Isolation of Bovine β -Lactoglobulin from Complexes with Chitosan

**Antonia Montilla, Enriqueta Casal, F. Javier Moreno*, Josefina Belloque,
Agustín Olano and Nieves Corzo**

Instituto de Fermentaciones Industriales (C.S.I.C.), C/ Juan de la Cierva 3, 28006 Madrid,
Spain

* Author to whom correspondence should be addressed.

Telephone number: (34) 91 562 29 00

Fax number: (34) 91 564 48 53

e-mail: j.moreno@ifi.csic.es

Address: Instituto de Fermentaciones Industriales (C.S.I.C.), C/ Juan de la Cierva 3,
28006 Madrid, Spain

ABSTRACT

A simple, economical and non-toxic method is described for the solubilization of undenatured β -lactoglobulin from their complexes with chitosan. The effect of pH (8-10), ionic strength (0.08-0.3M) and volume ratio between sodium acetate solutions and whey on the dissociation of β -lactoglobulin-chitosan complexes was evaluated. Following a single extraction step with 10 mL of 0.1M sodium acetate solution at pH 9, a recovery of 90% of β -lactoglobulin with a protein purity of 95% was achieved, suggesting that electrostatic interactions play a key role in the complexation of β -lactoglobulin with chitosan. The presence of chitosan free in solution was ruled out according to GC-FID analysis after acid hydrolysis. NMR spectroscopy showed that the β -lactoglobulin obtained and further subjected to dialysis showed a structural features very similar to the native protein. This method could offer a promising alternative to the traditional processes used for purification of β -lactoglobulin in the dairy industry.

Key words: chitosan, β -lactoglobulin, electrostatic interactions, cheese whey.

INTRODUCTION

Bovine β -lactoglobulin (β -lg) constitutes approximately 50% of the protein present in whey. Native β -lg is a small globular protein with defined secondary and tertiary structure and is widely used as a food ingredient because of its nutritional value and its ability to form gels (Allain, Paquin and Subirade, 1999; Lefevre and Subirade, 2000), emulsions (Dickinson, 1997; Beaulieu, Savoie, Paquin and Subirade, 2002; Lefevre and Subirade, 2003; Leung Sok Line, Remondetto and Subirade, 2005) and foams (Rantamaki, Tossavainen, Outinen, Tupasela, Koskela and Kaunismaki, 2000; Bals and Kulozik, 2003). Thus, much attention has been given to the development of techniques to isolate and purify this protein. β -lg has been purified using various techniques including ultrafiltration (Kinekawa and Kitabatake, 1996; Sannier, Bordenave and Piot, 2000), ion-exchange chromatography (Imafidon and Ng-Kwai-Hang, 1992) and precipitation or complexing with reagents such as carboxymethyl cellulose (Hidalgo and Hansen, 1969) among others. However, most of these processes have not been widely implemented for large-scale purification because of their complexity, high cost, low overall yield, poor selectivity and / or unacceptable product degradation associated with harsh conditions, such as high temperature used during the process (Zydney, 1998; Savant and Torres, 2000; Cheang and Zydney, 2003).

Chitosan, a polysaccharide comprising copolymers of glucosamine and *N*-acetylglucosamine, is positively charged at acidic pH values and interact with the negatively charged polyelectrolytes such as proteins (Remuñan-López and Bodmeier, 1996; Ausar et al., 2001; Casal, Corzo, Moreno and Olano, 2005). Chitosan has been used to

produce destabilization and coagulation of casein micelles from milk (Ausar et al., 2001), reduction of protein, fat and ash contents in whey wastes (Mukhopadhyay, Talukdar, Chatterjee and Guha, 2003) and to isolate caseinmacropeptide (Casal et al., 2005; Nakano, Ikawa and Ozimek, 2005). Recently, we reported the complex formation between chitosan and β -lg, in order to remove the β -lg from whey to simulate human milk protein (Casal, Montilla, Moreno, Olano and Corzo, 2006). The same procedure could be used to recover β -lg, providing an additional step to separate the protein from the chitosan complex.

Since protein binding capacity of chitosan is affected by ionic strength and pH, the aim of this work was to establish conditions for maximal extraction of β -lg from their complexes with chitosan to recover high purity β -lg.

MATERIALS AND METHODS

Chemicals. Low-molecular-weight chitosan (LMWC), average MW 120 kDa, with 85% deacetylation was supplied by Aldrich (Milwaukee, WI). Rennet powder was obtained from Hansen's Laboratorium (Denmark) and starter culture *Lc. Lactis ssp lactis* and *Lc. Lactis ssp cremoris* MA0 11 from EZAL (Rhodia Iberia, Spain). Acetic acid was purchased from Scharlau Chemie (Barcelona, Spain), chloride acid from VWR Prolabo (Fontenay sous Bois, France) and sodium hydroxide from J. T. Baker (Deventer, Holland).

Reagents employed for GC analysis including sugar standards (glucose, galactose, glucosamine), internal standard (β -phenyl-glucoside) and derivatising reagent (*N*-trimethylsilylimidazole) were obtained from Sigma (St. Louis, USA). Dried pyridine was from Merck (Darmstadt, Germany). Ultrapure water quality with 1 – 5 ppb TOC and <

0.001 EU/mL pyrogen levels (Milli-Q) were produced in-house using a laboratory water purification Milli-Q Synthesis A10 system (Millipore, Bellerica, Mass., USA) and was used throughout.

Rennet whey preparation. Pasteurised skimmed cow's milk inoculated with 2% of starter culture was warmed to 35°C. When the pH reached 6.3, CaCl₂ (0.25 g/L), rennet powder (0.1 g/L) was added. After coagulation the curd was cut to the adequate grain size (approximately a rice grain), and the mixture of curd particles and whey was gently stirred and heated to 45°C to reduce the moisture content in the curd. Finally, whey was filtered through glass wool.

Obtention of β -lg-chitosan complexes. Clarified whey obtained after treatment with 0.25 mg chitosan / mL at pH 4.5 to remove lipids and suspended solids was, then, subjected to treatments with 1% (w:v) chitosan in 0.1 M acetic acid at pH 6.2 at a concentration of 1.4 mg chitosan / mL of whey. The mixtures were vigorously stirred for 1 min to reach a complete interaction and coagulation, then allowed to stand for 30 min with gentle stirring and centrifuged at 5000 g for 10 min to separate the pellets from supernatants. Under these conditions, around 85% of β -lg was precipitated whereas, at least, 90% of the rest of whey proteins remained in solution (Casal et al., 2006). A control sample, containing no added chitosan, was treated under the same conditions.

Isolation of β -lg from complexes with chitosan. The pellet obtained from 1 mL of the chitosan-treated whey was mixed with 5 or 10 mL of sodium acetate solutions at different pHs (8, 9 or 10) and concentrations (0.08, 0.10, 0.15, 0.2 and 0.3 M), vigorously stirred for

30 min and centrifuged at 10000 g for 10 min to separate undissolved chitosan from solubilized β -lg.

For total solubilization of both chitosan and β -lg, a solution of 0.1 N acetic acid at pH 4 was used.

Determination of β -lg. Analyses of whey proteins in solution were performed by RP-HPLC-UV using 0.1% (w/v) trifluoroacetic acid in double-distilled water as solvent A and 0.085% (w/v) trifluoroacetic acid in double-distilled water/acetonitrile (10:90, v/v) as solvent B. The chromatographic conditions were those described by Casal et al. (2006). Protein contents obtained in the supernatants after extraction with sodium acetate solutions were expressed as a percentage of the total protein contained in the pellets. All treatments were replicated three times. The identity of whey proteins was confirmed by comparison of their retention times with those of the protein standards purchased from Sigma (St. Louis, MO).

Determination of chitosan. The quantitation of chitosan was done by means of their glucosamine content determined by GC-FID after acid hydrolysis. The optimum hydrolysis conditions that led to a high depolymerization degree of chitosan were those achieved with 3 N HCl at 110°C for 40 hours. Thereby, either chitosan solutions (0.01-0.2 mg/mL) or supernatants containing the redissolved β -lg after extraction with 10 mL of 0.1M sodium acetate at pH 9 were subjected to acid hydrolysis before subsequent GC-FID analysis.

Prior to derivatisation and GC analysis, 1 mL of filtered hydrolysates and 0.2 mL of a solution of 0.01% (w:v) phenyl- β -D-glucoside in methanol/water (70:30, v/v) (internal

standard) were mixed and dried at 38-40°C in a rotavapor. Then, 100 µL of pyridine and 100 µL of N-trimethylsilylimidazole were added to silylate the carbohydrates and the derivatisation reaction was completed in 30 minutes at 70°C. The reaction was stopped by cooling and the silylated carbohydrates extracted with 0.1 mL of hexane and 0.2 mL of water. Volumes in the range of 1-2 µL of the organic phase containing silyl derivatives were injected onto the column. The trimethylsilyl ethers were separated using a commercial 8 m x 0.25 mm inside diameter, 0.25 µm film fused silica capillary column CP-SIL 5 CB (100% dimethylsiloxane, non polar) (Chrompack, Middelburg, The Netherlands). The temperatures of injector and detector were 300°C, the oven temperature was programmed from 140°C to 180°C at 5°Cmin⁻¹, then at 50°Cmin⁻¹ to 250°C and keeping this temperature for 5 min, followed for an increase up to 300°C at rate of 50°Cmin⁻¹, and finally keeping this temperature for 7 min. Injections were made in the split mode (1:10). Data acquisition and integration was done using HP ChemStations software (Hewlett-Packard, Wilmington, USA).

GC analysis of glucosamine gave rise to two anomers as previously shown (Karkkainen and Vihko, 1969). The quantitation of glucosamine was performed with the more retained anomer as it did not overlap with other peaks. This anomer represented 39.3% of the glucosamine. GC analyses were performed in quadruplicate. Linear correlation analyses between glucosamine and hydrolysed chitosan contents were performed using the MS Excel programme.

NMR analysis of β-Ig. NMR was used to compare the spectra of the chitosan-purified β-Ig and a commercial and native β-Ig (Sigma, St. Louis, MO, USA). Prior to analysis, the

chitosan-purified protein was subjected to membrane filtration through an Amicon Ultra-15 centrifugal filter unit, 10 KDa MW cut-off (Millipore Corporation, Bedford, MA, USA), followed by freeze-drying, to remove sodium acetate that may overlap with the NMR protein resonances. The commercial β -lg was used directly from the container. The two protein samples were dissolved in D₂O at a concentration of 20-30 mg/mL, and the pH was adjusted to 2.0 with DCl.

Spectra were recorded in a 500 MHz Varian ^{UNITY}INOVA spectrometer (Varian NMR Instruments, Palo Alto, CA, USA). All spectra were taken at 35 °C, with a spectral width of 6000 Hz and a delay time of 0.5 s. The water line was suppressed using presaturation for 1.5 s. One-dimension ¹H spectra were recorded with 3.7 s acquisition time and 512 transients. DQF-COSY and NOESY (100 ms mixing time) spectra were obtained by accumulation of 32-64 transients, using 2048 and 512 points in the t₂ and t₁ dimensions respectively. Raw data were processed and visualised using Varian software.

RESULTS AND DISCUSSION

Optimisation of the isolation of β -lactoglobulin from chitosan complexes

Electrostatic protein-polyelectrolyte complexation sensitively depends on the solution pH and ionic strength (de Vries, 2004). The pK_a of chitosan is about 6.5 (Claesson and Ninham, 1992; Nyström, Kjoniksen and Iversen, 1999), thus, at pH values below 6.5 chitosan is positively charged along its backbone and is soluble in dilute aqueous solution, whereas at higher pH dissolved chitosan usually flocculates due to deprotonation of the

175 amino groups (Claesson et al., 1992; Guzey and McClements, 2006). With the aim to keep
176 chitosan undissolved and recover the β -lg free in solution, all assayed conditions were
177 performed at pH values above the pK_a of chitosan using sodium acetate solutions as
178 extracting solvent. Sodium acetate is a food additive recognized as safe and non-toxic by
179 the U. S. Food and Drug Administration (FDA) and European Union.

180 Fig. 1 shows the percentage of β -lg recovered from chitosan complexes following a
181 single extraction step with 5 or 10 mL of 0.1M sodium acetate at pH 8, 9 and 10. The
182 highest recoveries were obtained extracting with 10 mL instead of 5 mL, regardless pH.
183 Thus, around 90% of precipitated β -lg could be recovered by using 10 mL of sodium
184 acetate solutions at pH 9 and 10 and only 60% of the precipitated protein was resuspended
185 at pH 8, whilst hardly 35% of β -lg was extracted with 5 mL of sodium acetate solutions.
186 Additionally, a second extraction step did not increase the recovery of β -lg due to pellets
187 lost its compactness and were extremely difficult to handle.

188 In alkaline solution, the amino groups of the chitosan were neutralized and the β -lg
189 remained negatively charged, leading to the lost of electrostatic linkages between the
190 polymer and protein. The low recovery of protein observed with the sodium acetate
191 solution at pH 8 could be due to some chitosan segments remained positively charged since
192 the final pH of the solution was 7.0 which is pretty close to the pK_a of chitosan.

193 On the other hand, to evaluate the effect of ionic strength on the dissociation of β -lg
194 from chitosan complexes, sodium acetate solutions at pH 9 with molarities within the range
195 0.08-0.3 were assayed (Fig. 2). With 5 mL of sodium acetate solutions, only 60% of β -lg
196 was recovered at the highest molarities (0.2 and 0.3) assayed. Nevertheless, using 10 mL of
197 extracting solvent, molarities between 0.1 and 0.3 gave rise to the recovery of at least 90%

of the complexed protein, whereas 0.08 only allowed the dissociation of 55% of the precipitated β -lg. At higher ionic strengths, the charges on both the β -lg and the chitosan are shielded by the counterions (Mattison, Brittain and Dubin, 1995). As consequence of these shielding effects, electrostatic interactions between chitosan and β -lg were truncated allowing, thus, the presence of free β -lg in solution. Either molarities below 0.1 or reduced volumes (5 mL) of sodium acetate solutions seem to be insufficient to dissociate the β -lg - chitosan complexes at a noticeable level.

Once achieved a high recovery of the precipitated β -lg, we proceed to estimate the protein purity of the supernatant by RP-HPLC analysis. The supernatant comprised of 94.5% β -lg, 3% of α -lactalbumin, 2% of caseinmacropeptide and 0.3% of BSA (Fig. 3). The minor presence of some whey proteins might be attributed to an incomplete phase separation occurred during the precipitation of β -lg with chitosan and, therefore, some remnants of other whey proteins could be absorbed on the pellet. Unsuccessful attempts were made to obtain a better phase separation between the complexed β -lg with chitosan and the rest of the whey proteins remaining in solution by increasing the centrifugation speed from 5,000 to 10,000 g and/or performing several rinses of the pellet with water and sodium hydroxide prior to the solubilization of β -lg (results not shown).

Determination of chitosan by GC-FID following acid hydrolysis

With the aim to rule out the presence of chitosan in the supernatant obtained during the recovery of β -lg, a gas chromatography method after acid hydrolysis of chitosan was developed for the determination of glucosamine. The graph depicted in Fig. 4A shows the

linear correlation between the response of glucosamine detected and levels of chitosan hydrolysed. Prior to quantification of the glucosamine in the supernatants obtained during the recovery of β -lg, the accuracy of the method was evaluated. Known amounts of chitosan (0.0165 - 0.165 mg), within the range of chitosan added to whey for selective removal of β -lg, were added to 1 mL of the supernatants and, then, subjected to acid hydrolysis as described in Materials and Methods section. As shown in Fig. 4B, excellent recoveries (~100%) were obtained for the whole range studied.

The concentration of glucosamine found in the supernatants of the solubilized β -lg was 0.25 ng / mL supernatant ($n=10$) which is equivalent to 4 μ g of chitosan per mL of supernatant. To elucidate the origin of the glucosamine, i.e. whether it comes from the chitosan or from whey, β -lg was precipitated by decreasing the pH with acetic acid down to 4.5, followed by heating at 80°C for one hour, and then was redissolved with 0.04% sodium hydroxide (w:v). This sample showed a content of 0.54 ng of glucosamine / mL supernatant ($n=2$) thus, it can be concluded that the low levels of glucosamine detected in the supernatant of the resuspended β -lg were not derived from the chitosan.

NMR analysis of the recovered β -lactoglobulin

NMR is a powerful tool that has been used for structural analysis of milk peptides and proteins and, particularly β -lg (Belloque, 2006). Modifications in the spectral pattern indicate structural changes such as misfolding, denaturation or aggregation. To inspect the structural integrity of chitosan-purified β -lg, NMR spectra of the isolated and dialysed protein were taken. These spectra were then compared to spectra of the native protein from

a commercial source. The 1D and COSY spectra of the chitosan-purified and the native β -lg were very similar, regarding intensity and chemical shift of resonances (results not shown). This indicates that the chitosan-purified protein presented a similar structure to that of the native β -lg. In addition, no line broadening was observed, consistent with a non-aggregated protein. Finally, NOESY spectra cross-peaks, that represent the “structural fingerprint” of the protein, were also very similar to the native protein (Fig. 5). It has to be considered that the isolated protein was not tested under the same conditions that it was recovered, i. e. 0.1M sodium acetate. However, if lowering the pH and removing the salts led to a protein with a structure very close to that of native β -lg; it can be deduced that the structure of the sodium acetate extracted protein was also able to reverse any conformational changes induced by the alkaline pH or the presence of salts. This also opens up the possibility of obtaining a native-like β -lg by removal of solvent co-solutes.

CONCLUSIONS

In summary, from these results it can be inferred the key role of electrostatic interactions on the complexation of β -lg and chitosan. This can explain the easy solubilization, and consequently high yield of β -lg just by a simple extraction step with a solution of sodium acetate. To conclude, these findings point out that the precipitation of β -lg with chitosan and subsequent solubilization may be an economical and feasible method for the recovery of β -lg present in whey. Moreover, the reversibility of the β -lg precipitation with chitosan could open up the possibility of re-using the chitosan, which

would undoubtedly make more economical the industrial application of this method to
purify native β -lg.

ACKNOWLEDGEMENTS

This work was supported by the Comisión Interministerial de Ciencia y Tecnología
(CICYT), Project number AGL2004-07227-C02-02 and AGL2004-03322.

REFERENCES

Allain, A. F, Paquin, P., & Subirade, M. (1999). Relationships between conformation of β -
lactoglobulin in solution and gel states as revealed by attenuated total reflection Fourier
transform infrared spectroscopy. *International Journal of Biological Macromolecules*, 26,
337-344.

Ausar, S. F., Bianco, I. D., Badini, R. G., Castagna, L. F., Modesti, N. M., Landa, C. A., &
Beltramo, D. M. (2001). Characterization of casein micelle precipitation by chitosans.
Journal of Dairy Science, 84, 361-369.

Bals, A., & Kulozik, U. (2003). Effect of pre-heating on the foaming properties of whey
protein isolate using a membrane foaming apparatus. *International Dairy Journal*, 13, 903-
908.

Beaulieu, L., Savoie, L., Paquin, P., & Subirade, M. (2002). Elaboration and characterization of whey protein beads by an emulsification/cold gelation process: application for the protection of retinol. *Biomacromolecules*, 3, 239-248.

Belloque, J. (2006). High-resolution NMR of milk and milk proteins. In G. A. Webb (Ed.), *Modern Magnetic Resonance, volume 3, Applications in Materials, Food and Marine Sciences*. (pp. 1-7). Springer, The Netherlands.

Casal, E., Corzo, N., Moreno, F. J., & Olano, A. (2005). Selective recovery of glycosylated caseinmacropeptide with chitosan. *Journal of Agricultural and Food Chemistry*, 53, 1201-1204.

Casal, E., Montilla, A., Moreno, F. J., Olano, A., & Corzo, N. (2006). Use of chitosan for selective removal of β -lactoglobulin from whey. *Journal of Dairy Science*, In press.

Cheang, B., & Zydney, A. L. (2003). Separation of α -lactalbumin and β -lactoglobulin using membrane ultrafiltration. *Biotechnology and Bioengineering*, 83, 201-209.

Claesson, P. M., & Ninham, B. W. (1992). pH-dependent interactions between adsorbed chitosan layers. *Langmuir*, 8, 1406-1412.

De Vries, R. (2004). Monte Carlo simulations of flexible polyanions complexing with whey proteins at their isoelectric point. *Journal of Chemical Physics*, 120, 3475-3481.

313 Dickinson, E. (1997). Properties of emulsions stabilized with milk proteins: Overview of
 314 some recent developments. *Journal of Dairy Science*, 80, 2607-2619.

315

316 Guzey, D., & McClements, D. J. (2006). Characterization of β -lactoglobulin-chitosan
 317 interactions in aqueous solutions: A calorimetry, light scattering, electrophoretic mobility
 318 and solubility study. *Food Hydrocolloids*, 20, 124-131.

319

320 Hidalgo, J., & Hansen, P. M. T. (1969). Interactions between food stabilizers and β -
 321 lactoglobulin. *Journal of Agricultural and Food Chemistry*, 17, 1089-1092.

322

323 Imafidon, G. I., & Ng-Kwai-Hang, K. F. (1992). Isolation and purification of β -
 324 lactoglobulin by mass ion-exchange chromatography. *Journal of Dairy Research*, 59, 101-
 325 104.

326

327 Kärkkäinen, J., & Vihko, R. (1969). Characterisation of 2-amino-2-deoxy-D-glucose, 2-
 328 amino-2-deoxy-D-galactose, and related compounds, as their trimethylsilyl derivatives by
 329 gas-liquid chromatography-mass spectrometry. *Carbohydrate Research*, 10, 113-120.

330

331 Kinekawa, Y. I., & Kitabatake, N. (1996). Purification of β -lactoglobulin from whey
 332 protein concentrate by pepsin treatment. *Journal of Dairy Science*, 79, 350-356.

333

334 Lefevre, T., & Subirade, M. (2000). Molecular differences in the formation and structure of
 335 fine-stranded and particulate β -lactoglobulin gels. *Biopolymers*, 54, 578-586.

336

337 Lefevre, T., & Subirade, M. (2003). Formation of intermolecular β -sheet structures: a
338 phenomenon relevant to protein film structure at oil-water interfaces of emulsions. *Journal*
339 *of Colloid and Interface Science*, 263, 59-67.

340

341 Leung Sok Line, V., Remondetto, G. E., & Subirade, M. (2005). Cold gelation of β -
342 lactoglobulin oil-in-water emulsions. *Food hydrocolloids*, 19, 269-278.

343

344 Mattison, K. W., Brittain, I. J., & Dubin, P. L. (1995). Protein-polyelectrolyte phase
345 boundaries. *Biotechnology Progress*, 11, 632-637.

346

347 Mukhopadhyay, R., Talukdar, D., Chatterjee, B. P., & Guha, A. K. (2003). Whey
348 processing with chitosan and isolation of lactose. *Process Biochemistry*, 39, 381-385.

349

350 Nakano, T., Ikawa, N., & Ozimek, L. (2005). Use of chitosan as an adsorbent to isolate
351 kappa-casein glycomacropeptide from sweet whey: Preliminary study. *Milchwissenschaft*,
352 60, 62-64.

353

354 Nyström, B., Kjoniksen, A.-L., & Iversen, C. (1999). Characterization of association
355 phenomena in aqueous systems of chitosan of different hydrophobicity. *Advances in*
356 *Colloid and Interface Science*, 79, 81-103.

357

Rantamäki, P., Tossavainen, O., Outinen, M., Tupasela, T., Koskela, P., & Kaunismäki, M. (2000). Functional properties of the whey protein fractions produced in pilot scale processes. Foaming, water-holding capacity and gelation. *Milchwissenschaft*, 55, 569-572.

Remuñán-López, C., & Bodmeier, R. (1996). Effect of formulation and process variables on the formation of chitosan-gelatin coacervates. *International Journal of Pharmaceutics*, 135, 63-72.

Sannier, F., Bordenave, S., & Piot, J. M. (2000). Purification of goat β -lactoglobulin from whey by an ultrafiltration membrane enzymic reactor. *Journal of Dairy Research*, 67, 43-51.

Savant, V. D., & Torres, J. A. (2000). Chitosan-based coagulating agents for treatment of cheddar cheese whey. *Biotechnology Progress*, 16, 1091-1097.

Zydney, A. L. (1998). Protein separations using membrane filtration: New opportunities for whey fractionation. *International Dairy Journal*, 8, 243-250.

FIGURE CAPTIONS

Figure 1. β -lactoglobulin (β -lg) solubilized from chitosan complexes following a single extraction step with 5 or 10 mL of 0.1M sodium acetate solution at pH 8, 9 and 10,

respectively. β -lg was analysed by RP-HPLC following the method described in Materials and Methods section. Vertical bars represent standard deviation values ($n=3$).

Figure 2. β -lactoglobulin (β -lg) solubilized from chitosan complexes following a single extraction step with 5 or 10 mL of 0.08, 0.1, 0.15, 0.2 and 0.3M sodium acetate solutions at pH 9. β -lg was analysed by RP-HPLC following the method described in Materials and Methods section. Vertical bars represent standard deviation values ($n=3$).

Figure 3. RP-HPLC profile of the β -lactoglobulin (β -lg) solubilized from chitosan complexes following a single extraction step with 10 mL of 0.1M sodium acetate solution, pH=9. α -LA: α -lactalbumin; CMP: caseinomacropptide.

Figure 4. Linear correlation between (A) chitosan hydrolyzed with 3N HCl and glucosamine determined by GC-FID, and (B) known amounts of standard chitosan added to the supernatant containing the solubilized β -lactoglobulin and chitosan concentration determined by GC-FID.

Figure 5. Partial view of NOESY spectra (100 ms mixing time) of (A) chitosan purified β -lactoglobulin (β -lg) and (B) commercial β -lg in D₂O, at pH 2 and 35 °C. For more details, see Materials and Methods section.

401 **Figure 1. Montilla et al.**

402

403

404

Figure 2. Montilla et al.

Figure 3. Montilla et al.

Figure 4. Montilla et al.

Figure 5. Montilla et al.