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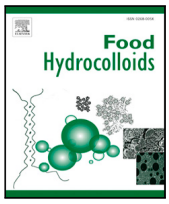

Oat protein *in vitro* digestion is not influenced by pectin in dispersion or gel systems

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ABSTRACT

The in-vitro digestion of oat protein isolate (OPI) was studied in the presence of increasing concentrations (0.5, 1.0, 1.5 % w/w) of low methoxy pectin (LMP). The in-vitro digestion of OPI was further studied upon incorporation within a calcium LMP network. The OPI and pectin, as well as the calcium - pectin and OPI gels were characterized using rheology, mechanical properties and microstructure. The in vitro digestion of the OPI was performed using the static INFOGEST protocol, and the digestion was analysed based on degree of protein hydrolysis and amino acid composition. Both the rheology and mechanical properties, as well as results obtained upon digestion indicate no or little interaction between pectin and OPI at neutral pH values. The OPI - pectin dispersions show higher than expected viscosities, suggesting phase separated system. The addition of OPI in calcium pectin network has small impact on small deformation but diminish the mechanical strength, as compared to calcium pectin gels. The presence of pectin, as dispersion or gel, does not alter the protein hydrolysis nor the amino acid composition of the supernatant obtained after gastric and intestinal digestion phase; thereby providing opportunities for oat protein - dietary fibre formulations that preserve protein digestion integrity while offering potential prebiotic effects.

1. Introduction

Oats show potential as a protein source due to their relatively high protein content, which ranges from 12 to 20 grams per 100 grams of dry weight, and their beneficial composition of essential amino acids (Boukid, 2021).

Oat proteins have a high heat stability, resulting in heat induced gels requiring high temperatures, > 95 °C, and alkaline conditions for strong gels, the latter not commonly used in food applications (Ma, Khanzada, & Harwalkar, 1988). Partial enzymatic hydrolysis, increases the gel strength of oat proteins, however, gels were still studied at high temperatures (110 °C) and with the strongest gels formed at alkaline conditions (Nieto-Nieto, Wang, Ozimek, & Chen, 2014). Addition of polysaccharides is another approach to overcome the limiting gelling properties of oat proteins.

Nieto-Nieto and co-workers showed that inulin increases the gel strength of oat proteins (15% oat proteins and 0.5%, or below, of inulin). The authors argued that the inulin and oat protein formed a phase separated system where inulin filled the voids of the protein network (Nieto-Nieto, Wang, Ozimek, & Chen, 2015). The same group further investigated the impact of dextrin, carrageenan, and chitosan on the thermal gelation of oat proteins. Similarly, the authors observed an increase in mechanical properties of the gels, particularly at neutral pH values (Nieto Nieto, Wang, Ozimek, & Chen, 2016). The authors attributed the results to an apparent increase in protein concentration induced by the phase separation process arising from the incompatibility of the biomolecules used (Nieto Nieto et al., 2016; Tolstoguzov, 2007).

The addition of polysaccharides to protein can also impact digestion of protein. Addition of polysaccharides to dairy drinks increases feeling of satiety (Peters et al., 2011) and time of gastric emptying (Benini et al., 1995), results believed to be partly related to increased viscosity (Marciani et al., 2000) of digesta and gel strength (Hoad et al., 2004; Ström, Boers, Koppert, Melnikov, Wiseman, & Peters, 2009). The viscosity increase of the digesta can also lead to reduced post-prandial blood glucose (Dikeman, Murphy, & Fahey, 2006) and reduced blood serum cholesterol (Vuksan et al., 2011).

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The mechanisms behind the effect of polysaccharides on blood glucose and serum cholesterol is believed to be related to reduced gastric emptying rate but also to reduced diffusion rate of enzymes, nutrients or direct interaction between polysaccharides with proteins. Xanthan, guar gum, and locust bean gum reduce the digestion rate of whey protein and starch (Chen et al., 2020). Chen and co-workers found a negative linear function between viscosity and digestion rate, as well as reduction in release of glucose as a function of viscosity (Chen et al., 2020). Gum arabic, xylan and low methoxy pectin (LMP) were further shown to reduce digestibility of peanut protein, as assessed by nitrogen release content through dialysis bags (Mouécoucou, Villaume, Sanchez, & Méjean, 2004). The reduction in nitrogen release content was observed at polysaccharide concentrations of > 10% for LMP and gum arabic and > 20% for xylan. For gum arabic and xylan, the reduction in digestibility was attributed to decreased protein hydrolysis, while for LMP, the interaction between LMP and low molecular weight peptides was identified as a contributing factor. In another study, combinations of soy proteins and xanthan and soy protein and carrageenan were studied for structural changes under *in vitro* gastric conditions. The acidic environment of the gastric phase favoured soy — polysaccharide gel formation, consequently delaying protein digestibility (Hu et al., 2017). Similar results have been reported for milk proteins and alginate, high methylated pectin and guar gum (Markussen, Madsen, Young, & Corredig, 2021), whey protein and pectin (Zhang & Vardhanabhuti, 2014), whey protein and guar, xanthan and carrageenan (Zhang, Zhang, & Vardhanabhuti, 2014) as well as casein incorporated within agar and *kappa*-carrageenan gels (Fontes-Candia, Jiménez-Barrios, Miralles, Recio, López-Rubio, & Martínez-Sanz, 2022).

Here, our objective is to explore the impact of pectin, dispersions and gels on oat protein hydrolysis and amino acid composition, following the INFOGEST, static *in vitro* digestion protocol. Pectin, is an anionic polysaccharide, recognized for its role as a food thickener and gelling agent. The structural composition of commercially available pectins comprises of homogalacturonan. Homogalacturonan consists of α-(1,4)-D-galacturonic acid units (GalA), where GalA can be esterified with methyl groups to different degrees (Fellah, Anjukandi, Waterland, & Williams, 2009; Ström, Ribelles, Lundin, Norton, Morris, & Williams, 2007). The degree of methylation (DM) categorizes the pectin as high methoxyl pectin with DM > 50 or low methoxy pectin (LMP) with DM < 50. LMP readily forms gel in the presence of multivalent ions (except for Mg^{2+}), where especially the calcium induced LMP gels are well explored (Grant, Morris, Rees, Smith, & Thom, 1973; Ström et al., 2007). We have thus chosen to study the impact of LMP addition to oat protein dispersion and incorporation of oat protein in calcium pectin on gels on oat protein digestion.

2. Materials and methods

2.1. Materials

Dry hulled oat groats of "Active" variety were provided by Lantmännen, Sweden. The LMP, with product number Classic Au-L 018/22 was provided by Herbstreith & Fox, Germany. The apple pectin had a DM of 32% and a GalA content of 82%, as given by the producer. The pectin was used without further purification. $CaCO_3$ (< 50 μm particle size), glucono -δ-lactone (GDL), pepsin from porcine mucosa, pancreatin from porcine pancreas 8 × UPS, Pefabloc SC, L-serine, phthaldialdehyde, dl-dithiothreitol, sodium tetraborate decahydrate and sodium dodecyl sulphate were purchased from Merck, Sweden and bile bovine powder and amino acid standards H from Fisher Scientific, Sweden.

2.2. Oat protein extraction

The protein extraction was conducted according to the protocol reported by Ma 1983, with minor modifications (Ma, 1983). The oat groats were ground to a particle size of 400 μm. To produce defatted flour, oat flour was mixed with hexane at a ratio of 1:2 (w/v) for one hour at 500 rpm. Centrifugation at 2000 rcf for 15 min was used to separate the hexane phase from the solid fraction. This process was repeated three times. The resulting defatted flour was left to dry under a fume hood overnight to ensure complete evaporation of the hexane. Next, the defatted ground oat was dispersed in deionized water at a ratio of 1:8 (w/v), and the pH was adjusted to 9 using 1 M NaOH. After stirring the mixture for one hour at 500 rpm, it was wet sieved through 150 μm and 50 μm and centrifuged at 2000 rcf for 15 min. The pellet was separated from the supernatant and subjected to one more round of alkaline extraction and wet sieving. The proteins were precipitated from the supernatant by adjusting the pH between 4.5 and 5.0 using 1 M HCl. After stirring for 1 h at 500 rpm, the dispersion was centrifuged at 2000 g for 15 min and the pellet was collected and re-suspended in deionized water followed by pH adjustment to 7 and freeze-drying (LabconcoTM console freeze dryer with a stoppering tray dryer (Missouri, USA) at −10 °C and < 1 mbar pressure, with the collector chamber at −40 °C). The process was repeated until 100 g of protein isolate were produced. The various batches of oat protein isolate (OPI) were combined and stored in a desiccator at room temperature.

2.3. Oat protein isolate composition

The protein content of the extracted oat protein was determined using the Kjeldhal method (Barbano, Lynch, & Fleming, 1991) with a nitrogen to crude protein conversion factor of 5.83 (Tontisirin, MacLean, Warwick, Food, & Agriculture Organization of the United Nations, 2003). The total fat content was determined using the Manjonnier method (Lunder, 1971). The moisture content was determined using approximately 0.5 g of protein powder. The samples were placed in porcelain capsules and weighed, and then dried in an oven at 105 °C for 24 h. The dry residues were weighed after being cooled in a desiccator. The dry residues obtained from the moisture content measurements were used to determine the ash content. The samples were combusted in a furnace for 4 h at 550 °C. After cooling the oven to 100 °C, the samples were transferred to a desiccator and weighed at room temperature.

2.4. Preparation of dispersions

Stock dispersions: The OPI was dispersed in deionized water at a concentration of 12% w/w and stirred overnight at room temperature. In addition, LMP was dispersed in deionized water at a concentration of 4% w/w. The pectin dispersion was stirred for 30 min at 80 °C, followed by overnight stirring at room temperature. The pH of both stock dispersions was adjusted with NaOH 1 M and maintained within the pH range of 6.8 to 7.2.

LMP and OPI dispersions: A volume of the stock OPI dispersion and stock LMP dispersion were mixed to achieve a final fixed OPI concentration of 6% w/w and LMP concentrations of 0.5% w/w, 1.0% w/w, or 1.5% w/w, respectively.

2.5. Preparation of gels

A freshly prepared GDL solution was added into a 20 mL container containing $CaCO_3$ and the mixture was stirred for a few seconds. Subsequently, a volume of the stock OPI dispersion was added to the mixture, followed by the addition of the stock LMP dispersion using a multi-dispenser equipped with a ViscoTip (Eppendorf, Germany) achieving a final fixed OPI concentration of 6% w/w and LMP concentrations of

0.5% w/w, 1.0% w/w, or 1.5% w/w. For the control gels, the volume of OPI stock dispersion was substituted with water. The concentrations of Ca^{2+} corresponded to 100% theoretical crosslinking of the GalA of the pectin, assuming a DM of 32% and complete dissociation of $CaCO_3$. That is, all gels had a fixed R-value of 1, where R is defined as $R = 2[Ca^{2+}]/[COO^-]$. Additionally, GDL was added in stoichiometric equivalence to the $CaCO_3$ (e.g. 15 mM $CaCO_3$ and 30 mM GDL) to achieve complete dissolution of the $CaCO_3$ (Ström & Williams, 2003). The mixtures were stirred for 3 min, and added to the rheometer or poured into cylindrical moulds. The gels poured into the moulds were set at room temperature for 15 h before mechanical testing or use in *in vitro* digestion. The dimensions of the moulds used for assessing the mechanical properties of gels had a diameter of 10 mm and height of 10 mm, whereas the ones used for *in vitro* digestion had a diameter of 3 mm and height of 2.5 mm.

2.6. Rheology

Shear viscosity: The shear viscosity was measured using a DHR-2 rheometer from TA Instruments, DE, USA, equipped with a cone and plate geometry. The cone had a diameter of 40 mm and an angle of 1.9 °C. The gap used was 57 μm. Viscosity measurements were conducted at 20 °C, across shear rates ranging from 0.5 s^{-1} to 1000 s^{-1}. The experiments were performed in triplicates.

Small amplitude oscillatory shear measurements: The evolution of the storage (G') and loss (G'') modulus of the samples were measured at a strain of 0.5% and frequency of 6.28 $rads^{-1}$, using a parallel plate geometry of 40 mm in diameter and a gap of 200 μm. The parallel plate was equipped with a solvent trap to reduce solvent evaporation. Subsequently, the gels were subjected to a frequency sweep from 0.1 to 200 $rads^{-1}$ at t = 60 min and at a strain of 0.5%. All tests were performed at 20 °C. The temperature was controlled using a Peltier plate. The frequency dependence of the gels was evaluated through the linear fitting of the G' curves during frequency sweep, according to Eq. (1):

$$\log G' = n \log \omega + K \tag{1}$$

Where n is the slope and shows the degree of frequency dependency, ω the angular frequency and K the intercept.

2.7. Large deformation

The compression tests were performed using a mechanical tester Instron 5565 A, MA, USA with a 5 kN load cell. The compression rate was 0.5 mm/s. The true stress (σ(t)) and true strain (ε(t)) were calculated using Eqs. (2) and (3), respectively. The Young's modulus (E) was calculated from the slope of the stress - strain curve in the strain region 0.01 to 0.1.

$$\sigma(t) = \frac{Fh}{A_0 h_0} \tag{2}$$

$$\varepsilon(t) = \ln \frac{h}{h_0} \tag{3}$$

Where F is the force, h, the height, h_0 the initial height and A_0 the initial area of the sample. The stress–strain diagrams were plotted and the stress and strain at the break determined from the maximum peaks from the diagrams. The experiment was performed in triplicates in at least two different batches of gels.

2.8. Electron microscopy

The gels were cut into pieces (2–3 mm), fixed in 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in 1% (w/v) osmium tetroxide and dehydrated using a series of ethanol solutions with increasing concentrations. For scanning elecron microscopy (SEM) samples were dried using a Critical Point Dryer (BALTEC CPD 030, Leica, Wetzlar, Germany). The dried gels were then sputter-coated with gold (BALTEC SCD 005, Leica, Wetzlar, Germany) and viewed in a scanning electron microscope (JEOL 6360-LV, Tokyo, Japan) at an accelerating voltage of 20 kV. Multiple SEM images were taken at different magnification (× 6000 and × 40,000) on different parts of each gel.

For transmission electron microscopy (TEM), after dehydration samples were embedded in EPON and ultra-thin sections were prepared, contrasted with lead citrate and uranyl acetate. Samples were observed using a transmission electron microscope (JEOL JEM-1011, Tokyo, Japan) at 80 kV . Multiple TEM images were taken at different magnification (× 20,000 and × 25,000) on different parts of each gel.

2.9. In vitro static digestion protocol

In vitro protein digestion experiments were conducted according to INFOGEST protocol (Brodkorb, Egger, Alminger, et al., 2019) with minor modifications. Initially, 1 g of dispersions or gels was mixed with 1 mL of simulated salivary fluid, water and $CaCl_2$ (3 M). Salivary α-amylase was omitted. Subsequently, the bolus was mixed with 2 mL of simulated gastric fluid, $CaCl_2$ (3 M), HCl (1 M), water and porcine pepsin (1871 U/ mg solid) to achieve an activity of 2000 U/mL in the final mixture. The intestinal phase was simulated by adding 4 mL of simulated intestinal fluid $CaCl_2$ (3 M), NaOH (1 M), water, bovine bile (2.48 mmol bile acids/g) and porcine pancreatin (5.9 U of trypsin/ mg solid) to achieve a trypsin activity of 100 U /mL in the final mixture. The pancreatin mixture was prepared according to Sousa et al. (2023), wherein the pancreatic suspension was treated with ultrasound (45 Hz, 130 W) at room temperature for 5 min and centrifuged at room temperature at 2000 g for 5 min. The supernatant was collected and used. The pH of the samples was recorded before and at the end of the gastric and intestinal phases. To rectify proteolysis originating from enzyme autolysis of the proteases, blank samples using water were included. The experiments were performed at 37 °C. An 0.5 mL aliquot was withdrawn at the end of the intestinal phase, and the enzyme deactivation was achieved using Pefabloc CS with final concentration 5 mmol/ L and heat treatment of 100 °C for 2.5 min. The samples were stored at −20 °C before further analysis. The experiments were performed in at least three different batches of gels.

2.10. Degree of protein hydrolysis

The degree of protein hydrolysis was assessed using the o-phthaldialdehyde (OPA) assay according to Nielsen, Petersen, and Dambmann (2001) with minor modifications. The thawed intestinal digesta were centrifuged at room temperature for 5 min at 9000 g, and the resulting supernatant collected and diluted approximately 90 times.

A reagent was prepared, consisting of 18.8 mL of 50.8 mg/mL sodium tetraborate, 1.3 mg/mL sodium dodecyl sulphate, 0.9 mg/mL dithiothreitol and 0.5 mL of 40 mg/mL OPA in methanol. The final volume was adjusted to 25 mL with distilled water. The assay was performed by mixing 0.4 mL of the diluted supernatant with 3.0 mL of OPA reagent. The mixture was held at room temperature for two minutes, after which absorbance was measured at 340 nm using spectrometer (Cary 60, Agilent Inc., CA, USA). The calibration curve for L-Serine ranged from 0.09 to 0.94 mmol/L. Water was used as blank.

The degree of proteolysis was calculated using Eq. (4). The h value was determined by Eq. (5) and the h_{tot} for oat proteins was found to be 7.74 ± 0.22 mmol of $serine_{(eqv)}$/g of protein after acid hydrolysis without enzymes. The acid hydrolysis was performed by diluting 5 to 10 mg/mL of protein in HCl 6 M and heating at 100 °C for 24 h using a dry block heater (Grant BT3, Cambridge, UK). The parameters α and β were assigned the values of 1.0 and 0.4, respectively, according to Nielsen et al. (2001).

$$DH = \frac{h}{h_{tot}} * 100 \tag{4}$$

The primary amino groups of the samples, expressed as serine equivalent ($ser_{(eqv)}$ NH_2), were calculated using a serine calibration curve. The results were expressed in mmol of serine/ L of digesta after subtracting the blank (204.3 ± 24.9 mmol $ser_{(eqv)}$/ L). This value was then divided by the protein content of the sample.

$$h = \frac{ser_{(eqv)} NH_2 - \beta}{\alpha} \quad (5)$$

2.11. Determination of amino acids

The supernatant of the thawed and centrifuged intestinal digesta (200 μL) were mixed with 2 mL HCl at a final concentration of 6 M HCl in glass tubes. Similarly, 50 mg dry oat protein was mixed with 4 mL HCl at a final concentration of 6 M HCl in glass tubes. After replacing the air inside the tubes with nitrogen, the samples were hydrolysed at 110 °C for 24 h using a heat block. The hydrolysed samples were filtered (0.22 μm, PES, Fisherbrand) and diluted in 0.2 M acetic acid prior injection into LC-MS. The LC-MS was an Agilent 1100 HPLC equipped with a Phenomenex column (C18 250 μm ×4.6 μm × 3 μm), coupled to an Agilent 6120 quadrupole in the SIM positive mode. External calibration curves of 17 mixed amino acids (Amino Acid Standard H, Thermo Scientific) were used for quantification of the amino acids. The separation was conducted at 0.7 mL/min for 40 min using different ratios of mobile phase A (3% methanol, 0.2% formic acid, and 0.01% acetic acid) and mobile phase B (50% methanol, 0.2% formic acid and 0.01% acetic acid). Due to the use of acidic hydrolysis, (i) tryptophan and cysteine could not be recovered and (ii) glutamine and asparagine were co-determined with glutamic and aspartic acid, respectively. Collected data were analysed using the MassHunter Quantitive Analysis software (version B.09.00, Agilent Technologies).

2.12. Statistical analysis

All experiments were conducted in at least two batches, and the results are presented as the mean ±standard deviation. The statistical analysis was performed using two-way ANOVA with the Tukey test, considering pectin content and matrix (dispersion or gel) selected as factor variables for degree of proteolysis and amino acid content. Significance was established at the 5% level ($p < 0.05$). Data analysis was carried out using OriginPro 2022b.

3. Results and discussion

The composition of the extracted protein isolate was; 86.5 ± 0.2% of protein, 8.0 ± 0.1% of fat, 5 ± 0.2% of moisture and 2.6 ± 0.1% of ash. All % in w/w. The pectin concentrations of 0.5, 1 and 1.5% wt were chosen as the concentration range corresponded to mechanically weak gels disappearing during digestion (0.5% wt) and strong gels remaining throughout digestion (1 and 1.5% wt). In addition, the concentration range is typical for calcium induced pectin gels see for example (Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2005; Fraeye et al., 2010b; Vincent & Williams, 2009).

3.1. Rheological properties of dispersions and gels

Pectin dispersions, irrespective of concentration (0.5 to 1.5 w/w%), exhibited close to Newtonian behaviour (Fig. 1). Oat protein dispersion of 6% displayed a shear-thinning behaviour, which suggests the presence of weak protein - protein interactions. The viscosity at 10 s^{-1} of the single pectin and oat dispersions are outlined in Table 1.

The mixed pectin and oat protein dispersions show shear thinning behaviour independent of pectin concentration and at fixed protein concentration of 6%. The viscosity values for only pectin and oat protein dispersions as well as their blends, at shear rate of 1 s^{-1}, are outlined in Table 1. Table 1 outlines the experimental viscosity (η_{exp}) as well as the theoretical values of viscosity (η_{theo}), where the

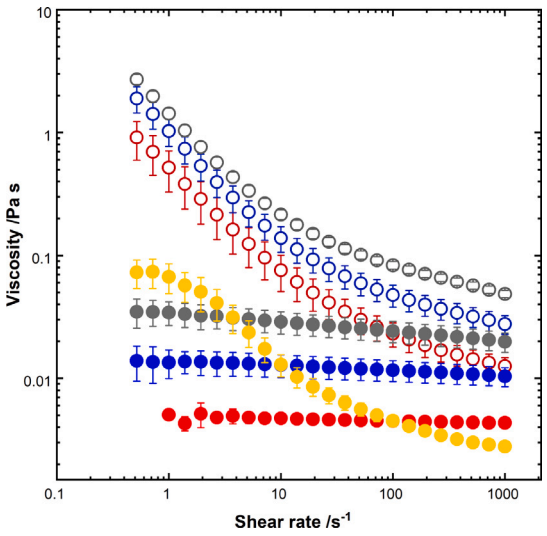

Fig. 1. Flow curves of pectin (closed symbols), oat protein (closed yellow) and pectin - oat protein mixed dispersions (open symbols) where oat protein is fixed at 6% w/w and pectin added to achieve concentrations of 0.5% w/w (red), 1.0% w/w (blue) and 1.5% w/w (grey). The samples were measured at 20 °C.

Table 1
Viscosity measured experimentally of single systems of LMP and OPI, as well as LMP - OPI blends at shear rate of 10 $rads^{-1}$ (η_{exp}), as well as η_{theo} of the LMP-OPI blends where η_{theo} was obtained from the sum of the η_{exp} of the single systems. Δ denotes the difference between η_{exp} and η_{theo}. N.a. stands for non applicable.

LMP - OPI /%	η_{exp} /Pas	η_{theo} /Pas	Δ /Pas
0.5 – 0.0	0.005 ± 0.0004	n.a.	n.a.
1.0 – 0.0	0.015 ± 0.003	n.a.	n.a.
1.5 – 0.0	0.03 ± 0.002	n.a.	n.a.
0.0 – 6.0	0.02 ± 0.0015	n.a.	n.a.
0.5 – 6.0	0.12 ± 0.02	0.025	0.18
1.0 – 6.0	0.2 ± 0.03	0.035	0.17
1.5 – 6.0	0.35 ± 0.02	0.05	0.3

values of η_{theo} are obtained from the sum of the η_{exp} of the single pectin (at different concentrations) and OPI dispersions. The symbol Δ denotes the difference between the viscosity measured (η_{exp}) of the blends and the η_{theo} as obtained considering the viscosity of the single systems. The results show that the viscosity of the pectin - oat protein blends is higher than the sum of the corresponding systems of only pectin and OPI (η_{theo}). Such synergistic behaviour has been observed for whey protein - pectin systems at pH values close to neutral, as well as carrageenan and whey protein (Lizarraga, Piante Vicin, González, Rubiolo, & Santiago, 2006) and attributed to associative interactions between pectins and whey protein. Similar behaviour has however also been observed between pea protein and carrageenan and attributed to segregative phase separation (Musampa, Alves, & Maia, 2007). The viscosity values measured for all samples are below those reported to affect gastric emptying, satiety or nutrient transport to the small intestine (Hoad et al., 2004; Marciani et al., 2000).

G' and G'' of calcium pectin and calcium pectin and OPI, as a function of frequency, and strain sweeps are shown in Fig. 2 (a,b). Both frequency and strain sweeps show that the gels behave as strong networks with little frequency dependence and an abrupt or brittle breakage of the gels at strains above 20%. G' is about one magnitude higher than G'' independent of OPI addition or not. In addition, the G'' of calcium - pectin showed a minimum at intermediate frequencies, a characteristic often associated with strong gels (Clark, 1991). Fitting G' obtained during frequency sweep, according to Eq. (1), gives overall low values of n, but where the value of n is reducing (0.042 ± 0.002;

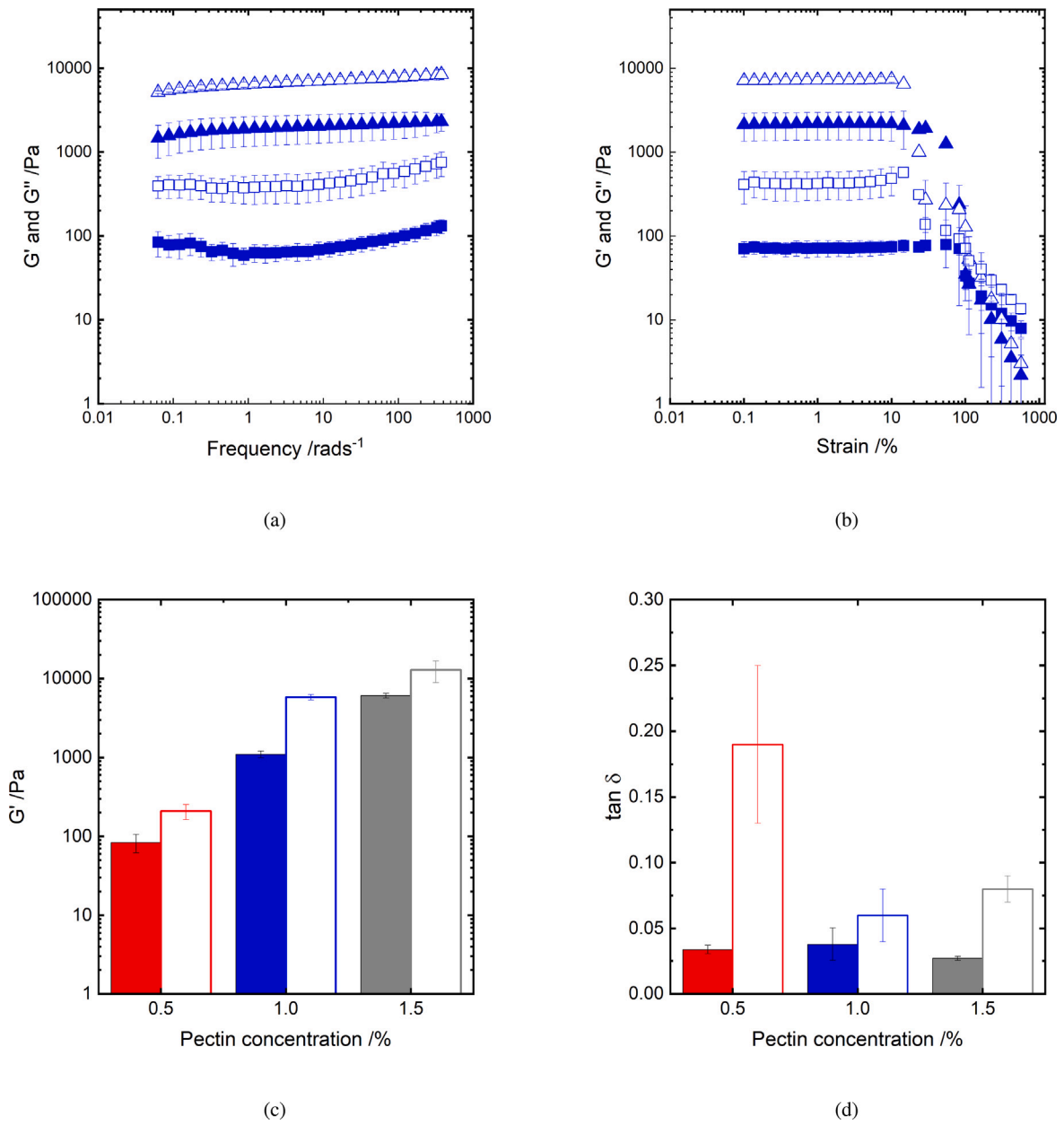

Fig. 2. G' (triangles) and G'' (squares) of gels made of calcium-pectin (closed symbols) and calcium - pectin - OPI (open symbols) as a function frequency (a) and strain (b) where pectin concentration is 1% and OPI 6%. G' of calcium - pectin (closed bars) and calcium - pectin - OPI (open bars) as a function of pectin concentration (c) and tan δ as a function of pectin concentration (d) at t = 60 min. Measurements performed at γ = 0.5% (a), f = 6.28 rads $^{-1}$ (b) f = 6.28 rads $^{-1}$ and γ = 0.5% (c,d), all at$T$ = 20 ° C. Error bars based on three measurements.

0.038 ± 0.002; 0.030 ± 0.001) with increasing pectin concentration (0.5, 1.0 and 1.5% respectively). The values of n increases upon incorporation of 6% OPI to the calcium pectin gels, (0.104 ± 0.004; 0.049 ± 0.001; 0.058 ± 0.001) where pectin concentration increases from 0.05, 1.0 and 1.5%. All fits with (R^2 > 0.94). The addition of OPI to the calcium pectin network is thus increasing the frequency dependence of the calcium pectin network. G' of the calcium - pectin - OPI are higher in absolute values in both the frequency and strain sweeps compared to calcium pectin gels. Fig. 2 c and d show G' and tan δ as a function of pectin concentration at fixed R-value, with and without addition of OPI, at t = 60 min. Fig. 2 c shows that G' increases consistently with pectin concentration, but also with addition of 6% OPI. Tan δ is consistently low for all pectin concentrations (Fig. 2 d), but increased upon addition of OPI, especially for the gel containing the lowest amount of pectin. The data confirms that all gels behave as strong gels, independent of addition of 6% OPI, although OPI addition interrupt the calcium pectin gel, as indicated by the increase in n of the frequency sweep and increased tan δ, especially prominent at low pectin concentrations (0.5%). It should be noted that the addition of calcium to only oat protein did not form a gel.

3.2. Mechanical properties of gels

The Young's modulus, stress and strain at break of the gels increased with increasing pectin concentration at constant R value Fig. 3, and are summarized in Table 2. The results are in line with findings for calcium induced apple pectin (Fraeye et al., 2010a). Increased stress at break of calcium-alginate gels has been correlated with the junction zones themselves, rather than with the polymer segments between the junction zones (Zhang, Daubert, & Foegeding, 2005), which is determined in small deformation. Increased stress at break can instead be related to distribution of force over more load bearing junctions or

Table 2
Mechanical properties of calcium - pectin and calcium - pectin oat protein gels. Mean values of Young's modulus (E), stress at break (σ_{Br}), and strain at break (ϵ_{Br}).

LMP - OPI % w/w	E (kPa)	σ_{Br} (kPa)	ϵ_{Br} (-)
0.5 – 0.0	17.3 ± 6.0	6.4 ± 1.6	0.26 ± 0.04
1.0 – 0.0	67.2 ± 6.9	26.9 ± 5.0	0.31 ± 0.04
1.5 – 0.0	125.3 ± 8.2	70.0 ± 9.2	0.43 ± 0.01
0.5 – 6.0	14.2 ± 1.9	2.3 ± 0.3	0.12 ± 0.03
1.0 – 6.0	69.4 ± 14.1	12.2 ± 2.8	0.19 ± 0.01
1.5 – 6.0	128.5 ± 4.8	29.6 ± 1.0	0.23 ± 0.01

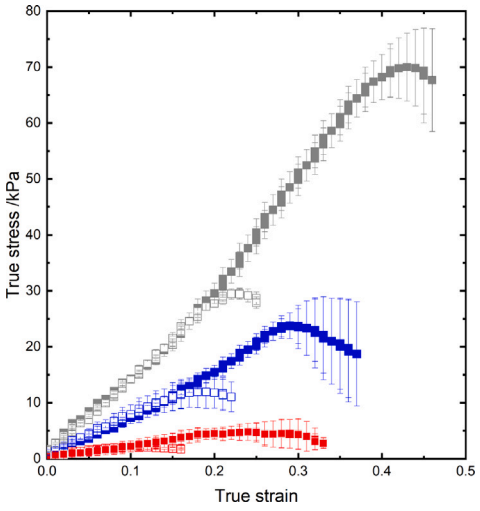

Fig. 3. Stress - strain curves of calcium - pectin gels (closed squares) and calcium - pectin - OPI gels (open squares) with pectin concentrations of 0.5% w/w (red), 1.0% w/w (blue) and 1.5% w/w (black).

related to the gel having junction zones that are stronger, for example, via increased lateral aggregation.

The addition of OPI to the calcium pectin gels reduced both stress and strain at break without affecting the Young's modulus (Table 2, Fig. 3). The alterations in the strength and deformation capacity of the gels suggest that oat proteins act as disruptive particles upon large deformation of the calcium - pectin network. This aligns with findings in other studies, such as inulin - whey protein gels, where the addition whey (1%–4% w/w) disrupts the formation of a firm inulin gel (Glibowski, 2009). The strength of the calcium-pectin oat protein gels at pectin concentrations of 1.5% are equivalent to gel strengths shown to impact satiety and gastric emptying (Hoad et al., 2004; Ström et al., 2009), whereas the gel strength of the calcium-pectin oat protein gels at pectin concentration of 1 and 0.5% is below.

3.3. Microstructure of gels

In Fig. 4 the SEM (a–b) and TEM (c–d) images depict the microstructure of calcium - pectin gel (1.0% w/w) and a representative calcium - pectin - OPI gel (1.0 – 6.0% w/w). The calcium pectin gel morphology observed by SEM showed no distinctive structural features (Fig. 4 a). The addition of oat proteins forms distinctive clusters in the calcium pectin gels, appearing as a group of relatively spherical entities with an approximate size of 1 μm (Fig. 4 b). TEM imaging offers insights into the nanostructure of the gels. The calcium - pectin gel, Fig. 4 c, reveals thin, elongated strands with a high degree of inter-connectivity. This open gel structure agrees with that previously shown for this type of gels (Löfgren, Guillotin, Evenbratt, Schols, & Hermansson, 2005; Mansel et al., 2015). In the case of the calcium - pectin - OPI gels (Fig. 4 d), spherical clusters with a relatively low degree of connectivity were observed, which were attributed to oat protein, indicating limited network formation by the protein itself. Protein particles are larger than the pectin strands and are distributed unevenly throughout the matrix.

The results from rheology, mechanical properties and microstructure of the calcium - pectin - OPI gels suggest that the OPI act as disruptive fillers within the calcium - pectin gel, as judged by the reduction in mechanical strength and strain at break. The oat protein particles are considerably larger than the voids in the calcium - pectin network, as observed in the microscopy images. The incorporation of OPI in calcium - pectin gels is less detrimental for smaller deformations where the higher G' can be explained by a higher dry content or segregative phase separation between the oat protein and pectin, as indicated by the increased viscosity of the pectin - OPI dispersions, than expected from the viscosity obtained by the single biopolymer dispersions. This needs to be changed in case we show the confocal images.

3.4. Effect of pectin on in vitro digestion of oat proteins

Free amino acids and oligopeptides released at the end of the *in vitro* gastrointestinal digestion were quantified utilizing the OPA assay on the liquid fraction of the digesta. The OPA assay estimates the quantity of primary amino groups released during protein hydrolysis (Church, Porter, Catignani, & Swaisgood, 1985), which correlates with the proportion of amide bonds cleaved.

Fig. 5 shows that the degree of proteolysis of only OPI is close to 45%. The result of degree of proteolysis of the OPI cannot be directly compared with existing literature due to (a) few studies available on oat protein digestion using the static INFOGEST protocol (Darewicz, Pliszka, Borawska-Dziadkiewicz, Minkiewicz, & Iwaniak, 2022; Sánchez-Velázquez, Cuevas-Rodríguez, Mondor, Ribéreau, Arcand, Mackie, & Hernández-Álvarez, 2021), and (b) few studies using INFOGEST and the degree of proteolysis to follow digestion. However, Sanchez-Velazquez and co-workers studied oat protein digestion using the INFOGEST protocol and they found a high proportion of peptides (50%) with molar mass > 1300 g/mol remaining after digestion (Sánchez-Velázquez et al., 2021). The authors used electrophoresis and size exclusion chromatography to analyse the digestive products (Sánchez-Velázquez et al., 2021), and their results indicate a non complete oat protein hydrolysis, similar to our results. Similarly, Darewicz and co-workers showed 53% of intact proteins from gastric phase and 10% of intact oat protein after duodenal phase using the INFOGEST protocol on oat proteins (Darewicz et al., 2022), again indicating incomplete hydrolysis.

The addition of pectin as dispersion or the incorporation of OPI in calcium pectin gels did not change (at statistical significance level of $p > 0.05$) the degree of proteloysis of the oat protein (Fig. 5), except for the sample with oat proteins incorporated into a calcium-pectin gel of 1% pectin. The results show that the presence of pectin as dispersion, or oat protein incorporated within the calcium pectin network of 0.5 and 1.5% pectin, is not hindering digestion of the protein, as evaluated using the static INFOGEST model. The peptides present in the supernatant can *invivo* be absorbed, if small enough, or further hydrolysed to amino acids by brush border enzymes (the latter not included in this study).

Visual observations of the gels (Fig. 6) supported the findings of the mechanical strength of the gels, where 0.5% pectin - OPI gels displayed a weakness and rapid disintegration during early stages of gastric processing. In contrast, protein gels containing 1% and 1.5% pectin showed a greater firmness and maintained their shape and appearance throughout gastric and intestinal stages, nevertheless, this did not alter the degree of proteolysis of the OPI for the 1.5% pectin gel. Although there is a statistical significant difference in proteolysis of the gel containing 1% pectin, the results from amino acid analysis are similar for all gels (see discussion further down).

Other studies have shown that whey protein emulsion gels with different mechanical properties disintegrated differently under simulated gastric dynamic conditions. The "soft" gels dissolved faster,

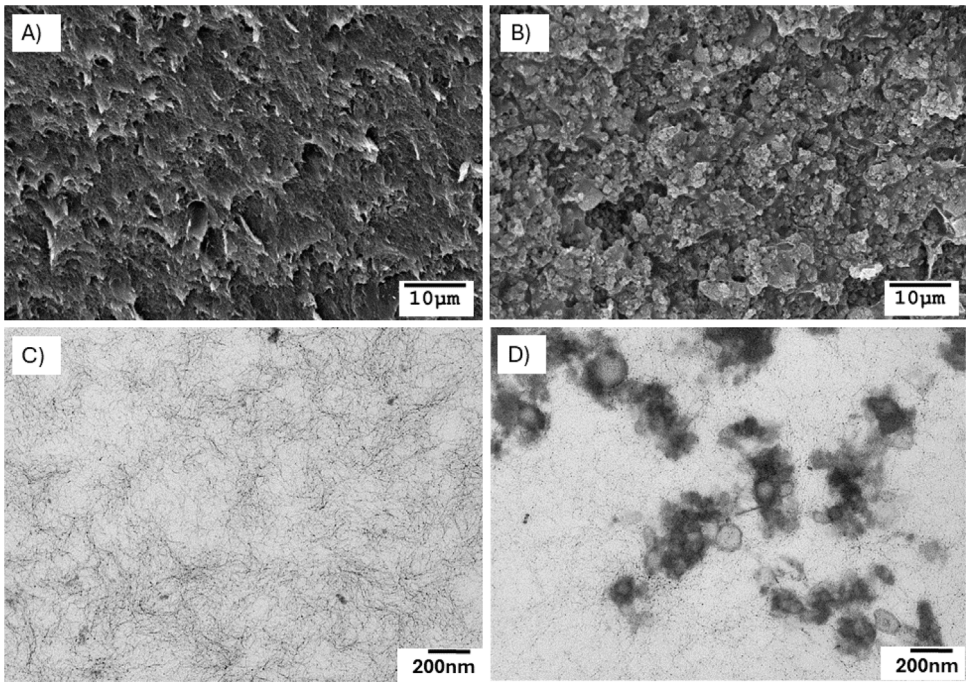

Fig. 4. Electron microscopy images depicting a representative calcium - pectin (1.0 pectin % w/w) and calcium - pectin - oat protein gel (1.0 – 6.0% w/w), scanning electron microscopy images on the top row (a–b) and transmission electron microscopy images on the bottom row (c–d).

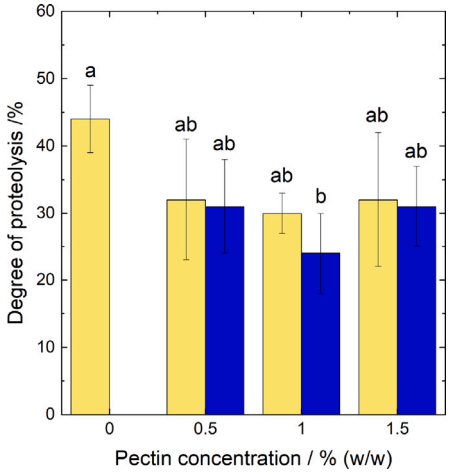

Fig. 5. Degree of oat protein proteolysis with varying pectin content (0.5% w/w, 1.0% w/w, 1.5% w/w). Dispersion (yellow bars) and gel systems (blue bars). * Letters a and b indicate that values that do not share a letter are significantly different ($p < 0.05$).

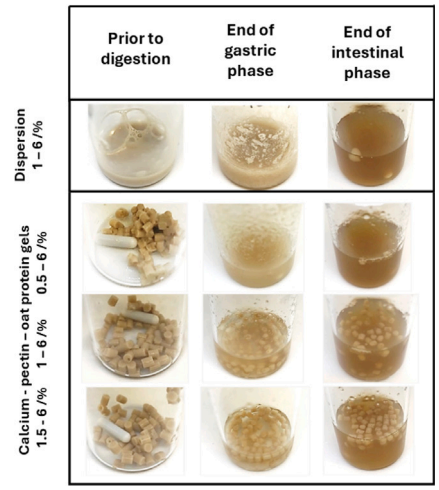

Fig. 6. Visual appearance of calcium - pectin - oat protein dispersion and gels (3 × 2.5 mm) prior digestion, at the end of the gastric phase and at the end of the intestinal phase.

leading to the formation of smaller peptides and the release of free oil droplets (Guo, Ye, Lad, Dalgleish, & Singh, 2014). Furthermore, research investigating whey and egg protein gels and dispersions under gastric conditions showed that the rate of protein hydrolysis is significantly higher in dispersions compared to gel matrices (Luo, Boom, & Janssen, 2015). The authors suggested that the protein becomes immobilized within the gel matrix (Luo et al., 2015), thereby restricting the deep penetration of the pepsin and the diffusion of the digestion products out of the matrix (Luo, Borst, Westphal, Boom, & Janssen, 2017). The mentioned studies primarily focus on the gastric phase without extending their analysis to the intestinal phase to assess final digestibility. In contrast, our study excludes gastric time points and evaluates only the final digestion products.

Our results show that digestive enzymes can act on proteins incorporated within calcium - pectin gels, and resulting peptides diffuse out of the gel. Indeed, the electron microscopy images show an open calcium - pectin and calcium - pectin - oat protein gel, with visible voids of 10^{ths} of nm or more, well above the reported hydrodynamic radii of pepsin and trypsin of 3.0 nm and 1.9 nm, respectively (Gtari, Bey, Aschi, Bitri, & Othman, 2017). Sánchez-Velázquez et al., whom used the INFOGEST protocol to study oat protein digestion as a function of cooking, showed that 90% of the peptides were of a size of < 6 500 $gmol^{-1}$ after intestinal digestion (Sánchez-Velázquez et al., 2021). This molar mass of a peptide corresponds to a physical size of < 1.4 nm (Erickson, 2009), thus similarly to the digestive enzymes well below the pore size of the calcium pectin oat protein gel. The results suggest that digestive enzymes and peptides can diffuse through the calcium pectin network.

The calcium - pectin network itself remains intact in case of the higher pectin concentrations (Fig. 6). The intact calcium pectin network

is expected given their mechanical strength but the results also indicate that there is not enough ion exchange to deplete the calcium from the network. Similar results have been obtained for calcium - alginate networks (Lopez-Sanchez, Fredriksson, Larsson, Altskär, & Ström, 2018). It should be noted that the size of the gels (3 × 2.5 mm) create a large surface area for the enzymes to act. However, even if the degree of proteolysis is not affected by the gel matrix, gastric emptying rate (which was not studied) may be affected, especially for the gel with pectin concentration of 1.5%.

The type of amino acid, and their amount, obtained after digestion of only OPI, pectin and OPI incorporated in calcium pectin network were compared to the amino acid composition of non digested OPI. The results show that the amino acid composition is similar in all samples, independent of OPI being digested on its own, in presence of pectin or incorporated in a calcium pectin gel. The results (Tables 3 and 4) show that there is no interaction between oat protein or peptide and pectin or retention of a specific peptide or protein within the calcium pectin gel, that is strong enough to alter the amino acid or peptide profile in the supernatant of the intestinal digesta obtained using the static INFOGEST protocol.

The amino acid profile and quantity of each amino acid agree relatively well with literature where glutamine concentration in the OPI is present at high concentrations, followed by leucine and aspartic acid. The value we obtained (20.7%) for the glutamine is however a little lower than the value of 25% reported by Pettersson and co-workers (Pettersson, Lindberg, Thomke, & Eggum, 1996), but similar to the value reported by Runyon and co-workers (20.4%) for defatted oat flour (Runyon, Sunilkumar, Nilsson, Rascon, & ahl, 2015). Among the essential amino acids we obtain similar values for histidine, threonine, valine, iso-leucine, leucine, lysine and methionine but somewhat higher values of phenyl alanine than those reported by Runyon and co-workers (Runyon et al., 2015).

The absolute amount of the amino acids present in the supernatant of the intestinal digesta was corrected for the enzymes added to the INFOGEST protocol and compared to the absolute amount of amino acids obtained from the non pre - digested OPI. The results show no statistical difference between the quantity of amino acid obtained from the digested OPI, OPI in presence of pectin and OPI incorporated in calcium pectin gels after acid hydrolysis, Fig. 7. Our interpretation of the results from degree of proteolysis of the OPI, and quantification of the amino acid analysis of the supernatant obtained after gastric and intestinal digestion, is that the majority of the initial OPI is present in the supernatant, predominantly as peptides given the degree of proteolysis. The results from the amino acid composition suggest that even if proteolysis is lower in the OPI pectin gel of 1%, the peptides can be released similarly from the gels independent of pectin concentration. The peptides present in the supernatant can *invivo* be absorbed, if small enough, or further hydrolysed to amino acids by brush border enzymes (the latter not included in this study).

The results obtained from the rheology indicate a weak associative or segregative interaction between oat protein and pectin based on viscosity data. Mechanical properties and microscopy images show the oat protein acts as disruptive fillers within the calcium pectin gel. The *invitro* digestion analysis agree well with an open calcium pectin network, not hindering proteolysis of the oat proteins.

4. Conclusions

The viscosity of LMP and OPI dispersions is higher than expected from the viscosity of only LMP and OPI, respectively. The incorporation of OPI at 6% in calcium pectin gels of pectin concentrations up to 1.5% show a behaviour attributed to strong gels, typical of calcium - LMP gels, even if tan δ and frequency dependence of G' increased. The mechanical properties of the calcium - pectin - OPI gels are reduced in terms of both stress at break and fracture at break, as compared to calcium pectin gels, suggesting the oat protein being present as inactive fillers. The degree of proteolysis of the OPI is independent of pectin addition as dispersion or OPI being incorporated within the calcium - pectin gel, suggesting no strong interaction between pectin and OPI, and a calcium - pectin - OPI network being large enough for both digestive enzymes to diffuse into the gel, to be able to act on the protein and resulting peptides or amino acids to diffuse out of the gel during the time of the gastric and intestinal *in vitro* digestion. Weak or no interaction between pectin and OPI is confirmed by the rheology and mechanical properties of the gels. The results show the successful incorporation of oat protein into calcium pectin gels, while maintaining digestibility of the protein as evaluated using the INFOGEST static protocol.

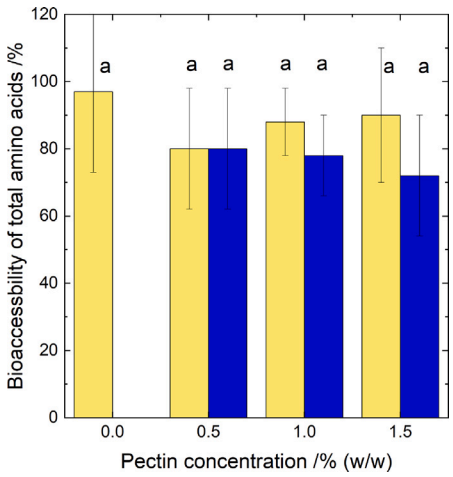

Fig. 7. Bioaccessibility (%) of total amino acids from dispersion (yellow bars) and gel systems (blue bars) with different pectin concentrations (0.5, 1.0, 1.5% w/w) at the end of the intestinal phase. * Letter a indicate that values are not significantly different ($p < 0.05$).

CRediT authorship contribution statement

Marina Marinea: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Patricia Lopez-Sanchez:** Writing – review & editing, Supervision, Methodology, Investigation. **Diana Ortiz:** Investigation. **Karin Larsson:** Writing – review & editing, Investigation, Formal analysis. **Anna Ström:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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.

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Table 3
Concentration (g/100 g of protein) of non - essential amino acids of non digested OPI and that present in the supernatant of *in vitro* digested OPI, pectin - OPI dispersions and calcium - pectin - OPI gels.

	Asp	Glu	Ser	Gly	Arg	Ala	Tyr	Pro
Acid Hydrolysate								
OPI	7.7 ± 0.2[a]	20.7 ± 0.7[a]	4.5 ± 0.2[a]	4.1 ± 0.4[a]	5.7 ± 0.3[a]	4.2 ± 0.3[a]	4.0 ± 0.2[a]	4.2 ± 0.2[a]
Dispersions								
0.0 – 6.0%	7.7 ± 2.3[a]	19.6 ± 3.5[a]	4.2 ± 1.2[a]	4.0 ± 1.2[a]	4.8 ± 0.7[ab]	4.2 ± 1.6[a]	5.8 ± 0.3[a]	3.6 ± 0.8[a]
0.5 – 6.0%	6.1 ± 1.8[a]	18.7 ± 1.8[a]	3.3 ± 1.1[a]	2.8 ± 1.7[a]	4.5 ± 1.0[ab]	3.3 ± 0.9[a]	3.8 ± 0.2[a]	3.6 ± 0.8[a]
1.0 – 6.0%	7.8 ± 1.4[a]	19.4 ± 0.6[a]	4.2 ± 0.9[a]	3.7 ± 1.6[a]	4.8 ± 0.5[ab]	3.9 ± 0.8[a]	3.5 ± 0.6[a]	3.4 ± 0.4[a]
1.5 – 6.0%	8.1 ± 2.4[a]	20.2 ± 2.5[a]	4.4 ± 1.4[a]	4.1 ± 2.1[a]	4.6 ± 0.3[ab]	3.9 ± 1.0[a]	3.9 ± 1.3[a]	3.4 ± 0.8[a]
Gels								
0.5 – 6.0%	5.7 ± 1.1[a]	20.8 ± 2.7[a]	3.3 ± 0.2[a]	2.5 ± 0.7[a]	4.5 ± 0.7[ab]	3.4 ± 0.2[a]	3.6 ± 1.5[a]	3.2 ± 0.4[a]
1.0 – 6.0%	5.4 ± 1.4[a]	19.4 ± 2.2[a]	3.3 ± 0.2[a]	2.6 ± 0.5[a]	3.0 ± 1.5[b]	3.4 ± 0.1[a]	3.8 ± 1.8[a]	3.0 ± 0.6[a]
1.5 – 6.0%	5.2 ± 1.6[a]	18.7 ± 2.2[a]	3.2 ± 0.6[a]	2.2 ± 0.6[a]	3.9 ± 0.2[ab]	3.0 ± 0.3[a]	3.1 ± 1.7[a]	2.8 ± 0.6[a]

* Letters a – b indicate that values not sharing a letter in the same column are significantly different ($p < 0.05$).

Table 4
Concentration (g/100 g of protein) of essential amino acids of non digested OPI and that present in the supernatant of *in vitro* digested OPI, pectin - OPI dispersions and calcium - pectin - OPI gels.

	His	Thr	Val	Phe	Ile	Leu	Lys	Met
Acid Hydrolysate								
OPI	2.2 ± 0.1[a]	3.4 ± 0.1[a]	5.1 ± 0.2[a]	5.3 ± 0.1[a]	4.0 ± 0.1[a]	7.9 ± 0.1[a]	3.9 ± 0.1[a]	1.7 ± 0.0
Dispersions								
0.0 – 6.0%	1.8 ± 0.3[ab]	3.5 ± 0.9[a]	5.3 ± 1.8[a]	5.9 ± 0.9[a]	4.3 ± 1.1[a]	7.7 ± 1.8[a]	3.7 ± 1.0[a]	<LOQ
0.5 – 6.0%	1.4 ± 0.4[b]	3.2 ± 0.4[a]	4.4 ± 0.8[a]	4.9 ± 0.6[a]	3.5 ± 0.4[a]	6.6 ± 0.7[a]	3.4 ± 0.1[a]	<LOQ
1.0 – 6.0%	1.8 ± 0.2[ab]	3.4 ± 0.6[a]	4.8 ± 0.9[a]	4.9 ± 0.2[a]	3.8 ± 0.7[a]	6.9 ± 1.3[a]	3.6 ± 0.7[a]	<LOQ
1.5 – 6.0%	1.6 ± 0.1[ab]	3.4 ± 0.8[a]	4.9 ± 1.3[a]	5.2 ± 0.8[a]	3.7 ± 1.0[a]	7.1 ± 1.7[a]	3.7 ± 1.4[a]	<LOQ
Gels								
0.5 – 6.0%	1.4 ± 0.1[b]	2.6 ± 0.4[a]	4.0 ± 0.4[a]	5.1 ± 1.1[a]	3.2 ± 0.5[a]	6.5 ± 0.5[a]	3.1 ± 0.8[a]	<LOQ
1.0 – 6.0%	1.5 ± 0.2[b]	2.6 ± 0.5[a]	4.1 ± 0.3[a]	4.7 ± 1.0[a]	3.2 ± 0.5[a]	6.3 ± 0.6[a]	3.1 ± 0.8[a]	<LOQ
1.5 – 6.0%	1.3 ± 0.2[b]	2.6 ± 0.7[a]	3.6 ± 0.5[a]	4.1 ± 0.9[a]	2.7 ± 0.6[a]	5.6 ± 0.8[a]	3.1 ± 1.0[a]	<LOQ
Nutritional Recommendations								
WHO/FAO/UNU[1]	1.5	2.3	3.9	3.8	3.0	5.9	4.5	1.6

* Letters a – b indicate that values not sharing a letter in the same column are significantly different ($p < 0.05$).
** Abbreviation <LOQ means "below the Limit Of Quantification".
[1] Mean nitrogen requirement of 105 mg nitrogen/kg per day (0.66 g protein/kg per day) (WHO/FAO/UNU, 2007).

Data availability

Data will be made available on request.

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