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In vitro gastric digestion of polysaccharides in mixed dispersions: Evaluating the contribution of human salivary α-amylase on starch molecular breakdown

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ABSTRACT

The aim of this work was to investigate the impact of the addition of salivary α-amylase on starch hydrolysis in protein-containing dispersions during an *in vitro* digestion process. *In vitro* digestion provides useful insights on the fate of nutrients during gastro-intestinal transit in complex food matrices, an important aspect to consider when developing highly nutritious foods. Many foods contain polysaccharides, and as their disruption in the gastric stage is limited, salivary α-amylase is often neglected in *in vitro* studies. A reference study on the effect of salivary α-amylase using one of the most advanced and complex *in vitro* digestion models (INFOGEST) is, however, not available. Hence, this work reports the gastrointestinal breakdown of three mixed dispersions containing whey protein isolate with different polysaccharides: potato starch, pectin from citrus peel and maize starch. The latter was also studied after heating. No polysaccharide or salivary α-amylase-dependent effect on protein digestion was found, based on the free NH_2 and SDS-PAGE. However, in the heat-treated samples, the addition of salivary α-amylase showed a significantly higher starch hydrolysis compared to the sample without α-amylase, due to the gelatinization of the starch granules, which improved the accessibility of the starch molecules to the enzyme. This work demonstrated that the presence of different types of polysaccharides does not affect protein digestion, but also it emphasizes the importance of considering the influence of processing on food structure and its digestibility, even in the simplest model systems.

1. Introduction

Starch is an important source of dietary energy for the human body. Evaluation of its digestibility is key in designing novel foods for human consumption (Singh et al., 2010). Recent years have seen great interest in describing various foods during *in vitro* digestion using harmonized methods such as the standardized INFOGEST digestion model (Brodkorb et al., 2019). It is becoming increasingly clear how various factors can affect the digestive behavior of macronutrients. In this sense, ingredient's processing, composition and structure of the food matrix are important parameters to consider when assessing the digestive behavior of macronutrients (Gallego-Lobillo et al., 2021; Krause et al., 2022; Lucas-González et al., 2018; Pälchen et al., 2021). It is moreover important to stress that the design of the *in vitro* digestion conditions is also critical in obtaining results that are relevant to *in vivo* conditions.

The choice of enzymes and their concentration is a key step in the choice of the *in vitro* digestion method, as their role in the human digestive system consists in breaking down macromolecules. During digestion, macromolecules are hydrolyzed by enzymes in a well-coordinated process that breaks down complex molecules into simpler and absorbable components. Thus, preparing representative enzymatic solutions is essential to ensure maximum *in vivo* relevancy (Guerra et al., 2012). However, in spite of the attempts to harmonize *in vitro* digestion methods to improve inter-laboratory comparisons, and to be able to screen and reference the digestibility of various foods, challenges remain. Examples exist in the literature of methodological differences between *in vitro* digestion studies. One of these differences is in regard to the inclusion or exclusion of different digestive enzymes in the experimental design and use of different enzyme concentrations (Dávila León et al., 2024). A source of current debate is the common practice of neglecting the effect of salivary α-amylase (sAA) during the oral phase, justified by the short residence time of the bolus in the oral cavity. This is

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reflected in the variation in α-amylase concentrations used, and the complete exclusion of the enzyme or of the complete oral phase during *in vitro* digestion experiments of samples containing starch (Gallego-Lobillo et al., 2021; Krause et al., 2022). Moreover, most static *in vitro* digestion simulations use fixed parameters from the beginning of each phase, and this may also be an issue for the activity of the α-amylase due to its pH-dependent activity (Xavier and Mariutti 2021).

In the gastric phase, sAA inactivation is caused by the low pH (pH 3.0), while activation of the proteolytic enzyme pepsin occurs below pH 4.0 (Pälchen et al., 2021). A fixed pH during the static *in vitro* digestion method does not reflect the dynamic postprandial changes of the gastric pH occurring *in vivo* (Brodkorb et al., 2019). Recent work confirmed that this gradual acidification influences both protein hydrolysis and sAA activity (Freitas et al., 2018; Freitas and Le Feunteun 2019).

To further complicate comparisons, the structure of different foods, as modified during processing, can result in various alterations of the macronutrients release during digestion. In the case of starch, this is particularly important as when starch molecules are heated in excess of water, their native semi-crystalline structure is disrupted. The disruption is caused by water imbibition and swelling of the starch granules, with final gelatinization of starch and exposure of its amylose and amylopectin chains (Singh et al., 2010). The amorphous and disordered structure of gelatinized starch leads to a greater availability of α-amylase binding sites, which makes starch more susceptible to enzymatic hydrolysis (Dhital et al., 2017).

The aim of this work was to study the fate of polysaccharides exposed to sAA during static *in vitro* gastric digestion. To evaluate the potential interactions with protein digestibility, a mix containing starch and whey protein was used. A citrus peel pectin and whey protein mix was used as a control for starch hydrolysis as it is not digested by α-amylase. The study hypothesized that including sAA would not have any effect on protein hydrolysis. To evaluate the effect of sAA on starch hydrolysis in more *in-vivo*-relevant pH conditions, two different pH levels (pH 6.0 and pH 3.0) during the gastric phase were studied. A careful study of polysaccharides, and, in particular, of starch degradation in the presence of sAA, will contribute to a better tuning of *in vitro* digestion conditions in complex food matrices containing polysaccharides.

2. Materials and methods

2.1. Materials

Three complex polysaccharides were evaluated in this work: potato starch (PS), waxy maize starch (MS), and pectin from citrus peel (PC). PS (S5651) and PC (P9135) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The composition of these two polysaccharides, expressed on a dry matter basis, was as follows: PS consisted of 94% carbohydrates, 0.1% protein and 5.9% moisture. PC consisted of ≥74% galacturonic acid, 4% protein and 7% moisture. MS was kindly provided by Roquette Frères (Lestrem, France) and consisted of 86% carbohydrates (as is), 0.3% protein (as is) and 12.5% moisture (as is) (according to manufacturer's specification). In addition, a whey protein isolate (WPI, Arla Foods Ingredients, Viby J, Denmark) was studied. On dry matter basis **it** consisted of 0.1% carbohydrates, 92% protein, 0.1% fat, 3.8% ash and 4% moisture (according to manufacturer's specification). Milli-Q water used for experiments was obtained using a Merck Millipore synergy (SYNS0HF00, MB13221H) water filtration system. Chemicals and reagents used were of analytical grade and purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany.

2.2. Preparation of mixed dispersions

PS, PC and MS were prepared in separate beakers at a final concentration of 30 mg mL^{-1} (3%, w/w) in Milli-Q water. WPI was added to each beaker containing the polysaccharides at a final concentration of 30 mg mL^{-1} (3%, w/w) in Milli-Q water. The beakers were stirred on a magnetic stirrer for 1 h. In addition, a fourth dispersion was prepared from the MS-WPI mixture but heated to 80 °C while stirring for 3 min, followed by cooling to room temperature in ice water. This heated mixed dispersion was named GMS due to the gelatinization of the maize starch.

2.3. Saliva collection and α-amylase activity

Saliva was voluntarily collected by the author using a 3 min cycle in accordance with Sharma et al. (2020). First, the subject rinsed the mouth with water and saliva was collected while chewing a piece of 5 cm square of Parafilm. Saliva from the first minute was discarded. Chewing was conducted in a cycle with intermittent breaks after 30 s (Sharma et al., 2020). The saliva was collected in a 50 mL falcon tube on ice. Aliquots were transferred to 1.5 mL tubes and centrifuged at 10,000 g at 4 °C for 30 min. The supernatant was collected and snap frozen using liquid nitrogen and stored at −18 °C until use.

The activity of sAA was quantified using the DNS assay. This assay is a colorimetric method which uses 3,5-dinitrosalicylic acid (DNS) to quantify the production of reducing sugars (Gusakov et al., 2011). The DNS working solution was prepared by slowly mixing 8 mL of 5.3 M sodium potassium tartrate in 2 M NaOH with 12 mL Milli-Q water and 20 mL of 96 mM 3,5-dinitrosalicylic acid solution. The DNS working solution was stirred on a magnetic stirrer at 60 °C until complete dissolution and stored in an amber flask at room temperature until use. The substrate solution was prepared by dissolving potato starch (1% w/v) in a 20 mM sodium phosphate solution with 6.7 mM NaCl (pH 6.9, 20 °C). The substrate solution was stirred and heated just below boiling temperature for 15 min, cooled to room temperature, and the volume was made up to 25 mL with Milli-Q water.

The α-amylase activity in the collected saliva was measured according to the INFOGEST protocol (Brodkorb et al., 2019). In brief, four tubes were incubated at 20 °C for 5 min each containing 1 mL substrate solution (1% w/v potato starch). The four tubes were then mixed with 0, 0.5, 0.7 or 1 mL of thawed saliva, and incubated at 20 °C for exactly 3 min. The enzyme activity was stopped by addition of 1 mL DNS working solution, and the saliva volume was made up to 1 mL. For the blank test, Milli-Q water was added instead of saliva. The tubes were then incubated using a heating mixer (Thermo Fisher Scientific, CA, U.S.A.) at 100 °C for 15 min, and then immediately cooled to room temperature and diluted four times with Milli-Q water. The samples were measured in a microplate with a Synergy 2, Biotek spectrophotometer (Thermo Scientific, Massachusetts, U.S.A.) at 540 nm. The α-amylase activity was calculated from a maltose calibration curve (0–2 mg/mL. Eq. (1)):

$$\alpha - amylase\ activity \left(\frac{U}{mL}\right) = \frac{maltose\ (mg)\ released\ in\ 3\ min}{saliva\ (mL)} \tag{1}$$

The α-amylase activity was expressed as units of activity per mL of saliva. One unit refer to the amount of α-amylase containing saliva required to liberate 1.0 mg of maltose from potato starch in 3 min at pH 6.9 and 20 °C (Sharma et al., 2020). α-amylase activity of thawed saliva was 360 U/mL.

2.4. INFOGEST in vitro digestions

Digestion of PS, PC, MS, and GMS was performed in accordance with the INFOGEST standardized protocol, with some modifications as outlined in Fig. 1. The simulated salivary fluids (SSF), simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) were prepared as previously described (Brodkorb et al., 2019). Prior to the actual digestion of the samples, a set of control digestions were performed to determine the amounts of HCl and NaOH needed for reaching the set pH of the gastric chyme.

For the oral phase, the food dispersions (10 g) were mixed with SSF, 0.3 M $CaCl_2(H_2O)_2$, sAA (thawed saliva with activity of 360 U/mL of saliva), and Milli-Q water, as described by Brodkorb et al. (2019).

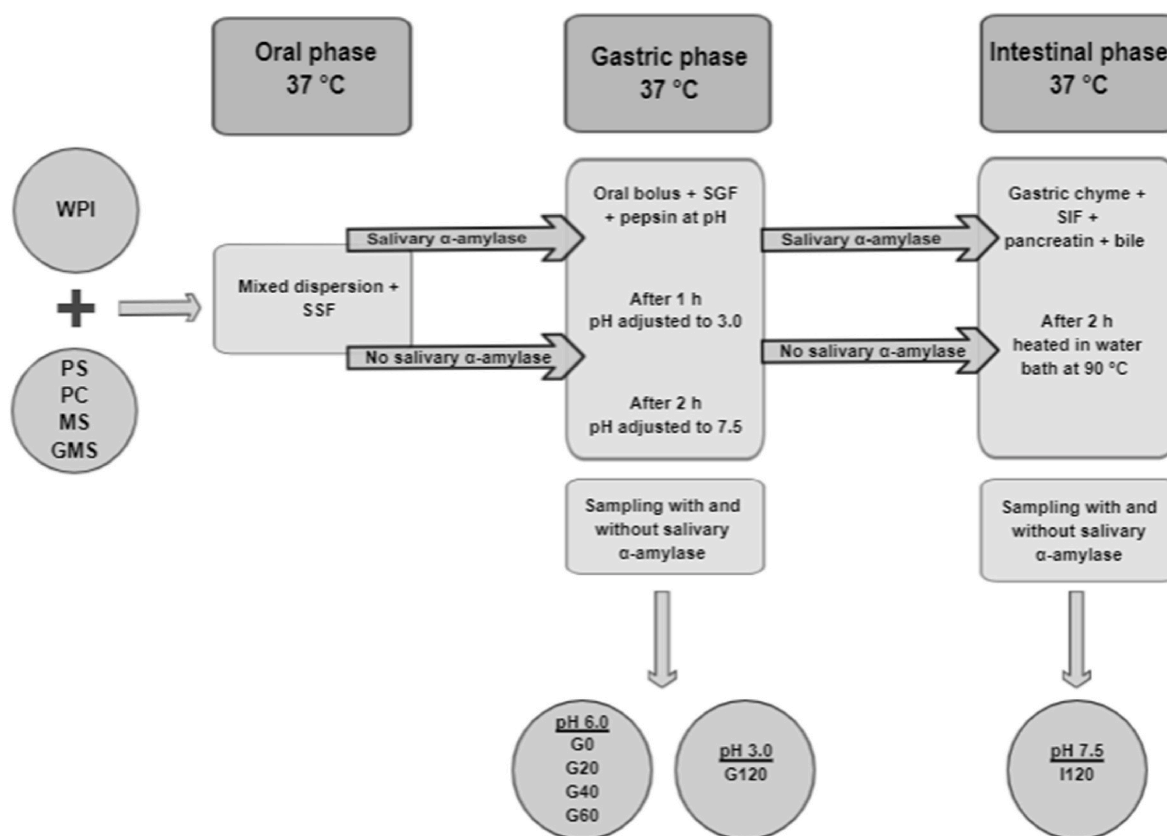

Fig. 1. Overview of the *in vitro* INFOGEST method employed to examine the effect of sAA on four mixed dispersions, outlining the sampling time, and the steps carried out during digestion. Sampling in the gastric phase after 0, 20, 40, 60 and 120 (min). Sampling in the intestinal phase after 120 (min).

Control samples were produced by replacing saliva (sAA) with Milli-Q water for all the dispersions studied. After incubation at 37 °C for 2 min, the oral bolus was diluted 1:1 (volume ratio) with SGF, 0.3 M $CaCl_2(H_2O)_2$, pepsin with activity of 2000 U/mL in final digesta (from porcine gastric mucosa ≥250 units/mg solid, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 2.0 M HCl to reach pH 6.0 and Milli-Q water. The samples were incubated and mixed at 37 °C with a rotator (Cole-Parmer Stuart Rotator Disk, Thermo Fisher Scientific, CA, U.S.A.) at 40 rpm. To uncover possible time-dependent changes of the dispersion components during the gastric phase, samples were collected after 0, 20, 40, 60 and 120 min of gastric digestion. Pepsin activity was stopped by placing the tubes in ice-water and adding NaOH to reach pH 7.5. To include the impact of food buffering capacity in the stomach, the samples were adjusted to pH 3.0 with 2.0 M HCl after 60 min of gastric digestion and incubated for an additional hour (120 min in total).

After 120 min of gastric digestion, the samples intended for intestinal digestion were diluted 1:1 (vol/vol) with SIF, 0.3 M $CaCl_2(H_2O)_2$, 10 mM (in final digesta) bile salts (bile extract from porcine, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), pancreatin with trypsin activity in 100 U/mL (from porcine pancreas, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and Milli-Q water. The samples were incubated for 120 min as described above. At the end of the intestinal digestion, enzyme activity was stopped by heating in a 90 °C water bath for 5 min followed by immediate cooling in ice water. The samples were stored at −18 °C until further analysis. Digestion experiments were carried out in triplicate.

2.5. Determination of the release of reducing sugars

Release of reducing sugars in the various digesta was measured using the same method as in section 2.3 measuring the activity of α-amylase in saliva. In brief, the release of reducing sugars was measured by mixing 0.1 mL digesta with 0.100 mL DNS working solution and heating them in a heating mixer at 100 °C for 15 min. The samples were then cooled to room temperature, diluted four times with Milli-Q water, and measured in a microplate with a Synergy 2, Biotek spectrophotometer 540 nm. The initial free reducing sugar content was measured for the untreated dispersions and subtracted from the data obtained after the digestion experiments. Free reducing sugars were measured in the dispersions, digested with and without sAA, and reported as maltose equivalents (mg/mL). In the case of starch, the reducing sugars were also converted to starch using 0.947 (Krause et al., 2022; Shriner 1932), and a relative increase in digested starch was determined calculating the ratio of the polysaccharide in the digesta to the initial polysaccharide concentration of the dispersions.

2.6. Determination of oligosaccharides by anion exchange chromatography

High performance anion exchange chromatography, coupled with pulsed amperometric detection (HPAEC-PAD, ICS-6000, Dionex, USA), was employed for quantification of maltose and glucose released during *in vitro* gastrointestinal digestion. Before extraction, 5 mL digesta were evaporated using a vacuum concentrator (Genevac EZ-2, Gardiner, NY, USA) at 50 °C. From the evaporated digesta, ~50–60 mg was collected (exact weights were noted) and mixed with 950 μL 80% EtOH and incubated at 80 °C for 1 h with constant stirring. The samples were then centrifuged at 16,000 g at 1 °C for 10 min. The pellets were then collected and re-extracted in 950 μL 80% EtOH, incubated and centrifuged as previously described. The supernatants from both extractions were combined and separated from the pellet and placed in a freeze dryer at −110 °C down to 1 bar overnight. The pellet was kept for SEC-MALS analyses (section 2.7). The ethanolic supernatants were collected and re-evaporated using a vacuum concentrator (Genevac, EZ-2,

Gardiner, NY, USA) at 50 °C, followed by freeze-drying at −110 °C down to 1 bar overnight. After drying, the samples were reconstituted in 1 mL Milli-Q water and centrifuged at 16,000 g at 4 °C for 5 min. Supernatants were injected into a Dionex ICS-6000 ion chromatograph system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a CarboPac-PA1 column (2 × 250 mm) and PA-1 Guard precolumn (2 × 50 mm) set at 25 °C. The mobile phase consisted of solvent A: deionized water; solvent B: 200 mM NaOH. Glucose and maltose were separated working in isocratic mode with 91% solvent A and 9% solvent B (i.e., 18 mM NaOH) at a flow rate of 0.25 mL/min. The quantity of saccharides was determined by means of calibration curves using glucose and maltose standards, injected, and analyzed under the same conditions described above. Obtained data represents μM saccharide (glucose and maltose) per mg digesta.

2.7. Molecular size distribution of native and digested starch samples (SEC-MALS)

To further evaluate potential differences in the starch digestibility, the size distribution of starch molecules, before and after digestion were analyzed using a high performance size exclusion chromatography (HPSEC) (Agilent 1260 Series SEC system, Agilent Technologies, Waldbronn, Germany) equipped with a 1260 Infinity II diode array detector (DAD, Agilent, California, USA), a Shodex RI-501 refractive index detector (Showa Denko K. K., Japan) and an 18 angle DAWN multi-angle light scattering (MALS) detector (Wyatt Technology, California, USA).

Briefly, potato and maize starch samples (12 mg) were solubilized in 1.5 mL of dimethyl sulfoxide (DMSO) solution containing 0.5% (w/w) lithium bromide (LiBr) at 80 °C in a thermomixer (Thermomixer Comfort, Eppendorf, Hamburg, Germany) for 24 h. Afterwards, samples were let to cool down and centrifuged at 10,000 g at 25 °C for 15 min. Supernatant was collected and injected in the HPLC system, running at the conditions previously reported (Roman et al., 2019).

For the digested samples, the ethanol extracted pellets saved from preparation of supernatants for ion chromatography of section 2.6 were used. The pellets were frozen at −80 °C and freeze dried at −110 °C down to 1 bar overnight to obtain a dry residue. The dry residues were collected and solubilized in DMSO/LiBr as described above for the non-digested samples before their injection in the HPLC.

2.8. Quantification of free amino groups (R–NH_2)

As an indication of protein hydrolysis, the amount of free amino groups in the various samples was compared, using the o-phthaldialdehyde (OPA) method. This method estimates the concentration of primary amines as mmol of L-glutamic acid equivalents in the soluble fraction of the digested samples. The whole dispersion samples were precipitated with MeOH (80%, v/v, 1:4) at −18 °C for 1 h and thereafter centrifuged at 4000 g (4 °C, 15 min). The supernatants were collected and stored at −18 °C until further analysis. The OPA working solution was prepared by mixing: 12.5 mL of 0.1 M of sodium tetraborate decahydrate, 2.5 mL of sodium dodecyl sulfate (SDS, 10%, w/w), 0.5 mL of OPA in EtOH (4%, w/w), 0.5 mL of 2-mercaptoethanol (Na-MES, 20%, w/w), 1.25 mL of Terigitol™ 15-S-9 (10%, w/w) and 25 mL Milli-Q water. The assay was performed by mixing 232 μL of OPA working solution with 8 μL of sample, blank (perchloric acid, 0.5 M) or standards (L-glutamic acid, 0–8 mM) in a 96-well microplate and incubating at 30 °C for 10 min. The absorbance was measured at 335 nm and expressed as free L-glutamic acid equivalent (mM) (Thermo Scientific, Massachusetts, U.S.A.).

2.9. Distribution of polypeptides by SDS-PAGE electrophoresis

To assess the distribution of polypeptides after *in vitro* digestion of the mixed dispersions Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was conducted. The samples were prepared in reducing conditions by mixing 13 μL dispersion with 5 μL NuPage LDS sample buffer (4X) (Invitrogen, Thermo fisher Scientific, CA, U.S.A) and 2 μL dithioerythritol (1 M DTE), making a total volume of 20 μL. Samples were incubated using a heating mixer (Thermo Fisher Scientific, CA, U.S.A.) at 95 °C for 5 min. For analysis, a Bis-Tris-gradient (4–12%) gel of 1.0 mm X 12 wells (Invitrogen, Thermo Fisher Scientific, CA, U.S.A.) was used. Each well was loaded with either 10 μL sample or 5 μL pre-stained protein ladder standard (PageRuler Prestained Protein Ladder, Thermo Fisher Scientific, Vilnius, Lithuania). Protein separation was performed at 200 V for 35 min using MES-SDS running buffer according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, CA, U.S.A.) using a XCell SureLock™ Mini Cell (Invitrogen, Thermo Fisher Scientific, CA, U.S.A.). The bands were stained with SimplyBlue (Invitrogen) and image analysis performed using ChemiDoc XRS + and Image Lab software (Bio-Rad lab., CA, U.S.A.).

2.10. Statistical analysis

All data analysis were treated using two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test using GraphPad Prism version 9.5.1 with inclusion/exclusion of sAA and sampling time as independent variables. Minimal significance was set at 5% ($P < 0.05$). The following represents three independent measurements: reducing sugar content by DNS, concentrations of glucose and maltose by HPAEC-PAD. Quantification of free R–NH_2 by OPA and the size distribution of starch molecules by SEC-MALS were results of two independent measurements.

3. Results and discussion

3.1. Reducing sugar content after in vitro digestion

The release of reducing sugars was monitored after oral digestion (G0), during the course of the gastric phase (G20, G40, G60 and G120) and at the end of the intestinal phase (I120). Fig. 2 shows reducing sugar contents determined as maltose equivalents with and without sAA during *in vitro* digestion. In general, the three unheated polysaccharide dispersions showed low levels of reducing sugar (8–32 mg mL^{-1}) after the oral stage (G0). The heated, gelatinized starch (GMS) also showed low amounts of reducing sugars at G0. Further, throughout digestion, potato starch (PS) and pectin (PC) showed higher levels of reducing sugars compared to maize starch (MS), which consistently had the lowest levels of hydrolysis throughout gastric digestion. Furthermore, time of incubation and pH showed little to no effect during the gastric phase. This was somehow unexpected, as the α-amylase is known to be more active around neutral pH, so a higher starch hydrolysis would have been expected at pH 6.0 (Marini 2005).

There was a significant increase in hydrolysis ($P < 0.05$) for PS and MS after 120 min of intestinal digestion compared to levels at the beginning of gastric digestion, regardless of the addition of sAA to the digesta. In accordance with literature, this increase can be attributed to the action of pancreatin (Woolnough et al., 2010). As expected, no change was observed for PC during *in vitro* gastrointestinal digestion. Pectin is mostly composed of galacturonic acid linked by α-(1, 4)-glucosidic bonds, which can be hydrolyzed by pectinases (Pedrolli et al., 2009). Since pectinases are not present in the simulated gastrointestinal juices, there should be no hydrolysis of PC.

In the case of starch, the relative digested starch molecules were low after the gastric phase across all dispersions (3–16%). These low levels of hydrolysis are in line with prior reports (Gallego-Lobillo et al., 2021). The authors also reported only 1–2% of polysaccharide hydrolysis after gastric digestion, followed by a significant increase after the intestinal phase, using the INFOGEST protocol. However, the levels of hydrolysis in the present work were generally a bit higher than those reported earlier (Gallego-Lobillo et al., 2021).

The hydrolysis of polysaccharides during the gastric phase has

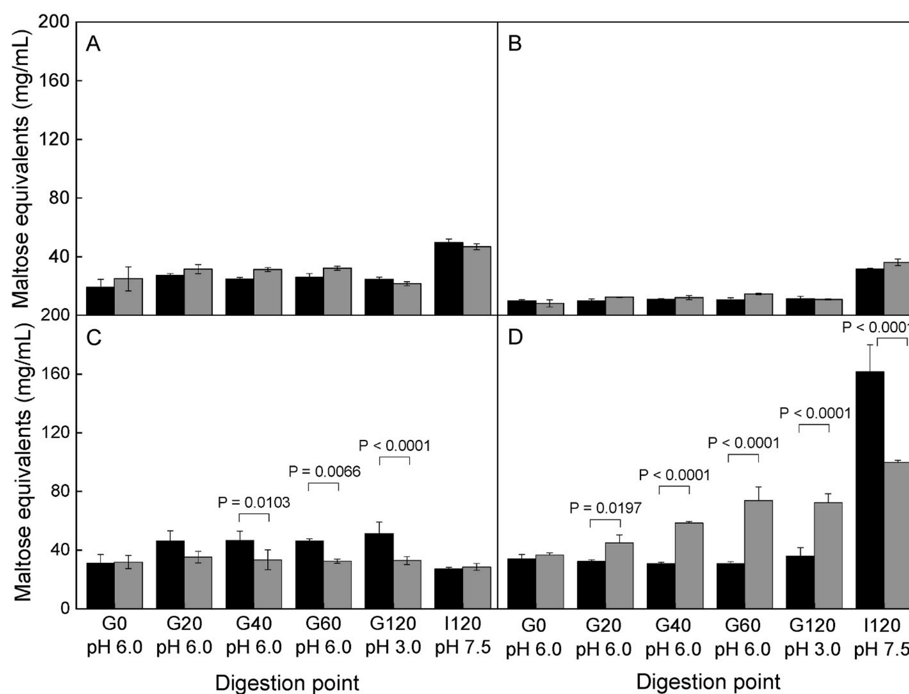

Fig. 2. Reducing sugar content during *in vitro* digestion expressed as maltose equivalents (mg/mL) determined by DNS assay. Statistical differences (P-values) between the maltose equivalents in digesta with (light grey columns) and without sAA (black columns) for PS (A), MS (B), PC (C), and GMS (D) are shown. Gastric phase (G) samples: 0 min at pH 6.0, 20 min at pH 6.0, 40 min at pH 6.0, 60 min at pH 6.0 and 120 min at pH 3.0. Intestinal phase (I) samples: 120 min and at pH 7.5. Bars indicate standard deviations.

previously been linked to the low pH of the gastric environment. It has been previously hypothesized that these conditions may provoke alterations to the surface of starch molecules, through partial breakdown of protein found on the surface and inside the starch granules (Fernandes et al., 2020; Pérez and Bertoft 2010). In addition, the molecular structure and supramolecular assemblies that characterize these polysaccharides contribute to low extents of hydrolysis, for example, in MS compared to PS, although previous work reports higher digestibility of maize starch when compared to potato starch (Dhital et al. 2010, 2017).

In the gelled starch sample (GMS) a significant effect of the addition of sAA ($P < 0.05$) was observed for the samples undergoing gastric digestion (G20 to G120). This contrasts with the other dispersions investigated. Furthermore, in the GMS digests containing sAA, a gradual increase of starch digestion was observed from G0 to G60 (pH 6.0) while no increase was observed from G60 and G120 (pH 3.0). This is conformed with the literature, which has shown how a gradual rather than an instant decrease in gastric pH (pH 6.3–2.5) during *in vitro* digestion of isolated cotyledon cells from chickpeas (~62% starch and ~17% protein per 100 dry weight) resulted in higher levels of starch hydrolysis (Pälchen et al., 2021). The authors reported 21% hydrolyzed starch in the gastric phase and 92% hydrolyzed starch in the intestinal phase using gradual decrease in gastric pH. Furthermore, the authors reported no hydrolysis of starch in the gastric phase and 69% after intestinal digestion using static pH 3.0 conditions in the gastric phase (Pälchen et al., 2021).

In both cases, with (51%) and without sAA (32%), the GMS showed a higher susceptibility to digestion, displaying the highest amounts of relative hydrolyzed starch compared to the potato or its non-gelatinized counterpart post-intestinal digestion. Heating starch in excess of water causes swelling and gelatinization of the granules, exposing the α-glucan chains, improving enzyme (sAA) accessibility (Singh et al., 2010). Although employing a different digestion protocol, Liu et al. (2021) showed strong correlations between the degree of gelatinization (DG) and starch hydrolysis for potato starch, wheat starch and lotus seed starch. The authors reported a gradual increase in starch hydrolysis as DG increased from 0 to 100% (Liu et al., 2021).

None of the investigated starch polysaccharides reached a high extent of digestion. Other literature has reported as much as 80–100% starch hydrolysis into reducing sugars after the intestinal phase (Krause et al., 2022; Teng et al., 2016). However, the levels reached by the GMS samples point to the importance of processing-induced structural changes in starch hydrolysis during digestion. Recently, Krause et al. (2022) examined sponge cakes. In these systems, starch was exposed to high temperatures (170 °C) and moisture over a long period of time (25 min), resulting in high enzyme accessibility (Singh et al., 2010). Longer digestion times also have been shown to be highly effective in digesting starch. For example, Teng et al. (2016) reported 100% digested waxy maize starch after 8 h, and ~70% digested potato starch after 48 h of digestion. However, such lengths of digestion are not physiologically relevant. Furthermore, it is possible that in this study, GMS is not completely gelatinized, as the maize starch-protein dispersion was only heated to 80 °C for 3 min. Although this temperature was above the gelatinization temperature of waxy maize starch, the short heating time may have only resulted in partial swelling and gelatinization of the starch granules.

3.2. Oligosaccharides analysis

The release of reducing sugars at the end of the intestinal phase measured using HPAEC-PAD for the different polysaccharide-protein dispersions is summarized in Fig. 3. The graphs show the concentrations of glucose (A) and maltose (B) after the intestinal phase for PS, MS, and GMS. In this case, pectin was not analyzed, as changes in the backbone with digestion was not anticipated. As expected, the glucose and maltose (starch degradation products) concentrations were below detection in the gastric phase for all dispersions. The results presented in Fig. 3 demonstrate a detectable increase in glucose and maltose concentrations post-intestinal digestion, consistent with the findings in Fig. 2. The low amounts of starch degradation products in the gastric phase followed by a noticeable increase after intestinal digestion is

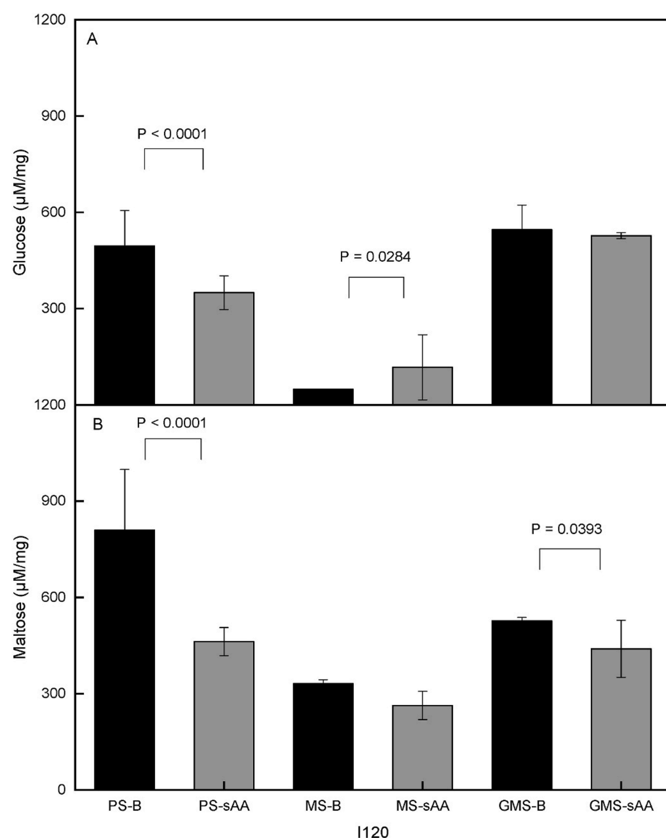

Fig. 3. Release of glucose (A) and maltose (B) expressed as μM/mg digesta after intestinal digestion of PS, MS and GMS. Black columns: without sAA, grey columns: with sAA. Bars indicate standard deviations.

consistent with the findings from previous studies (Gallego-Lobillo et al., 2021; Krause et al., 2022). Based on these results, there was no significant effect of sAA in the digestion phase. Both DNS and HPAEC-PAD provide data which can describe in detail starch degradation. However, studies consistently report higher values with DNS compared to those measured with HPAEC-PAD. With the latter, short oligosaccharides are excluded from the measurement, and those are a more likely product of digestion based on the hydrolysis mechanism of α-amylase, and also contributing to the color development of the DNS method.

3.3. Size distribution of molar mass of starch-digestion products

The degradation patterns of the three starch dispersions (PS, MS and GMS) were also analyzed at the different digestion stages, using size exclusion chromatography coupled to multi angle light scattering detectors, to evaluate potential differences in the size distribution of the polysaccharide chains. Fig. 4 shows the molar mass (g/mol) and the differential refractive index (dRI) of the heat-treated maize starch (GMS) dispersion after gastric digestion: 0 min at pH 6.0 (A), 60 min at pH 6.0 (B) and 120 min at pH 3.0 (C), and after intestinal digestion: 120 min at pH 7.5 (D). These results are in full agreement with the reducing sugar data measured in the supernatants shown in Fig. 2. While no difference was observed for PS and MS during gastric digestion (data not shown), there was a distinct effect of sAA for GMS. The initial stage of gastric digestion (G0) showed that the sample treated with sAA eluted later and with lower molar mass compared to the samples digested without sAA. This suggests that the addition of sAA in the oral phase caused more fragmentation of the polysaccharide chains. Hence, it was clearly shown that the salivary amylase, in spite of the brief time allocated for its optimal activity, already caused some molecular hydrolysis at the early stages of gastric digestion. The gastric phase showed no discernible time-dependent differences regardless of treatment (B and C). Thus, there was no indication of pH-dependent degradation of the polysaccharides, in agreement with the results for the reducing sugars release.

The observed increase in hydrolysis of polysaccharides following intestinal digestion is consistent with previous studies and highlights the role of pancreatic α-amylase in starch breakdown (Freitas and Le Feunteun 2019). No difference was observed between treatments for any of the starch dispersions after intestinal digestion (PS and MS data not shown). This lack of difference between I120 with and without sAA is probably due to the applied method (SEC-MALS). For the SEC-MALS analyzes, only the pellet fraction was used, which might not have had

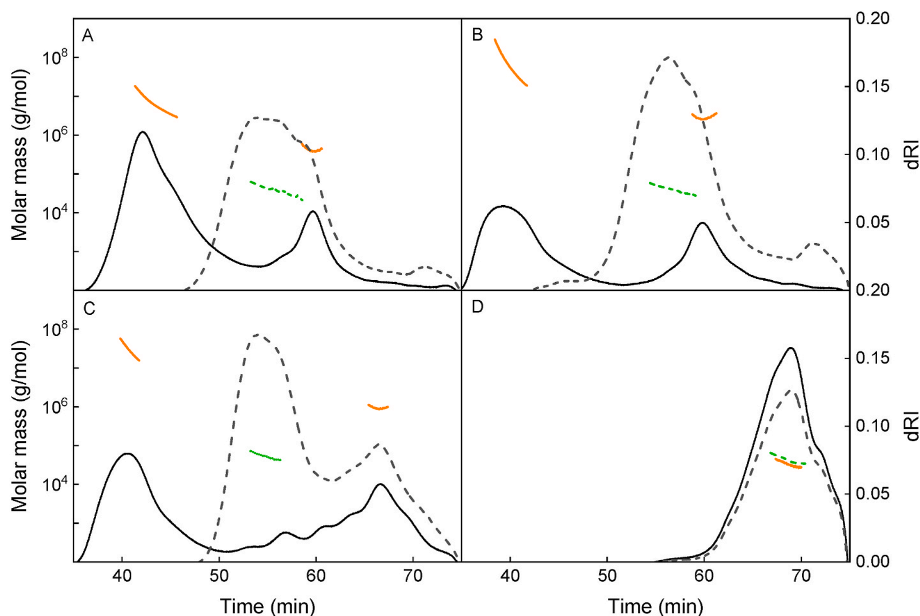

Fig. 4. SEC-MALS-RI chromatograms of GMS during *in vitro* gastrointestinal digestion. Solid lines represent refractive index signals of samples digested without sAA and dashed lines represent samples digested with sAA. Orange and green lines represent molar mass of samples digested without and with sAA, respectively. Gastric phase (G) samples: 0 min at pH 6.0 (A), 60 min at pH 6.0 (B) and 120 min at pH 3.0 (C) min. Intestinal phase (I) sample at time: 120 and at pH 7.5 (D). Note different y-axis for molar mass (left y-axis) and differential refractive index (dRI) (right y-axis).

any high molecular starch digestion products. Thus, it was concluded that while there was a notable effect of sAA on starch degradation during the gastric stage digestion of the gelled system, the action of the pancreatic α-amylase levels out any distinguishable differences between treatments (with/without sAA) during the intestinal stage.

3.4. Effect of polysaccharide on in vitro digestion of whey proteins

The primary amines were measured as mmol of L-glutamic acid equivalents in the soluble fraction of the digested samples, in orderto uncover any polysaccharide and/or sAA-dependent proteolysis of WPI during gastrointestinal digestion (G0, G120 and I120),. The extent of proteolysis for the various polysaccharides and treatments are summarized in Fig. 5. Low concentrations (6.6–9.6 mM) were measured at G0 and with no further significant change throughout the gastric stage, indicating no time and pH-dependent proteolysis of WPI.

At the end of the *in vitro* intestinal phase, the concentrations of L-glutamic acid equivalents were more than 7 times as high for all dispersion and treatment combinations compared to their respective concentrations measured in the gastric phase. There was no significant sAA-dependent effect on proteolysis for any of the dispersions during the gastrointestinal digestion.

This low degree of free amino groups during the gastric stage, followed by a significant increase post-intestinal digestion was expected, since pepsin is an endopeptidase estimated to be responsible for the digestion of only ~15% of dietary protein (Smith and Morton 2010). It has previously been shown that pepsin has a preparatory function important for the subsequent trypsin and chymotrypsin-catalyzed hydrolysis in the small intestine, which the results of this work also suggested (Moran 2016; Rivera Del Rio et al., 2021). In this study only the endpoint of intestinal digestion was investigated. It is, however, important to note that previous studies have reported the majority of proteolysis to occur within the first minutes of the intestinal phase (Hiolle et al., 2020; Jiménez-Munoz et al., 2023).

Literature has shown how the different macronutrients can affect the digestibility of one another, with multiple influencing factors such as the type, source, and concentration of the individual macronutrient together with their processing history (Atallah et al., 2020; Krause et al., 2022; Opazo-Navarrete et al., 2019; Yang et al., 2019). It was hypothesized that during digestion, polysaccharides may slow down proteolysis by acting as a physical barrier between pepsin and the protein. Additionally, they absorb gastric juice, thereby delaying acidification and pepsin activation (Opazo-Navarrete et al., 2019; Wahbeh and Green 2021). In this work no significant polysaccharide-dependent effect could be measured, however, these were simple formulated suspensions, and not complex foods. These results are in line with those reported by Krause et al. (2022), who found no difference in relative amounts of readily bio-accessible protein after *in vitro* intestinal digestion between sponge cakes formulated with either wheat flour, pea flour or pea starch. However, prior work clearly demonstrated that structuring of food by polysaccharides can affect protein digestion (Markussen et al., 2021). Thus, the results suggested that in suspensions, the hydrolysis of WPI was not influenced by the type of polysaccharide.

To confirm this result and evaluate potential kinetics or mechanistic differences in the hydrolysis, changes in polypeptide composition and size were analyzed by SDS-PAGE as summarized in Fig. 6. The bands corresponding to the control WPI were observed at ~14 kDa (α-lactalbumin), ~18 kDa (β-lactoglobulin) and ~66 kDa (Bovine serum albumin, BSA) in accordance with the literature (Madureira et al., 2007). As expected, there was no effect of the salivary amylase treatment in the digested PC-WPI mixture. β-lactoglobulin showed resistance towards degradation, whereas the bands of α-lactalbumin and BSA disappeared completely after 120 min of gastric digestion. This confirms that the acidification introduced after 60 min of gastric digestion is indeed essential to pepsin activity (Pälchen et al., 2021). The perseverance of β-lactoglobulin is in agreement with the literature, as native β-lactoglobulin is known to be resistant to pepsin hydrolysis at gastric pH (1.0–3.0) due to its highly ordered β-sheet core structure. In contrast, α-lactalbumin is known to be susceptible to pepsin hydrolysis under gastric conditions (Kim et al., 2007; Molinari et al., 1996; Schmidt and Poll 1991). This resistance exhibited by β-lactoglobulin towards gastric degradation is also current for a polysaccharide-containing dispersion.

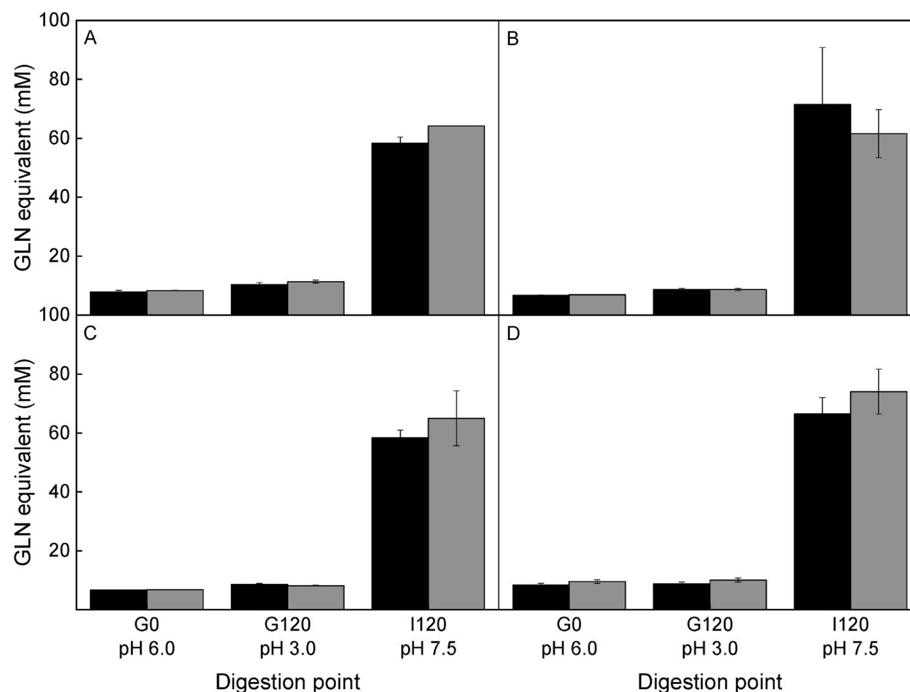

Fig. 5. Free R–NH_2 expressed as free L-glutamic acid equivalent (mM) during *in vitro* gastrointestinal digestion of PS (A), MS (B), PC (C) and GMS (D). Gastric phase (G) samples: 0 min at pH 6.0 and 120 min at pH 3.0. Intestinal phase (I) samples: 120 min and at pH 7.5. Black columns: without sAA, grey columns: with sAA. Bars indicate standard deviations. The addition of sAA was not significant for any of the investigated polysaccharides ($P > 0.05$).

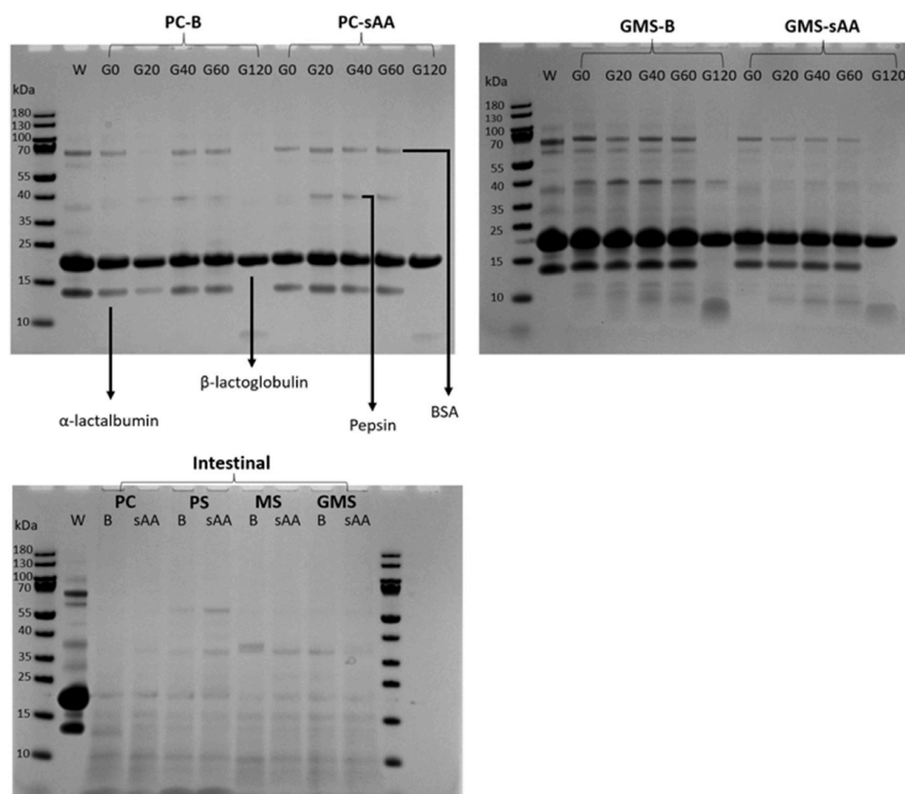

Fig. 6. SDS-PAGE analysis of the digesta at the various stages during *in vitro* gastric digestion of WPI mixed with either PC or GMS with (sAA) and without (B) sAA. The electrophoretic profile after intestinal digestion of WPI with either PC, PS, MS, and GMS with (sAA) and without (B) sAA is also shown. Gastric phase (G) samples: 0 min at pH 6.0, 20 min at pH 6.0, 40 min at pH 6.0, 60 min at pH 6.0 and 120 min at pH 3.0. Intestinal phase samples: 120 (min). W: WPI. Molecular weight markers are shown in kDa. Equal volumes of digesta were loaded in all wells.

The current results are in contrast with previous results of Markussen et al. (2021), who demonstrated a difference in the electrophoretic profile depending on the type of protein-polysaccharide dispersion under semi-continuous *in vitro* gastric digestion. However, in their work structural changes were induced to the matrix by the presence of polysaccharide, so this discrepancy points to the importance of structure in imparting differences in kinetics of digestion, not the polysaccharide, *per se*.

In the case of mixtures containing gelatinized maize starch (GMS), a small difference in the degradation of WPI seems to be present in the sAA treated samples during gastric digestion. Although all the bands representing α-lactalbumin, β-lactoglobulin and BSA can be identified, they were more intense in the dispersions digested without sAA. These results would once again demonstrate the importance of food structure in digestion, suggesting that more intact starch molecules may have a protective effect on WPI (Opazo-Navarrete et al., 2019). Furthermore, the salivary amylase may have released small carbohydrate chains, which when interacting with the proteins, would induce structural changes leading to a change in protein hydrolysis.

Previous studies have reported a decreased protein breakdown with increasing polysaccharide content (Fontes-Candia et al., 2022; Ma et al., 2021). Although heat can cause denaturation of β-lactoglobulin and make it susceptible to pepsin activity, it appeared not to be the case in this work. The brief heating of the dispersions did not fully denature β-lactoglobulin, potentially leaving a fraction of native β-lactoglobulin. Ozel et al. (2020) reported that β-lactoglobulin has been found resistant towards pepsin activity in a hydrogel solution subjected to heat treatment at 90 °C for 30 min, which is consistent with the present findings.

In contrast, Hiolle et al. (2020) investigated the degradation of four different microstructures with identical compositions and found no considerable effect of the microstructure with respect to hydrolysis of neither protein nor carbohydrates. However, they reported that lipolysis in a biscuit was four-times higher than in a custard. Nevertheless, the authors reported a food-structure-dependent bio-accessibility of micronutrients, stressing the potential of designing food systems with tailored nutrient release.

The polypeptide composition at the end of intestinal digestion in this work showed no distinct differences. All dispersions exhibited extensive protein hydrolysis, including β-lactoglobulin degradation, indicating that polysaccharide type does not impact WPI proteolysis after *in vitro* gastrointestinal digestion. In line with the results obtained by the OPA method, no sAA-dependent proteolysis was shown after the intestinal phase. Since only one type of polysaccharide was subjected to heating in this work, a polysaccharide-dependent effect in a heated system cannot be foreclosed. In addition, the polysaccharide-protein ratio has previously been shown to influence proteolysis, as higher polysaccharide concentration leads to the formation of stronger gel structures, creating better protective barriers against enzymatic action (Fontes-Candia et al., 2022; Koutina et al., 2018). It could therefore be relevant to investigate the impact of polysaccharide concentration on protein digestibility.

4. Conclusions

The investigation of polysaccharide containing dispersions led to a better understanding of the relevance of using salivary α-amylase (sAA) in the oral phase, within the INFOGEST method. The results demonstrated that adding sAA did not significantly influence the degradation of the polysaccharides unless the system was heated. The heated system containing maize starch (GMS) showed higher polysaccharide fragmentation in the gastric phase, whereas the results indicated an equalization between treatments at the end of the intestinal phase. Furthermore, a pH-dependent degradation of starch was only shown for

GMS, as after acidification of the gastric pH the starch hydrolysis plateaued. In the case of protein hydrolysis, the results confirmed that there was no polysaccharide or sAA-dependent release of free NH_2 groups. In addition, no significant difference was shown by comparison between the start and end point of gastric digestion. This corroborates that in the gastric phase pepsin has a preparatory function rather than actual digesting protein. The electrophoretic profiles supported that heating of the system is necessary to observe an effect of adding sAA. While no difference was observed for the polypeptide distribution of pectin and whey containing dispersion, there seemed to be an effect of sAA treatment of the gelatinized system containing maize starch and whey protein. The gelled structure appeared to form a physical barrier, providing protection of the proteins against pepsin activity. However, at the end of intestinal digestion, regardless of polysaccharide type, the protein in all dispersions showed similar polypeptide distributions. Thus, this work demonstrated that the relevance of sAA addition does not depend on the polysaccharides in question, but rather on supramolecular structures, such as heat-induced gels.

While this study provides valuable insights into the relevancy of including sAA in *in vitro* gastrointestinal studies, there are some limitations. SEC-MALS excludes short oligosaccharides, which are a likely product of hydrolysis by α-amylase. These short oligosaccharides might also contribute to the color development in the DNS method, resulting in an overestimation. Future research could focus on the specific polysaccharide-protein interactions during *in vitro* digestion experiments at various concentration ratios using state of the art technologies.

Ethical statement

The saliva used in this work was that of the main author, and not subjected to ethical committee approval.

CRediT authorship contribution statement

M. Torp Nielsen: Conceptualization, Investigation, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **L. Roman:** Conceptualization, Investigation, Methodology, Writing – review & editing. **M. Corredig:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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