kit into 30 ml of MOPS buffer (50 mM, pH 7), and 3 ml of this solution was added to each tube, before incubating in a boiling water bath for a further 12 min. The tube was allowed to cool and 4 ml of sodium acetate buffer (200 mM, pH 4.5) was added. Amyloglucosidase solution (0.1 ml) (as provided by the manufacturer) was added, and the tubes were incubated in a water bath at 50°C with shaking for 30 min. From these tubes, 0.1 ml was removed, and diluted to 1 ml using deionised water, before centrifuging at 1180 g for 10 min. A 0.1 ml volume of

supernatant was removed and analysed for glucose as described by the manufacturer. Semolina protein was determined using in-house calibrations on a NIRSystem 6500 spectrophotometer (Foss, Hillerød, Denmark) as a single scan. The NIR system was calibrated using the method described in Sissons, Osbourne & Sissons 2006. The moisture content was measured, in triplicate, by drying the samples in an oven at 105 ℃ overnight and recording the weight loss of moisture, following AACCI method 44-40.01. The composition percentage of the three durum wheat semolina samples is shown in Table S1.

2.4. Enzyme solutions

Pepsin with a concentration of 1.0 mg / ml was dissolved in hydrochloric acid (0.02 M); porcine α-amylase/amyloglucosidase enzyme mixture was prepared composed of 135.26 U porcine α-amylase and 1.23 U amyloglucosidase per 5.0 ml in a 0.2 M sodium acetate buffer (pH 6.0) containing calcium chloride (200 mM) and magnesium chloride (0.49 mM).

2.5. In vitro digestion

In vitro starch digestion was carried out in duplicate using a slight modification of the method of Muir, Birkett, Brown, Jones & O'Dea (1995). Semolina, purified starch derived from the semolina, one whole spaghetti strand of length around 35 mm and pasta powder samples containing 90 mg of starch each as determined in Section 2.3, were cooked in a flask with 6.0 ml of deionised water at 100 ℃ for 10 min. After cooling to 37.0 ℃ in a water bath, 5.0 ml of pepsin solution (1 mg/ml) in 0.02 M HCl was added to the samples. Controls with 5.0 ml of 0.02 M HCl (without added pepsin) were also prepared. After incubation at 37.0 ℃ for 30 min, 5.0 ml of

0.02 M NaOH was added to neutralise the solution, followed by addition of 5.0 ml of porcine α-amylase/amyloglucosidase enzyme mixture (in acetate buffer, pH 6) to the flask. The total 21.0 ml reaction solution was incubated at 37.0 ℃ in a water bath in a sealed flask, stirred with a magnetic stirrer bar at 50 rpm, with 100 µl aliquots removed at a range of time points and dispersed into 900 µl of absolute ethanol to terminate the reaction.

2.6. Measuring the amount of starch digested

Digestion solution from the above section (100 µl, containing 90% ethanol) was added to 3.0 ml of glucose oxidase/peroxidase determination reagent (GOPOD Reagent - Megazyme). Samples were then incubated at 50 ℃ for 20 min. A 100 µl sample of D-glucose solution (1.0 mg/ml) was used as a standard and 100 µl of ethanol was used as a blank. After cooling to ambient temperature, the absorbance at 510 nm was recorded by a UV-1700 Pharma Spectrophotometer (Shimadzu), subtracting the absorbance of the blank. The ratio of starch digested was calculated using the following equation:

$$\%\text{Digested} = \Delta A\ (\text{Sample}) \times \frac{100\ \mu\text{L} \times 1.0\ \text{mg/ ml}}{\Delta A\ (\text{D-Glucose Standard})} \times 10 \times 210 \times \frac{100\ \%}{90\ \text{mg}} \times \frac{162}{180} \quad (1)$$

Here the absorbance at each time point is denoted Δ*A* (Sample), and the absorbance from the standard D-glucose solution is given as Δ*A* (D-Glucose Standard). The value 10×210 is the computational multiple from 100 µl aliquots to 21.0 ml reaction solution, and 162/180 is the transformation coefficient from starch (monomer unit anhydroglucose) to glucose in weight.

2.7. Fitting to first-order kinetics

Starch digestion data have often been fitted to a first-order equation:

$$C_t = C_\bullet (1 - e^{-kt}) \quad (2)$$

where C_t is the percentage of starch digested at a given time (t), $C_\bullet$ is the estimated percentage of starch digested at the end point of the reaction, and k is the starch digestion rate coefficient. In order to obtain the values of k and $C_\bullet$, eqn.2 can be transformed into a LOS plot where there is a linear relationship between $\ln(dC_t/dt)$ and k, as shown in eqn.3:

$$\ln(dC_t/dt) = -kt + \ln(C_\bullet k) \quad (3)$$

k and $C_\bullet$ are calculated from the slope ($-k$) and intercept ($\ln(C_\infty k)$), respectively. The slope in this study was estimated from the second-order finite-difference formula $\ln[(C_{i+1} - C_{i-1})/(t_{i+1} - t_{i-1})]$ as functions of $(t_{i+1} - t_{i-1})/2$ for all except the first and last points, which were ignored. The resulting k and $C_\bullet$ were used to construct model-fit starch digestion curves according to eqn.2, and residuals analysis was employed to compare experimental data to the starch digestion curves generated by the model fit.

For substrates containing starch fractions digested at a single rate, the LOS plot is linear, while others may have multiple distinct linear phases. Therefore the whole starch digestion can be expressed by a piecewise function:

$$C_t = \begin{cases} C_1 + C_{1\infty}(1 - e^{-k_1 t}), & 0 \le t \le t_1 \\ C_2 + C_{2\infty}(1 - e^{-k_2 t}), & 0 \le t \le t_2 \\ \ldots\ldots & \\ C_n + C_{n\infty}(1 - e^{-k_n t}), & t_{n-1} \le t \le t_n \end{cases}$$

(4)

where n depends on the number of phases. In each phase, k_n and $C_{n\infty}$ represent corresponding starch digestion rate coefficients and estimated percentages of starch digested at the reaction end-point. The starting percentage of starch digestion in each phase is represented by C_n and t_n is the corresponding terminal time (Edwards, et al., 2014).

2.8. Measuring the amount of protein hydrolysed

Semolina, one whole spaghetti strand and pasta powder samples containing 90 mg of starch each were cooked in a flask with 5.0 ml of deionised water at 100 ℃ for 10 mins. After cooling to 37.0 ℃ in a water bath, 5.0 ml of pepsin solution (1 mg/ml) in 0.02 M HCl was added to the samples. The reaction was halted by adding 5.0 ml of 0.02 M Na_2CO_3 at 0, 5, 10, 20 and 30 mins. The reaction mixture was centrifuged and 500 µl aliquots of supernatant were removed and diluted with 500 µl water. The amount of protein components solubilised was measured by the Thermo Scientific™ Pierce™ BCA Protein Assay Kit using BSA as a reference standard. A 0.1 ml volume of each standard or sample was added into 2.0 ml working reagent, and incubated at 37°C for 30 min. After the samples were allowed to cool to room temperature, the absorbance was measured by a UV-1700 Pharma Spectrophotometer (Shimadzu) set to 562 nm. The ratio of protein hydrolysed was calculated using the following equation:

$$\text{Protein Hydrolysed} = \Delta A\,(\text{Sample}) \times \frac{C(\text{BSA,mg/ml})}{\Delta A\,(\text{BSA})} \times 10 \times 30 \times \frac{100\ \%}{M(\text{Protein,mg})} \quad (5)$$

Here the absorbance at each time point is denoted ΔA (Sample), and the absorbance from the standard BSA solution with certain concentration (C (BSA, mg/ml)) is given as ΔA (BSA). The value 10×30 is the computational multiple from 100 µl aliquots to 15.0 ml reaction solution, and M (Protein, mg) is the total mass of protein in each sample.

2.9. Confocal Scanning Laser Microscopy (CSLM)

Cooked pasta samples were sectioned as thin as ~ 1 mm using a razor blade; cooked purified starch, cooked semolina and cooked pasta powder samples were sampled as ~ 0.5 ml of solution (90 mg starch/6.0 ml distilled water) prior to *in vitro* starch digestion (see Section 2.5), and was pipetted onto a microscope slide. Sections and solution samples were stained for 30 min with fluorescein isothiocyanate (FITC) (1.0 ml, 0.05% *w/v*) and Rhodamine B (1.0 ml, 0.05% *w/v*), respectively (Kim, et al., 2008). Rhodamine stains the protein red and FITC stains both protein and starch granules green. Samples were rinsed in distilled water and then mounted in water on glass cavity slides and sealed with a cover slip and nail varnish, before they were viewed promptly using a ZEISS LSM700 confocal microscope with dual excitation. A FITC and a tetramethylrhodamine isothiocyanate filter block were used for the excitation of the two dyes under wavelengths of 488 and 555 nm, respectively.

α-Amylase labelled with FITC using the procedure of Dhital et al. (Dhital, Warren, Zhang, & Gidley, 2014) was employed to digest the cooked pasta. The FITC fluorophore can be introduced with retention of activity of the α-amylase, as detailed

in Dhital, Warren, Zhang, & Gidley, 2014, and allows the movement of the enzyme to be tracked as it diffuses and binds to the substrate. The same activity of labelled α-amylase (30 U/ml; assayed after FITC labelling) was employed as was used in the *in vitro* digestion experiments (Section 2.5). Rhodamine B was employed as an additional stain to identify gluten in the pasta sections as described above. The Rhodamine B (1.0 ml, 0.05% w/v), was added following digestion by the FITC labelled α-amylase. Sections were viewed, as described previously, after ~10% of the starch had been digested.

2.10. Statistical analysis

The statistical significance of starch digestion rate constants was analysed using one-way ANOVA and multiple comparison test with least significant difference adjustment at p value <0.05. Initial data analysis and linear regression fitting was carried out in Microsoft Excel. Further statistical analysis of the data was carried out in IBM SPSS Statistics version 21.

3. Results

3.1. Modelling of starch digestion curves

All k and C_∞ values are presented in Table S2-S4, and applied to construct model-fit curves according to eqn.4, to check that the experiment data is well fitted by the kinetic parameters. Low mean residual values and SDs were observed (Fig. S2), indicating that the experimental data are well fitted and thus k and C_∞ values obtained by the LOS fitting procedure may be considered reliable.

Typical experimental starch digestion curves are shown for Jandaroi durum semolina and its derived processed samples: purified starch, pasta and pasta powder (Fig.1). A visual comparison of k and C_∞ values for starch digestion of different samples can be seen in Fig.2. Starch digestion is initially rapid, then slows with time following an exponential curve. After transformation of the data into LOS plots, the purified starch shows one linear step with one rate coefficient k which did not alter significantly when pepsin hydrolysis was introduced prior to starch digestion (Fig.1 g and h, Fig.2 and Table S5-S7). Similarly, a single linear step was observed for starch digestion of semolina samples with a single k value that was not significantly different to that of purified starch (Fig.1 e and f, Fig.2 and Table S5-S7). However, for pasta, k values were decreased significantly compared to the semolina (Fig.1 a and b, Table S5-S7). There are two distinct linear steps for the starch digestion of pasta, where ~20% of starch is digested in the first phase with a quicker rate constant (k_1) and the remainder of the starch is digested in the second stage, with a slower rate constant (k_2), almost 10 fold lower than for semolina (Fig.2 and Table S2-S4). Pepsin hydrolysis of pasta brought about a significant increase of the k_1 value but no significant increase of the k_2 value (Table S5-S7). It has previously been suggested that there may be two distinct steps for starch digestion in pasta (Fardet, et al., 1998), but these workers identified these steps through visual inspection of starch digestion curves and were unable to provide quantifiable evidence of two separate starch digestion rate coefficients.

After the pasta is ground into pasta powder, the k values in the absence of pepsin hydrolysis increase significantly compared to pasta; with pepsin hydrolysis, prior to *in vitro* starch digestion, pasta powder was only digested at a single rate constant,

similar in magnitude to the k_1 rate constant for digestion of pasta following pepsin hydrolysis (although it should be noted that the majority of pasta digestion following pepsin hydrolysis occurred at a second, significantly slower, rate constant) (Table S5-S7). Two distinct steps may be observed in the pasta powder without pepsin digestion (Fig.1 c), with as much as ~50-60% of the starch being digested in the first step at a faster rate than the remaining starch digested in the second stage (Fig.2 and Table S2-S4). This was not observed in the pepsin treated sample (Fig.1 c). More starch was degraded in pasta powder compared to semolina and this may be due to the smaller particle size in the pasta powder due to finer grinding than occurs for the production of semolina. Following pepsin hydrolysis, the total amount of starch hydrolysed (C_∞) remained unchanged for the whole pasta sample, but it significantly increased in the semolina and to a lesser degree in the pasta powder samples.

3.2. Starch and gluten structure observed by CSLM

Morphological differences of the starch and protein components in cooked semolina, pasta and pasta powder are revealed using CSLM (Fig.3). In Fig.3, the three upper panes represent cooked semolina, pasta and pasta powder, respectively; the other panes indicated by letter and number represent central (a), intermediate (b) and external regions (c) of a cooked pasta strand with a starch digestion extent of 0% (1), 20% (2) and 80% (3). The starch is stained by FITC in green while the protein components stained by FITC and Rhodamine B are yellow. The protein components in semolina had not hydrated and did not form a gluten network, which is why there appears to be no clear protein network (Fig. 3 upper-left pane). However, in the cooked pasta sample, a clear protein matrix is visible which forms during the pasta manufacturing process. The starch granules in cooked semolina swelled completely,

showing no clear granular structure but those in cooked pasta remained largely intact and not fully swollen. Once the pasta structure was degraded by grinding into a powder, starch granules were no longer protected from swelling and readily gelatinised, with no clear granular structure.

The appearance of the starch granules varied from the surface to the central regions of the whole intact pasta: completely swollen in the external region (Fig.3 j-l), partially swollen in the intermediate region (Fig.3 g-i) and almost intact in the central region of the pasta strand (Fig. 3 d-f). Similar morphological differences among the three regions were observed independent of the degree of starch digestion, from 0% to 20% and 80%.

A section of cooked pasta hydrolyzed by FITC-labelled α-amylase (FITC labelling in green) is shown in Fig.4. It can be seen that starch granules in the central region (Fig.4a) of cooked pasta were entrapped firmly and were inaccessible to α-amylase, with very little FITC labelling, whereas starch granules that had not been entrapped were being digested. As for starch in the external (Fig.4c) and intermediate (Fig.4b) regions, they were mostly digested, as there were few intact starch granules that could be observed, and little FITC labelling; however, α-amylase-labelled FITC was still attached to the gluten network (indicated by the yellow colour, resultant from the co-localisation of rhodamine b- and FITC- labelled α-amylase in the gluten).

3.3. Hydrolysis of protein components

The percentages of protein hydrolyzed by pepsin for each wheat sample can be seen in Fig.5. The gluten in pasta was only hydrolysed very slowly by pepsin, with no gluten digested by 30 min; whereas the protein components in semolina (gliadin and

glutenin proteins) and pasta powder (disrupted gluten network) were degraded much more rapidly and to a greater degree, up to 45%.

3.4. Comparing starch digestion of pasta between genotypes

The three genotypes of durum wheat semolina employed in the present study differ in their starch, protein and moisture contents. They have the same low molecular weight glutenin subunit (LMW-GS) allele. However, Caparoi semolina has high molecular weight glutenin subunits (HMW-GS): Glu-A1 null, Glu-B1 7+8, different from Jandaroi or Yawa semolina which have HMW-GS: Glu-A1 null, Glu-B1 6+8 (Table S1) (Determined by SDS-PAGE, personal communication, Dr. M. Sissons). It can be seen in Table S8 that there are no clear differences in the starch digestion rates of purified starch, pasta and pasta powder between the three different genotypes; Jandaroi and Yawa semolina had similar starch digestion rates which were not altered significantly by pepsin hydrolysis; however, Caparoi semolina showed significantly higher starch digestion rates than Yawa semolina (P value 0.025) and somewhat higher starch digestion rates than Jandaroi semolina, although not quite reaching statistical singificance (P value 0.061). Pepsin hydrolysis reduced the Caparoi semolina k to a value insignificantly different from Jandaroi or Yawa semolina.

4. Discussion

4.1. Effect of the compact structure of pasta on starch digestion rate

The starch granules of semolina, pasta and pasta powder were shown to swell to different degrees after cooking. As seen in Fig.3, most of the starch granules in semolina and in pasta powder were able to swell completely after cooking, and the

starch was rapidly digested; this indicates that these starch granules were hydrolysed at the same time as they were available to α-amylase. However, the starch granules in pasta were not degraded evenly, with different structural features present between the external and central region of the pasta strand; the starch granules were degraded completely in the external region (Fig.3 j-l), partially degraded in the intermediate region (Fig.3 g-i) and almost intact in the central region (Fig.3 d-f). Similar phenomena were also reported in previous studies, with the internal structure of pasta after cooking being divided into three regions: external, intermediate and central regions (Cunin, et al., 1995; Heneen, et al., 2003; Petitot, Abecassis, & Micard, 2009). The external region of cooked pasta was clearly distinguished from the intermediate and central regions, as starch granules in the external region were largely deformed during cooking and were therefore characterised by having larger swollen starch granules surrounded by thin protein films (Petitot, et. al., 2009). In contrast, starch granules in the intermediate region were only partly swollen and were embedded in a coagulated and dense protein network (Fardet, et al., 1998; Heneen, et al., 2003). The starch granules in the centre of the strand were not fully gelatinised, because of restrictions to swelling of the starch granules due to the compact structure of the gluten network, as well as unavailability of water due to competition with the gluten network (Cunin, et al., 1995). Notably, it can be seen from our results that even when starch digestion proceeds from 20% to 80%, there were still these morphological differences between starch granules located in different regions of the pasta strand (Fig.3 e,h,k and f,i,l), which indicates that the starch granules in pasta are digested layer by layer from the external region towards the central region.

Fig.1 illustrates that the starch granules in pasta, unlike those in semolina, are digested asynchronously. Specifically, in the early stages of digestion, starch granules in the external region were digested quickly, as they were completely swollen and closer (and thus more available) to enzymes; the starch granules in the intermediate region were digested slowly, as they were partially swollen and only had contact with a small amount of the enzymes; the starch granules in the central region remained undigested as they were almost intact and so inaccessible to enzymes. However, given enough time, these middle and central regions will eventually degrade, as under CSLM (Figure 3), all starch granules are seen to disappear.

To summarise, as the enzymes penetrated the pasta, starch granules showing different degrees of swelling were digested sequentially from the external region towards the central region, as observed by CLSM in Figure 3. This sequential digestion through the pasta structure reduces the reaction area and contributes to the significantly different reaction rates observed during the two starch digestion phases. The dramatic increase in digestion rate constant when the pasta is ground prior to cooking provides evidence in support of this conclusion (Fig.1c and d). After destruction of the pasta structure by grinding, many of the starch granules were no longer fully encapsulated with gluten, making them more like the starch granules in semolina (Fig. 3); the granules are more accessible to enzymes and can swell completely, accelerating the starch's digestibility.

The compact structure of pasta is also able to protect the gluten network from being degraded by pepsin. As is shown in Fig.5, very little of the gluten, in pasta, was degraded during 30 min of pepsin hydrolysis. In contrast, the protein components in

semolina and in pasta powder were degraded to a high degree within the first 5 min of the pepsin hydrolysis. Pepsin hydrolysis brought about a significant increase in the value of k_1, while only producing a slight increase in the value of k_2, suggesting that pepsin hydrolysis of intact pasta is confined to the external regions of the pasta, and that the pepsin did not penetrate the inner gluten network where the starch is firmly entrapped. Pepsin hydrolysis of the whole pasta did not bring about a significant difference in the C_∞ value, presumably as there was not enough of the gluten network hydrolysed to alter the amount of starch available to the enzyme. Semolina and pasta powder samples, where more of the protein components (gluten and gliadin proteins in semolina, and mechanically disrupted gluten in pasta powder) were hydrolysed, showed a significant increase in C_∞ following proteolysis (a larger increase was observed for semolina than pasta powder). This may be attributed to the protein components, which may reduce accessibility of the starch to amylase hydrolysis, being degraded by proteolysis.

4.2. Effect of different high molecular weight glutenin subunits on starch digestion rate

The starch digestion rates of purified starch, pasta and pasta powder did not show significant differences between Jandaroi, Caparoi and Yawa, even though the semolina used to make these samples differed in their HMW-GS composition. However, the different HMW-GS composition observed may affect the starch digestion rates of semolina. The natural protein components in Jandaroi and Yawa semolina (with HMW-GS: Glu-A1 null, Glu-B1 6+8) appear to exert little influence on starch digestibility. In the present study the starch digestion rate constants of the two semolina following pepsin hydrolysis treatment remained almost unchanged

(Fig.1e and f and Table S5, S7), even though a large proportion of the protein components in the semolina were degraded (Fig.5). These two semolina samples were similar in their starch digestion behaviour to that of purified starch (Table S5 and S7), which has had almost all the non-starch components and protein removed. In contrast, the natural protein components in Caparoi semolina (with HMW-GS: Glu-A1 null, Glu-B1 7+8) appear to exert significant influence on starch digestibility, as the Caparoi semolina following no pepsin hydrolysis had a significantly higher k value than the one with a large proportion of the protein components degraded by pepsin (Table S6).

It should also be noted that the estimated percentages of starch digested at the reaction endpoint (C_∞) were around or less than 80% for starch digestion in all three semolina, while (C_∞) of around 90% or greater was observed for starch digestion of purified starch, pasta, pasta powder and semolina hydrolysed by pepsin (Fig.2 and Table S2-S4). While from the present data we can only speculate on the reasons for this, it may be inferred that there could be a fraction of the starch granules that are combined with natural protein components in the semolina in such a way as to inhibit the activity of α-amylase. Another possibility might be that the larger particles of semolina include interior endosperm cells with intact cell walls (Edwards, et al., 2014). As for HMW-GS, Glu-B1 7+8 seems more able than Glu-B1 6+8 to inhibit the activity of α-amylase, possibly by interacting with the starch granules, reducing the availability of starch for digestion. However this conclusion remains speculative due to the limited number of durum cultivars available in the present study. Meanwhile other factors, such as polymeric molecular weight

distribution of the glutenin may also play a vital role, which should be considered in future research.

4.3. Effect of gluten entrapment on starch digestion rate

After the semolina was kneaded and extruded into pasta, the protein components were hydrated and energy was imparted through mixing to form a gluten network which entraps the starch granules. In Fig.1, Fig.2 and Tables S2-S4 quantitative evidence for the role and mechanism of the gluten network in reducing the rate of starch digestion in pasta is provided. Pepsin hydrolysis of whole pasta brings about a significant increase in the value of k_1 for subsequent starch digestion, indicating that degradation of the gluten network increases the access of starch degrading enzymes to the more rapidly digested fraction of the starch in pasta; pepsin hydrolysis also leads to the disappearance of the second, slower, starch digestion phase for pasta powder, resulting in similar digestion kinetics to the unprocessed semolina. In this case, it appears that the second, slower stage of starch digestion in pasta powder without pepsin was as a result of entrapment by the gluten network. The break-up of the compact structure of pasta through grinding to a powder increased the susceptibility of the gluten network to pepsin hydrolysis. As a result, when starch digestion was carried out following pepsin hydrolysis, the gluten network was fully degraded by the pepsin, freeing the entrapped starch, such that the starch digestion proceeded at a rapid rate in a single step.

4.4. Mechanism of gluten entrapment on slowing starch digestion

The mechanisms by which the gluten network slows digestion rates of entrapped starch are not fully understood. The most common explanation is that the gluten

network entrapping starch granules acts as a barrier to inhibit the accessibility of enzymes. It may also limit water absorption by starch granules, limiting the degree to which the starch is able to swell and hence gelatinise during pasta cooking in excess water, and limiting the ability of enzymes to access available starch and therefore decreasing the rate of starch digestion (Colonna, et al., 1990). It is also possible that effects on starch digestion rate may be imposed by other components (non-starch polysaccharides (NSP) and lipid components), but this is beyond the scope of the present study, which focuses on the protein components of pasta. The limits imposed on the diffusion of amylase by the gluten network cannot be the sole reason for the slow digestion kinetics observed for pasta, since the porosity of the gluten network can be as high as 0.5-40 μm (Fardet, et al., 1998), large enough to allow α-amylase (size generally reported to be in the range 7–10 nm (Larson, Greenwood, Cascio, Day, & McPherson, 1994; Strobl, et al., 1998)) to diffuse freely. Therefore, some authors have suggested that the low starch digestion rates of pasta may be attributed to the tortuosity of the gluten network, which lengthens the pathway α-amylase must take to reach its substrate (Fardet, et al., 1998). An alternative suggestion is the possibility that α-amylase has a weak binding interaction with the gluten network, which retards the penetration of the enzyme into the gluten network. This hypothesis is supported by the finding that α-amylase from different origins could be effectively retained by wheat albumins, but this binding is reversed by adding maltose or gelatinised starch (Buonocore, Poerio, Gramenzi, & Silano, 1975); from this it can be inferred that α-amylase may also interact with protein components of gluten through a weak binding that can be reversed by maltose or soluble starch. More direct evidence can be seen in Fig.4, supporting the

hypothesis that FITC-labelled α-amylase was attached to the gluten network as the enzyme penetrated to hydrolyze the starch of cooked pasta. This kind of attachment may be attributed to α-amylase forming a weak interaction with the gluten network, in such a way as to retard the penetration of α-amylase and hence reduce starch digestion.

5. Conclusions

LOS plots and confocal laser microscopy were used to characterise the digestion of starch in granules with different structures typical of pasta. Quantitative rate and morphological evidence were obtained to understand the role which gluten entrapment and compact microstructure play in reducing starch digestion rates. The natural protein components in semolina do not have a significant influence in altering starch digestion rates until they are hydrated to form a gluten network entrapping starch granules, and this network is further developed during pasta making and drying. Besides gluten entrapment, the compact microstructure of pasta is another key factor in reducing starch digestion rates. It is able to prevent the starch granules in the central region from swelling during thermal gelatinisation, because of both confined space and a lack of water availability due to competition with the gluten network as water penetrates from external to central regions. From the data in the present study, we cannot rule out the influence of other, non-protein, components in pasta on starch digestion rates, but it is clear that the gluten matrix has a major influence on the rate of starch digestion. The compact microstructure is also able to reduce the accessibility of starch granules to enzymes by lowering the reaction area. Moreover, the compact structure can prevent pepsin from hydrolysing the inner gluten network in the pasta structure and subsequently reduce the digestion

rate of the entrapped starch. Thus, *in vitro* starch digestion rates and morphological visualisation obtained for different structural features together explain the lower starch digestibility of pasta.

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Figure legends

Figure.1. Typical starch digestion curves, model-fit curves and LOS plots from Jandaroi pasta (a, b), pasta powder (c, d) and durum wheat semolina (e, f) and purified starch (g, h) with pepsin treatment (a, c, e, g) and without pepsin treatment (b, d, f, h). All of the points in the LOS plots are linearly treated by least-squares fit. For a, b and d, the LOS plots can be divided into two parts with linear lines of different slope. The R-squared values relate to the LOS plots. The part of the LOS plot describing k_1 is shown in red, and the part describing k_2 is shown in green. Digestion data are shown in blue and model-fit curves in a black dotted line.

Figure.2. Values of starch digestion rate constants (k min^{-1}) at each phase and corresponding estimated percentage of starch digested (C_∞ %). Starch digestion following pepsin hydrolysis in red. Starch digestion following no pepsin hydrolysis in blue.

Figure.3. Confocal scanning laser microscopy of cooked semolina, cooked pasta and cooked pasta powder. The samples were stained with FITC and Rhodanmine B and the starch granules (S) and gluten network (G) are shown in green and yellow, respectively. Panes a, b and c show cooked semolina, cooked pasta and cooked pasta powder, respectively. Panes d-l refer to cooked pasta: d-f represent the central, g-i the intermediate and j-l the external regions of the sample, respectively. Labels d, g and j represent 0%; e, h and k represent 20%; and f, I and l represent 80% of the sample starch having been digested.

Figure.4. Confocal scanning laser microscopy of a section of cooked pasta hydrolyzed by α-amylase labelled with FITC and stained by Rhodamine B. Labels a, b, and c

represent central, intermediate and external regions, respectively. Starch granules are indicated with S; partially digested starch granules with SD; FITC labelled α-amylase with α-F; and gluten with G.

Figure.5. Percentage of protein hydrolysed by pepsin digestion of semolina, pasta and pasta powder.

Fig.1

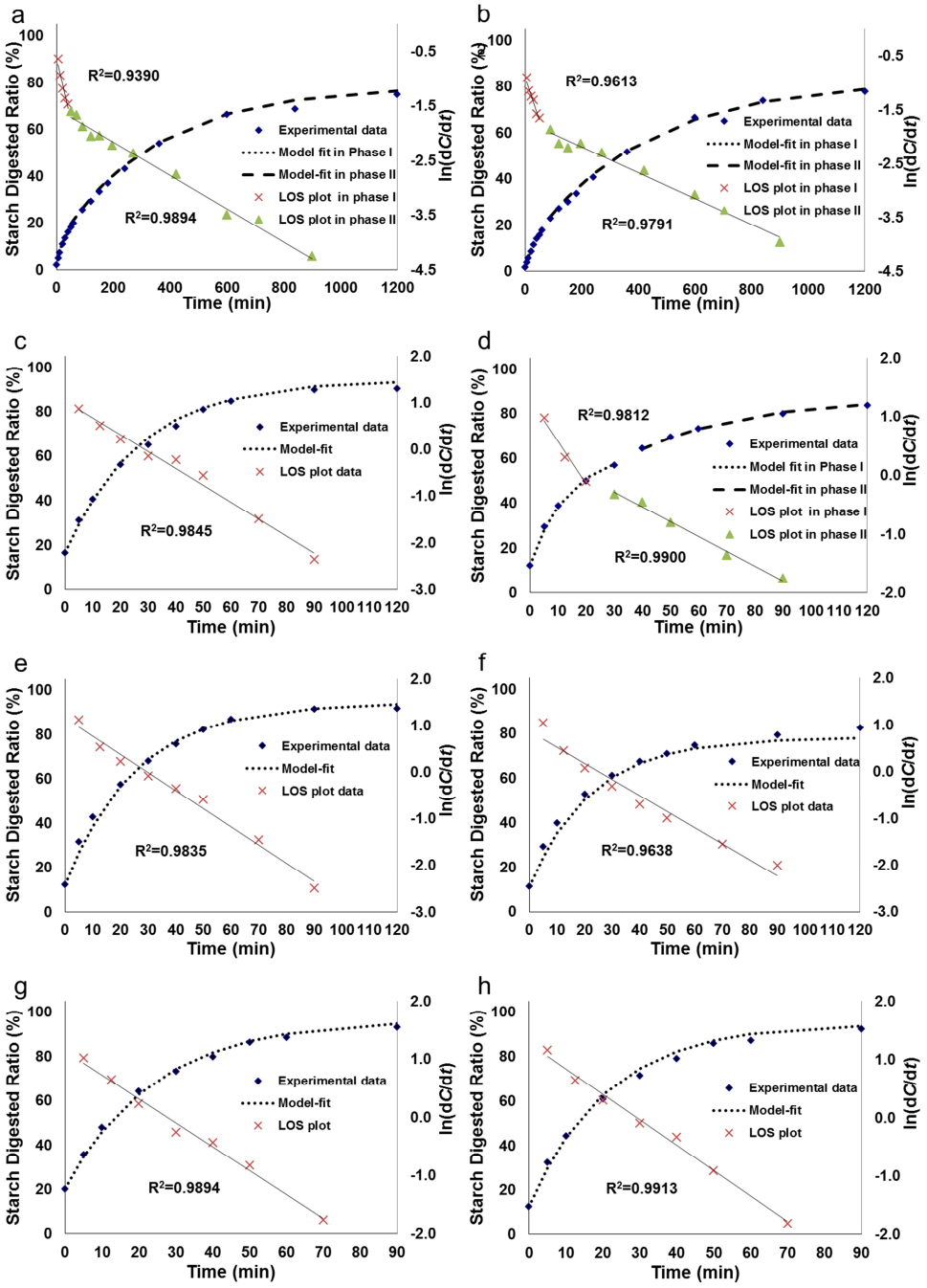


Fig.2

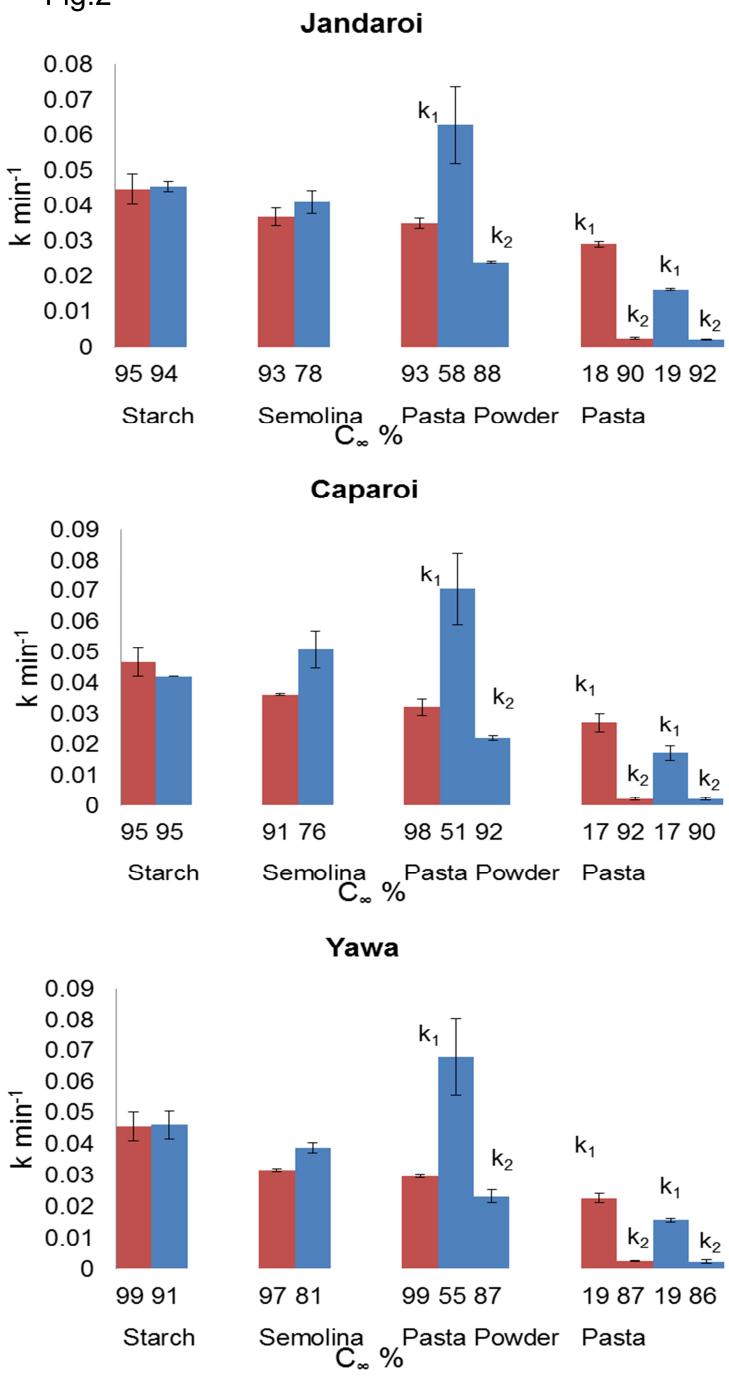


Fig.3

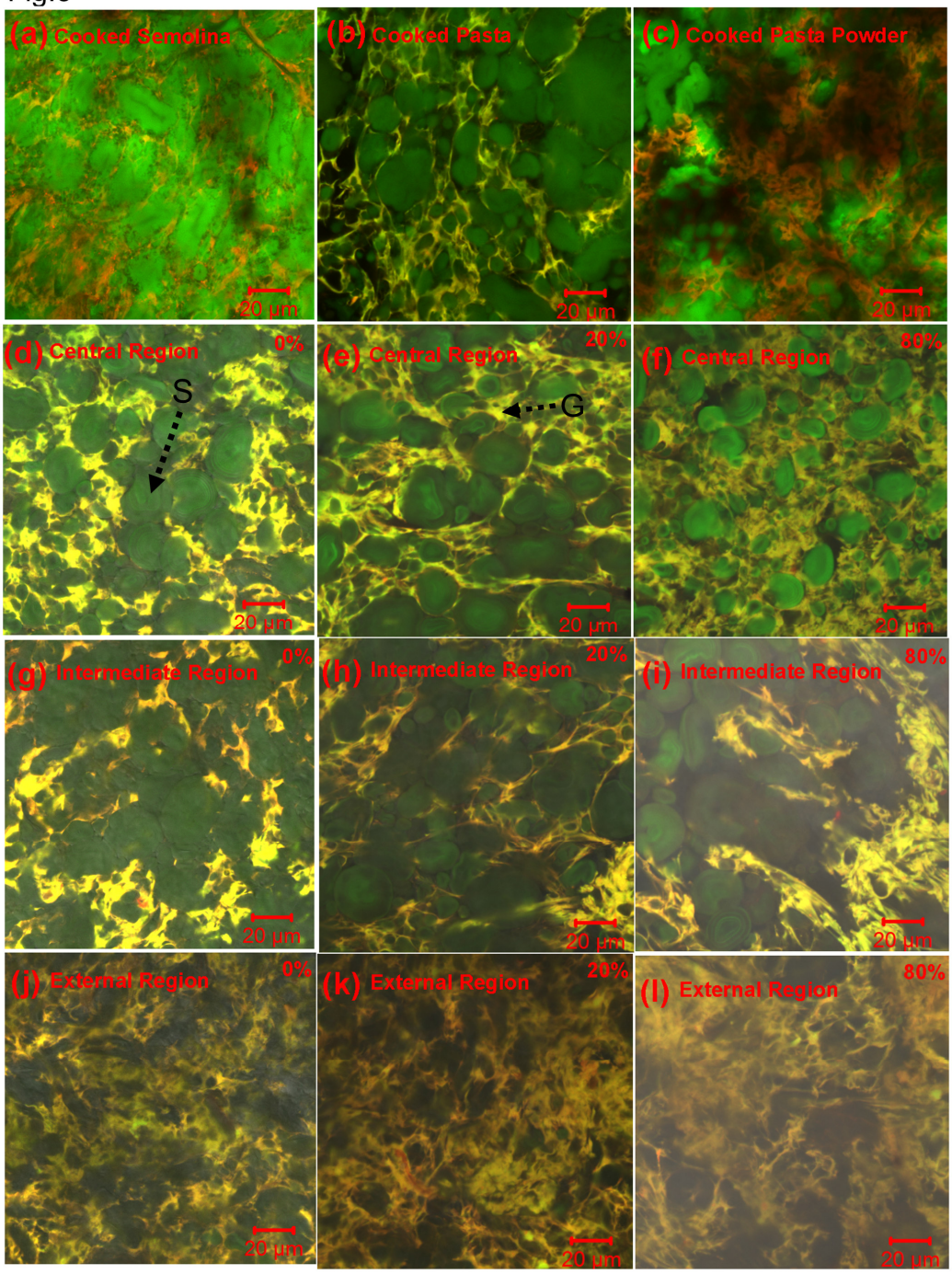

(a) Cooked Semolina
(b) Cooked Pasta
(c) Cooked Pasta Powder
(d) Central Region
0%
S
(e) Central Region
20%
G
(f) Central Region
80%
(g) Intermediate Region
0%
(h) Intermediate Region
20%
(i) Intermediate Region
80%
(j) External Region
0%
(k) External Region
20%
(l) External Region
80%
20 µm

Fig.4

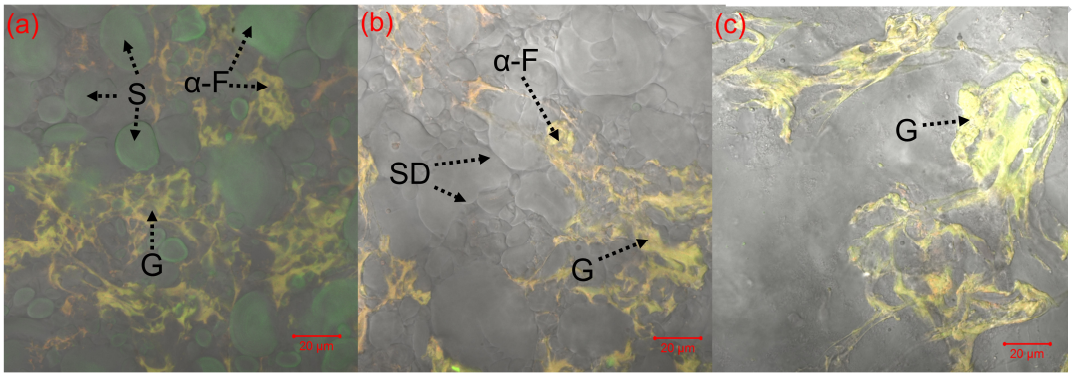
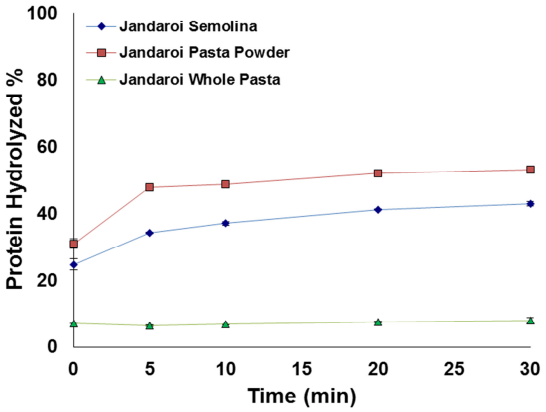
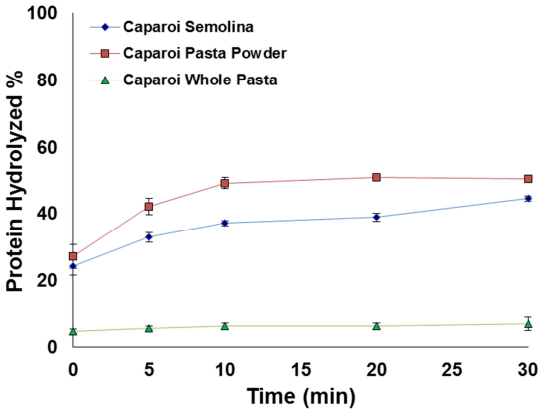
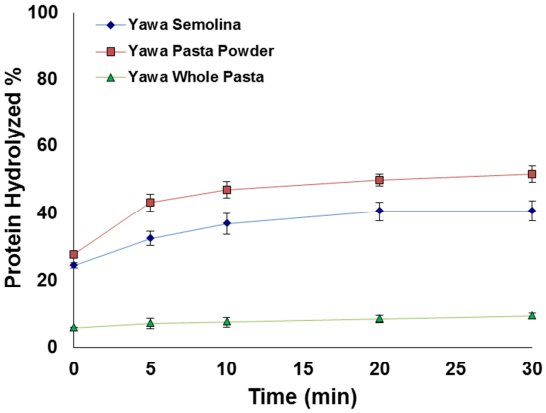


Fig.5







Highlights

- Sequential steps in pasta hydrolysis are quantified
- The observed hydrolysis rates are related to structural features
- The compact structure of pasta protects the gluten from proteolysis
- The intact gluten network reduces the rate of starch hydrolysis
- Evidence is presented for interactions between α-amylase and gluten