**Compact structure and proteins of pasta retard *in vitro* digestive evolution of
branched starch molecular structure**

Wei Zou^{a,b}, Mike Sissons^c, Frederick J. Warren^b, Michael J. Gidley^b, and Robert G. Gilbert^{a,b,*}

^aJoint International Research Laboratory of Agriculture and Agri-Product Safety, College of
Agriculture, Yangzhou University, Yangzhou 225009, Jiangsu Province, P.R. China

^bThe University of Queensland, Centre for Nutrition and Food Sciences, Queensland Alliance for
Agriculture and Food Innovation, Brisbane, QLD 4072, Australia

^cNSW Department of Primary Industries, Tamworth Agricultural Institute, 4 Marsden Park Road,
Calala, NSW 2340, Australia

*Corresponding author at: The University of Queensland, Centre for Nutrition and Food Sciences,
Building 83, Brisbane 4072, QLD, Australia. Email: b.gilbert@uq.edu.au. Phone: +61 7 3365 4809.
Fax: +61 7 3365 1188.

Abstract

The role that the compact structure and proteins in pasta play in retarding evolution of starch molecular structure during *in vitro* digestion is explored, using four types of cooked samples: whole pasta, pasta powder, semolina (with proteins) and extracted starch without proteins. These were subjected to *in vitro* digestion with porcine α -amylase, collecting samples at different times and characterizing their weight distribution of branched starch molecules using size-exclusion chromatography. Measurement of α -amylase activity showed that a protein (or proteins) from semolina or pasta powder interacted with α -amylase, causing reduced enzymatic activity and retarding digestion of branched starch molecules with hydrodynamic radius (R_h) < 100 nm; this protein(s) was susceptible to proteolysis. Thus the compact structure of pasta protects the starch and proteins in the interior of the whole pasta, reducing the enzymatic degradation of starch molecules, especially for molecules with R_h > 100 nm.

Key words:

Pasta; Proteins; Starch; Molecular structure; Enzyme activity; GPC

33 1. Introduction

34 Pasta is considered to be among the more healthy carbohydrate-based foods, since it has been
35 shown by many *in vitro* (Berti, Riso, Monti & Porrini, 2004; Colonna et al., 1990; Fardet et al., 1998)
36 and *in vivo* (Berti et al., 2004; Granfeldt & Björck, 1991; Jenkins et al., 1981; Monge, Cortassa,
37 Fiocchi, Mussino & Carta, 1990) experiments that starch digestion in pasta proceeds more slowly
38 than **in** most other starchy foods, resulting in attenuated glycemic response. The relatively slower
39 starch digestion of pasta can be generally attributed to two aspects of pasta structure. The first is the
40 compact structure of pasta, which reduces the reaction area where starch granules can be accessed by
41 digestive enzymes (Jenkins et al., 1983; Zou, Sissons, Gidley, Gilbert & Warren, 2015), prevents the
42 starch granules from being thermally swollen (Heneen & Brismar, 2003; Sissons, Aravind & Fellows,
43 2010; Zou et al., 2015) and inhibits pepsin from hydrolyzing the gluten network, thus reducing the
44 digestion rates of entrapped starch (Zou et al., 2015). As a result, the starch digestion of pasta
45 proceeds with sequential kinetic steps, at a slower rate than purified starch or deconstructed pasta
46 (Zou et al., 2015). **The second is the presence of a continuous gluten network that entraps the starch**
47 **granules, as suggested by many workers (Colonna et al., 1990; Cunin, Handschin, Walther & Escher,**
48 **1995; Dexter, Dronzek & Matsuo, 1978; Favier, Samson, Aubled, Morel & Abecassis, 1996; Singh**
49 **& MacRitchie, 2004; Sissons et al., 2010) who think it reduces the accessibility of α -amylase to the**
50 **starch** because the tortuosity (Ghanbarian, Hunt, Ewing & Sahimi, 2013) of the gluten network may
51 increase the length of the pathway the enzyme has to take to access the starch substrate (Fardet et al.,
52 1998). It has also recently been shown that α -amylase may form weak binding interactions with the
53 gluten network, which retards the penetration of the enzyme into the gluten network (Zou et al.,
54 2015).

55 Current experimental data for characterizing starch digestion, as affected by these two aspects of
56 pasta structure, involves recording the amount of reducing sugars produced at different times. There
57 are as yet no data either examining the evolution of starch molecular structure or giving direct

58 experimental evidence indicating whether the activity of starch digestive enzymes would be inhibited
59 by the presence of proteins. The aim of this study is to remedy this lack by characterizing the
60 evolution of starch molecular structure during *in vitro* digestion for semolina, pasta, pasta powder
61 and purified starch, for pastas with various combinations of compact physical structure and protein
62 composition. In addition, the activity of porcine α -amylase in the digestive solution is measured. **The**
63 **overall aim of these studies is to develop an improved mechanistic understanding of the slower**
64 **digestion of pasta, exploring if pasta proteins are capable of reducing the activity of α -amylase to**
65 **retard *in vitro* digestion of starch molecules, and clarifying the evolution of starch molecular sizes**
66 **during the digestive process.**

67 **2. Materials and methods**

68 *2.1. Materials*

69 Two commercial durum wheat varieties (Caparoi and Yawa) were chosen, for which information
70 regarding their production and chemical composition is described in a previous publication (Zou et
71 al., 2015). Semolina was prepared from the two varieties according to procedures described
72 elsewhere (Sissons, Gianibelli & Batey, 2002). **Obtaining semolina composition, and the methods for**
73 **processing and characterizing purified starch, whole pasta (spaghetti, diameter around 1.8 mm) and**
74 **pasta powder (ground using coffee grinder at room temperature for 30 s into a powder of size range**
75 **100 – 1000 μ m), were as described elsewhere** (Zou et al., 2015). The following combinations are
76 studied for each of the two Durum wheat varieties used here. The terms in quotes are the codes used
77 subsequently.

- 78 • “WP” means whole pasta (spaghetti) with its intact gluten network and compact structure,
79 which was cooked (100 °C for 10 min) and **treated** (37 °C for 30 min) by 0.02 M HCl, and
80 “WP-Pepsin” comprises WP hydrolyzed by 0.02 M HCl with pepsin added to hydrolyze
81 proteins in digestive solution, before they were both hydrolyzed by porcine α -amylase;

“WP-Pepsin-Pancreatin” means WP-Pepsin that was later hydrolyzed by porcine pancreatin instead of purified α -amylase. All of these were studied to understand whether the digestive evolution of branched starch molecules could be retarded by pasta in the presence of the unperturbed gluten network and compact structure.

- “SE” means semolina without protein removal, which was cooked (100 °C for 10 min) and **treated** (37 °C for 30 min) by 0.02 M HCl, and “SE-Pepsin” comprises SE hydrolyzed by 0.02 M HCl with pepsin added to hydrolyze proteins in digestive solution, and **which were then** both hydrolyzed by porcine α -amylase. These were studied to understand whether the digestive evolution of branched starch molecules could be retarded by proteins present in semolina.
- “PP” means pasta powder with its inherent gluten network but ground from whole pasta to break up the compact structure, which was cooked (100 °C for 10 min) and **treated** (37 °C for 30 min) by 0.02 M HCl, and “PP-Pepsin” comprises PP hydrolyzed by 0.02 M HCl with pepsin added to hydrolyze proteins in digestive solution, before they were both hydrolyzed by porcine α -amylase. These were studied to understand whether the digestive evolution of branched starch molecules could be retarded by the unperturbed gluten network without the presence of the compact structure.
- “ST” means starch purified from semolina by removing proteins, which was cooked (100 °C for 10 min) and then **treated** (37 °C for 30 min) with 0.02 M HCl, after which it was hydrolyzed by porcine α -amylase. This was studied to understand the digestive evolution of branched starch molecules in the absence of proteins.

Other reagents were pepsin (Sigma P-6887, from gastric porcine mucosa), α -amylase (Sigma A-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), pancreatin (Chem-supply, PL378, from porcine pancreas) and **protease**

107 (Megazyme, subtilisin A. from *Bacillus licheniformis*, ~ 6 U/mg of protein; 350 U/mL (40°C, pH 8.0,
108 casein as substrate)). All other chemicals were of analytical grade.

109 2.2. Enzyme solutions

110 Pepsin with a concentration of 1.0 mg/mL was dissolved in hydrochloric acid (0.02 M); porcine
111 α -amylase was prepared with 135.26 U porcine α -amylase per 5.0 mL in a 0.2 M sodium acetate
112 buffer (pH 6.0) containing calcium chloride (200 mM) and magnesium chloride (0.49 mM); porcine
113 pancreatin with a concentration of 2.0 mg/mL was dissolved in acetate buffer.

114 The following method was used to measure the activity of α -amylase. The soluble protein
115 concentration for the α -amylase (Sigma A-6255) is 21 mg/mL. The rate of reducing-sugar release by
116 the enzyme was measured with maltose as standard, and transformed into standard enzymatic units:
117 U/mg, units contained in 1 mg soluble protein, and one unit liberates 1.0 mg of maltose from soluble
118 starch in 3 min at pH 7.0 at 37°C.

119 2.3. *In vitro* digestion

120 *In vitro* starch digestion (schematic given in Fig. S1 in the Supporting Information) was carried
121 out for SE, ST, WP and PP using a slight modification of the method of (Muir, Birkett, Brown, Jones
122 & O'Dea, 1995). SE, ST, WP and PP containing 90 mg of starch were cooked in a flask with 6.0 mL
123 of deionized water at 100 °C for 10 min. After cooling to 37.0 °C in a water bath, 5.0 mL of pepsin
124 solution (1 mg/mL) in 0.02 M HCl was added to the samples. Controls with 5.0 mL of 0.02 M HCl
125 (without added pepsin) were also prepared. After incubation at 37.0 °C for 30 min, 5.0 mL of acetate
126 buffer (pH 6) was added to adjust the solution to ~pH 6.0, followed by addition of 5.0 mL of porcine
127 α -amylase or porcine pancreatin (in acetate buffer, pH 6.0) to the flask. The total 21.0 mL reaction
128 solution was incubated at 37.0 °C in a water bath in a sealed flask, stirred with a magnetic stirrer bar
129 at 50 rpm, with 100 μ L aliquots removed at a range of times and dispersed into 900 μ L of sodium
130 carbonate (0.3 M) to terminate the reaction. The mixed solution was centrifuged at 5000 g for 10 min.

131 An aliquot of 100 μ L was transferred into 1.0 mL 4-hydroxybenzoic acid hydrazide (PAHBAH)
 132 solution (0.5 % w/v, dissolved in 0.5 M HCl followed by adding 9 times this volume of 0.5 M
 133 NaOH), before the mixed solution was incubated at 100°C in a water bath for 5 min. When the
 134 solution was cooled to ambient temperature, the absorbance was recorded by a UV-1700 Pharma
 135 Spectrophotometer (Shimadzu) at 410 nm. The absorbance of a series of maltose concentrations (0 –
 136 1.0 mM) was used to generate a standard curve. The concentration of reducing sugars produced in
 137 digestive solution was calculated by the slope of a plot of concentration of maltose (C_{maltose} , mM) vs.
 138 absorbance (Fig. S2a), before it was transformed into concentration of starch digested by multiplying
 139 by 324/342 (the disaccharide residue molecular weight ratio from maltose to starch). The starch
 140 digestion curves are presented as percentage of starch digested vs. time.

141 2.4. Fitting to first-order kinetics

142 Starch digestion data collected above were fitted to a first-order equation:

$$143 \quad C_t = C_{\infty} (1 - e^{-k t}) \quad (1)$$

144 Here C_t (%) is the percentage of starch digested at time t (min), and C_{∞} (%) is the estimated
 145 percentage of starch digested in the end of reaction; k (min^{-1}) represents the starch digestion rate
 146 coefficient, which was measured using a logarithm-of-slope (LOS) analysis described in details
 147 elsewhere (Edwards, Warren, Milligan, Butterworth & Ellis, 2014) through a transformed equation:

$$148 \quad \ln (d C_t / dt) = -k t + \ln (C_{\infty} k) \quad (2)$$

149 All k and C_{∞} values obtained were applied to construct model-fit curves according to a piecewise
 150 function (see (Zou et al., 2015)), to ensure the experimental data were well fitted by the kinetic
 151 parameters.

152 2.5. Collection of digesta

153 Digestion was terminated for SE, ST, and PP by adding absolute ethanol of quadruple volume into
 154 the flask; starch digestion was terminated for pasta by incubating the flask in a 100°C water bath for

155 10 min, then the WP separated and transferred into a new flask and crumbed with tweezers before
156 ethanol of the same volume was added. After waiting for 30 min, the starch pellet was obtained at the
157 bottom of the flask by centrifuging at 4000 g for 10 min; the ethanol supernatant was poured out
158 carefully and the pellet was then placed in an oven and dried at 40°C overnight.

159 2.6. Size-exclusion chromatography

160 Size-exclusion chromatography (SEC, also called GPC or HPLC-SEC) is a technique separating
161 molecules based on molecular hydrodynamic radius, R_h (see, e.g. (Vilaplana & Gilbert, 2010)). The
162 sample preparation for SEC is briefly as follows. Proteins were removed from SE, ST, WP and PP
163 using protease and sodium bisulfite solution, followed by addition of 40 mL absolute ethanol and
164 centrifuged, according to a slightly modified method described elsewhere (Vansteelandt & Delcour,
165 1999). Starch samples obtained were dissolved in 2.0 mL of DMSO–0.5% (w/w) LiBr solution at
166 80°C in a water bath overnight; afterwards starch was precipitated after addition of 12.0 mL of
167 absolute ethanol to separate from ethanol-soluble non-starch polysaccharides. The resulting starch
168 was dissolved in DMSO/LiBr at 80 °C overnight, and the concentration of soluble starch molecules
169 was measured using a Megazyme total starch assay kit, before the final concentration was diluted to
170 2 mg/mL for SEC analysis.

171 The SEC weight distribution, $w(\log R_h)$, of whole branched starch was characterized using an
172 Agilent 1100 SEC system with a refractive index detector (RID; ShimadzuRID-10A, Shimadzu
173 Corp., Kyoto, Japan), as described previously (Cave, Seabrook, Gidley & Gilbert, 2009; Vilaplana et
174 al., 2010). A GRAM pre-column, GRAM 100 and GRAM 3000 columns (PSS. Mainz, Germany)
175 were used with DMSO/LiBr eluent at 0.3 mL/min. Calibration used pullulan standards with a range
176 of molecular weights (342 to 2.35×10^6), to convert elution volume to R_h through the Mark–
177 Houwink equation, with parameters K and α for pullulan in DMSO/LiBr solution at 80 °C taken as
178 2.424×10^{-4} dL g⁻¹ and 0.68 respectively, while the dn/dc value is 0.0853 mL/g (Vilaplana et al.,

2010). The SEC weight distribution of whole branched starch molecules from the RID are presented as $w_{br}(\log R_h)$.

2.7. Measuring α -amylase activity

Starch digestions for SE, ST, WP and PP were carried out using the procedures of Section 2.3. A blank flask was also prepared following the same procedures but without any starch-containing samples. The procedure used for activity measurements is shown schematically in Fig. S3. After incubation for 60 min for all flasks, 50 μ L solution from flask I was added to flask II containing 4.0 mL gelatinized maize starch (15 mg/mL) with magnetic stirring at 37°C in a water bath. A 300 μ L aliquot was collected at 0, 4, 8 and 12 min into tube III (Eppendorf, 2.5 mL) containing 300 μ L of sodium carbonate solution (0.3 M) to halt α -amylase activity. The tubes were centrifuged at 5000 g for 10 min. 100 μ L of supernatant was transferred into a new tube IV each containing 1.0 mL PAHBAH solution (preparation as in section 2.3), and the resulting solution was incubated at 100°C in a water bath for 5 min. When the solution was cooled to room temperature, the absorbance at 410 nm was recorded. The activity of α -amylase for hydrolysis of maize starch into reducing sugar was obtained from the slope of the linear least-squares fit of the plot of concentration of reducing sugar against time, as shown in Fig. S2b (SI). As this rate is always measured with the same added concentration of enzyme, it is proportional to the activity; the slope was converted to rate per unit volume of the digestive solution by dividing by the 50 μ L of added enzyme solution.

In order to observe the activity of α -amylase after hydrolyzing soluble proteins in digestive solution, starch digestions for PP, PP-Pepsin, SE and SE-Pepsin were carried out as described in Section 2.3. Protease (1.0 mL) was added into each flask after digestion for 60 min and proteolysis allowed to proceed for an additional 10 min.

201 2.8. *In vitro* digestion after addition of α -amylase

202 SE and PP were used for *in vitro* starch digestion following the procedures described in Section
203 2.3. After starch digestion for ~ 60 min, porcine α -amylase solution (100 μ L sodium acetate buffer
204 containing 135.26 U porcine α -amylase) was added to the digestive solution (~21.0 mL), and the
205 percentages of starch digested vs. time were obtained as in Section 2.3.

206 2.9. Statistical analysis

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

211

212 3. Results

213 3.1. Starch digestion data

214 Typical experimental starch digestion curves and LOS plots for SE, SE-Pepsin, ST, PP, PP-Pepsin,
215 WP, WP-Pepsin and WP-Pepsin-Pancreatin can be seen in Figs. 1 and S4; a visual comparison of k
216 values can be seen in Fig. S5, in which one sees there is an initial linear step with a significantly
217 larger rate coefficient, denoted k -f, and a following linear step with a significantly smaller rate
218 constant, denoted k -s, (Tables S1 and S2) for all starchy samples, indicating starch digestion
219 proceeded successively with a fast and slow step.

220 The reason why the starch digestion did not start at 0% is because the cooked samples were
221 digested very quickly once α -amylase was added. During the very short time for the digestion
222 solution to be pipetted into the Na_2CO_3 solution, part of the starch would have been degraded into

223 reducing sugar. This phenomena is especially evident for the cooked semolina, pasta powder and
224 purified starch, since these did not have compact structure and would be much easier to be digested.

225

Fig. 1. Typical starch digestion curves, model-fit curves and LOS plots from Caparoi SE-Pepsin (c), PP-Pepsin (d), WP-Pepsin (g) and WP-Pepsin-Pancreatin (h) that were cooked and hydrolyzed by 0.02 M HCl with pepsin added to hydrolyze proteins in digestive solution, and from Caparoi SE (a), PP (b), ST (e) and WP (f) that were cooked and hydrolyzed by 0.02 M HCl with proteins in digestive solution unaltered. All of the points in the LOS plots are linearly treated by least-squares fit. All the LOS plots can be divided into two parts with straight lines of different slope: k -f and k -s are starch digestion rates for the initial fast step and the later slow step, respectively. The R-squared values relate to the LOS plots. The part of the LOS plot describing k -f is shown in red, and the part describing k -s is shown in green. Digestion data are shown in black points and model-fit curves in a black dotted line.

239

240 There was an initial fast step (a few minutes) in which ~ 40 – 60% of starch was digested from SE
241 (Figs. 1a and S4a) or PP (Figs. 1b and S4b) in the presence of proteins, while more than 80% starch
242 was digested from ST (Figs. 1e and S4e), SE-Pepsin (Figs. 1c and S4c) and PP-Pepsin (Figs. 1d and
243 S4d) that had proteins hydrolyzed by pepsin prior to α -amylase digestion. This was followed by a
244 slow step over several hours for SE, PP and ST, with significantly lower k -s values (Tables S1 and
245 S2) for SE (Fig. 2 a-1 and b-1) and PP (Fig. 2 a-3 and b-3) with proteins unaltered, compared for SE-
246 Pepsin (Fig. 2 a-2 and b-2), PP-Pepsin (Fig. 2 a-4 and b-4) or ST (Fig. 2 a-5 and b-5) with proteins
247 hydrolyzed. In contrast, the starch digestion rate for WP was much slower (Fig. 1f & g). Adding
248 pepsin (WP-Pepsin) or porcine pancreatin (WP-Pepsin-Pancreatin) led to slightly but significantly
249 different k -f values for the fast step but no significant changes for the k -s values for the slow step
250 (Figs. S5 and Tables S1 and S2) compared to WP.

251

Fig. 2. Values of starch digestion rate constants at slow step ($k-s$, min⁻¹) for Caparoi (a) and Yawa (b) samples. SE-Pepsin and PP-Pepsin were cooked and hydrolyzed by 0.02 M HCl with pepsin added to **hydrolyze** proteins in digestive solution; SE, PP and ST were cooked and hydrolyzed by 0.02 M HCl with proteins in digestive solution unaltered.

259

260 For SE or PP, where there was no alteration of the original proteins, when starch digestion entered
261 the latter slow step, at about 60 min, adding fresh porcine α -amylase at about this time resulted in a
262 transient acceleration in the rate of starch digestion, which slowed again within ~ 2 min (Fig. 3). The
263 increased percentage of starch digested during the transient acceleration was much less than the
264 starch digested in the initial two minutes.

265

Fig. 3. Starch digestion curves for Caparoi SE and PP that were cooked and **treated by 0.02 M HCl**. After the starch digestion had proceeded for ~ 60 min, an additional aliquot of the same units of porcine α -amylase was added into the digestive solution.

272

273 3.2. *Classifying branched starch molecular size distributions based on different regions of R_h*

274 Typical SEC weight distributions for branched starch molecules for Caparoi and Yawa SE are
275 classified based on various regions of R_h , as shown in Fig. 4. Note that fully-branched SEC data such
276 as these should not be used for finding amylose content, because of the effect of shear scission in
277 SEC, but instead the amylose content is best obtained from debranched SEC data (Vilaplana, Hasjim
278 & Gilbert, 2012). Whole starch molecules can generally be divided into three groups of different R_h
279 ranges: large size, group I ($R_h > 100$ nm), intermediate size, group II (10 nm $< R_h < 100$ nm) and
280 small, group III ($R_h < 10$ nm). Amylose accounts for most native starch molecules with R_h less than
281 100 nm while amylopectin accounts for most starch molecules of R_h more than 100 nm (Syahariza,
282 Sar, Hasjim, Tizzotti & Gilbert, 2013). Compared to Caparoi SE, Yawa SE has slightly less starch
283 molecules of intermediate size and more of the large size. Notably, a greater proportion of starch
284 molecules of $R_h > 1000$ nm is found for Yawa SE.

285

Fig. 4. SEC weight distributions for branched starch molecules of Caparoi and Yawa semolina (SE).

291

292 3.3. *In vitro* digestive evolution of weight distribution of branched starch molecules

293 To observe how the compact structure of pasta and wheat proteins can exert influence on the
294 digestive evolution of branched starch molecules, the evolution of the SEC weight distribution of
295 branched starch molecules during *in vitro* digestion is presented in Figs. 5 and S6. The weight
296 distributions of branched starch molecules for each sample type were seen to be slightly different
297 prior to the addition of α -amylase (“0%” data), caused during the procedures converting SE to ST
298 and WP, and grinding WP to PP. With digestion by HCl and porcine α -amylase, starch from SE (Figs.
299 5a, c and S6a, c), PP (Figs. 5b, d and S6 b, d) and ST (Figs. 5e and S6e) shows a rapid digestive
300 change of $w_{br}(\log R_h)$. When starch was more than 30% digested, many small molecules still
301 remained undigested, with fewer intermediate-size ones, while large starch molecules had been
302 hydrolyzed completely. For SE-Pepsin or PP-Pepsin (with proteins hydrolyzed by pepsin), there were
303 fewer small starch molecules, and practically none of intermediate size (Figs. 5a, b and S6a, b); by
304 contrast, in SE and PP (with proteins unaltered), there were many more starch molecules of
305 intermediate and small size, with the intermediate size being quite abundant (Figs. 5c, d and S6c, d).
306 Hydrolyzing proteins (ST, Figs. 5e and S6e compared with SE-Pepsin, Figs. 5a and S6a) also
307 resulted in fewer small starch molecules and practically no intermediate-size ones following
308 digestion.

309

Fig. 5. Digestive evolution of weight distributions of branched starch molecules during *in vitro* digestion for Caparoi SE (a, c), PP (b, d), ST (e) and WP (f-h).

315

316 Compared to that from SE, PP or ST, starch from WP (Figs. 5f-h and S6f-h) showed a much
317 slower digestive evolution of $w_{br}(\log R_h)$. When starch from WP was more than 30% digested, small
318 starch molecules showed an increase in relative number, and intermediate-size became relatively
319 fewer while large ones became more abundant relative to no digestion (Fig. 5f). For WP (Fig. 5f and
320 Fig. S6f), WP-pepsin (Fig. 5g and Fig. S6g) and WP-Pepsin-Pancreatin (Fig. 5h and Fig. S6h) (with
321 proteins in digestive solution **hydrolyzed** by pepsin or protease included in pancreatin), the starch
322 digestive evolution of $w_{br}(\log R_h)$ were similar, with abundant small, intermediate-size and large
323 starch molecules undigested.

324 3.4. Activity of α -amylase in digestive solution

325 Fig. 6 compares the activity of α -amylase added into solution to digest starch mixed with different
326 protein structures. Compared to the blank without starch-containing samples added (h), the activity
327 of α -amylase shows a significant reduction for SE (b), PP (d) and WP (f) wherein proteins were not
328 hydrolyzed, whereas there was no significant reduction in enzymatic activity for SE-Pepsin (a), PP-
329 Pepsin (c) and WP-Pepsin (e), and similarly no significant reduction for ST (g), wherein proteins had
330 been removed before digestion. In addition, the solution for WP, with compact structure (f), had
331 much less reduction in enzymatic activity compared to powdered SE (b) or PP (d).

332 Fig. 7 shows data obtained to see if the reduced activity of α -amylase incubated with samples SE
333 and PP could be increased by treatment with extra pepsin. As expected, in the blank, α -amylase
334 activity decreases due to proteolysis of α -amylase (e-1 to e-2). This reduction in activity was also
335 observed for SE-Pepsin (a-1 to a-2) and PP-Pepsin (c-1 to c-2). However, for SE and PP treated with
336 extra protease, an increase in α -amylase activity was observed, more so for the SE sample
337 (comparing b-1 to b-2 and d-1 to d-2), where the enzymatic activity reached a level comparable to
338 SE-Pepsin (a-2) and PP-Pepsin (c-2). SE and PP showed a reduced activity of α -amylase, because of
339 the presence of proteins. However, once the proteins were hydrolyzed by added protease, an

340 increased enzymatic activity was observed (comparing b-1 to b-2 and d-1 to d-2), and the enzymatic
341 activity a level comparable to that for SE-Pepsin (a-2) and PP-Pepsin (c-2).

342

Fig. 6. Activity of α -amylase in digestive solution of SE-Pepsin (a), PP-Pepsin (c) and WP-Pepsin (e), and for SE (b), PP (d), WP (f) and ST (g). Blank contains only α -amylase (h).

Fig.7. Activity of α -amylase in digestive solution of SE-Pepsin (a-1), SE (b-1), PP-Pepsin (c-1), PP (d-1) and blank containing only α -amylase (e-1); after adding extra protease, activity of α -amylase in digestive solution measured again for SE-Pepsin (a-2), SE (b-2), PP-Pepsin (c-2), PP (d-2) and blank (e-2).

355

356 4. Discussion

357 4.1. Proteins slow starch digestion

358 The following discussion considers to what extent wheat proteins present in SE and PP account
359 for decreased starch digestion rates and for changes in the size distribution of branched molecules
360 during digestion.

361 The starch molecules of large and intermediate-size ($R_h > 10$ nm) for ST were more easily
362 digested by α -amylase, since they were quickly degraded at significantly greater k -f values (Fig. S5,
363 Tables S1 and S2) into numerous fragments of small size ($R_h < 10$ nm) in the initial fast step (Figs.
364 5e and S6e). However the small size of starch digestive fragments were more difficult to be further
365 digested by α -amylase, because much lower k -s values (Fig. S5, Tables S1 and S2) and abundant
366 fragments of R_h around 1–10 nm were seen in the latter slow step (Figs. 5e and S6e). The slower
367 starch digestion is probably because the substrates not digested by α -amylase, such as α -limit dextrin,
368 small linear oligomers along with larger α -glucans (Dona, Pages, Gilbert & Kuchel, 2010), were
369 produced by hydrolysis of amylopectin with α -amylase in the slow step. Although the similar
370 phenomena can also be seen for SE (Figs. 5c and S6c), SE-Pepsin (Figs. 5a and S6a), PP (Figs. 5d
371 and S6d) and PP-Pepsin (Figs. 5b and S6b), this should not be the sole reason resulting in the
372 subdued starch digestion for SE or PP with proteins unaltered, as their k -s values in the slow step
373 were significantly lower (Fig. 2, Tables S1 and S2) than the comparable ST, SE-Pepsin or PP-Pepsin
374 with a reduction of proteins, suggesting that, except the effect of starch substrate, the slowed starch
375 digestion must also have partially resulted from the proteins present in SE and PP.

376 Supporting this inference, the presence of protein changed the *in vitro* digestive evolution of the
377 size distribution of branched starch molecules. Proteins unaltered in the digestive solution from SE
378 (Figs. 5c and S6c) and PP (Figs. 5d and S6d) would have protected the residual intermediate-size and

379 small starch molecules from further degradation, whereas removing proteins for SE-Pepsin (Figs. 5a
380 and S6a) and PP-Pepsin (Figs. 5b and S6b) resulted in fewer residual small starch molecules and
381 practical disappearance of intermediate-size starch molecules. The possible mechanisms by which
382 protein components retard the digestive evolution of branched starch molecules are discussed in the
383 following.

384 We put forward two hypotheses for the retarded digestive evolution of branched starch molecules in
385 the presence of proteins. The first is that certain starch-protein interactions, such as gluten
386 entrapment of the starch for PP, residual tissue (e.g. cell walls) or putative protein-starch complex
387 structure for SE, may act as a physical barrier to inhibit the accessibility of enzymes. This
388 explanation is consistent with previous reports (Zou et al., 2015) that the residual gluten network in
389 PP entraps starch granules, so as to slow starch digestion rates. However, there are problems with
390 this explanation, because neither gluten entrapment, residual cell walls nor a putative protein-starch
391 complex structure can explain why large starch molecules ($R_h > 100$ nm) could not be inhibited from
392 being degraded by digestive enzymes, as all of them had been digested in SE and PP following
393 addition of α -amylase (Figs. 5c-d and S6c-d).

394 Another hypothesis is that there are proteins in SE and in PP which are able to reduce the activity
395 of α -amylase. This suggestion is consistent with a previous finding that the gluten network in WP
396 may be able to bind with α -amylase molecules as they migrate from the exterior to the interior of the
397 pasta particle (Zou et al., 2015). Further evidence for this is seen in Fig. 6, showing that α -amylase
398 added into digestive solution with proteins (SE, PP and WP) showed a significantly reduced activity,
399 while the activity of α -amylase remained unaltered for SE-Pepsin (Fig. 6a), PP-Pepsin (Fig. 6c) and
400 WP-Pepsin digestive solutions (Fig. 6e), in which proteins had been hydrolyzed. All these
401 phenomena indicate that proteins may diffuse into the aqueous phase and remain capable of reducing
402 the activity of α -amylase after cooking and incubation with HCl. The addition of α -amylase results in
403 it interacting with these proteins (e.g. by forming a complex) so as to reduce activity. This would

404 explain the more reduced activity of α -amylase added to SE (Fig. 6b) and to PP (Fig. 6d) compared
405 to WP (Fig. 5f). This is probably because SE or PP are not protected by the compact pasta protein
406 structure, consistent with a previous finding that as much as ~60% of total proteins became
407 hydrolyzed for SE and PP after cooking and pepsin hydrolysis, whereas less than ~10% were
408 hydrolyzed for WP (Zou et al., 2015). This also explains why adding fresh α -amylase to SE or PP at
409 ~60 min results in an immediate but transitory acceleration in starch digestion (Fig. 3). Since the
410 degree of product inhibition by maltose product can be ignored under conditions used in most kinetic
411 studies of amylase action on starch (Dona, Pages, Gilbert & Kuchel, 2011; Warren, Butterworth &
412 Ellis, 2012), it is therefore likely that the added α -amylase would combine with those proteins and its
413 activity thus reduced. Consistent with this, adding fresh α -amylase does not cause significant
414 increase in digestion, as seen within the first 2 min, presumably because the starch concentration had
415 been largely reduced (Figs. 1 a, b, and S4 a, b) and also the starch had already been degraded by this
416 stage (Figs. 5c, d, S6c and d) for SE or PP, so that fresh α -amylase had less probability of interacting
417 with residual starch substrate before losing activity.

418 Some additional experiments were performed to help understand if α -amylase was inhibited or
419 deprived of enzymatic activity after interacting with proteins in SE or PP. Protease was added at 60
420 min, and the ensuing proteolysis resulted in a reduced activity of α -amylase for the blank (containing
421 only α -amylase; Fig. 7e), and also for SE-Pepsin (Fig. 7a) and PP-Pepsin (Fig. 7c), from which
422 proteins had been hydrolyzed. This is expected, since α -amylase would be hydrolyzed by protease
423 and therefore would lose enzymatic activity. However, for SE (Fig. 7b-1) and PP (Fig. 7d-1) whose
424 α -amylase activity had been reduced to a low level because of the presence of wheat proteins, the
425 ensuing proteolysis did not further decrease the activity, but instead partially restored it (Fig. 7b-2
426 and Fig. 7d-2) to a comparable level as that for SE-Pepsin (Fig. 7a-2), PP-Pepsin (Fig. 7c-2) and
427 blank (e-2). These observations firstly suggest that the wheat proteins may be more susceptible to
428 proteolysis than α -amylase. After proteolysis of proteins for SE and PP, the α -amylase that had

429 interacted with wheat proteins would be liberated to continue to act to hydrolyze starch and therefore
430 result in some restoration of enzymatic activity. In addition, the data also suggest that the wheat
431 proteins could interact to inhibit the activity of α -amylase, but not enough to completely remove this
432 activity.

433 4.2. Compact structure of pasta retards the digestive evolution of branched starch molecules

434 WP (with its compact structure) showed slower starch digestion rates (Figs. 1, S4 and S5) than SE
435 and PP, and a slower digestive evolution of branched starch molecules following addition of α -
436 amylase (Figs. 5 and S6). This is firstly because the compact structure of WP inhibited the
437 accessibility of interior starch to α -amylase. For SE and PP (Figs. 5 c, d and S6 c, d), large and
438 intermediate-size starch molecules mostly degraded quickly into a dramatically increased number of
439 small ones; however, WP, with intact compact structure (Figs. 5 f and S6 f), was capable of retaining
440 most large and intermediate-size starch molecules. Although a small relative increase of smaller
441 starch molecules is seen, most intermediate-size and large starch molecules remain undigested.

442 In addition to exerting a physical barrier for protection of starch from enzyme access, the compact
443 structure of pasta can also protect the proteins in the interior of the pasta. SE-Pepsin and PP-Pepsin
444 (Figs. 5a, b and S6a, b) show disappearance of essentially all intermediate-size starch as a result of
445 starch digestion following hydrolysis of proteins; however, WP-Pepsin (Figs. 5g and S6g) and WP-
446 Pepsin-pancreatin (Figs. 5h and S6h), with compact structure, had an almost unaltered weight
447 distribution of intermediate-size and especially large starch molecules as a result of starch digestion
448 following proteolysis either by added pepsin or by protease included in added pancreatin.
449 Presumably this is because WP (with compact structure) is also less accessible to the protease. The
450 protease must also migrate from the exterior region to the interior region of the pasta particle.
451 Therefore, the compact structure of WP would protect the interior proteins from being hydrolyzed by
452 added protease. This is consistent with the observation of a lower reduction in α -amylase activity for
453 WP (Fig. 6f) compared to SE (Fig. 6b) or PP (Fig. 6d), presumably because only part of proteins had

diffused into solution to reduce enzymatic activity, while most of them were located inside and protected by the compact structure. We showed previously that less than 10% proteins were hydrolyzed from cooked WP, and also that subsequent addition of pepsin did not result in hydrolysis of more proteins from cooked WP (Zou et al., 2015). This suggests that the proteins protected in the central region of cooked pasta interact with the α -amylase going from the external to central regions, to reduce enzyme activity and thus to slow starch digestion. This is consistent with the observation that there is only a slight acceleration of starch digestion in the external regions (compare k -f values in Fig. S5 b, d and Tables S1, S2) but no significant acceleration of starch digestion in the central regions (compare k -s values in Fig. S5 b, d and Tables S1, S2) for cooked WP by addition of pepsin or pancreatin, and also why cooked WP has a slow digestive evolution of branched starch molecules (Figs. 5 f-h and S6 f-h).

5. Conclusions

The digestive rate and *in vitro* evolution of starch molecular structure were characterized for a range of pasta-derived cooked substrates: semolina, whole pasta, powdered pasta and extracted starch, with various combinations of treatments with acid and with protein-digesting enzymes. Starch digestion for all these samples showed a distinct initial fast step with significantly larger rate constant, and a later slow step with significantly lower rate constant. The significantly lower starch digestion rate coefficients in the slow step were observed for semolina and pasta powder with proteins unaltered, which suggests that proteins also probably account for the slowed starch digestion, except the effect of less accessible starch substrates to α -amylase. Further analysis revealed that the activity of porcine α -amylase was reduced; retarded digestion for branched starch molecules of intermediate/small sizes was seen for samples which contain proteins in the digestive solution, but rapid digestion for branched starch molecules of small/intermediate/large sizes was seen for samples where these proteins were removed. The combined observations strongly support the hypothesis that protein(s) present in cooked semolina, powdered and whole pasta interact with α -amylase to reduce

its enzymatic activity, and thus retard the digestive evolution of branched starch molecules. Data also suggest that enzyme activity can be at least partially recovered once the protein(s) are hydrolyzed by protease. The compact structure of pasta protects the inner region of a pasta fragment from protein-degrading and starch-degrading enzymes, therefore protein(s) were retained to reduce activity of α -amylase and also the remaining gluten network may be able to prevent the leaching of large amylopectin. All these reduce the enzymatic degradation of the starch, especially for larger molecules.

Acknowledgements

We gratefully acknowledge the support of an Australian Research Council grant (DP130102461), the 1000-Talents Program of the Chinese State Administration of the Foreign Experts Bureau and a National Science Foundation of China grant (31561143008). Wei Zou gratefully acknowledges the support of an International Postgraduate Research Scholarship from the Australian government.

References

- Berti, C., Riso, P., Monti, L. D., & Porrini, M. (2004). *In vitro* starch digestibility and *in vivo* glucose response of gluten-free foods and their gluten counterparts. *European Journal of Nutrition*, 43(4), 198-204.
- Cave, R. A., Seabrook, S. A., Gidley, M. J., & Gilbert, R. G. (2009). Characterization of starch by size-exclusion chromatography: The limitations imposed by shear scission. *Biomacromolecules*, 10(8), 2245-2253.
- Colonna, P., Barry, J.-L., Cloarec, D., Bornet, F., Gouilloud, S., & Galmiche, J.-P. (1990). Enzymic susceptibility of starch from pasta. *Journal of Cereal Science*, 11(1), 59-70.
- Cunin, C., Handschin, S., Walther, P., & Escher, F. (1995). Structural changes of starch during cooking of durum wheat pasta. *LWT-Food Science and Technology*, 28(3), 323-328.
- Dexter, J., Dronzek, B., & Matsuo, R. (1978). Scanning electron microscopy of cooked spaghetti. *Cereal Chemistry*, 55, 23-30.

504 Dona, A. C., Pages, G., Gilbert, R. G., & Kuchel, P. W. (2010). Digestion of starch: *In vivo* and *in*
505 *vitro* kinetic models used to characterise oligosaccharide or glucose release. *Carbohydrate Polymers*,
506 80(3), 599-617.

507 Dona, A. C., Pages, G., Gilbert, R. G., & Kuchel, P. W. (2011). Starch granule characterization by
508 kinetic analysis of their stages during enzymic hydrolysis: ¹H nuclear magnetic resonance studies.
509 *Carbohydrate Polymers*, 83(4), 1775-1786.

510 Edwards, C. H., Warren, F. J., Milligan, P. J., Butterworth, P. J., & Ellis, P. R. (2014). A novel
511 method for classifying starch digestion by modelling the amylolysis of plant foods using first-order
512 enzyme kinetic principles. *Food & Function*, 5(11), 2751-2758.

513 Fardet, A., Hoebler, C., Baldwin, P., Bouchet, B., Gallant, D., & Barry, J.-L. (1998). Involvement of
514 the Protein Network in the *in vitro* Degradation of Starch from Spaghetti and Lasagne: a Microscopic
515 and Enzymic Study. *Journal of Cereal Science*, 27(2), 133-145.

516 Favier, J., Samson, M., Aubled, C., Morel, M., & Abecassis, J. (1996). Thermal kinetics of dough
517 proteins insolubilisation determined by size exclusion high performance liquid chromatography.
518 *Sciences des Aliments*, 16.

519 Ghanbarian, B., Hunt, A. G., Ewing, R. P., & Sahimi, M. (2013). Tortuosity in Porous Media: A
520 Critical Review. *Soil Science Society of America Journal*, 77(5), 1461-1477.

521 Granfeldt, Y., & Björck, I. (1991). Glycemic response to starch in pasta: a study of mechanisms of
522 limited enzyme availability. *Journal of Cereal Science*, 14(1), 47-61.

523 Heneen, W. K., & Brismar, K. (2003). Structure of cooked spaghetti of durum and bread wheats.
524 *Starch-Stärke*, 55(12), 546-557.

525 Jenkins, D., Wolever, T., Taylor, R. H., Barker, H., Fielden, H., Baldwin, J. M., Bowling, A. C.,
526 Newman, H. C., Jenkins, A. L., & Goff, D. V. (1981). Glycemic index of foods: a physiological
527 basis for carbohydrate exchange. *The American Journal of Clinical Nutrition*, 34(3), 362-366.

528 Jenkins, D. J., Wolever, T. M., Jenkins, A. L., Lee, R., Wong, G. S., & Josse, R. (1983). Glycemic
529 response to wheat products: reduced response to pasta but no effect of fiber. *Diabetes Care*, 6(2),
530 155-159.

531 Monge, L., Cortassa, G., Fiocchi, F., Mussino, G., & Carta, Q. (1990). Glyco-insulinaemic response,
532 digestion and intestinal absorption of the starch contained in two types of spaghetti. *Diabetes*,
533 *Nutrition and Metabolism - Clinical and Experimental*, 3(3), 239-246.

534 Muir, J. G., Birkett, A., Brown, I., Jones, G., & O'Dea, K. (1995). Food processing and maize variety
535 affects amounts of starch escaping digestion in the small intestine. *The American Journal of Clinical*
536 *Nutrition*, 61(1), 82-89.

537 Singh, H., & MacRitchie, F. (2004). Changes in proteins induced by heating gluten dispersions at
538 high temperature. *Journal of Cereal Science*, 39(2), 297-301.

539 Sissons, M., Aravind, N., & Fellows, C. M. (2010). Quality of Fiber-Enriched Spaghetti Containing
540 Microbial Transglutaminase. *Cereal Chemistry*, 87(1), 57-64.

541 Sissons, M., Gianibelli, M., & Batey, I. (2002). Small-scale reconstitution of durum semolina
542 components. *Cereal Chemistry*, 79(5), 675-680.

543 Syahariza, Z., Sar, S., Hasjim, J., Tizzotti, M. J., & Gilbert, R. G. (2013). The importance of amylose
544 and amylopectin fine structures for starch digestibility in cooked rice grains. *Food Chemistry*, 136(2),
545 742-749.

546 Vansteelandt, J., & Delcour, J. A. (1999). Characterisation of Starch from Durum Wheat (*Triticum*
547 *durum*). *Starch - Stärke*, 51(2-3), 73-80.

548 Vilaplana, F., & Gilbert, R. G. (2010). Two-dimensional size/branch length distributions of a
549 branched polymer. *Macromolecules*, 43(17), 7321-7329.

550 Vilaplana, F., Hasjim, J., & Gilbert, R. G. (2012). Amylose content in starches: towards optimal
551 definition and validating experimental methods. *Carbohydrate Polymers*, 88(1), 103-111.

552 Warren, F. J., Butterworth, P. J., & Ellis, P. R. (2012). Studies of the effect of maltose on the direct
553 binding of porcine pancreatic α -amylase to maize starch. *Carbohydrate Research*, 358, 67-71.

554 Zou, W., Sissons, M., Gidley, M. J., Gilbert, R. G., & Warren, F. J. (2015). Combined techniques for
555 characterising pasta structure reveals how the gluten network slows enzymic digestion rate. *Food*
556 *Chemistry*, 188, 559-568.

557