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Interactions between β-Lactoglobulin and Pectins during in Vitro Gastric Hydrolysis

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This paper deals with the influence of different levels of three pectins, low-methylated pectin (LMP), high-methylated pectin (HMP), and low-methylated and amidated pectin (LMA), on the in vitro gastric hydrolysis of β-lactoglobulin (β-lg). Proteolysis by pepsin consisted of a 2-h progressive reduction of pH. A turbidity measurement of β-lg−pectin mixtures was carried out during the proteolysis. The influence of pectins on pepsin enzymatic activity was also evaluated. β-Lg was resistant to peptic digestion. The presence of each of the three pectins at a concentration of 50 wt % increased the N release at all pH values considered, despite a significant inhibition of the pepsin enzymatic activity with the pectins. The turbidity of β-lg solutions during proteolysis was reduced by the addition of pectins, because of the formation of electrostatic complexes between this protein and pectins. The increase of N release could be a false positive result due to the difficulty of precipitating protein by trichloroacetic acid because of the formation of electrostatic complexes demonstrated by the decrease of turbidity.

KEYWORDS: β-Lactoglobulin; pectin; pepsin; N release; turbidity; pepsin enzymatic activity

INTRODUCTION

Plant polysaccharides (or hydrocolloids) are widely used in dairy products to improve their texture, give body to liquid formulations, produce milk gel, and increase their shelf life (*1*−*3*). Pectins are the most important polysaccharides used in acidic dairy products. These polysaccharides interact with milk proteins, caseins, and whey proteins such as β-lactoglobulin (β-lg) (*4*−*6*).

β-Lg is the major whey protein of cow's milk. At physiological pH, β-lg is a dimer with a molecular weight of 36400 Da (*7*) and with a pH isoelectric (pHi) around 5.2 (*8*). It consists of 162 amino acids with four disulfide bonds per dimer and one free cysteine group. Intramolecular bonds that maintain the structural integrity of protein ensure its stability against hydrolysis and heat (*7*). Its three-dimensional structure consists of nine strands of antiparallel β-sheet, eight of which form a hydrophobic barrel on one side with an α-helix (*9*). This structure explains the resistance of β-lg to digestive enzymes in the gastrointestinal tract (*10*, *11*). However, cleavage of disulfide bonds by technological treatments of milk such as sterilization, heating, and high pressure enhances digestibility of whey proteins in milk, particularly β-lg (*10*, *12*−*15*).

Pectin is a structural polysaccharide of plant origin. It is defined as a mixture of heteropolysaccharides consisting predominantly of partially methoxylated galacturonic acid residues. Its structure is based on 1,4-linked α-D-galacturonic acid, interrupted by L-rhamnose residues with side chains of neutral sugars (mainly D-galactose and L-arabinose). Uronic acid is responsible for the anionic character of the gum; its pHi is around 3 (*1*). Pectins are widely used in the food industry for their gelling, thickening, and stabilizing properties. Commercially, they are mainly extracted from citrus peel (lemon, lime, and grapefruit) and apple pomace (*16*, *17*). Their ability to form viscoelastic solutions and structured networks is widely exploited in jams, jellies, and marmalades. Their rheological properties are exploited in texture control of emulsions and dispersions in low-pH food products (*1*, *18*). Their functional properties are sensitive to the degree of esterification (DE). High-methoxyl pectins (>50% DE), low-methoxyl pectins (<50% DE), and low-methoxyl and amidated pectins are the principal commercial pectins.

Pectins, just like other soluble fibers, play an important role in the gastrointestinal tract, leading to a low macronutrient hydrolysis, reduction of nutrient absorption, and animal growth (*19*−*26*). This is due to their physicochemical properties; in particular, their ability to retain water is related to their viscosity

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Table 1. Influence of Different Levels of Low-Methylated Pectin (LMP) on the Peptic Digestibility of β-Lactoglobulin (Percent)[a]

pH	levels of LMP					
	0 wt %	1 wt %	10 wt %	20 wt %	30 wt %	50 wt %
5	1.3 ± 0.15aA	0.98 ± 0.09aA	1.05 ± 0.01bA	1.91 ± 0.08cA	2.43 ± 0.02dA	2.24 ± 0.02eA
4	1.32 ± 0.04aA	1.00 ± 0.05bA	1.23 ± 0.06aB	0.78 ± 0.01cB	0.83 ± 0.02cB	2.42 ± 0.02dB
3	1.61 ± 0.04aB	1.28 ± 0.05bB	1.12 ± 0.01bB	1.19 ± 0.09bC	1.48 ± 0.04aC	3.58 ± 0.03cD
2	2.07 ± 0.14aC	1.36 ± 0.02bB	1.31 ± 0.01bB	1.15 ± 0.02cC	1.85 ± 0.05dD	4.44 ± 0.09eE

[a] Values are means ± EC ($n = 6$). Values not sharing a common letter are significantly different at $P < 0.05$; first letter is related to rows, second to columns.

and their interactions with other food molecules. The viscosity of soluble polysaccharides is often responsible for the reduction of nutrient availability (*19*), although some authors have obtained also a reduction of nutrient bioavailability with no viscous fibers (*26*). Interactions between polysaccharides and proteins are often assessed on diluted systems and speculated to explain the decrease of protein digestibility. No experiments studying the interactions between pectins and β-lg during digestion have been reported.

The aim of this work is to study the influence of three different pectins, low-methylated pectin (LMP), high-methylated pectin (HMP), and low-methylated and amidated pectin (LMA), during the in vitro gastric phase on the digestibility of β-lg by pepsin, on the enzymatic activity of pepsin, and on the ability of β-lg to form complexes with pectins.

MATERIALS AND METHODS

Materials. Acid-processed bovine β-lg powder (lot 838) was kindly provided by Lactalis (Retiers, France). It contained 89.75% protein (N × 6.38). Pectins were kindly provided by Degussa Texturant Systems (Boulogne-Billancourt, France). LMP (lot 0B800), DE 37−41%, contained 87% polysaccharides. HMP (lot AYD 250), DE 69−74%, contained 88% polysaccharides, and LMA (lot AMP 805), DE 30−37%, degree of amidation (DA) 14−18%, contained 87% polysaccharides.

Pepsin (activity 3800 units/mg of protein, 1:60000, EC 3.4.23.1), bovine hemoglobin (Hb), and thimerosal were purchased from Sigma.

Methods. *Preparation of β-Lg/Polysaccharide Mixtures.* β-Lg and pectin (LMP, HMP, and LMA) mixtures were prepared in percent by weight (wt %). Powdered β-lg containing 40 mg of nitrogen (1.7 wt % of protein, N × 6.38) was taken in triplicate and dissolved in 15 mL of sodium phosphate buffer (0.17 M, pH 7). Polysaccharides were dissolved in the same buffer in the same concentration (1.7 wt %). Dispersions were left overnight at 4 °C to allow complete hydration of the macromolecules. The β-lg and polysaccharide stock dispersions were blended so as to obtain 0 (β-lg without polysaccharide), 1, 10, 20, 30, and 50 wt % of relative polysaccharide concentration (as compared to the β-lg concentration). Thimerosal (50 μgL) was added to all of the solutions to prevent bacterial contamination.

Peptic Hydrolysis. One milliliter of pepsin in 0.02 N HCl (1 mg/mL) was added to 15 mL of the β-lg or β-lg−polysaccharide mixed dispersions described above (E/S = 1/250). For simulating in vivo gastric digestion, the pH of the suspension was progressively reduced from pH 7 to 2 by adding 0.02 N HCl with a peristaltic pump (Ecoline, Ismatec, Zurich, Switzerland) (flow rate = 80 μL min^{-1}), for 2 h at 37 °C. Digestion made in triplicate was stopped at pH 5, 4, 3, and 2 by adding 30% trichloroacetic acid (TCA) (v/v). Samples were centrifuged at 5000*g* for 20 min using a Beckman Coulter centrifuge (Villepinte, France). Pellets were discarded, and 10 mL of supernatant was taken for soluble nitrogen analysis.

Nitrogen Analysis. The total nitrogen content in the supernatant was analyzed by the Kjeldhal method according to AOAC methods 47.021 and 47.023. Protein digestibility estimated by the nitrogen (N) release was calculated as follows:

$$\text{N release (\%)} = \text{N in supernatant (mg)} \times 100/\text{N in protein sample (mg)}$$

Turbidimetric Measurement. Turbidity measurements were either carried out or not (control samples) during peptic hydrolysis with the following substances: β-lg, pectins, β-lg−pectin (50 wt %), and pepsin mixtures.

One hundred and fifty milliliter samples containing β-lg, pectins, and mixtures of protein and polysaccharides or pepsin in sodium buffer (0.17 M, pH 7) were put in a water bath at 37 °C and shaken by a magnetic stirrer. A turbidimetric probe (Mac Van Instruments, Melbourne, Australia) was put into the sample, and the turbidity of solution was measured at 600 nm. Five milliliters of pepsin in 0.02 N HCl was added (E/S = 1/250). HCl (0.2 N) was progressively added as previously described with the peristaltic pump (Ecoline, Ismatec) (flow rate = 80 μL min^{-1}), for reducing the pH from 7 to 2 (pH-meter, Knick, Portameter 751, Labo Standa, Caen, France). The turbidity of the solutions was recorded every 10 s for 2 h with a data recorder (Data Logger CA 100, Chauvin Arnoux, France). Measurements made in duplicate were stopped when the mixture reached pH 2.

Enzymatic Pepsin Activity Measurement. Enzymatic pepsin activity was measured according to the method of Ryle (*27*) using bovine hemoglobin (Hb) as substrate. Hb (2 g) was dissolved in 100 mL of a solution containing 75 mL of sodium buffer (0.17 M, pH 7) and 25 mL of 0.07 N HCl. The pH was raised to 2 with 2 N NaOH or 1 N HCl. Pectins (2 g) were dispersed in the same solution. Mixtures of Hb−pectins were made with 0, 1, and 50 wt % of pectins. Pepsin (0.2 mL; 0.03 mg/mL in 0.02 N HCl) was added to 1 mL of solutions of Hb or Hb−pectins. After mixing, samples were incubated in a water bath at 37 °C. The pepsin hydrolysis reaction was stopped every minute for 10 min by the addition of 5 mL of 4% TCA. After 10 min of centrifugation at 1000*g*, absorbance was measured on the supernatant at 280 nm against water, using a spectrophotometer (Beckman, Villepinte, France). All samples were made in triplicate. The calculation of enzymatic activity was made as follows:

$$\text{enzymatic activity } (\mu\text{mol/min}) = \Delta A/\epsilon \times \Delta t$$

where ΔA = absorbance variation, ϵ = absorption coefficient, and Δt = time variation (min).

Statistical Analysis. The results are given as mean ± standard deviation and were analyzed by a one-way analysis of variance (ANOVA, Statview V). Differences between means at $P < 0.05$ were analyzed using the Fisher test.

RESULTS

Pepsin Hydrolysis. The effect of the three pectins used on the N release after a 2-h β-lg proteolysis by pepsin is reported in **Tables 1−3**. The digestibility of β-lg alone was very low at all of the pH values considered, with N release values between 1 and 2%. The presence of pectins modified the N release, depending on the type and concentration of pectins in the mixture.

Addition of pectins at low levels (1−10 wt %) caused a decrease of the β-lg N release, significant ($P < 0.001$) only with LMP (**Table 1**). At 20 and 30 wt %, only LMA provoked a significant increase of β-lg N release ($P < 0.001$) compared with β-lg alone (**Table 3**). In all cases, N release increased significantly when pectins were present at 50 wt % at all of the pH values considered.

Table 2. Influence of Different Levels of High-Methylated Pectin (HMP) on the Peptic Digestibility of β-Lactoglobulin (Percent)[a]

	level of HMP					
pH	0 wt %	1 wt %	10 wt %	20 wt %	30 wt %	50 wt %
5	1.3 ± 0.15aA	1.96 ± 0.44aA	1.52 ± 0.25aAB	1.29 ± 0.07aA	1.47 ± 0.21aA	2.04 ± 0.08bA
4	1.32 ± 0.04aA	1.55 ± 0.05bA	1.19 ± 0.10aB	1.25 ± 0.04aA	1.43 ± 0.03aA	2.68 ± 0.20cB
3	1.61 ± 0.04aB	1.25 ± 0.16bB	1.52 ± 0.14aAB	1.64 ± 0.24aA	1.81 ± 0.63abAB	3.94 ± 1.13cC
2	2.07 ± 0.14aC	1.85 ± 0.03aA	1.71 ± 0.11abA	2.81 ± 0.46dC	1.97 ± 0.31aB	5.22 ± 0.24eD

[a] Values are means ± EC ($n = 6$). Values not sharing a common letter are significantly different at $P < 0.05$; first letter is related to rows, second to columns.

Table 3. Influence of Different Levels of Low-Methylated and Amidated Pectin (LMA) on the Peptic Digestibility of β-Lactoglobulin (Percent)[a]

	levels of LMA					
pH	0 wt %	1 wt %	10 wt %	20 wt %	30 wt %	50 wt %
5	1.3 ± 0.15aA	1.29 ± 0.13aA	1.53 ± 0.09aA	1.94 ± 0.16bA	2.78 ± 0.43bA	3.90 ± 0.08cA
4	1.32 ± 0.04aA	1.20 ± 0.04bA	1.44 ± 0.04aA	1.86 ± 0.11cA	2.34 ± 0.06dA	3.83 ± 0.35eA
3	1.61 ± 0.04aB	1.21 ± 0.07bA	1.58 ± 0.09aA	1.91 ± 0.16cA	2.62 ± 0.13dA	4.51 ± 0.27eA
2	2.07 ± 0.14aC	1.61 ± 0.14bB	2.05 ± 0.05aB	2.33 ± 0.07cB	3.10 ± 0.21dA	6.65 ± 0.82eD

[a] Values are means ± EC ($n = 6$). Values not sharing a common letter are significantly different at $P < 0.01$; first letter is related to rows, second to columns.

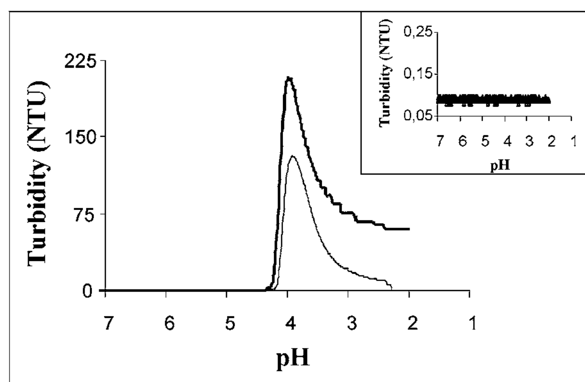

Figure 1. β-Lactoglobulin turbidity as a function of pH and time: β-lg–pepsin (**—**), β-lg (–). Insert: pepsin turbidity.

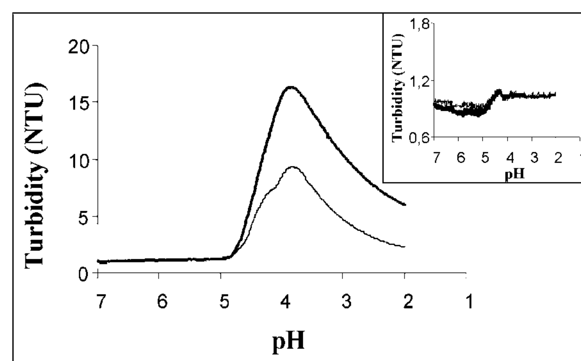

Figure 2. Effect of low-methylated pectin (LMP) on β-lg turbidity as a function of pH and time: β-lg–pectins–pepsin (**—**); β-lg–pectins (–). Insert: pectin–pepsin (**—**); pectins (–). Each point is a mean of two measures.

Decrease or increase of β-lg N release was independent of the type of pectins, but the importance of these phenomena was related of the type of pectins. Reduction of N release by LMP was more important than that by HMP and LMA. Inversely, increase of β-lg N release was more important with LMA than with HMP and LMP.

Turbidity. Figures 1−4 present the variation of the turbidity as a function of pH decreasing from 7 to 2 over a period of time. The turbidity of the β-lg solution varied with the pH. The presence of pectins modified the turbidity.

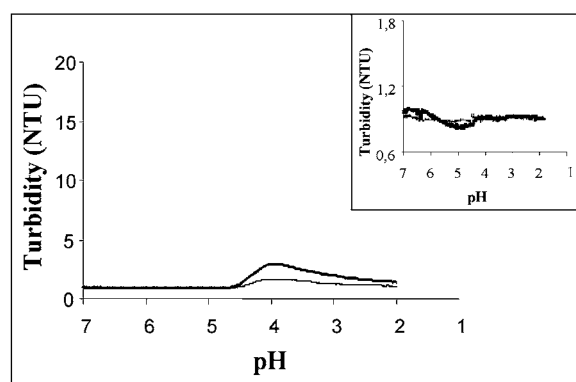

Figure 3. Effect of high-methylated pectin (HMP) on β-lg turbidity as a function of pH and time: β-lg–pectins–pepsin (**—**); β-lg–pectins (–). Insert: pectin–pepsin (**—**); pectins (–). Each point is a mean of two measures.

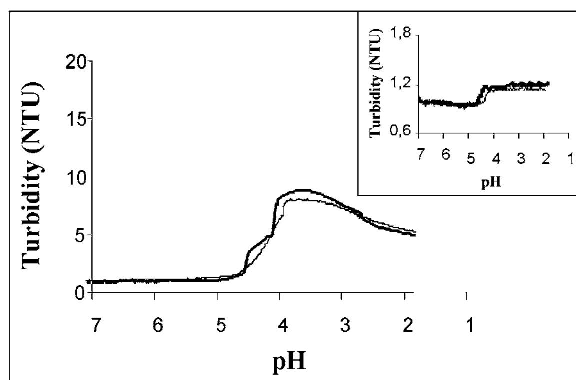

Figure 4. Effect of low-methylated and amidated pectin (LMA) on β-lg turbidity as a function of pH and time: β-lg–pectins–pepsin (**—**); β-lg–pectins (–). Insert: pectin–pepsin (**—**); pectins (–). Each point is a mean of two measures.

During the first hour (pH 7 to 5), the turbidity of the β-lg solution without pectins and with or without peptic digestion was close to zero (**Figure 1**). The turbidity of the β-lg solution alone without pepsin (**Figure 1**) increased when the pH was <5 and rapidly reached a maximum (125 NTU) at pH 3.8. Below this pH value, turbidity decreased and returned to zero. In the case of peptic digestion, the maximum of the turbidity

Table 4. Turbidity (NTU) of β-Lactoglobulin at pH 3.8: Effects of Pectins and Pepsin[a]

		LMP	HMP	LMA
β-lg	125 ± 10.46aA	9.3 ± 0.53bA	1.7 ± 0.11cA	8.01 ± 0.44bA
β-lg–pepsin	208 ± 39.6aB	16.32 ± 0.22bB	2.98 ± 0.04cB	8.84 ± 0.5dA

[a] Values are means ± EC (n = 2). Values not sharing a common letter are different at P < 0.05; first letter is related to rows, second to columns.

Table 5. Influence of Low-Methylated Pectin (LMP), High-Methylated (HMP), and Low-Methylated and Amidated Pectin (LMA) on Pepsin Enzymatic Activity (Micromoles per Minute)[a]

level of pectins	LMP	HMP	LMA
0 wt %	0.383 ± 0.021aA	0.383 ± 0.021aA	0.383 ± 0.021aA
1 wt %	0.327 ± 0.041aA	0.408 ± 0.037aA	0.361 ± 0.037aA
50 wt %	0.206 ± 0.023bA	0.274 ± 0.032bAB	0.315 ± 0.019bB

[a] Values are means ± EC (n = 3). Values not sharing a common letter are different at P < 0.05; first letter is related to rows, second to columns.

of the β-lg solution at pH 3.8 was higher (208 NTU). Turbidity decreased below pH 3.8 and did not return to zero until the end of the experiment (51 NTU at pH 2). The turbidity of the pepsin solution alone did not present any variation at any of the pH considered (**Figure 1**, insert).

The addition of pectins decreased the turbidity of the β-lg solutions (**Figures 2−4**; **Table 4**). The maximum turbidity of mixtures was significantly (P < 0.001) lower compared to β-lg without pectins during or not during pepsin digestion (**Table 4**). The decrease of the β-lg solution's turbidity was greater with HMP (**Figure 3**) than with LMA (**Figure 4**) and LMP (**Figure 2**). Hydrolysis by pepsin provoked an increase of the turbidity that was greater with the β-lg−LMP mixture than with the β-lg−LMA mixture and the β-lg−HMP mixture (**Table 4**).

The turbidity of pectin solutions alone did not present any significant variation at any of the pH values considered, with or without the pepsin hydrolysis (**Figures 2−4**, inserts).

Enzymatic Activity of Pepsin. The influence of pectins on the enzymatic activity of pepsin is reported in **Table 5**. The enzymatic activity of pepsin during Hb hydrolysis was 0.383 μmol/min. Addition of pectins at 1 wt % to Hb had no effect on the pepsin enzymatic activity. All pectins, added at 50 wt %, significantly inhibited (P < 0.05) the pepsin activity. The reduction of pepsin activity was greater with LMP than with HMP and LMA.

DISCUSSION

The effects of LMP, HMP, and LMA on the digestibility of β-lg were evaluated using an in vitro pepsin hydrolysis model in which the pH was progressively reduced from 7 to 2 over a period of 2 h, just like under in vivo conditions (*28*).

β-Lg was poorly hydrolyzed by pepsin at pH 2, at which its activity is maximum and β-lg is in a monomeric form. These results agree with those obtained by other authors. In this way, Reddy et al. (*10*) and Schmidt and Poll (*11*) reported an in vitro resistance of β-lg to degradation by pepsin with different methods. In vivo, β-lg was found intact after its passage through the stomach (*29*, *30*). Pepsin is an acidic endoproteinase that hydrolyzes bonds between aromatic amino acids (tyrosine and phenylalanine). The β-lg sequence displays around 50 bondings of potential peptic cleavage sites, but most of them are buried in the hydrophobic core of the molecule and are not accessible by pepsin (*31*). Kella and Kinsella (*32*) suggested that acid stability of β-lg could result from increased internal hydrogen bonding that arises between either two titrated carboxyl groups or one amide and one carboxyl group; thus, the resistance of β-lg to peptic digestibility may reflect its stable conformation at pH 2.

For each pH value, the presence of pectins had no effect at low concentrations (1 and 10 wt %) on N release, except with LMP. At the highest polysaccharide concentration (50 wt %), an increase of the N release related to the pH decrease was observed with the three pectins despite the reduction of pepsin enzymatic activity. This suggests that the increase with 50 wt % of pectins is not due to an increased ability of pepsin to hydrolyze β-lg but is due to an incomplete TCA precipitation of protein resulting from possible protein/polysaccharide interactions instead. β-Lg and pectins can interact at pH values used in this study, and increasing concentrations of polysaccharides promote these interactions and the N release erroneously increased. Electrostatic interactions between β-lg and pectins most likely occur in the pH range of 3−5, at which part of the gastric digestion experiments was carried out and the two macromolecules carry opposite charges.

The turbidity of the different solutions of β-lg containing or not containing pectins increased from pH 4.7 with a maximum at pH 3.8. The minimum of solubility of β-lg was found at pH 3.8. This pH value is lower than the theoretical pHi of β-lg (pH 5.2). Schmitt et al. (*8*), using the same β-lg sample, found a minimum of solubility at pH 4.75 when β-lg was dispersed in water. It is possible that our low pHi is due to the ionic strength of buffer solution (0.17 M) because increasing the ionic strength of solutions reduces pHi. The increase of turbidity was the greatest for β-lg alone and is an indication of the formation of aggregates. The formation of the aggregates at β-lg pHi is explained by the protein net charge close to zero, implying an absence of repulsion and, finally, protein precipitation. At pH below the pHi where β-lg is positively charged, an electrostatic repulsion exists between protein molecules, large enough to prevent self-association and diminishing turbidity. With β-lg alone, turbidity retrieves its initial value (zero) at pH 2. However, reversibility of the β-lg solution turbidity is incomplete when β-lg is hydrolyzed by pepsin. According to the results obtained for pepsin solution, its turbidity does not present any variation. Therefore, this high value of β-lg turbidity could possibly be related to a coaggregation of peptides of β-lg pepsin hydrolysis and β-lg molecules. This could also prevent the access of the enzyme on the cleavage bonds, preventing the hydrolysis of this protein.

The presence of pectins considerably reduced the peak of β-lg turbidity solution observed at pH 3.8. This indicates a reduction of the β-lg aggregate formation. This phenomenon is pH-dependent and is an indication of the electrostatic character of the insoluble complex formation between β-lg and pectin's ionized carboxylic groups in this range of pH where they carry opposite charges as previously described (*33*−*35*). Interactions between pectins and β-lg prevent protein association, reducing the turbidity of the solution. Girard et al. (*34*, *35*) demonstrated the β-lg−LMP or −HMP pectin complex formation due to electrostatic interactions and also to hydrogen bonding at pH 4. These authors have demonstrated that the complexes are formed in two steps passing from the molecular to the aggregate state. The first step would correspond to the formation of soluble intrapolymer complexes between β-lg and pectin molecules. The second steps would imply the aggregation of these intrapolymer complexes forming insolubles interpolymer complexes (*35*).

Formation of electrostatic complexes between pectins and casein at acidic pH has been reported and is the basis for stabilizing casein-coated emulsion in dairy acidified products (*1*, *4*, *33*). In practice, HMP is widely used for this application. LMP has a high charge density and causes high turbidity due to more interactions with proteins and a lesser stabilizing effect caused by a low viscosity (*1*, *3*). On the other hand, as opposed to LMP, HMP has a low charge density and establishes low interactions with proteins. Therefore, portions of free pectin that can interact with solvent increase and the viscosity of the solution as well, leading to a low precipitation of protein (*4*, *33*, *36*). LMA is slightly more charged than HMP, and the reduction of turbidity is slightly less effective.

Low pectin levels (1 wt %) had no effect on the enzymatic activity of pepsin, but N release by β-lg was reduced. Interactions between β-lg and the LMP polysaccharide with the highest charge density occur at low polysaccharide concentrations (*34*) and could reduce the proteolysis. Inhibition of digestive enzyme activity by viscous polysaccharides has already been demonstrated (*22*, *37*). Some authors found an increase of the pepsin activity by pectins (*38*). These authors linked the effects of pectins on enzymatic activity to their DE. Reduction of enzymatic activity could possibly be due to high viscosity, preventing the enzyme−substrate from binding (*21*), or direct interactions between pectins and enzymes (*38*).

CONCLUSION

These results confirm the low β-lg hydrolysis by gastric enzymes often observed. High pectins levels reduce the pepsin enzymatic activity and the ability of β-lg to form aggregates because of the formation of β-lg−pectin complexes depending of pH and increase the repulsion of β-lg molecules, which promote the protein solubility. Therefore, the increase of N release with the addition of pectins seems to be due to insoluble β-lg−pectin aggregates and not an increase of β-lg pepsin hydrolysis and agrees with the reduction of protein digestibility observed by others authors.

In vivo, as observed in this study mimicking gastric digestion, the action of pectins on the reduction of protein hydrolysis could be explained by the reduction of pepsin enzymatic activity, the protein−pectin complex formation, and probably other phenomena such as viscosity that is not studied here.

ABBREVIATIONS USED

β-lg, β-lactoglobulin; pHi, isoelectric point; DE, degree of esterification; DA, degree of amidation; LMP, low-methylated pectin; HMP, high-methylated pectin; LMA, low-methylated and amidated pectin; Hb, hemoglobin; NTU, nephelometric unit.

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