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

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Highlights

- Pasta has the nutritional advantage of being a slowly digested starchy food
- It contains both starch and significant amounts of protein in a matrix
- This examines how protein changes the evolution of starch molecular structure
- Data show that soluble proteins retard the digestive evolution of these molecules

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 soluble protein from semolina or pasta powder combined physically with α-amylase, causing reduced enzymatic activity and retarding digestion of branched starch molecules with hydrodynamic radius (R_h) < 100 nm; this protein was susceptible to proteolysis. Thus the compact structure of pasta protects the inner starch and proteins in the whole pasta, reducing the enzymatic degradation of starch molecules, especially for molecules with R_h >100 nm.

Key words:

1. Introduction

Pasta is considered to be among the more healthy carbohydrate-based foods, since it has been shown by many *in vitro* (Berti, Riso, Monti & Porrini, 2004; Colonna et al., 1990; Fardet et al., 1998) and *in vivo* (Berti et al., 2004; Granfeldt & Björck, 1991; Jenkins et al., 1981; Monge, Cortassa, Fiocchi, Mussino & Carta, 1990) experiments that starch digestion in pasta proceeds more slowly than those of most other starchy foods, resulting in attenuated glycemic response. The relatively slower starch digestion of pasta can be generally attributed to two aspects of pasta structure. The first is the compact structure of pasta, which reduces the reaction area where starch granules can be accessed by digestive enzymes (Jenkins et al., 1983; Zou, Sissons, Gidley, Gilbert & Warren, 2015), prevents the starch granules from being thermally swollen (Heneen & Brismar, 2003; Sissons, Aravind & Fellows, 2010; Zou et al., 2015) and inhibits pepsin from hydrolyzing the gluten network, thus reducing the digestion rates of entrapped starch (Zou et al., 2015). As a result, the starch digestion of pasta proceeds with sequential kinetic steps, at a slower rate than purified starch or deconstructed pasta (Zou et al., 2015). The second is the presence of a continuous gluten network that entraps the starch granules, as suggested by many workers. This reduces the accessibility of α-amylase to the starch (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), because the tortuosity of the gluten network may increase the length of the pathway the enzyme has to take to access the starch substrate (Fardet et al., 1998). It has also recently been shown that α-amylase may form weak binding interactions with the gluten network, which retards the penetration of the enzyme into the gluten network (Zou et al., 2015).

Current experimental data for characterizing starch digestion, as affected by these two aspects of pasta structure, involves recording the amount of reducing sugars produced at different times. There are as yet no data either examining the evolution of starch molecular structure or giving direct experimental evidence indicating whether the activity of starch digestive enzymes would be inhibited

by the presence of proteins. The aim of this study is to remedy this lack by characterizing the evolution of starch molecular structure during *in vitro* digestion for semolina, pasta, pasta powder and purified starch, for pastas with various combinations of compact physical structure and protein composition. In addition, the activity of porcine α-amylase in the digestive solution is measured. The overall aim of these studies is to develop an improved mechanistic understanding of the slower digestion of pasta, exploring hypotheses such as the existence of specific pasta proteins capable of reducing the activity of α-amylase to retard *in vitro* digestion of starch molecules, and clarifying the evolution of starch molecular sizes during the digestive process.

2. Materials and methods

2.1. Materials

Two commercial durum wheat varieties (Caparoi and Yawa) were chosen, for which information regarding their production and chemical composition is described in a previous publication (Zou et al., 2015). Semolina was prepared from the two varieties according to procedures described elsewhere (Sissons, Gianibelli & Batey, 2002). Semolina composition, and the methods for processing purified starch, whole pasta (spaghetti) and pasta powder are described elsewhere (Zou et al., 2015). The following combinations are studied for each of the two Durum wheat varieties used here. The terms in quotes are the codes used subsequently.

- "WP" means whole pasta (spaghetti) with its intact gluten network and compact structure, which was cooked (100 °C for 10 min) and hydrolyzed (37 °C for 30 min) by 0.02 M HCl with proteins unaltered, and "WP-Pepsin" comprises WP hydrolyzed by 0.02 M HCl with pepsin added to hydrolyze 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

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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 hydrolyzed (37 °C for 30 min) by 0.02 M HCl with proteins unaltered, and "SE-Pepsin" comprises SE hydrolyzed by 0.02 M HCl with pepsin added to hydrolyze proteins in digestive solution, and then 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 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 hydrolyzed (37 °C for 30 min) by 0.02 M HCl with proteins unaltered, 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 (Megazyme, subtilisin A. from *Bacillus licheniformis*). All other chemicals were of analytical grade.

2.2. Enzyme solutions

Pepsin with a concentration of 1.0 mg/mL was dissolved in hydrochloric acid (0.02 M); porcine α-amylase was prepared with 135.26 U porcine α-amylase 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); porcine pancreatin with a concentration of 2.0 mg/mL was dissolved in acetate buffer.

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

2.3. In vitro digestion

In vitro starch digestion (schematic given in Fig. S1 in the Supporting Information) was carried out for SE, ST, WP and PP using a slight modification of the method of (Muir, Birkett, Brown, Jones & O'Dea, 1995). SE, ST, WP and PP containing 90 mg of starch were cooked in a flask with 6.0 mL of deionized water at 100 °C for 10 min. After cooling to 37.0 °C 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 °C for 30 min, 5.0 mL of acetate buffer (pH 6) was added to adjust the solution to ~pH 6.0, followed by addition of 5.0 mL of porcine α-amylase or porcine pancreatin (in acetate buffer, pH 6.0) to the flask. The total 21.0 mL reaction solution was incubated at 37.0 °C 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 times and dispersed into 900 μL of sodium carbonate (0.3 M) to terminate the reaction. The mixed solution was centrifuged at 5000 g for 10 min. 100 μL was transferred into 1.0 mL 4-hydroxybenzoic acid hydrazide (PAHBAH) solution (0.5 % w/v, dissolved in 0.5 M HCl followed by adding 9 times this volume of 0.5 M NaOH), before the

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mixed solution was incubated at 100°C in a water bath for 5 min. When the solution was cooled to ambient temperature, the absorbance was recorded by a UV-1700 Pharma Spectrophotometer (Shimadzu) at 410 nm. The absorbance of a series of maltose concentrations (0 – 1.0 mmol/L) was used to generate a standard curve. The concentration of reducing sugar produced in digestive solution was calculated by the slope of a plot of concentration of maltose ($C_{maltose}$, mmol/L) *vs*. absorbance (Fig. S2a), before it was transformed into concentration of starch digested by multiplying by 324/342 (the disaccharide residue weight ratio from maltose to starch). The starch digestion curves are presented as percentage of starch digested *vs*. time.

2.4. Fitting to first-order kinetics

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

$$C_t = C_\infty \left(1 - e^{-kt}\right) \tag{1}$$

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

$$\ln (d\, C_t /dt) = - kt + \ln (C_\infty k) \tag{2}$$

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

2.5. Collection of digesta

Digestion was terminated for SE, ST, and PP by adding absolute ethanol of quadruple volume into the flask; starch digestion was terminated for pasta by incubating the flask in a 100°C water bath for 10 min, then the WP separated and transferred into a new flask and crumbed with tweezers before ethanol of the same volume was added. After waiting for 30 min, the starch pellet was obtained at the

bottom of the flask by centrifuging at 4000 g for 10 min; the ethanol supernatant was poured out carefully and the pellet was then placed in an oven and dried at 40°C overnight.

2.6. Size-exclusion chromatography

Size-exclusion chromatography (SEC, also called GPC or HPLC-SEC) separates on the basis of hydrodynamic radius, R_h (see, e.g. (Vilaplana & Gilbert, 2010)), which s a measure of the size of the molecularly disperse molecule in solution. The SEC technique was as follows. Proteins were removed from SE, ST, WP and PP and their digesta using protease and sodium bisulfite solution, each followed by an addition of absolute ethanol of 40 mL and centrifuged, according to a slightly modified method described elsewhere (Vansteelandt & Delcour, 1999). Starch samples obtained were dissolved in 2.0 mL of DMSO–0.5% (w/w) LiBr solution at 80°C in a water bath overnight; afterwards starch was precipitated after addition of 12.0 mL of absolute ethanol to separate from ethanol-soluble non-starch polysaccharides. The resulting starch was dissolved in DMSO/LiBr at 80 °C overnight, and the concentration of soluble starch molecules was measured using a Megazyme total starch assay kit, before the final concentration was diluted to 2 mg/mL for SEC analysis.

The SEC weight distribution, w (log R_h), of whole branched starch was characterized using an Agilent 1100 SEC system with a refractive index detector (RID; ShimadzuRID-10A, Shimadzu Corp., Kyoto, Japan), as described previously (Cave, Seabrook, Gidley & Gilbert, 2009; Vilaplana et al., 2010). A GRAM pre-column, GRAM 100 and GRAM 3000 columns (PSS) were used with DMSO/LiBr eluent at 0.3 mL/min. Calibration used pullulan standards with a range of molecular weights (342 to 2.35×10^6), to convert elution volume to R_h through the Mark–Houwink equation, with parameters K and α for pullulan in DMSO/LiBr solution at 80 °C taken as 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).

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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 µL 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 µL aliquot was collected at 0, 4, 8 and 12 min into tube III (Eppendorf, 2.5 mL) containing 300 µL of sodium carbonate solution (0.3 M) to halt α-amylase activity. The tubes were centrifuged at 5000 g for 10 min. 100 µL 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 µL 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 (subtilisin A., 1.0 mL containing 50 mg) was added into each flask after digestion for 60 min and proteolysis allowed to proceed for an additional 10 min.

2.8. In vitro digestion after addition of α-amylase

SE and PP were used for *in vitro* starch digestion following the procedures described in Section 2.3. After starch digestion for ~ 60 min, porcine α-amylase solution (100 µL sodium acetate buffer

containing 135.26 U porcine α-amylase) was added to the digestive solution (~21.0 mL), and the percentages of starch digested *vs.* time were obtained as in Section 2.3.

2.9. Statistical analysis

The statistical significance of starch digestion rate constants was analyzed 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. Starch digestion data

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

The reason why the starch digestion did not start at 0% is because the cooked samples were digested very quickly once α-amylase was added. During the very short time for the digestion solution to be pipetted into the Na_2CO_3 solution, part of the starch would have been degraded into reducing sugar. This phenomena is especially evident for the cooked semolina, pasta powder and purified starch, since these did not have compact structure and would be much easier to be digested.

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

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

3.2. Comparing weight distribution of branched starch molecule between genotypes

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

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

The evolution of the SEC weight distribution of branched starch molecules during *in vitro* digestion is presented in Figs. 5 and S6. With digestion by HCl and porcine α-amylase, starch from SE (Figs. 5a, c and S6a, c), PP (Figs. 5b, d and S6 b, d) and ST (Figs. 5e and S6e) shows a rapid digestive change of w_{br} (log R_h). When starch was more than 30% digested, many small molecules still remained undigested, with fewer intermediate-size ones, while large starch molecules had been hydrolyzed completely. For SE-Pepsin or PP-Pepsin (with proteins reduced by pepsin hydrolysis), there were fewer small starch molecules, and practically none of intermediate size (Figs. 5a, b and S6a, b); by contrast, in SE and PP (with proteins unaltered), there were many more starch molecules of intermediate and small size, with the intermediate size being quite abundant (Figs. 5c, d and S6c, d). Hydrolyzing proteins (ST, Figs. 5e and S6e compared with SE-Pepsin, Figs. 5a and S6a) also resulted in fewer small starch molecules and practically no intermediate-size ones following digestion.

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

3.4. Activity of α-amylase in digestive solution

The activity of α-amylase obtained from the digestive solution is shown in Fig. 6. When starch digestion had proceeded for 60 min, the α-amylase from the blank (only α-amylase present), ST, SE-Pepsin, PP-Pepsin or WP-Pepsin did not show a significantly reduced activity. However, a significantly reduced activity was seen in the digestive solution from SE, PP and WP. Furthermore, adding more protease resulted in a reduced activity of α-amylase (Fig. 7) in the blank containing only α-amylase, SE-Pepsin and PP-Pepsin. However an increased activity of α-amylase was seen in the digestive solution from SE and PP, where no proteins were altered in digestive solution.

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4. Discussion

4.1. Proteins slow starch digestion

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

Supporting this inference, the presence of protein changed the *in vitro* digestive evolution of the size distribution of branched starch molecules. Proteins unaltered in the digestive solution from SE (Figs. 5c and S6c) and PP (Figs. 5d and S6d) would have protected the residual intermediate-size and small starch molecules from further degradation, whereas removing proteins for SE-Pepsin (Figs. 5a and S6a) and PP-Pepsin (Figs. 5b and S6b) resulted in fewer residual small starch molecules and practical disappearance of intermediate-size starch molecules. The possible mechanisms by which

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protein components retard the digestive evolution of branched starch molecules are discussed in the following.

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

Another hypothesis is that there are soluble proteins in SE and in PP which are able to reduce the activity of α-amylase. This suggestion is consistent with a previous finding that the gluten network in WP may be able to bind with α-amylase molecules as they migrate from the exterior to the interior of the pasta particle (Zou et al., 2015). Further evidence for this is seen in Fig. 6, showing that α-amylase added into digestive solution with soluble proteins (SE, PP and WP) showed a significantly reduced activity, while the activity of α-amylase remained unaltered for SE-Pepsin (Fig. 6a), PP-Pepsin (Fig. 6c) and WP-Pepsin digestive solutions (Fig. 6e), in which proteins had been hydrolyzed. All these phenomena indicate that soluble proteins may diffuse into the aqueous phase and remain capable of reducing the activity of α-amylase after cooking and incubation with HCl. The addition of α-amylase results in it interacting with these soluble proteins (e.g. by forming a complex) so as to reduce activity. This would explain the more reduced activity of α-amylase added to SE (Fig. 6b) and to PP (Fig. 6d) compared to WP (Fig. 5f). This is probably because SE or PP are not protected by the compact pasta protein structure, consistent with a previous finding that as much as ~60% of total

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proteins became soluble for SE and PP after cooking and pepsin hydrolysis, whereas less than ~10% were soluble for WP (Zou et al., 2015). This also explains why adding fresh α-amylase to SE-HCl or PP-HCl at ~60 min results in an immediate but transitory acceleration in starch digestion (Fig. 3). Since the degree of product inhibition by maltose product can be ignored under conditions used in most kinetic studies of amylase action on starch (Dona, Pages, Gilbert & Kuchel, 2011; Warren, Butterworth & Ellis, 2012), it is therefore likely that the added α-amylase would combine with those soluble proteins and its activity thus reduced. Consistent with this, adding fresh α-amylase does not cause significant increase in digestion, as seen within the first 2 min, presumably because the starch concentration had been largely reduced (Figs. 1 a, b, and S4 a, b) and also the starch had already been degraded by this stage (Figs. 5c, d, S6c and d) for SE or PP, so that fresh α-amylase had less probability of interacting with residual starch substrate before losing activity.

Some additional experiments were performed to help understand if α-amylase was inactivated after combination with soluble proteins in SE or PP. Protease was added at 60 min, and the ensuing proteolysis resulted in a reduced activity of α-amylase for the blank (containing only α-amylase; Fig. 7e), and also for SE-Pepsin (Fig. 7a) and PP-Pepsin (Fig. 7c), from which proteins had been removed. This is expected, since α-amylase would be hydrolyzed by protease and therefore would lose enzymatic activity. However, a recovery of α-amylase activity was seen for SE (Fig. 7b) and PP (Fig. 7d). This was unexpected, because the α-amylase after protease hydrolysis also should have continued to reduce, not to increase, its activity. These observations firstly suggest that soluble proteins may be more susceptible to proteolysis than α-amylase. After proteolysis of soluble proteins, the added protease would continue to degrade the separated α-amylase and thus reduce its enzymatic activity to a similar level as for SE-Pepsin (see Fig. 7 a-2 and b-2) and PP-Pepsin (see Fig. 7 c-2 and d-2). Moreover they also suggest the soluble proteins in the digestive solution for SE and PP interact with α-amylase but this does not lead to a reduction of enzyme activity; once the soluble proteins

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were hydrolyzed by protease, α-amylase/soluble-protein complexes may separate, so that the activity is restored.

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

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

SE-Pepsin and PP-Pepsin (Figs. 5a, b and S6a, b) show disappearance of essentially all intermediate-size starch as a result of starch digestion following hydrolysis of proteins; however, WP-Pepsin (Figs. 5g and S6g) and WP-Pepsin-pancreatin (Figs. 5h and S6h), with compact structure, had an almost unaltered weight distribution of intermediate-size and especially large starch molecules as a result of starch digestion following proteolysis either by added pepsin or by protease including in added pancreatin. Presumably this is because WP (with compact structure) is also less accessible to the protease. The protease must also migrate from the exterior region to the interior region of the pasta particle. Therefore, the compact structure of WP would protect the soluble proteins from being hydrolyzed by added protease. This is consistent with the observation of a lower reduction in α-amylase activity for WP (Fig. 6f) compared to SE (Fig. 6b) or PP (Fig. 6d), presumably because only part of soluble proteins had diffused into solution to reduce enzymatic activity, while most of them were located inside the compact structure. We showed previously that less than 10% soluble proteins diffused 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 soluble proteins protected in the central region of cooked pasta combine 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 constants 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 soluble 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 soluble 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 this enzyme/soluble protein interaction is a physical one (e.g. entanglement or H bonding), because enzyme activity can be at least partially recovered. The compact structure of pasta protects the inner region of a pasta fragment from protein-degrading and

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starch-degrading enzymes, therefore soluble 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

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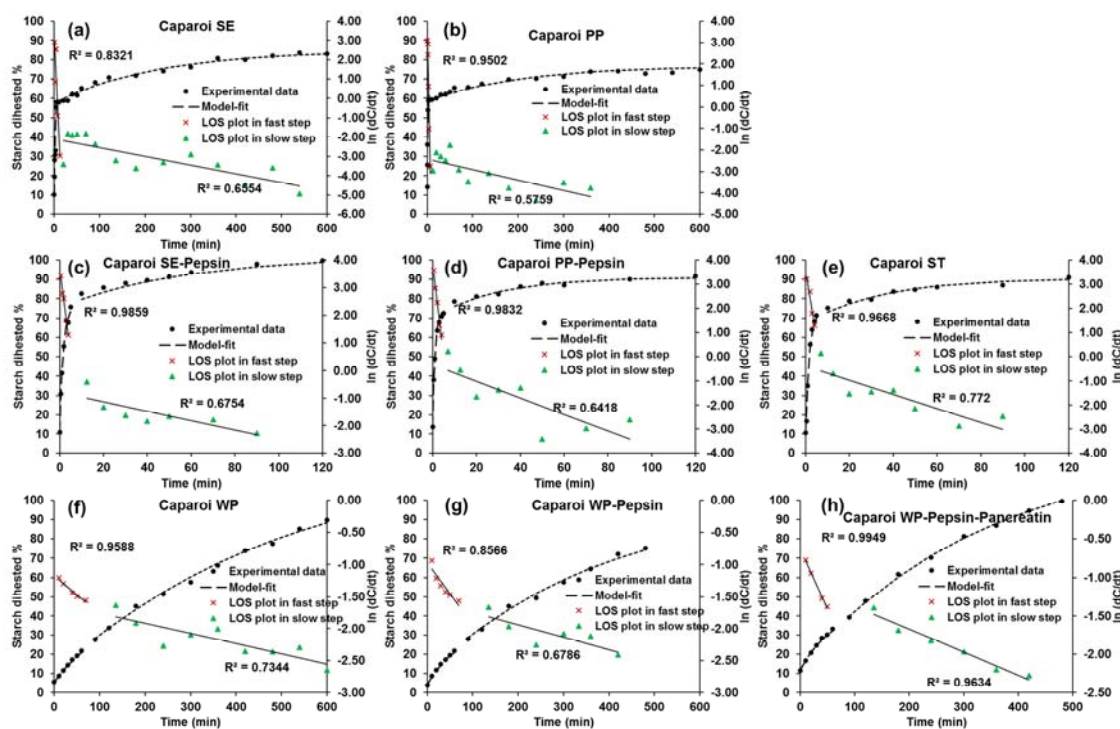
Fig. 1. Typical starch digestion curves, model-fit curves and LOS plots from Caparoi SE-Pepsin (c), PP-Pepsin (d), WP-Pepsin (g) and WP-Pepsin-Pancreatin (h) that were cooked and hydrolyzed by 0.02 M HCl with pepsin added to reduce proteins in digestive solution, and from Caparoi SE (a), PP (b), ST (e) 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 linear 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.
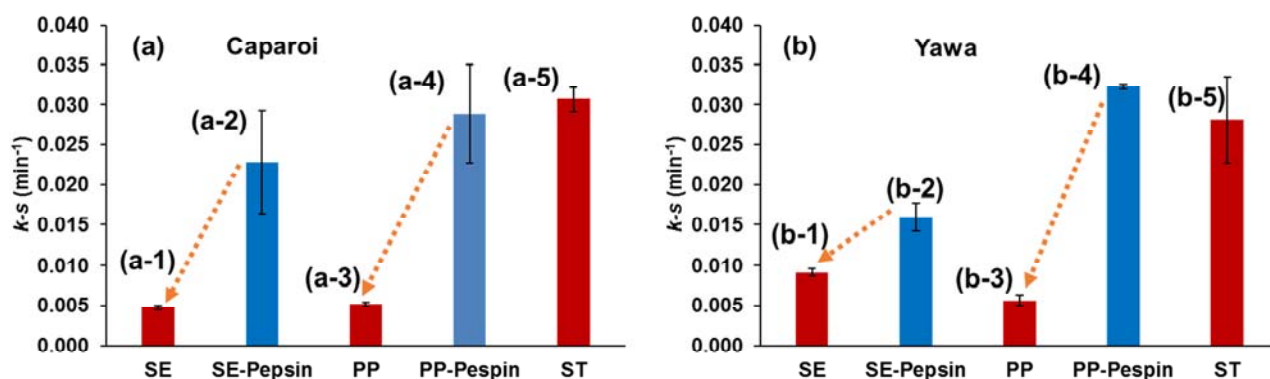
Fig. 2. Values of starch digestion rate constants at slow step (k-s, min^{-1}) for Caparoi (a) and Yawa (b) samples. SE-Pepsin and PP-Pepsin were cooked and hydrolyzed by 0.02 M HCl with pepsin added to reduce proteins in digestive solution; SE, PP and ST were cooked and hydrolyzed by 0.02 M HCl with proteins in digestive solution unaltered.
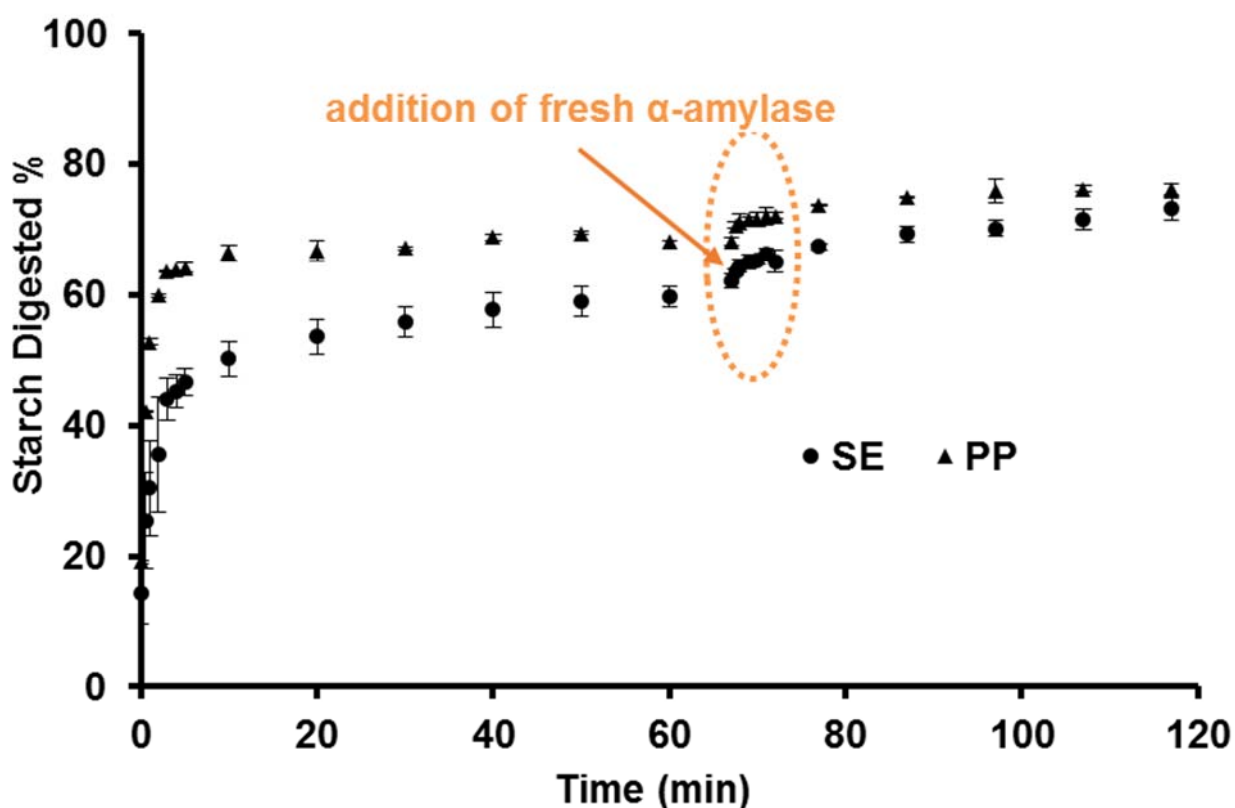

Fig. 3. Starch digestion curves for Caparoi SE and PP that were cooked and hydrolyzed by 0.02 M HCl with proteins unaltered in digestive solution. After the starch digestion had proceeded for ~ 60 min, an additional aliquot of the same units of porcine α-amylase was added into the digestive solution.
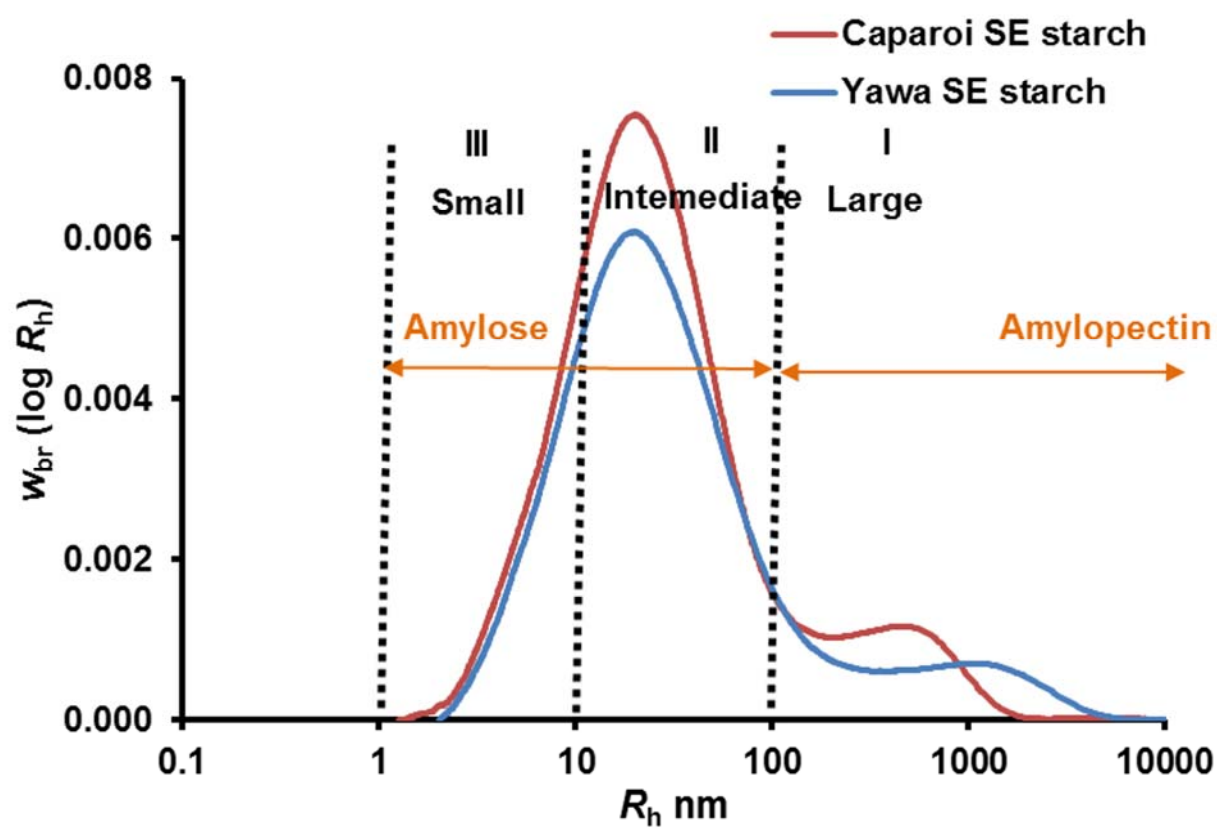
Fig. 4. SEC weight distributions (arbitrary normaization) for branched starch molecules of Caparoi and Yawa semolina (SE).
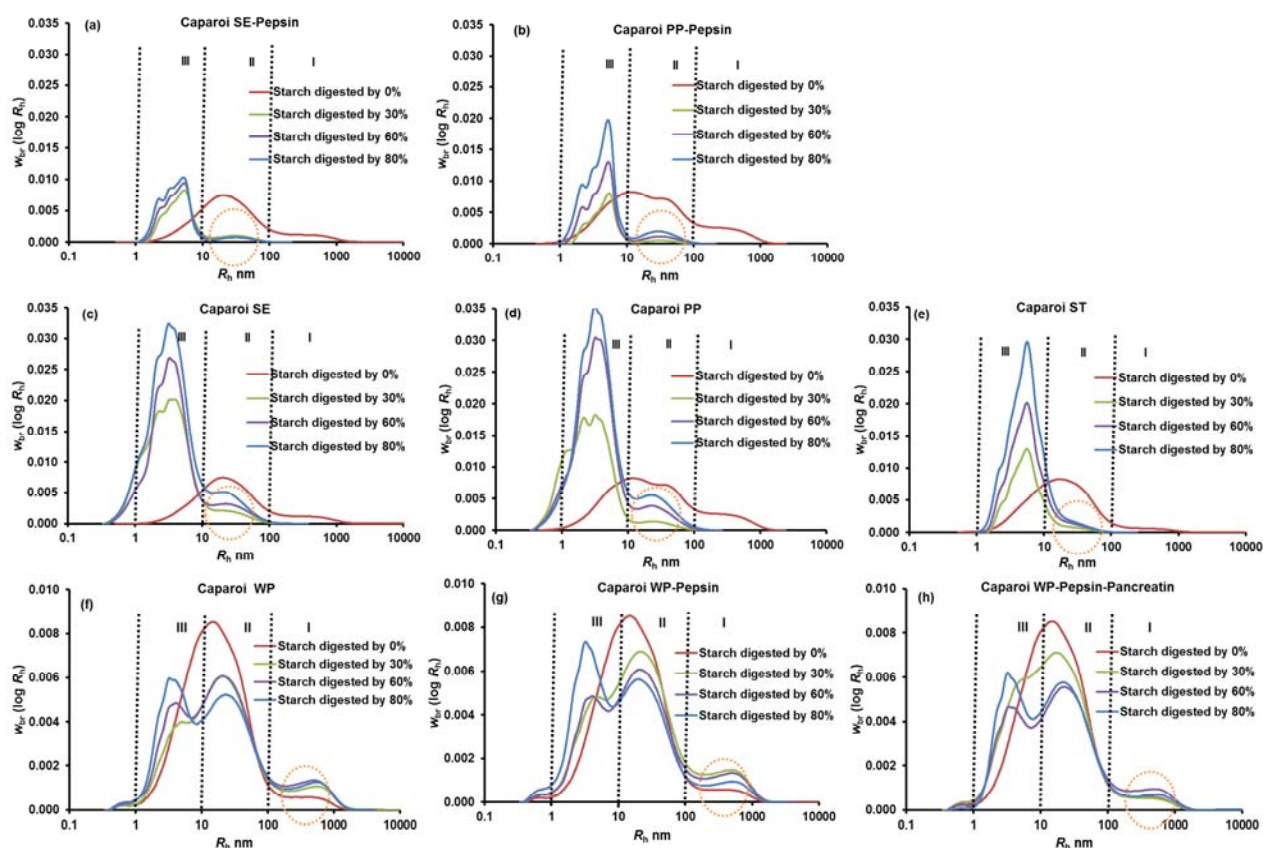
Fig. 5. Digestive evolution of weight distributions of branched starch molecules during *in vitro* digestion for Caparoi SE (a, c), PP (b, d), ST (e) and WP (f-h).
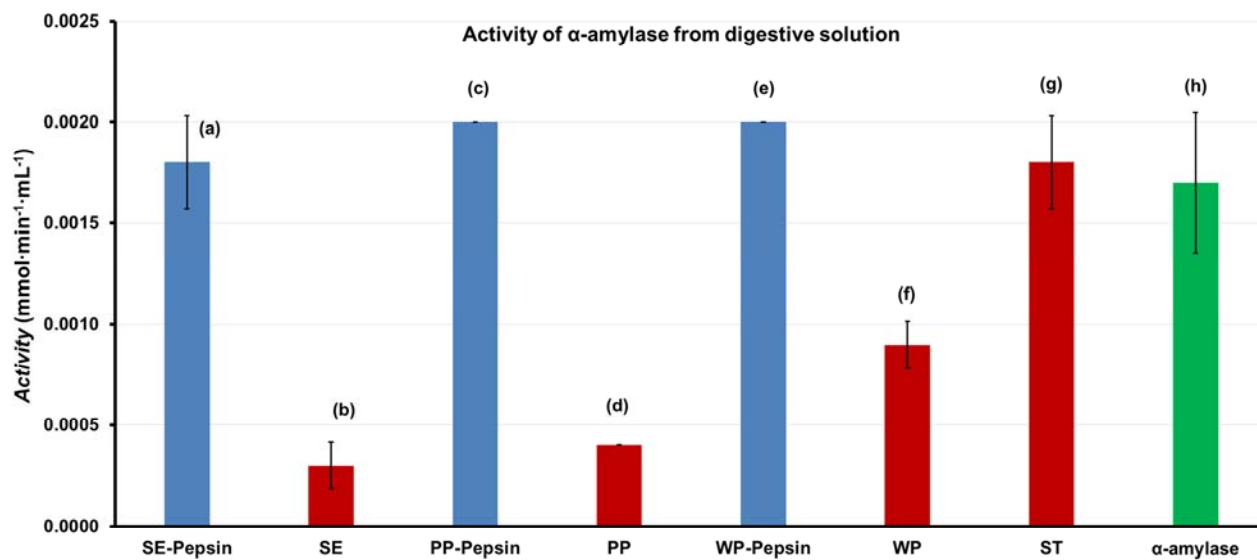

Fig. 6. Activity of α-amylase in digestive solution of SE-Pepsin (a), PP-Pepsin (c) and WP-Pepsin (e), and for SE (b), PP (d), WP (f) and ST (g). Blank contains only α-amylase (h).
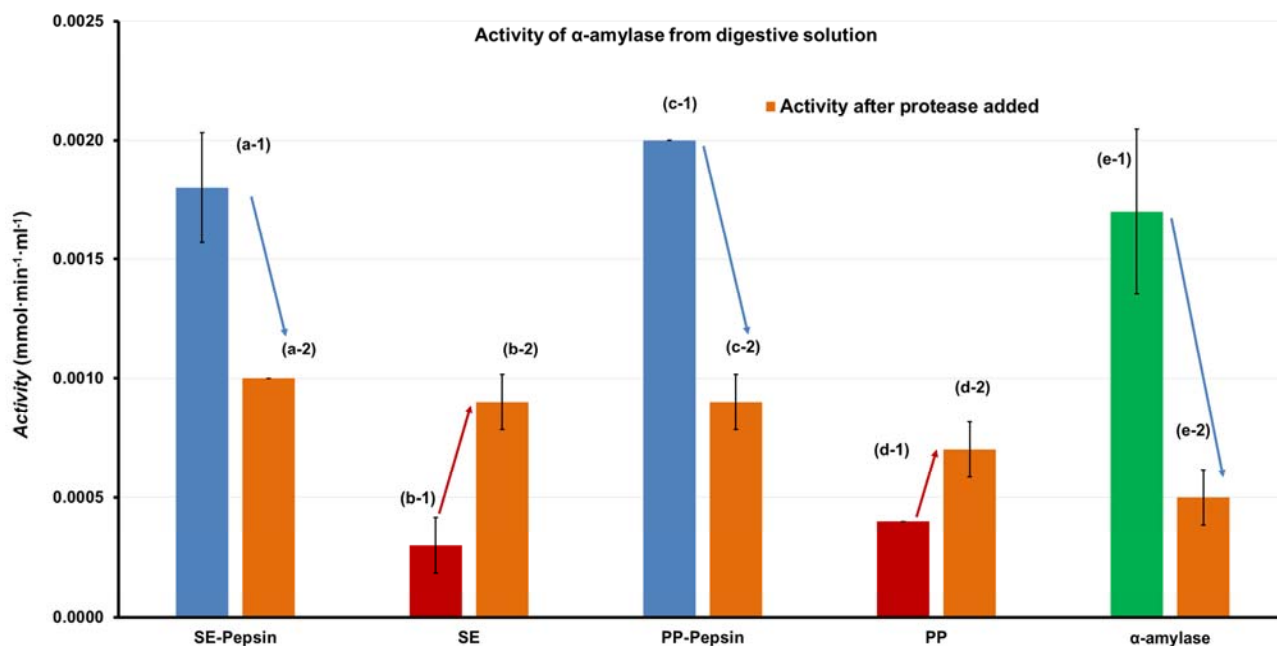
Fig.7. Activity of α-amylase in digestive solution of SE-Pepsin (a-1), SE (b-1), PP-Pepsin (c-1), PP (d-1) and blank containing only α-amylase (e-1) ; after adding extra protease, activity of α-amylase in digestive solution measured again for SE-Pepsin (a-2), SE (b-2), PP-Pepsin (c-2), PP (d-2) and blank (e-2).