

Impact of dietary fibers on the properties and proteolytic digestibility of lactoferrin nano-particles

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ABSTRACT

The application and development of biopolymer-based particulate delivery systems is receiving considerable attention from scientists and manufacturers seeking to engineer healthier and safer foods. Present work focused on the fabrication and proteolysis of bovine lactoferrin nano-particles onto which pectins, alginate and carrageenan were electrostatically deposited. DLS, CD and AFM were used to ascertain the formation of spherical structures with varying zeta potentials and modified protein folding. SDS-PAGE analyses of samples collected during simulated human gastro-duodenal digestion revealed that in the case of high and low methoxy pectin particle zeta potential values (−35.4 mV and −50.3 mV respectively) were found to be inversely linked to lactoferrin digestion rate. Moreover, the electrostatic binding of food grade iota-carrageenan onto lactoferrin nano-particles was found to yield highly charged particles (zeta = −69.2 mV) with low surface roughness and to enable a proportion of the lactoferrin to resist 1 h of simulated gastric digestion. Overall, these findings suggest that electrostatic biopolymer interactions may modulate protein digestion and possibly facilitate controlled protein delivery to the upper gastrointestinal tract or have implications to protein nutritional value.

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1. Introduction

The application and development of biopolymer-based particulate systems is receiving considerable attention from scientists and manufacturers in the food, personal care and pharmaceutical industries (Bengoechea, Jones, Guerrero, & McClements, 2011; Benshitrit, Levi, Tal, Shimoni, & Lesmes, 2012; Chen, Remondetto, & Subirade, 2006; Goldberg, Langer, & Jia, 2007; Jones & McClements, 2010; Lesmes & McClements, 2009; Matalanis, Jones, & McClements, 2011). To date, different particulate systems have been described in literature, varying in size, structure, composition and physico-chemical properties which offer numerous functionalities, particularly as vehicles for oral delivery of bioactive compounds. This novel and vivid field of research continuously seeks to overcome the limitations of current systems and establish the underlying principles behind their functionality, in order to facilitate their rational design, as recently reviewed (Jones & McClements, 2010; Lesmes & McClements, 2009; Livney, 2010; McClements, 2010; Ubbink,

Burbidge, & Mezzenga, 2008; Velikov & Pelan, 2008). Moreover, nanotechnologies and nano-delivery vehicles to be implemented in food manufacturing raise not only scientific and technological challenges but also an evident need for regulation and evaluation of their potential risks and biological fate, as recently advocated (Cushen, Kerry, Morris, Cruz-Romero, & Cummins, 2012; McClements & Rao, 2011).

To this end, the use of dietary proteins and carbohydrates has received great interest, as all ingredients are generally considered safe and provide an excellent platform to affect human health while delivering bioactive compounds such as nutraceuticals (Chen et al., 2006; Jones & McClements, 2010; Livney, 2010; Matalanis et al., 2011). Many pathways have been reported to lead to the successful fabrication of protein–polysaccharide particulate systems. Generally, the association of protein and polysaccharide molecules may occur chemically through covalent bonds (e.g., Maillard reaction) or by harnessing electrostatic biopolymer interactions to form biopolymer complexes, core–shell nano-particles and nano-laminated coatings (Dickinson, 2008; Jones, Lesmes, Dubin, & McClements, 2010; Jones & McClements, 2010; Peinado, Lesmes, Andrés, & McClements, 2010). Conceptually, this route utilizes the attractive forces between oppositely charged biopolymers to produce mixed structured particulate systems (Fig. 1).

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Recently, it has been shown, that electrostatic interactions between bovine lactoferrin (LF) and various food-grade anionic polysaccharides, such as pectin and alginate, can be harnessed to form various particulate systems and modulate emulsion functionality (Bengochea, Peinado, & McClements, 2011; Peinado et al., 2010; Tokle, Lesmes, & McClements, 2010). This iron binding glycoprotein has a molecular weight of 80 kDa, a relatively high isoelectric point (pI ~ 8.2) compared to whey proteins and various biological activities such as anti-microbial, anti-inflammatory, anti-tumor, immune-modulatory, anti-oxidant and enzymatic activities (Nagpal et al., 2011; Tomita et al., 2009; Wakabayashi, Yamauchi, & Takase, 2006). Moreover, LF has been found to be a source of bioactive peptides, e.g., lactoferricin that can be released during gastric digestion (Conesa et al., 2010; Elbarbary et al., 2010; Flores-Villasenor et al., 2010; Nagpal et al., 2011). Thus, LF digestion and the digestion of LF-based nano-particles in the alimentary tract is of importance as digestion is likely to be affected by various colloidal and physiological aspects, as previously shown for other proteins (Dupont et al., 2010; Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; Mackie & Macierzanka, 2010).

The objective of the present work was to fabricate and characterize LF-based nano-particles with anionic low methoxy pectin (LMP), high methoxy pectin (HMP), sodium alginate (ALG) and iota-Carrageenan (CAR). Characterization focused on evaluating the resistance to digestion of LF biopolymer complexes under simulated gastric and duodenal conditions. The main hypothesis was that the structural modifications of LF and its electrostatic binding with the anionic dietary fibers would alter LF susceptibility to enzymatic proteolysis under simulated human digestion conditions.

2. Experimental

2.1. Materials

Bovine LF was kindly donated by FrieslandCampina Dom, cat# 13230 (manufacturer determined product to contain 95% Protein, 91% LF purity). The polysaccharides (PS) used were GENU® Pectin type 121 slow set, GENU® Pectin type LM-12 CG and GENU® carrageenan type CJ were kindly donated by CPKelco. Alginic acid sodium salt from brown algae (Cat# A0682.) was purchased from Sigma. NaOH, HCl and all other reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation of biopolymer solutions and nano-particle suspensions

LF-based nano-particles were fabricated based on the protocol described previously (Peinado et al., 2010). In general, protein and polysaccharide solutions were prepared by dissolving weighed amounts of lactoferrin (LF), high-methoxypectin (HMP), low-methoxy pectin (LMP), sodium alginate (ALG) or iota-carrageenan

(CAR) in double distilled water (DDW) overnight under gentle stirring. Samples of 0.4% (w/w) LF solution were verified to be at pH 7 (pH corrected using HCl and NaOH as needed) and heated for 20 min at 90 °C, allowed to cool to ambient conditions and then pH was adjusted to pH 8. Polysaccharide solutions of various concentrations were carefully balanced to reach a pH of 8 and then added to the LF solution samples and equilibrated overnight before size, turbidity and ζ -potential analyses were performed.

2.2.2. Characterization of biopolymer particle properties

Solutions and suspensions of LF-based nano-particles were studied in terms of turbidity, particle size and charge (ζ -potential) using dynamic light scattering (DLS) and morphology using intermittent atomic force microscopy.

2.2.2.1. Turbidity measurements. The turbidity of the samples was analyzed using a UV/visible spectrophotometer (OPTIZEN POP, MECASYS) at a wavelength of 600 nm. Double distilled water was used as a blank reference.

2.2.2.2. Particle size and charge measurements. Particle size (Z-average diameter) and charge (ζ -potential) were determined using a combined DLS and particle electrophoresis instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK). Samples were diluted (if necessary) with filtered double distilled water then equilibrated for 1 min inside the instrument before dynamic light backscattering (detection angle = 173°) data was collected over at least 10 sequential readings. The Z-average particle diameter was calculated by the instrument using the Stokes–Einstein equation. The electrical charge (ζ -potential) of the particles was calculated using the Smoluchowski model through electrophoretic mobility measurements made in a capillary electrophoresis device inserted into the DLS instrument.

2.2.2.3. Structural characterization using Circular dichroism (CD) and Atomic Force Microscopy (AFM). Changes in secondary and tertiary structure of 0.2% (w/w) LF samples before and after processing (heat and pH adjustments) were studied by circular dichroism (CD). CD spectra were recorded using a CD spectropolarimeter (JASCO J710, Jasco Ltd., Japan) and the collected data was analyzed using Dichroweb: the online Circular Dichroism Website <http://dichroweb.cryst.bbk.ac.uk> accessed on October 11th, 2011 and described in (Provencher & Gloeckner, 1981; Sreerama & Woody, 1993; Whitmore & Wallace, 2008).

After production of the nano-particle suspension, samples were diluted with double distilled water 1:50 (v/v) and 2 μ L were placed on a freshly cleaved mica slide (SPiChemTM Mica, Grade V-4, 9.9 mm discs $\times$ 0.15 mm thick; West Chester, PA). Specimen slides were then stored in a desiccator before being placed on the AFM table for scanning. The air dried mica specimens were then scanned using JPK Nano Wizard II AFM (JPK Instruments Inc., Germany)

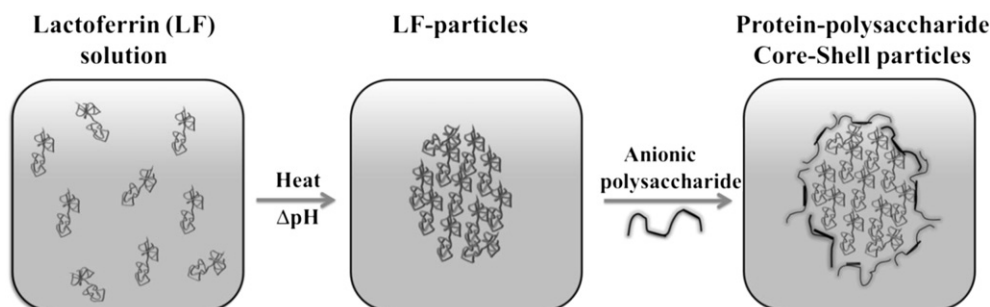


Fig. 1. A schematic illustration of the process for the fabrication of lactoferrin-polysaccharide nano-particles.

mounted with a silicone tip (μ Masch NSC21/NoAl, Spain; tip curvature $R_c < 10$ nm; typical force constant 17.5 N/m). Scanning of various scan areas ($0.5 \mu\text{m} \times 0.5 \mu\text{m}$, $1 \mu\text{m} \times 1 \mu\text{m}$, $2 \mu\text{m} \times 2 \mu\text{m}$, $5 \mu\text{m} \times 5 \mu\text{m}$, and $10 \mu\text{m} \times 10 \mu\text{m}$) was performed at intermittent mode in air and images were produced using Nano Wizard[®] control software v.4. Average and RMS surface roughness of samples was determined based on sixty cross sections performed on different $1 \mu\text{m} \times 1 \mu\text{m}$ height AFM measurements. Roughness values are presented as mean $\pm$ SD ($n = 60$) and statistical analyses of these values (one way ANOVA and *t*-test) were performed using excel Microsoft version 2010.

2.2.3. *In vitro* digestion of LF-based nano-particles

In line with the research aims, suspensions containing 0.2% (w/w) heat-treated LF supplemented with 0.2% (w/w) polysaccharide were subjected to *in vitro* digestion mimicking gastric and duodenal conditions of a healthy adult. Samples of 0.2% (w/w) native LF solutions or 0.2% (w/w) heat-treated LF were used as controls.

2.2.3.1. Simulated gastric digestion. 5 mL samples of LF 0.2%(w/w)+PS0.2%(w/w) were dissolved in 4 mL of a simulated gastric fluid (SGF) (0.3 M NaCl, pH 2.5), and then the pH was adjusted to 2.5 using 0.5 M HCl. After incubation at 37 °C for 10 min, 100 μ L of pepsin solution (77.5 mg/mL, pre-dissolved in SGF 0.15 M, pH 2.5) were added to reach a final concentration of 170 U of pepsin per mg of protein in the digestion mix. Samples were placed in an orbital shaking incubator (37 °C, 170 rpm) and aliquots of 0.1 mL were withdrawn before the addition of pepsin, right after pepsin addition, and 1, 2, 5, 10, 20, 40 and 60 min after the enzyme addition. Digestions were stopped by raising the pH to 7.5 by addition of 0.5 M ammonium bicarbonate (20 μ L) inactivating the pepsin (Macierzanka et al., 2009; Mandalari, Adel-Patient, et al., 2009).

2.2.3.2. Simulated duodenal digestion. Chyme acquired after 60 min of *in vitro* gastric digestion (7 mL) was used as the starting material for duodenal digestion following an inactivation step in which sample pH was elevated to 7.5 (using 1 M and 0.1 M NaOH) and maintained for 5–10 min. Samples of 7 mL of inactivated gastric sample were supplemented with Bis-Tris buffer, 1.0 M CaCl₂, solutions of bile salts (sodium taurocholate and sodium glycodeoxycholate), simulated duodenal fluid (SDF, pH 6.5, 0.15 M NaCl) and enzymes (α -chymotrypsin and trypsin) to reach a final volume of 9.993 mL at pH 6.5 with specified concentrations (7.4 mM bile salts, 0.4 U of bovine α -chymotrypsin per mg of test protein and 34.5 U of porcine trypsin per mg of test protein). Digestions were performed in a shaking incubator (37 °C, 170 rpm) for 30 min with 0.1 mL

aliquots collected at 0.3, 1, 2, 5, 10, 15 and 30 min. Aliquots were mixed with Phenylmethanesulfonyl fluoride solution (PMSF) and placed on ice to cease the proteolytic digestion (Macierzanka et al., 2009; Mandalari, Adel-Patient, et al., 2009; Mandalari, Mackie, et al., 2009). All digestion samples were stored in a -20 °C freezer until further analysis.

2.2.4. Characterization of proteolysis products SDS-PAGE

Comparison of the peptide breakdown profiles in the chyme formed during the various digestion samples was based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All chemicals and gels used for SDS-PAGE analysis were purchased from Invitrogen Ltd. (Paisley, UK). Gels were run for 35 min at 120 mA/gel and 200 V. A continuous buffer system was used, consisting of 50 mL of $20 \times$ NuPAGE MES SDS running buffer and 950 mL of ultra pure water. Afterward, gels were stained using a colloidal Coomassie stain (SimplyBlue SafeStain) (Macierzanka et al., 2009; Mandalari, Adel-Patient, et al., 2009; Mandalari, Mackie, et al., 2009).

3. Results and discussion

This study aimed to elucidate the potential impact of biopolymer electrostatic interactions on the formation and *in vitro* gastric and duodenal digestibility of lactoferrin-based nano-particles coated by anionic low methoxy pectin (LMP), high methoxy pectin (HMP), sodium alginate (ALG) and iota-Carrageenan (CAR). Overall, this study comprised of two main steps, first production and characterization of the various particles followed by *in vitro* gastric and duodenal digestion of samples.

3.1. Production and characterization of LF nano-particles

Initial experiments established the conditions and biopolymer concentrations required to form LF particulates with electrostatically deposited anionic polysaccharides. Based on previous work (Peinado et al., 2010) LF was subjected to heat and pH adjustments followed by incubation with increasing levels of polysaccharides. Samples were studied for their size and absorbance at 600 nm and the results are given in Fig. 2.

The data in Fig. 2A indicates that polysaccharide addition affected particle size to varying extent. Particularly, the addition of 0.01% (w/w) of LMP resulted in the appearance of visible particles with significant sedimentation which is likely to have been the result of bridging flocculation and is supported by the high sample absorbance at 600 nm (Fig. 2B). Higher LMP concentrations yielded stable particulates with size ranges below 1000 nm and

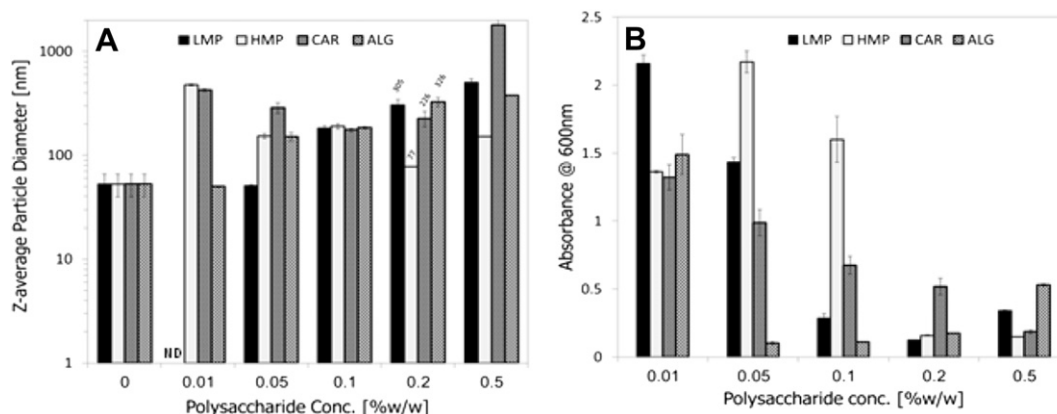


Fig. 2. (A) Z-average particle diameter [nm] and (B) Absorbance at 600 nm as a function of the polysaccharide concentration (%w/w) added. LMP: low methoxy pectin, HMP: high methoxy pectin, CAR: carrageenan and ALG: sodium alginate.

significantly reduced optical absorbance, which is consistent with this explanation.

In addition to size and turbidity analyses, the ζ -potential of the particles was measured as a function of the PS concentration (Fig. 3). These ζ -potential measurements revealed that the electrokinetic charge of the cationic protein particles became negative and reached saturation with increasing levels of anionic polysaccharides. This can be attributed to the surface deposition of the anionic polysaccharides onto the protein particulates. The results in Figs. 2 and 3 are in agreement with those recently reported (Bengoechea, Jones, et al., 2011; Bengoechea, Peinado, et al., 2011; Peinado et al., 2010). These experiments were used to determine with the optimum PS concentrations (0.2% w/w) for further experiments.

To further characterize the LF nano-particles CD and AFM analyses were performed and are depicted in Fig. 4 and Fig. 5, respectively. CD spectra (Fig. 4) demonstrated that the thermal processing leads to changes in the secondary and tertiary structure of LF. Moreover, the spectra revealed that these changes are manifested in a loss of strands and helical structures. Some thermal effects were also observed in turbidity and size measurements of LF samples before and after heating, in which turbidity (measured at 600 nm) increased from 0.0395 ± 0.0014 to 0.0647 ± 0.0019 and size increased from 42.9 ± 7.4 nm to 52.8 ± 12.9 nm (z-mean diameter). Moreover, AFM images (Fig. 5) confirmed that the production process leads to a marked change in protein organization resulting in the formation of nano-particles (Fig. 5B). Further AFM images were acquired in order to gain insights into the nature and morphology of the nano-particles with the various polysaccharides (Fig. 5C–F). These images reveal that all of the formed nano-particles were spherical in nature and varied in morphology. These results concur with recent work on LF-based nano-particles (Peinado et al., 2010).

AFM images were also used to study particle surface roughness (Fig. 6), similar to a recent report into the fine characteristics of β -lactoglobulin complexes with similar anionic polysaccharides (Jones et al., 2010). The surface roughness analyses (Fig. 6) revealed that both average and RMS roughness indicate similar trends in which polysaccharide addition altered particle roughness (one way ANOVA, $p < 0.001$). Moreover, particles augmented with

alginate or iota-carrageenan were found to exhibit a statistically significant ($p < 0.01$) reduction in average roughness compared to the treated LF.

Overall, this part of the work established that processing of LF and introduction of anionic polysaccharides was accompanied by a change in LF structure (Fig. 4), organization (Fig. 5) and particle morphology (Fig. 6). The differences shown and the intrinsic polysaccharide characteristics were suspected to confer some protective effects on LF during digestion. This hypothesis was tested using an *in vitro* digestion model mimicking the proteolytic environment of the stomach and duodenum of a healthy adult.

3.2. *In vitro* digestion of LF-based nano-particles

In these experiments, various LF-polysaccharide nano-particles were subjected to *in vitro* digestion as detailed above. Patterns of LF proteolysis were obtained via SDS-PAGE analyses of samples collected at designated time intervals during gastric and duodenal digestions. First, the protective effects of phosphatidylcholine (PC) on gastric and duodenal proteolysis (Mandalari, Mackie, Rigby, Wickham, & Mills, 2009) were studied as PC is an endogenous surface active compound found in the gastric compartment and is a major component of bile. Thus, the need for its inclusion in the *in vitro* digestion models had to be carefully evaluated (Fig. 7).

These results show that heating had no marked impact on the digestion of LF by pepsin (Fig. 7A and C). These experiments also revealed that irrespective of PC addition LF was immediately digested under gastric conditions (Fig. 7 B and D) which is in agreement with previous reports (Ono et al., 2011), although this has been shown to be strongly dependent on pH (Eriksen et al., 2010). Therefore, PC was excluded from all subsequent digestion experiments.

The various LF-based nano-particles were then subjected to *in vitro* digestions and the collected chyme was analyzed by SDS-PAGE (Figs. 8–10). Fig. 8 shows the digestion patterns of LF-based particles coated with pectins varying in charge (low and high

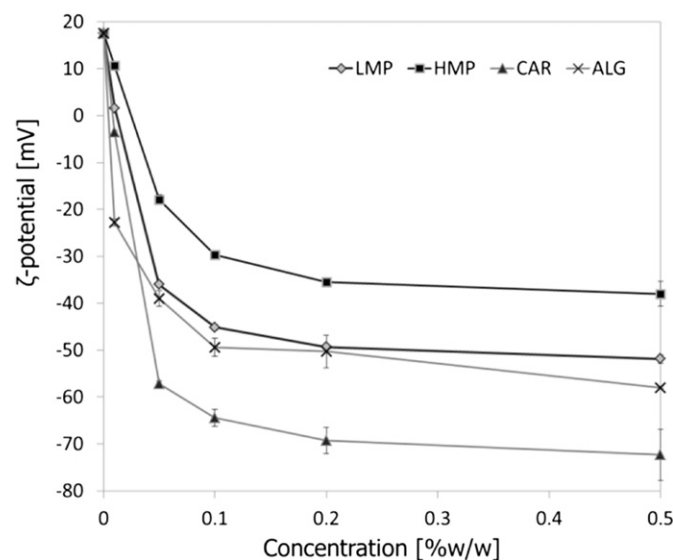


Fig. 3. Electro-kinetic charge (ζ -potential) of 0.2% (w/w) LF particles suspensions as a function of added polysaccharide concentration (%w/w). LMP: low methoxy pectin, HMP: high methoxy pectin, CAR: carrageenan and ALG: sodium alginate.

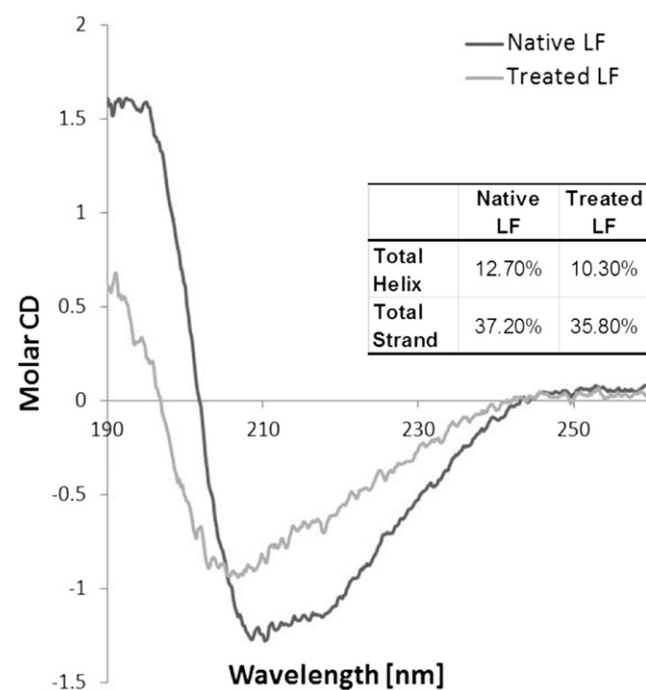


Fig. 4. CD spectra of native unheated lactoferrin (LF) and heated LF. Insert – quantitative analysis of total helices and strands found in the samples based on the CD spectra processing using dichroweb (<http://dichroweb.cryst.bbk.ac.uk>).

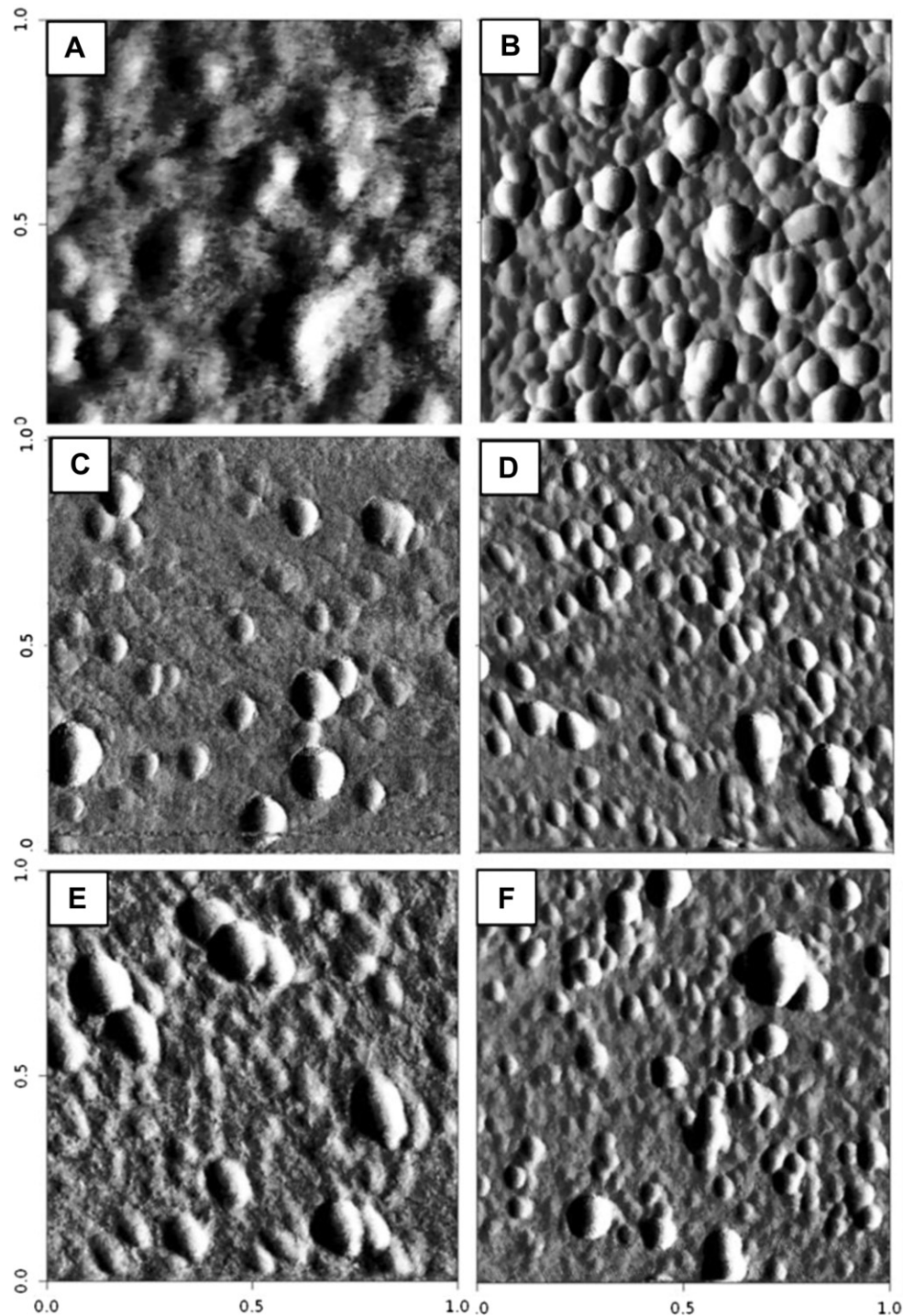


Fig. 5. $1\ \mu\text{m} \times 1\ \mu\text{m}$ AFM images of (A) native LF, 0.2 (%w/w), (B) Heat-treated LF, 0.2 (%w/w), (C) Heated LF + HMP, (D) Heated LF + LMP, (E) Heated LF + ALG and (F) heated LF + CAR.

methoxy pectins). Fig. 8A shows that LF-particles coated by HMP did not protect LF from immediate gastric proteolysis, as the intact LF band was not detectable even after short periods of digestion. In contrast, Fig. 8C revealed that during the digestion of LF-particles coated with LMP, traces of intact LF were detectable up to 10 min of gastric proteolysis but not at longer periods of digestion. This increased resistance to gastric proteolysis of LF is correlated with increased electro-kinetic charge of LF–LMP particles compared to those of LF–HMP particles (Fig. 3). Moreover, LF–LMP particles did

not have a markedly different ($p = 0.411$) surface roughness compared to LF–HMP particles (Fig. 6). Thus, it seems that the increased electrostatic interactions and ζ -potentials rather than surface roughness values are key parameters linked to the hindered susceptibility of LF to pepsin. However, pectin is a highly complex food ingredient and further investigation is needed to ascertain the impact of other pectin properties (e.g., molecular weight, polydispersity, degree of branching, amidation, ferulic acid content and residual metals) on proteolysis of protein hydrocolloids.

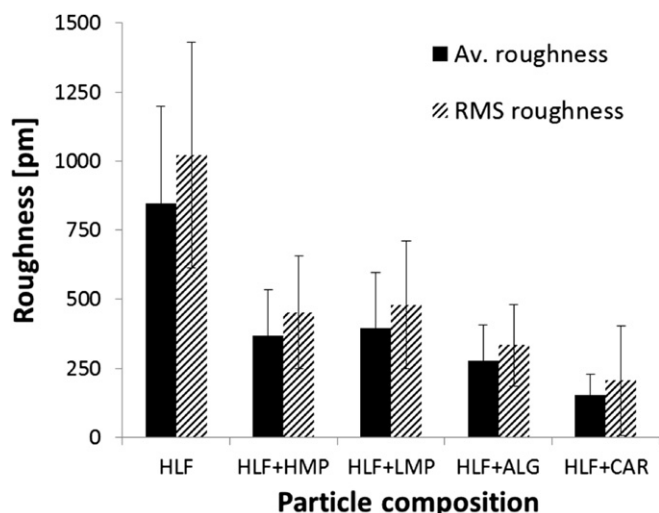


Fig. 6. Mean particle roughness measurements ($n = 60$) of heated lactoferrin (HLF) and its nano-particles with high methoxy pectin (HLF + HMP), low methoxy pectin (HLF + LMP), alginate (HLF + ALG) and iota-carrageenan (HLF + CAR).

The ζ -potential and surface roughness of LF–ALG nano-particles did not differ significantly from LF–LMP particles, hence alginate impact on LF proteolysis was also investigated (Fig. 9). Surprisingly, the results of these experiments (Fig. 9) indicate that alginate extends LF resistance to proteolysis beyond that of LMP, with the

intact LF band detected even after 40 min of gastric digestion. Moreover, the slow rate of LF gastric digestion led to different patterns of digestion and the formation of different levels of peptides (Fig. 9A) which persisted into the simulated duodenum where they were subsequently digested (Fig. 9B). LMP and ALG coated particles were found not to differ in either particle size (Fig. 2B), ζ -potential (Fig. 3) or surface roughness (Fig. 6) but to differ in LF gastric proteolysis. This would suggest other factors, such as biopolymer structure and architecture at the interface, may affect LF gastric proteolysis in LF-particles augmented with alginate. This could be attributed to the known differences between the highly heteropolymeric and branched nature of pectin compared to the relatively linear and homogenous nature of alginate. One can speculate that intrinsic and distinct features of alginate may be part of the underlying cause of such differences and could be linked to the observed modulation in oil lipolysis when encapsulated in alginate (Li & McClements, 2010). Further work is needed to establish the underlying cause/s of these observed differences in LF proteolysis.

Following the trend observed for LMP and HMP coated LF particles, carrageenan coated LF-particles were also subjected to digestion experiments, assuming the increased surface charge of ~ -70 mV (Fig. 3) and lower surface roughness (Fig. 6) of the LF–CAR particles compared to all other nano-particles could cause an even further delay in LF digestion. The SDS-PAGE results of these experiments (Fig. 10) corroborated this assumption, as LF–CAR nano-particles exhibited a significantly increased resistance to simulated gastric digestion with some intact LF continuing into simulated duodenal digestion (Fig. 10B).

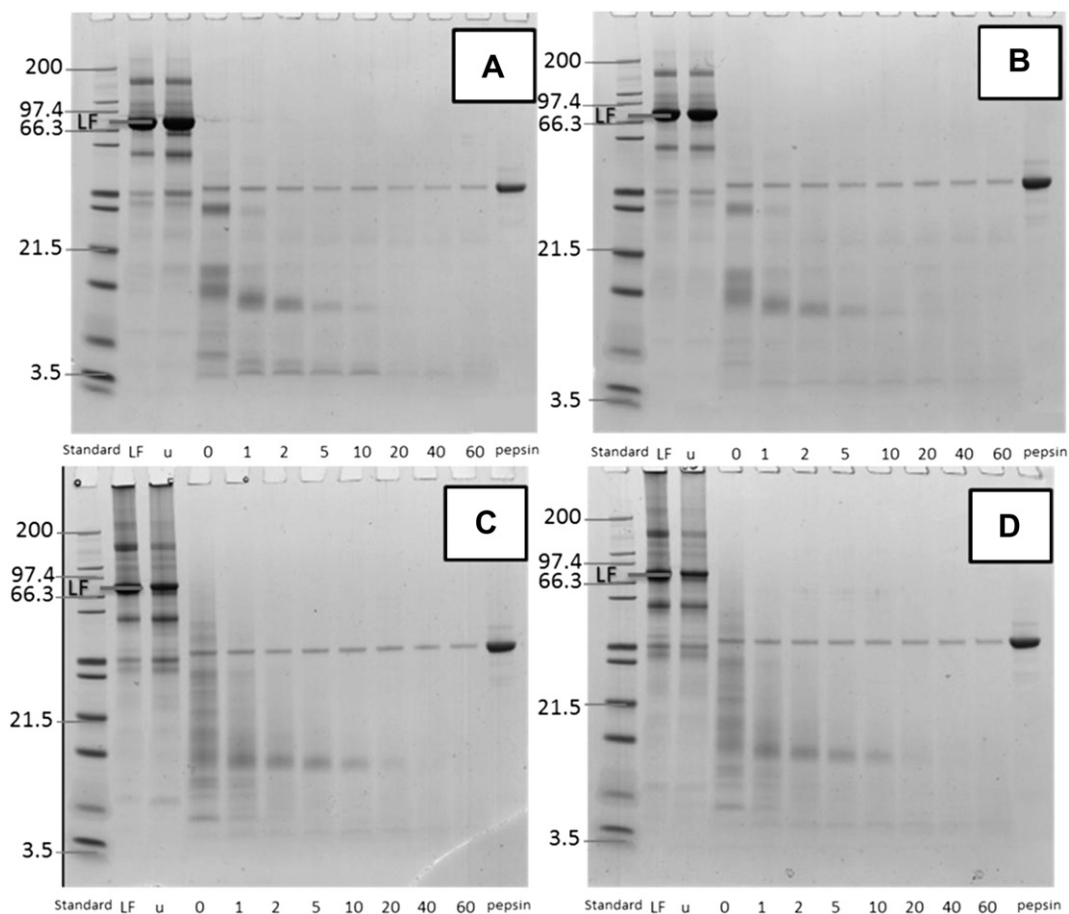


Fig. 7. SDS-PAGE gels of gastric digestion of (A) native LF (B) native LF with PC (C) heat-treated LF and (D) heat-treated LF with PC "LF" represents native LF "u" represents the sample before addition of pepsin, the numbers 0, 1, 2, 5, 10, 20, 40, and 60 represent the time of the sampling (min).

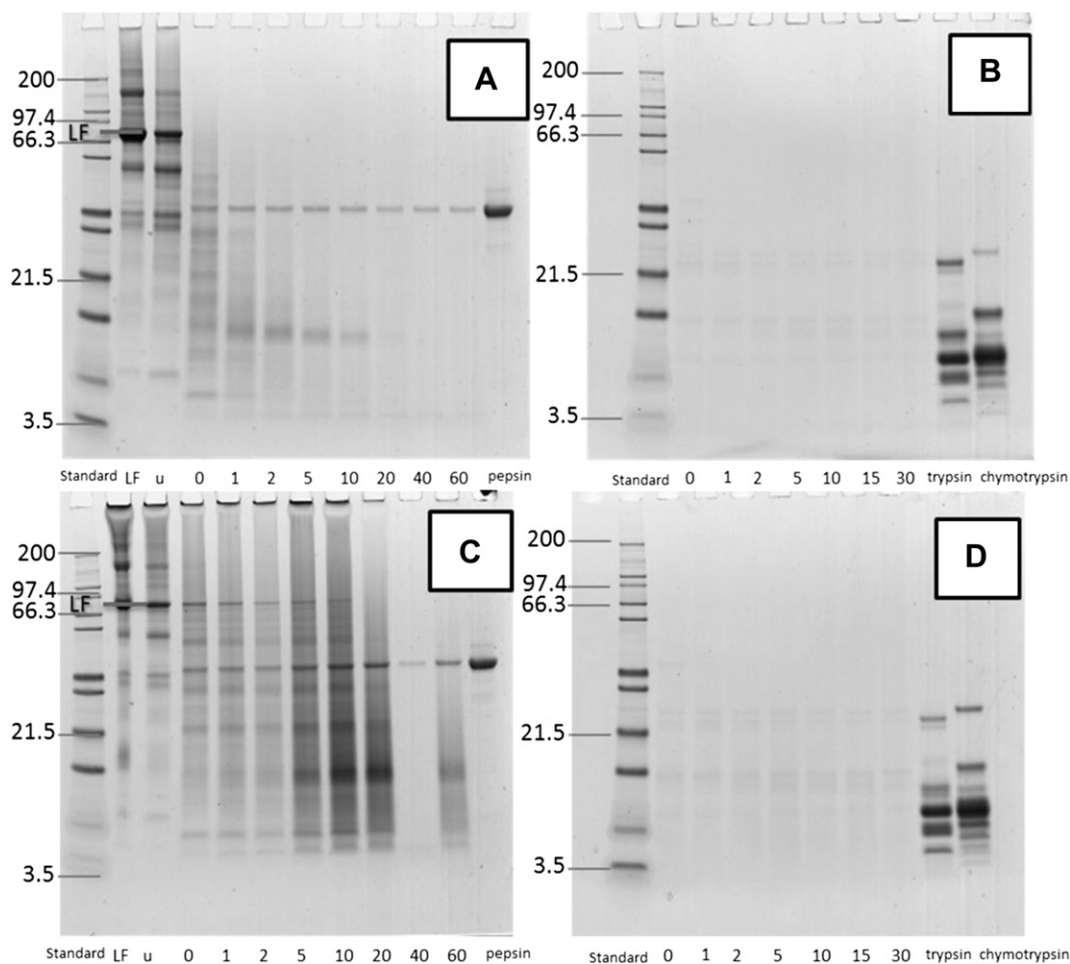


Fig. 8. SDS-PAGE gels of gastric digestion of (A and C) heat-treated LF + HMP 0.2% (w/w) and heat-treated LF + LMP 0.2% (w/w), respectively and for duodenal digestions of (B and D) heat-treated LF + HMP and heat-treated LF + LMP respectively. The numbers 0, 1, 2, 5, 10, 20, 40 and 60 represent the time of the sampling (min). In gastric digestion the numbers 0, 1, 2, 5, 10, 15 and 30 represent the time of the sampling (min.) in duodenal digestion. "u" represents the sample before addition of pepsin.

Specifically, these SDS-PAGE analyses revealed that intact LF may persist up to an hour of gastric digestion with certain specific peptide bands visible (Fig. 10A). Moreover, some intact LF reached the duodenum where it persisted for at least 10 min and

yielded some discrete peptide bands between 3.5 kDa and 21.5 kDa (Fig. 10B). In this case the extended protection against proteolysis may arise from a combination of high z-potential values and low surface roughness but one must not overlook the

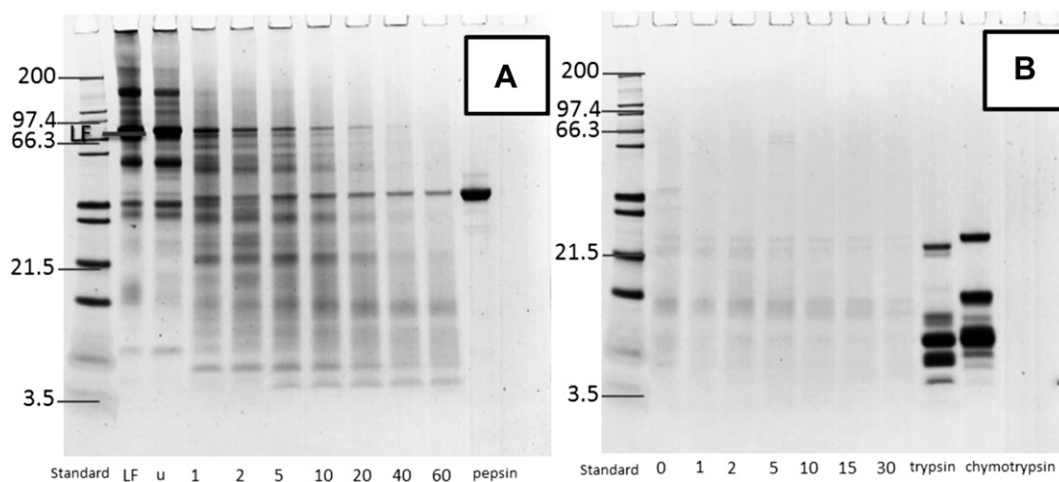


Fig. 9. SDS-PAGE gels of gastric digestion (A) and duodenal digestion (B) of heat-treated LF + ALG 0.2% (w/w). The numbers 0, 1, 2, 5, 10, 20, 40 and 60 represent the time of the sampling (min.) in gastric digestion. The numbers 0, 1, 2, 5, 10, 15 and 30 represent the time of the sampling (min.) in duodenal digestion. "u" represents the sample before addition of pepsin.

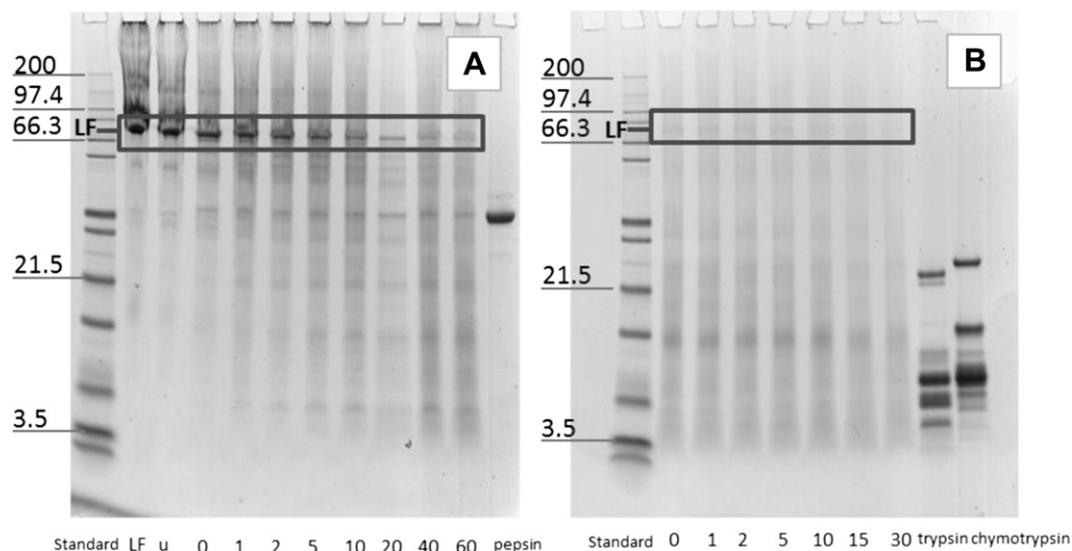


Fig. 10. SDS-PAGE gels of gastric digestion (A) and duodenal digestion (B) of heat-treated LF + CAR 0.2% (w/w). The numbers 0, 1, 2, 5, 10, 20, 40 and 60 represent the time of the sampling (min.) in gastric digestion. The numbers 0, 1, 2, 5, 10, 15 and 30 represent the time of the sampling (min.) in duodenal digestion. "u" represents the sample before addition of pepsin.

possibility iota-carrageenan may actively inhibit proteolytic enzymes. To this end, it should be noted that iota-carrageenan has been reported to be quite resistant to *in vitro* gastric proteolysis (Capron, Yvon, & Muller, 1996). However, further research is needed to establish the mechanism by which iota-carrageenan delays the proteolysis of LF, as this may have implications to food safety or alternatively be used for controlled delivery of proteins to the intestine.

In conclusion, the results of this study show that the formation of LF-based nano-particles does not significantly alter LF proteolysis under simulated gastric or duodenal conditions. However, polysaccharide supplementation affects particle charge and morphology resulting in reduced rate of LF proteolysis. The delay in LF proteolysis was found to be linked to particle ζ -potentials when using pectin so that increased electro-kinetic charge was concomitant with delayed proteolysis. In the case of similar ζ -potentials found for LF particles coated by alginate or low methoxy pectin it is believed that altered proteolysis rates may arise due to the different biopolymer structures and architectures. Thus, the extent of the protective effect of polysaccharides on LF against proteolysis has been shown to depend on polysaccharide physicochemical characteristics. Interestingly, the electrostatic binding of food grade iota-carrageenan onto LF particles was found to significantly increase LF resistance to proteolysis and helped some LF elude simulated gastric digestion altogether and reach the simulated duodenum. Moreover, all of the digestion experiments revealed LF proteolysis leads to the formation of peptides, with specific SDS-PAGE bands being relatively persistent and reaching even the duodenal experiments. These findings require further investigation as LF and LF-derived peptides have been documented to have biological activity (Conesa et al., 2010; Nagpal et al., 2011).

Overall, this work demonstrates electrostatic biopolymer interactions may be harnessed to slow LF digestion, acting as a natural means to control LF digestion and deliver intact LF to the upper gastrointestinal tract. Given the bioactive nature of LF and proteolysis-derived peptides, this work raises the possibility that electrostatic biopolymer interactions could be harnessed to modulate protein digestion and offer a natural platform to deliver bioactive proteins and peptides to the human gut.

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