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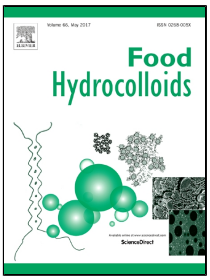

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- Addition of pectin slows starch digestion rate.
- Amylose to amylopectin ratio remains unchanged during digestion with pectin.
- Longer amylopectin chains digested slower with addition of pectin.
- Interaction between amyloglucosidase and pectin causes digestion rate reduction

Effects of pectin on molecular structural changes in starch during digestion

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Abstract

Starch digestion rate is strongly related to metabolic diseases such as obesity and diabetes. Starchy foods always contain non-starch components, which can affect starch digestibility. Mixtures of ungelatinized corn starch with a common non-starch component, pectin, were used to investigate pectin's effect on starch digestibility rate and evolution of starch molecular structure during digestion using amyloglucosidase and pancreatin. The whole-molecule size distribution and the chain-length distribution of chains were measured by size-exclusion chromatography and fluorophore-assisted carbohydrate electrophoresis. Digestion profiles and changes in molecular size distributions of whole and debranched digesta during digestion show that addition of pectin significantly decreased starch digestion rates. While pectin did not change the amylose/amylopectin ratio during most of the digestion, it decreased the digestion rate of short amylopectin chains compared to long ones. UV-visible spectral data suggested that a major contributor to this digestion rate change is from substantial pectin/amyloglucosidase interaction. This suggests an approach to designing nutritionally more beneficial starch-based foods by taking account of interactions between pectin and digestive enzymes.

Keywords: starch; pectin; starch digestibility; molecular structure; GPC

Chemical compounds studied in this article:

Amylose (PubChem CID: 53477771); Amylopectin (PubChem CID: 439207); Pectin (PubChem CID: 6857565).

Abbreviations

AM: amylose; AMG: amyloglucosidase; AP: amylopectin; AUC, area under the curve; CLD, chain length distribution; DMSO, dimethyl sulfoxide; DP, degree of polymerization; SEC, size-exclusion chromatography; FACE, fluorophore-assisted carbohydrate electrophoresis; RID, refractive index detector.

1. Introduction

Starch provides ~ 50% of the average human energy intake in developed countries and the percentage is even higher in many developing countries (Hoang et al., 2008). It is a branched homopolymer made up of glucose units extended with (1⟶4)-α linear glycosidic linkages and branched with (1⟶6)-α glycosidic linkages, comprising two main molecules: amylose (AM) and amylopectin (AP). AM is largely linear with a few long-chain branches and molecular weight ~ $10^{5–6}$, and AP is hyperbranched with numerous short chains and molecular weight ~ $10^{7–8}$. The structural features of AM and AP (including amylose fraction) in starch affect its cooking, eating, nutritional and other physiochemical properties.

Rapid digestion of starch by humans can cause a sharp increase of plasma glucose and results in very little starch reaching the lower bowel, which may lead to increased risk of obesity, type 2 diabetes and colorectal cancers (Dona, Pages, Gilbert, & Kuchel, 2010). The digestibility of starch can be affected by starch structure, including its molecular and granular structure, crystal type, and granule size (Zhang, Venkatachalam, & Hamaker, 2006), and by other types of causes such as food structure, texture, viscosity and interactions with other components (Singh, Dartois, & Kaur, 2010) in a food matrix.

Components such as non-starch polysaccharides are always present in starch-containing foods, and also can be added to starch-containing products during food processing to improve

the texture, water mobility, stability and viscosity. It has been suggested that additives which strongly increase the viscosity of starchy foods would decrease the hydrolysis rate of amylase, contributing to nutritional benefits (Brennan, 2005; Dhital, Warren, Butterworth, Ellis, & Gidley, 2015). One such additive is pectin, a cell-wall material mainly composed of galacturonic acid (Yapo, 2011) and commonly used as gelling agent and stabilizer (Willats, Knox, & Mikkelsen, 2006). Previous studies (Sasaki & Kohyama, 2012; Sasaki, Sotome, & Okadome, 2015) have reported that the addition of pectin increased the viscosity of starch suspensions and decreased the starch *in vitro* digestibility. They also suggested the pectin's suppressive effect on starch digestibility is related to a number of factors and is not only due to the rheological properties. It is noted that what matters for diffusion control of a reaction is the relative rate of diffusion of the reacting entities, which may or may not be related to the bulk viscosity. While a pectin solution has a high *bulk* viscosity, the enzymes involved are relatively small (~ 10 nm); at all except very high pectin concentration, the average space between pectin molecules is much larger than 10 nm: that is, the *local* viscosity for the enzyme is close to that of pure solvent. The hypothesis of the present paper is that a major effect in this slowed digestion rate will be from some sort of interaction between starch and pectin in the food matrix.

There have as yet been no studies of starch molecular structural changes during digestion in the presence of pectin. Since such studies have yielded useful mechanistic information for starch without additives, e.g. (Zhang, Sofyan, & Hamaker, 2008), it is useful to do the same to understand any effects of pectin addition. This is implemented here using size-exclusion chromatography (SEC, also known as GPC and HPLC-SEC) and fluorophore-assisted carbohydrate electrophoresis (FACE) to characterize both the molecular size distributions of whole starch molecules, using SEC, and the chain-length distributions (CLDs), using both SEC for the amylose chains and FACE (which gives a very accurate CLD, but currently is

confined to chains shorter than ~ 180 glucose units (Wu, Li, & Gilbert, 2014)) for the amylopectin chains.

In the present work, a suspension of ungelatinized corn starch is used as substrate, and *in vitro* digestion uses pancreatin and amyloglucosidase. Interactions between pectin and amyloglucosidase are investigated by UV/visible absorption spectroscopy. Only one starch and one pectin concentration are considered hre. However, the possible mechanism whereby pectin affects the digestion rate would most likely be the same for different starch and pectin concentrations. Sasaki and co-workers (Sasaki & Kohyama, 2012) showed that the same concentration of pectin as used here significantly affected the digestion rate of starch, but did not examine specific reasons for this effect. The present paper is based on this previous work and elucidates the underlying mechanisms by measuring molecular structural evolution.

2. Materials and methods

2.1 Materials

Corn starch (S4126), pectin from citrus peel (P9135) and porcine pancreatic pancreatin (P1750) were purchased from Sigma-Aldrich Co., US. Amyloglucosidase, isoamylase from *Pseudomonas sp.* and a glucose content assay kit were from Megazyme International Ireland Ltd., Ireland. Pullulan standards for SEC analysis were from Polymer Standards Service (PSS) GmbH, Germany, and cover the molecular weight range 342 to 2.35×10^6. GR grade dimethyl sulfoxide (DMSO) was from Merck Co. Other chemicals were reagent grade.

2.2 In vitro digestibility and fitting to first-order kinetics

The preparation of starch suspension and starch suspension mixed with pectin were carried out following a method modified from the literature (Sasaki & Kohyama, 2012). Pectin (125 mg on dry basis per group) was dispersed with 7.5 mL of distilled water in six 50 mL screw-capped tubes and stirred at 500 rpm for 35 min using a magnetic stirrer. Six tubes without

pectin were also prepared. After complete dispersion (judged by a clear appearance), the pectin solution was heated at 50°C for another 30 min with continuous stirring. Then 200 mg of corn starch was added to each tube and thoroughly dispersed by vortex mixing, and 2 mL of 0.125 M HCl solution was then added. The starch suspensions were incubated at 37°C with vigorous magnetic stirring for 30 min. The pH of the starch suspensions was adjusted to 5.2 by adding ~ 0.5 mL of 2.5 M sodium acetate solution. To prepare the enzyme solution for digestion, 20 mg of pancreatin (4 × USP specifications) and 1 mL of amyloglucosidase (3260 U/mL) were mixed and thoroughly dispersed in 50 mL of water. After centrifuging at 2000 *g* for 10 min, the supernatant of the enzyme solution was heated at 37°C in a water bath before use. The digestion of starch suspensions was then performed by adding 2.5 mL of enzyme solution in each tube with magnetic stirring. After 0, 2, 4, 6, 8, 24 and 48 h of incubation, absolute ethanol was added to each tube to stop the hydrolysis of starch. The glucose concentration in the supernatant was determined by using the Megazyme glucose assay content kit after centrifugation at 1500 *g* for 10 min.

Digestibility curves were then fitted to a first-order equation, which in integrated form is:

$$C_t = C_f(1 - e^{-kt}) \quad (1)$$

C_t is the percentage of starch digested at time t (min), C_f (where the subscript f is for "final") is the estimated percentage of starch digested by the end of reaction time and k (min^{-1}) is the starch digestion rate coefficient.

In practice, C_f and k are measured using a logarithm-of-slope (LOS) analysis described in detail elsewhere (Butterworth, Warren, Grassby, Patel, & Ellis, 2012) through a transformed equation:

$$\ln \frac{dC_t}{dt} = \ln (C_f k) - kt \quad (2)$$

The derivative dC_t / dt is obtained using second-order finite difference. From eq 2, a plot of the logarithm of this derivative against time yields C_f and k, if this plot is linear.

2.3. Size-exclusion chromatography

The whole-molecule SEC used an Agilent 1260 Infinity SEC system (Agilent, Santa Clara, CA, USA) with a refractive index detector (RID, Optilab UT-rEX, Wyatt, Santa Barbara, CA, USA) following a published method (Cave, Seabrook, Gidley, & Gilbert, 2009; Vilaplana & Gilbert, 2010). Starch samples were dissolved in dimethyl sulfoxide (DMSO) with 0.5% (w/w) LiBr at a concentration of 2 mg/mL. DMSO/LiBr is used as a solvent combination both to dissolve starch completely and to minimize degradation (Schmitz, Dona, Castignolles, Gilbert, & Gaborieau, 2009), and it is also used as the mobile phase for SEC analysis after being filtered through a 0.45 µm hydrophilic Teflon membrane filter. Starch samples were injected into a series of PSS separation columns (Polymer Standards Service, Mainz, Germany): SUPREMA pre-column, Gram 30 and Gram 3000. The injection volume was 100 µL, the flow rate 0.3 mL/min, and the column oven temperature 80°C. A series of pullulan standards with different peak molecular weights ranging from 342 to 2.35×10^6 was used to convert elution volume to molecular size (the SEC hydrodynamic volume, V_h, or the equivalent hydrodynamic radius, R_h, which is the separation parameter for SEC) using the Mark-Houwink equation. The Mark-Houwink parameters K and α of pullulan dissolved in DMSO-LiBr solution at 80°C are 2.424×10^{-4} dL g^{-1} and 0.68 (Cave et al., 2009).

To characterize the CLDs, the starch samples were firstly debranched using isoamylase in a 0.1 M acetate buffer solution (pH 3.5) and freeze-dried overnight as described elsewhere (Hasjim, Cesbron-Lavau, Gidley, & Gilbert, 2010; Tran et al., 2011). The starch samples after freeze drying were dissolved in DMSO/LiBr solution for 24 h in a thermomixer at 80°C with shaking at 350 rpm. The supernatant of samples were transferred to SEC vials for analysis after centrifugation at 4000 *g* for 10 min. The debranched-starch SEC distribution was measured using an Agilent 1100 SEC system with a refractive index detector (Shimadzu RID-

10A, Shimadzu Corp., Kyoto, Japan) and a series of PSS columns (GRAM precolumn, GRAM 30, and 1000 analytical columns) at a flow rate of 0.6 mL/min. As linear molecules, the V_h of debranched starch can be converted to X, the degree of polymerization (DP), by the Mark-Houwink relation. The SEC weight distribution of linear glucan molecules, $w(\log X)$, can also be converted to the number distribution, $N_{de}(X)$, which is the relative number of chains of DP X, using $N_{de}(X) = X^{-2}\, w(\log X)$ (Clay & Gilbert, 1995; Shortt, 1993). The Mark-Houwink parameters K and α for linear starch dissolved in DMSO-LiBr solution at 80°C are 1.5×10^{-4} dL g^{-1} and 0.743, respectively (Liu, Castro, & Gilbert, 2011).

2.4. Fluorophore-assisted carbohydrate electrophoresis

We use two complementary techniques for measuring the CLD. FACE provides very accurate CLD data for the amylopectin chains, which are relatively short. Longer chains, i.e. those of amylose, are characterized by SEC (which is not nearly as accurate as FACE for the amylopectin chains because of artifacts such as band broadening and inaccuracies in relating SEC elution volume to degree of polymerization for these short chains). The preparation of debranched samples for FACE was the same as for SEC analysis. The debranched starch after freeze drying was labelled with 8-aminopyrene-1, 3, 6, trisulfonic acid (APTS), following a procedure given elsewhere (Wu et al., 2014). The labelled starch was separated in an N-CHO-coated capillary using a voltage of 30 kV at 25°C with a carbohydrate separation buffer (Beckman-Coulter). A PA-800 Plus FACE System (Beckman-Coulter, Brea, CA, USA) equipped with a solid-state laser-induced fluorescence detector and an argon-ion laser, was used to obtain $N_{de}(X)$ as the peak area from the elugram. CLDs from FACE analysis are presented as $\log_{10}$ of peak area (i.e. $N_{de}(X)$ on a logarithmic axis) as function of X.

2.5 UV/visible absorption spectra of amyloglucosidase pectin solution

Interactions between macromolecules such as polysaccharides and proteins in solution are common (Tolstoguzov, 2003). The intermolecular association between biopolymers will affect

their molecular structures and properties. UV/visible spectroscopy is an effective method to study the interactions between polysaccharides and proteins (Kholiya, Chaudhary, Vadodariya, & Meena, 2016; Wu et al., 2017). The UV/visible absorption spectra of a range of mixed pectin and amyloglucosidase solutions in water were measured with a UV-6100s UV-Vis spectrophotometer (MAPADA instruments, Shanghai, China). The compositions of the control (A) and experimental groups (B, C, D, E and F) of amyloglucosidase/pectin solutions were shown in Table 1. In group A, the concentration of AMG, pH value and the ionic strength are the same as used in the *in vitro* digestion experiment. Group B had a higher concentration of AMG. No acetate buffer was used in group C and no AMG was added in group D. In groups E and F, the AMG was absent.

The pectin/AMG solutions were also prepared and incubated in the same procedure in the digestion part. The samples after incubation at 37°C for 0, 8, 24 and 48 h were centrifuged at 1000 rpm for 5 min to remove air bubbles, to which pectin-containing solutions are prone because of the significantly increased viscosity. The samples were then transferred to quartz cuvettes and UV-Vis spectra then measured.

2.6 Viscosity measurements

Samples viscosities were measured using a rotational viscometer (NDJ-5S, Changji Shanghai) at 37°C and 12 rpm with a No. 28 spindle. The suspensions with sodium acetate buffer were heated to 37°C before measurement.

2.7 Statistical analysis.

Analysis of variance (ANOVA) with the general linear model and Tukey's pairwise comparisons in Minitab 16 (Minitab Inc., State College, PA, USA) were used for statistical analysis. Significant differences of the mean values were determined at $p < 0.05$.

3. Results and discussion

3.1 Starch digestion profiles

Figure 1A shows the change in starch digestion ratio of the pure starch solution and the mixed solution of starch and pectin with digestion time; these plots are linear within experimental uncertainty. Although the value of C_f estimated from the digestibility curves can be unreliable, the rate coefficients (k values) encompass all the kinetic behaviour and reveal whether changes occur in digestion rate from rapid to slow as digestion proceeds (Butterworth et al., 2012). In Figure 1A, the pure starch and starch/pectin mixtures had similar digestion trends with a rapid digestion in the first 10 h digestion followed by a much slower digestion rate in the later digestion time, which follows first-order loss kinetics over the whole time course (Figure 1B). The digestion rates of the pure starch solutions were always higher than those of starch/pectin mixture, with first-order rate coefficients of 0.0016 and 0.0012 min^{-1} respectively, a reduction of 25%.

As mentioned above, the suppressive effect of pectin on digestion of both ungelatinized high-amylose corn starch and gelatinized potato starch has been reported in the literature (Sasaki & Kohyama, 2012; Sasaki et al., 2015). Sasaki and co-workers investigated the effects of several polysaccharides, including pectin, guar gum, konjac glucomannan and xanthan gum, on the starch's *in vitro* digestibility, and they found that the starch solutions with highest viscosities were not those with the slowest digestion rate, and that pectin with relatively low viscosity showed a moderate effect on starch hydrolysis (Sasaki & Kohyama, 2012). Further, pectin's suppressive effect on gelatinized potato starch's digestion was weakly dependent on its concentration, unlike other non-starch polysaccharides (Sasaki et al., 2015). However, pectin's effect on the digestibility could not only have arisen from higher viscosities and the resulting slowed diffusion. In the 2015 paper, pectin was found to significantly reduce starch

236 hydrolysis and the glucose level at 60 min after ingestion. This result may arise from an
237 interaction between pectin and amylase.

238 From the LOS plots shown in Figure 1B, the curves of the pure starch solutions and the starch
239 solutions in presence of the pectin were similar, although the actual slope was different. LOS
240 plots of processed starch products can have two or more linear regions, while only one has
241 been seen for natural starch (Zou, Sissons, Gidley, Gilbert, & Warren, 2015). Furthermore, it
242 has been shown that starch digestion enzymes (α-amylase, amyloglucosidase and pepsin)
243 could not hydrolyse pectin (CarvalhoMunarin, Tanzi, & Petrini, 2012). Thus the decrease of
244 starch digestion rate in the presence of pectin must be due to physical rather than chemical
245 factors. Two possible explanations for this are: 1) enzymes bound with pectin, hindering the
246 access of the enzymes to the inner starch granules; 2) the slower diffusion rate of the enzymes
247 due to the increased viscosity of the starch suspensions in the presence of pectin.

248 The viscosity of the starch/pectin sample was much higher (5100 mPa•s) than that of pure
249 starch (10 mPa•s). (Parenthetically, this increased viscosity of pectin suspension created some
250 experimental difficulties: for instance, it was difficult to fully disperse starch powder in pectin
251 solution, and vigorous agitation was required; the increased viscosity also made it hard to
252 accurately transfer a given volume, and thus such transfer was always done by weight, not
253 volume.) The disproportionate decrease in digestion rate with the increase in viscosity
254 indicated that any pectin-related viscosity effect was not likely to be the limiting factor in
255 starch digestion. As mentioned above, Sasaki & Kohyama (2012) reported that the starch
256 digestion rate was not changed with increasing viscosity above a critical value. It is
257 reasonable to suppose that hydrogen bonding and/or other interactions between the enzymes
258 and substrate is a major determinant of the decreased starch digestion rate.

259 The amount of enzymes used in this experiment was much less than that in some previous
260 digestion studies (Syahariza, Sar, Tizzotti, Hasjim, & Gilbert, 2013; Witt, Gidley, & Gilbert,

2010), leading to a slower starch digestion rate. This was done for easier correlation with starch molecular structure throughout the whole digestion time, especially at the rapid digestion stage.

3.2 SEC weight distributions and CLDs of starch in digesta

The time evolution of the debranched SEC weight distributions, $w(\log R_h)$, of samples with and without pectin, normalized to the starch content in the digesta, is given in Figure 2 in two different ways. Figure 2A and B show the data as line plots; C and D are three-dimensional plots of the same data for better presentation to show the progression of the digestion in time. As normally seen for native starch, two peaks were observed, arising from amylopectin (small R_h) and amylose (large R_h) respectively, with the separation point at $R_h \sim 5$ nm (corresponding to $X \sim 100$). This point (obtained from the RI signal of the debranched weight distribution data) was then used to define the region of amylose in the SEC distribution plot. The amylose fraction shown in Table 2 was calculated using a method described previously (Vilaplana, Hasjim, & Gilbert, 2012) as the ratio of amylose region's SEC debranched distribution area under the curve (AUC) to the entire SEC distribution plot's AUC (note that it was shown in this reference that the fully branched SEC distribution, Fig. 4, cannot be used for this purpose). In the starch/pectin group, the highest peak remained at $R_h \sim 1$ nm throughout the digestion, while a new peak around $R_h = 4$ nm gradually appeared during the digestion. The process is more clearly observed in Figure 2 D. In the pure starch group, the peak at $R_h \sim 1$ nm was the highest in the first 24 h. After 48 h digestion, the highest peak changed to $R_h \sim 4$ nm. The observation is consistent with a previous study (Witt et al., 2010), where it was found that a small starch species was formed during digestion. In Witt's paper, the R_h of the digestion-generated starch species was ~ 2 nm; the slightly different R_h seen here may be because of the different amylolysis procedure. The proportion of this digestion-generated small starch species in the pure starch group is higher than that in the starch/pectin

286 group, resulting in a higher proportion of amylose (~ 37%) in the starch group after 48 h
287 digestion, as shown in Table 2.

288 Debranched size distributions of digesta (Figure 2 and Table 2) show that the amylose
289 fraction and the debranched CLDs are similar for samples with and without pectin over the
290 first 24 h digestion. Thus whatever the nature of the interaction between starch and pectin, it
291 clearly had no significant effect on the great majority of individual chains. This is not
292 surprising, because of the low concentration of pectin relative to starch: an individual chain is
293 unlikely to have significant interaction with a portion of a pectin molecule. However, the
294 digesta of pure starch after 48 h of digestion contained higher proportions of AM than that of
295 starch with pectin (Figure 2); this late-digested material is sometimes termed "resistant
296 starch".

297 The FACE data, Figure 3, shows the features previously reported in the literature (Wu, Witt,
298 & Gilbert, 2013). Note that the normalization of such distributions is arbitrary; because the
299 amount of starch present in samples taken at later times is very small, we show the features in
300 these CLD data by normalizing all to have the same maximum value rather than to the amount
301 of starch. The main features are a first peak (DP ~6 – 35) corresponding to chains confined to
302 a single lamella, and a second peak (DP ~36 – 100) corresponding to chains spanning more
303 than one lamella (Wu, Morell, & Gilbert, 2013). It is explained in this reference that the
304 parameters obtained from this model fitting replace the more common method of defining
305 fractions from chains in arbitrarily defined regions. Chains longer than DP 100 are mostly
306 considered as amylose, although there also might be some extra-long amylopectin chains
307 (Hanashiro et al., 2008). During the digestion, the most interesting phenomenon is that the
308 peaks of longer amylopectin chains (DP > 60) in the mixture group are always lower than
309 those of the pure starch group. This result indicates that the presence of pectin has an effect on
310 the digestion of long amylopectin chains more than the shortest ones. In addition, the longest
311 chains in starch group with DP > 100 in the first 24 h were hydrolysed to about DP 70 at 48 h.

In comparison, with added pectin, the longest chains (DP > 100) were hydrolysed to DP 90 at 24 h and finally DP 60 at 48 h. CLDs after 48 h of digestion were significantly different from those of other time points, as is also seen in whole size distribution and debranched size distribution (Figures 2 and 4). For pure starch, the plots of longer AP chains to shorter chains was higher than that of the starch/pectin samples throughout the entire time range examined, showing the relative digestion rate of shorter chains, compared with longer chains, was reduced with the addition of pectin.

Figure 4 shows the time evolution of the SEC weight distribution of the whole molecules as functions of the hydrodynamic radius. Both groups show the usual bimodal features. The molecules with R_h up to 75 nm, with peak at R_h ~15 nm, are amylose, and the molecules with R_h larger than 75 nm, with peak at R_h ~200 nm, are amylopectin. There are no qualitative differences in the behaviour of starch compared to that of starch plus pectin. This behaviour is typical to that seen and discussed in detail previously (Witt et al., 2010).

The CLDs (Figures 2 and 3) show that a larger amount of longer AP chains were digested in the starch/pectin system than in pure starch, suggesting that the hydrolysis of amylopectin by amylase has chain length selectivity after adding pectin. The digestion of native starch undergoes an “inside-out” or “side-by-side” pattern (Zhang, Ao, & Hamaker, 2006), and it was reported that the molecular structure of amylopectin showed only minor changes during the digestion of native starch because of a relatively non-specific attack of digestive enzymes on amylopectin microstructure (Shrestha et al., 2012; Zhang et al., 2006). However, the observed preference of the hydrolysis of longer AP chains in this study suggests that the addition of pectin altered the hydrolysis pattern of the enzyme with starch. The effect might be due to chain-length-dependent interactions involving the enzyme and pectin.

3.3 UV/visible spectra of pectin/AMG solutions

The structure and properties of proteins can be affected by polysaccharides via various interactions (Turgeon, Beaulieu, Schmitt, & Sanchez, 2003; Turgeon, Schmitt, & Sanchez, 2007). For example, in a previous study (Dhital et al., 2015), cellulose was found to inhibit α-amylase's *in vitro* digestion activity, and the interaction was reversible and non-specific. The presence of interactions between pectin and AMG is checked here by UV/visible spectroscopy. UV/visible spectra of the six kinds of pectin/AMG solutions are presented in Figure 5. In Groups A, B and C, the absorbance intensity increased during the incubation. In Groups D, E and F, the absorbance was unchanged. The absorbance peak at ~ 100 nm is due to water and the peak at ~ 280 nm is attributed to phenyl groups in the enzymes.

Group A is the control group. The absorbance of Group A increased over the incubation period, indicating pectin and AMG interacted with each other in the buffer. Group B had higher concentration of AMG compared to Group A. The absorbance and the increment of absorbance of samples in group B were all higher than in group A, suggesting that the degree of interaction is in direct proportion to the AMG concentration. In group B, the absorbance peaks of protein (AMG) at 280 nm were the most obvious. The absorbance intensity of AMG rose during the incubation, but the symmetrical shape of the peak gradually disappeared. This is probably caused by the substantial increase of background absorbance. The maximum absorbance wavelength of AMG peak also decreased from 278 nm (0 h) to 272 nm (48 h). Group C had no acetate buffer, and the increase absorbance was less obvious than in groups A and B. This showed the extent of interaction between pectin and AMG decreased in the absence of sodium acetate, indicating the interaction is ionic-strength and/or pH dependent. The spectra of group D, E and F remained stable during the incubation, demonstrating the increase of absorbance in the first three groups came from the interaction between pectin and AMG but not self-aggregation of AMG or other possibilities.

The changes of the UV/visible absorption spectrum reflect interactions between AMG and pectin. The interactions leading to complex formation between polysaccharides and proteins can be classified into two kinds: specific interaction such as covalent linkages, and non-specific interactions such as electrostatic interactions, hydrophobic interactions, van de Waals forces and hydrogen bonding (Girard, Turgeon, & Gauthier, 2002). The non-specific interaction between AMG and pectin may be electrostatic interaction and/or hydrogen bonding. The interactions between pectin and AMG provides an explanation for at least some of the observed change in digestion kinetics and the decreased digestion rate of short amylopectin chains compared to long ones. The contribution from a viscosity effect is also a possibility, but previous studies have demonstrated that it could not be the only reason (Sasaki & Kohyama, 2012; Sasaki et al., 2015). The overall reduction in the digestion rate and fewer short AP chains in starch/pectin mixture are thus ascribed to the association between AMG and pectin, while viscosity effects may also play a role.

4. Conclusions

Addition of pectin to starch slows *in vitro* enzymatic starch digestion; first-order kinetics are followed both with and without pectin, with the rate coefficient for starch/pectin being much less than that for starch alone. The amylose to amylopectin ratio basically remains the same with and without pectin during most of the digestion but at later times with pectin, the digestion rate of longer amylopectin chains was slower than that of shorter amylopectin chains. An association between the digestion enzyme amyloglucosidase and pectin was seen in UV/visible spectra, ascribed to electrostatic complexation and and/or hydrogen bonding. The slowed digestion rate in starch/pectin might have a contribution from slowed diffusion because of the much greater bulk viscosity, but for reasons given above, this is unlikely to be the sole effect. Association between amyloglucosidase and pectin is highly likely to change

385 the conformation of the enzyme and/or hinder access of it to starch, and is seen as the
386 principal reason for the slowed rate. This is consistent with the slower rate of digestion of
387 longer amylopectin chains, because these may be harder to undergo interaction with the
388 binding site of a hindered or conformationally-altered enzyme. Complex formation between
389 proteins and polysaccharides, and how these might change interfacial enzymatic reactions or
390 selective delivery of protein peptides during food digestion, are of interest. One significance
391 of these findings is in methods of increasing the amount of resistant starch (which depends to
392 some extent on the presence of longer chains) in a food formulation through the presence of
393 pectin, which is a common food additive.

394

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398 and SEC analysis, and Wei Zou for discussions.

399

400 ## Figure captions

401 Figure 1. Digestion curves (A) and LOS plots (B) of pure starch (full line) and starch/pectin
402 mixture (dashed line). The finite-difference method used for the LOS plot does not calculate
403 the slopes at the initial and final points, and thus the range of the time axis for the LOS plots
404 are less than that of the digestion curve from which they are derived.

405 Figure 2. Two-dimensional (A and B) and three-dimensional (C and D) plots of debranched
406 digesta size distributions from pure starch solutions (A and C) and starch/pectin solutions (B
407 and D). Here and in subsequent figures, the same data are presented in two different ways to

help distinguish different aspects of the data: as multiple lines on the same plot (2D), and as surfaces at different times. All distributions have been normalized to starch content, which decreases as digestion proceeds.

Figure 3. Chain length distributions (CLDs) of starch in digesta from starch solutions with (full line) and without pectin (dashed line). Figure 2A to F represent the results of CLDs obtained from 0, 2, 4, 8, 24 and 48 h digestion time, respectively. All the distributions have been normalized to their highest peak.

Figure 4. Two-dimensional (A and B) and three-dimensional (C and D) plots of whole molecular size distributions of starch digesta from pure starch (A and C) and starch/pectin mixtures (B and D). All the distributions have been normalized to starch content.

Figure 5. The UV-Vis spectra of different solutions: (A) AMG (0.4 μL/mL)-pectin-buffer; (B) AMG (4 μL/mL)-pectin-buffer; (C) AMG-pectin-H_2O-9 μL 0.125 M HCl; (D) pectin-buffer; (E) AMG-buffer; (F) buffer.

Table captions

Table 1. The composition of samples for measurement of UV/visible spectra.

Table 2. The changes in amylose fraction (%) of pure starch and starch/pectin mixture during digestion.

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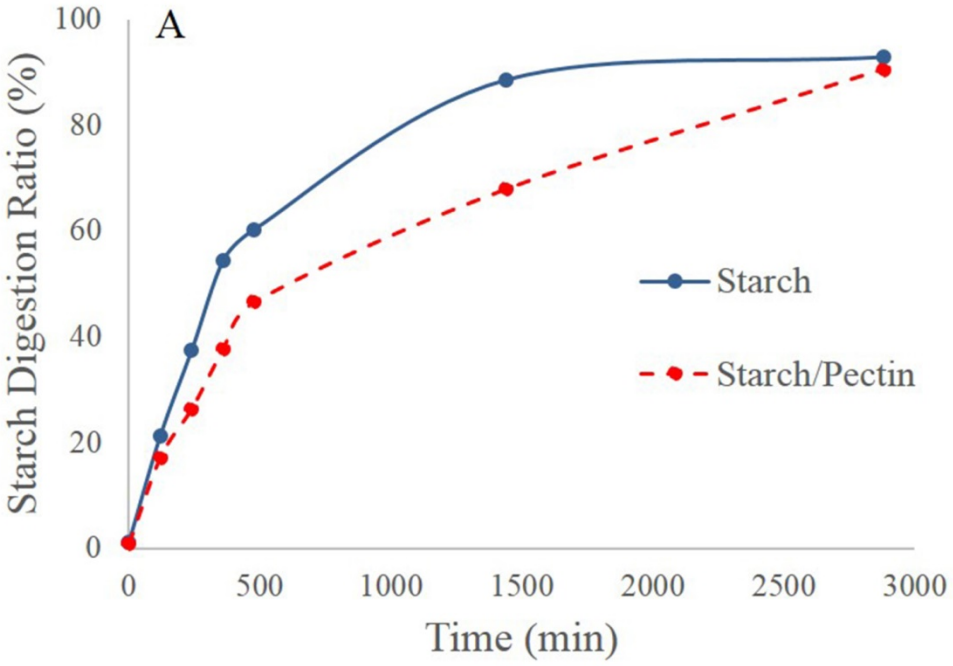
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40
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0
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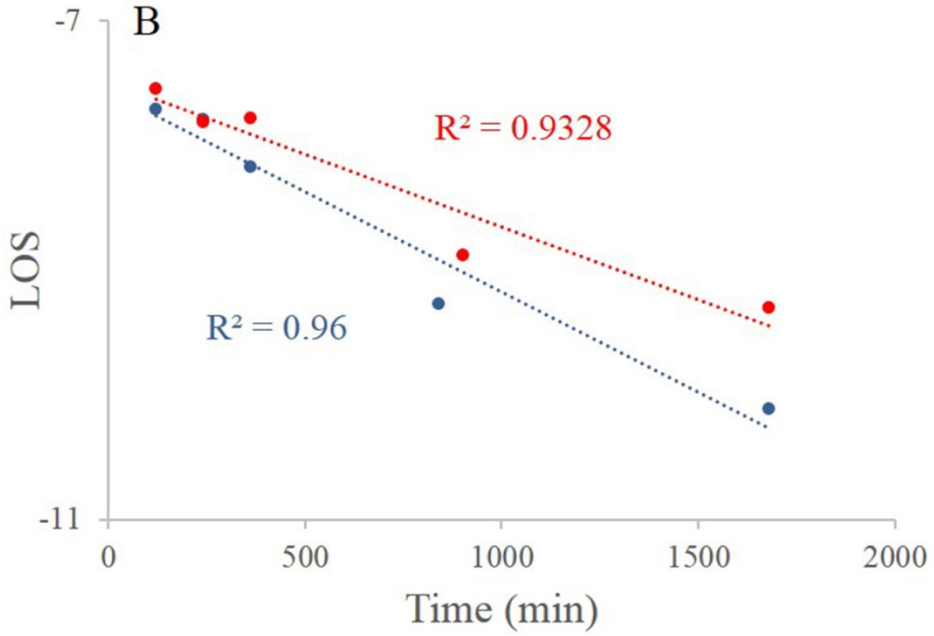
Time (min)
Starch
Starch/Pectin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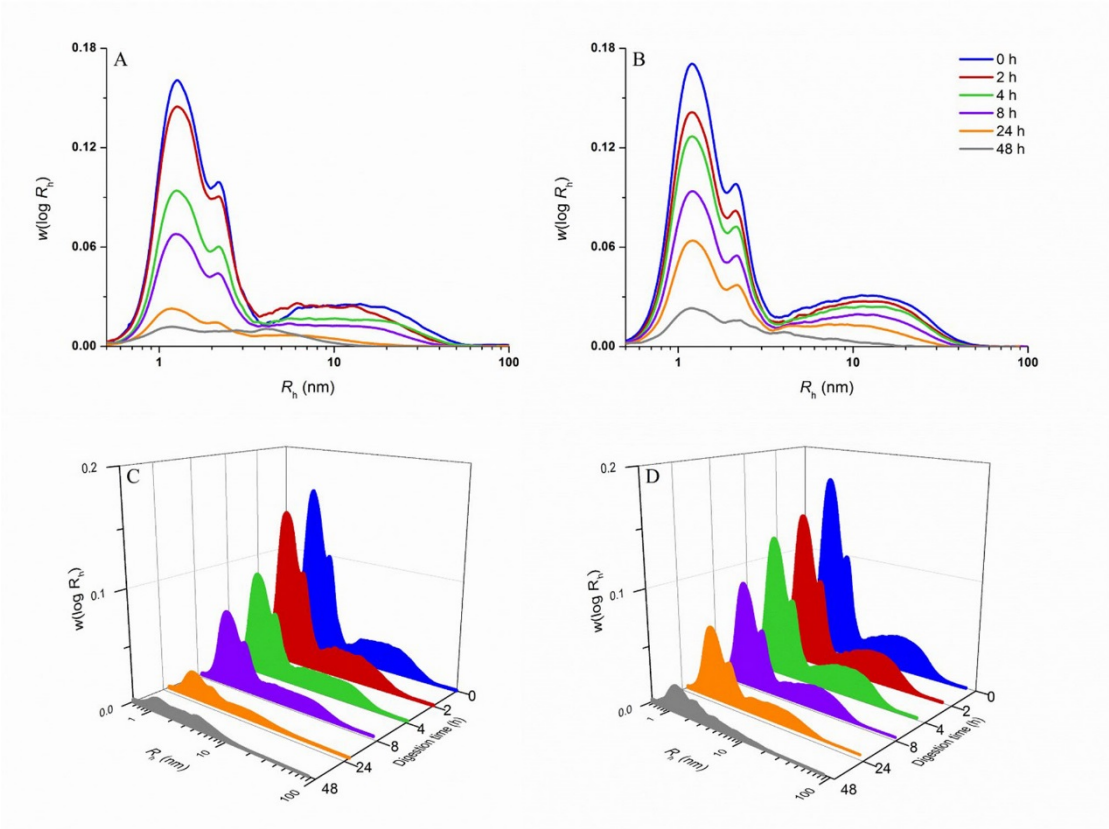

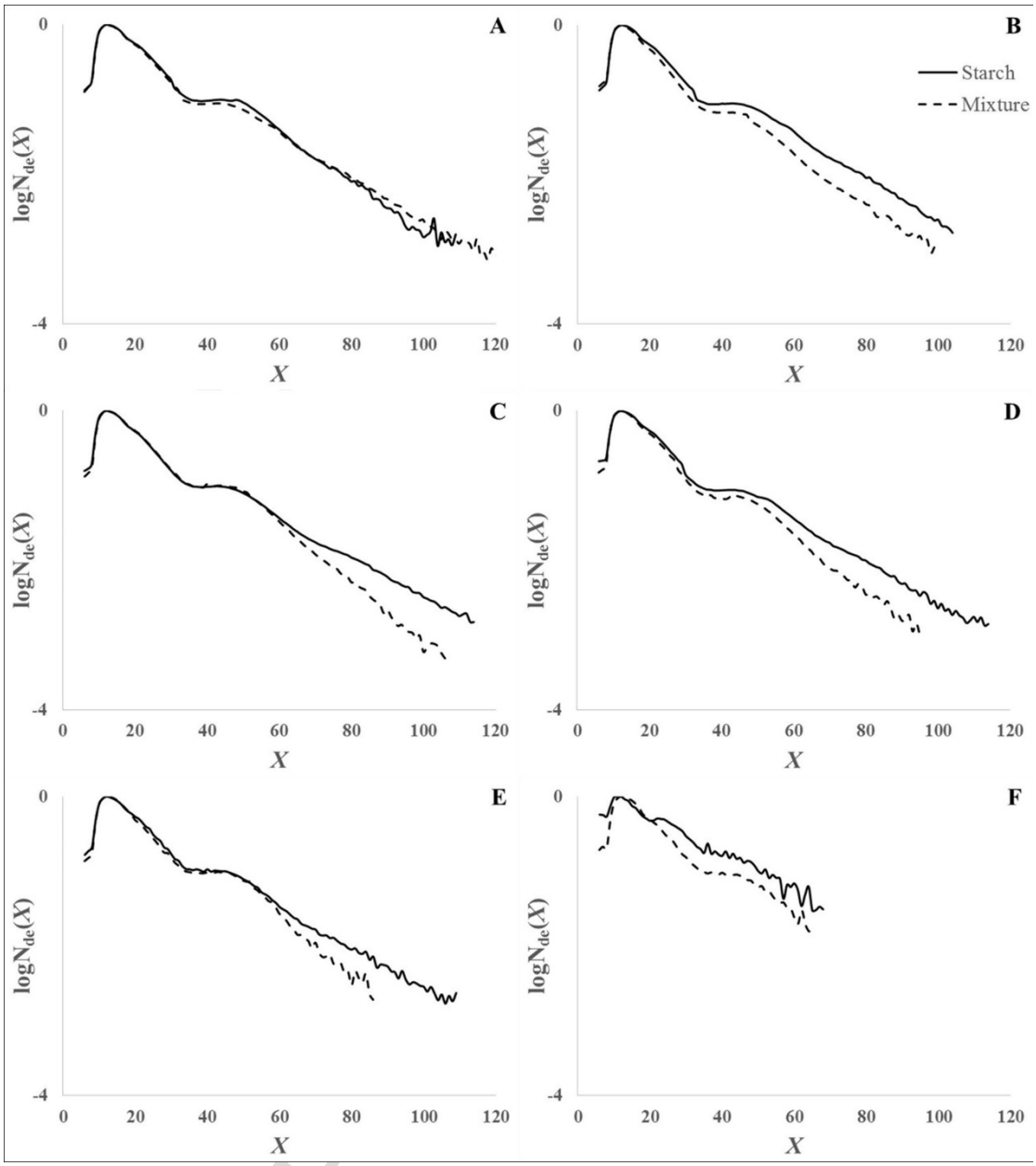

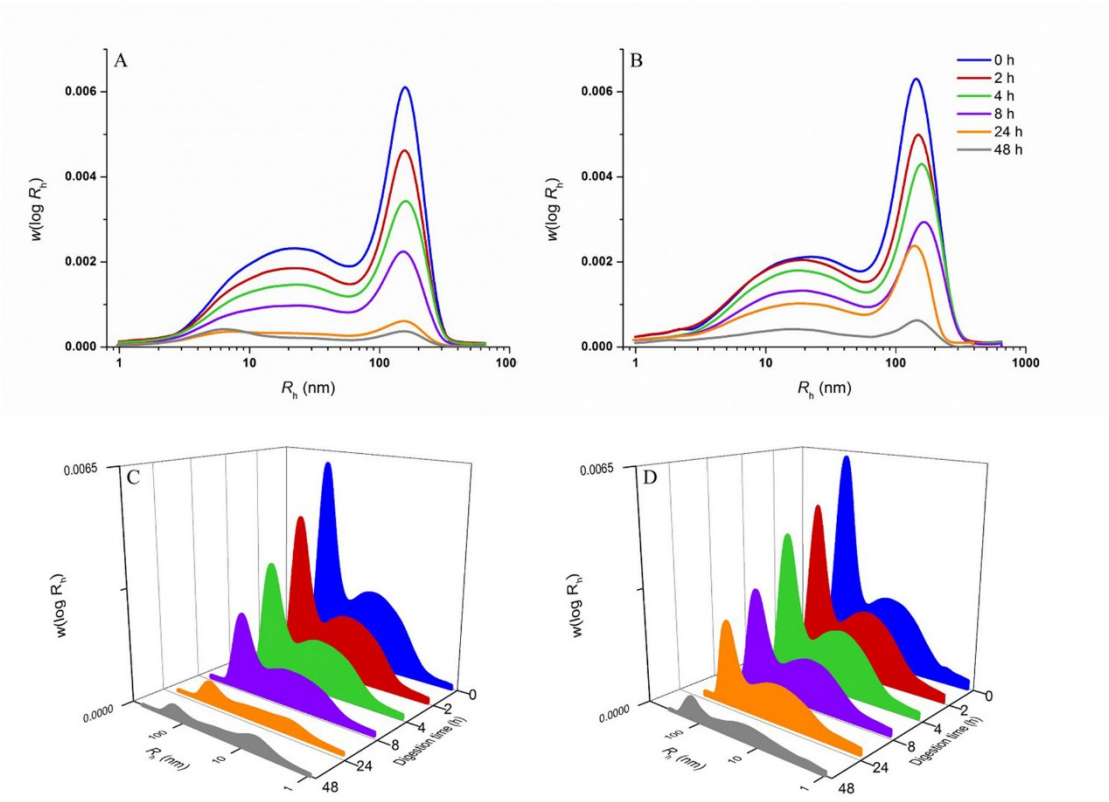

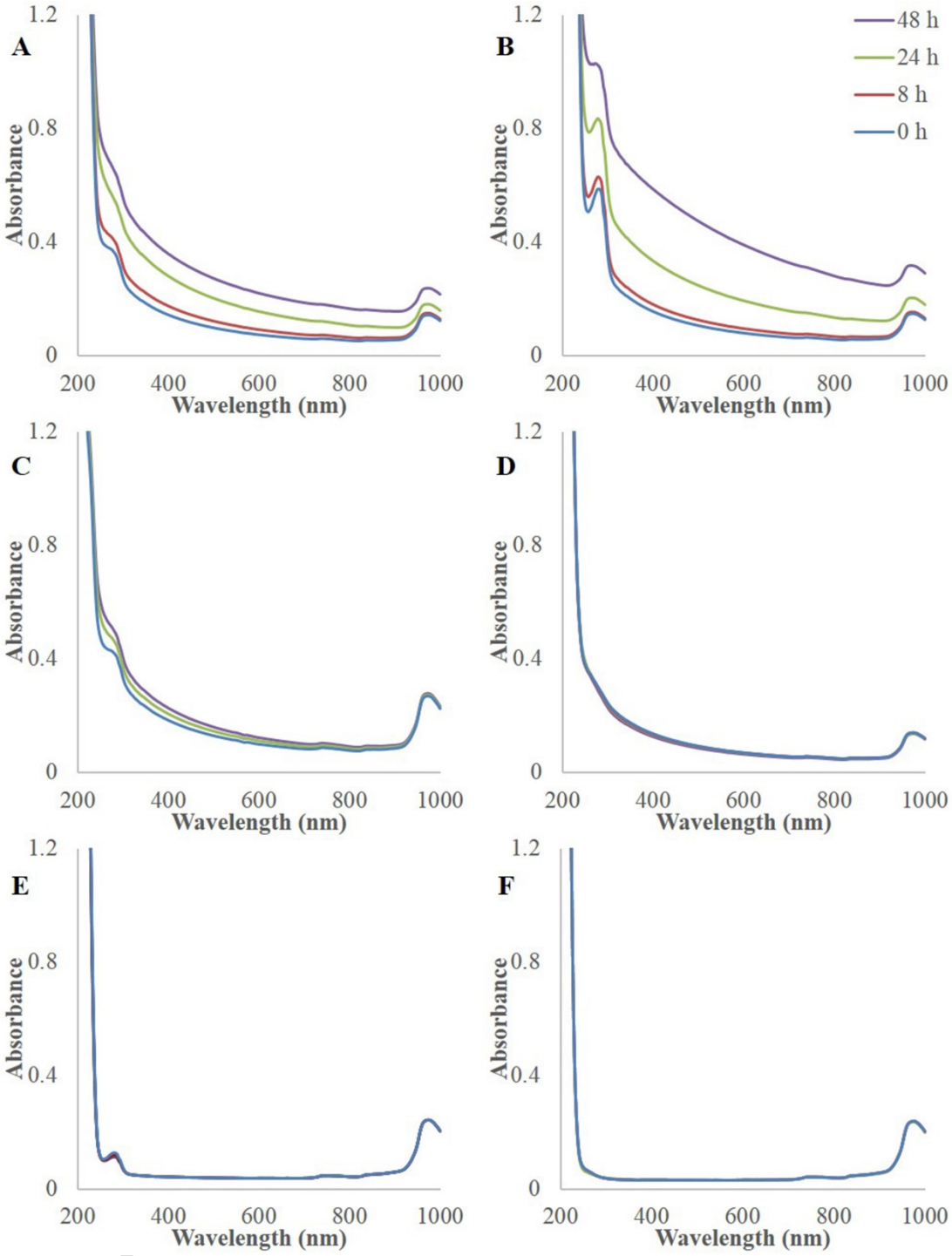
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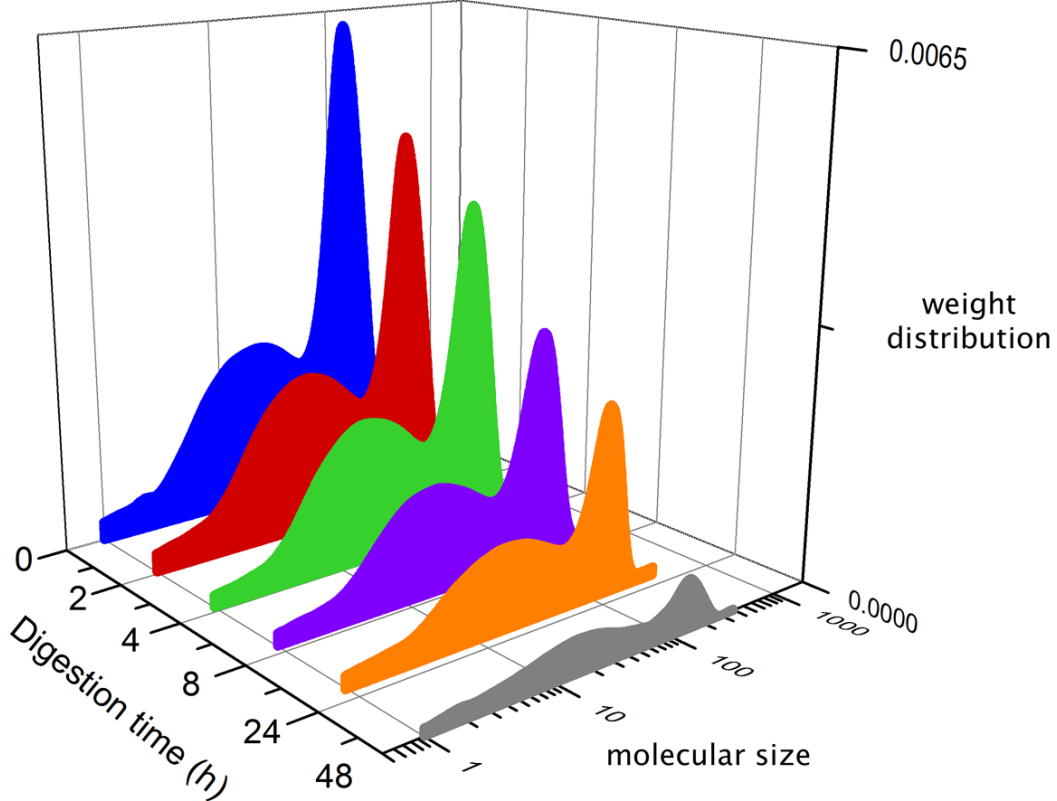
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2
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Digestion time (h)
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Table Error! Reference source not found.. The composition of samples for UV/visible spectra measurement.

Samples	Pectin (mg)	Concentration of AMG (μL/mL)	HCl-Sodium acetate buffer (mL)	Distilled water (mL)
Group A	5	0.4	2.5	7.5
Group B	5	4	2.5	7.5
Group C	5	0.4	0	10
Group D	5	0	2.5	7.5
Group E	0	0.4	2.5	7.5
Group F	0	0	2.5	7.5

Table Error! Reference source not found.. The changes in amylose fraction (%) of pure starch and starch/pectin mixture during digestion.

Sample	Amylose fraction (%)					
	0 h	2 h	4 h	8 h	24 h	48 h
Starch	25.2 ± 1.7	25.2 ± 0.9	27.4 ± 2	27.4 ± 0.5	28.2 ± 0.9	37.0 ± 2.7
Starch/pectin	26.0 ± 0.3	26.7 ± 2.7	27.6 ± 0.7	26.6 ± 2.6	28.4 ± 1.8	27.3 ± 7.8