Isolation of Bovine β-Lactoglobulin from Complexes with Chitosan

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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, $CaCl_2$ (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

106 30 min and centrifuged at 10000 g for 10 min to separate undissolved chitosan from
107 solubilized β-lg.
108 For total solubilization of both chitosan and β-lg, a solution of 0.1 N acetic acid at pH 4
109 was used.
110
111 ***Determination of β-lg.*** Analyses of whey proteins in solution were performed by RP-
112 HPLC-UV using 0.1% (w/v) trifluoroacetic acid in double-distilled water as solvent A and
113 0.085% (w/v) trifluoroacetic acid in double-distilled water/acetonitrile (10:90, v/v) as
114 solvent B. The chromatographic conditions were those described by Casal et al. (2006).
115 Protein contents obtained in the supernatants after extraction with sodium acetate solutions
116 were expressed as a percentage of the total protein contained in the pellets. All treatments
117 were replicated three times. The identity of whey proteins was confirmed by comparison of
118 their retention times with those of the protein standards purchased from Sigma (St. Louis,
119 MO).
120
121 ***Determination of chitosan.*** The quantitation of chitosan was done by means of their
122 glucosamine content determined by GC-FID after acid hydrolysis. The optimum hydrolysis
123 conditions that led to a high depolymerization degree of chitosan were those achieved with
124 3 N HCl at 110ºC for 40 hours. Thereby, either chitosan solutions (0.01-0.2 mg/mL) or
125 supernatants containing the redissolved β-lg after extraction with 10 mL of 0.1M sodium
126 acetate at pH 9 were subjected to acid hydrolysis before subsequent GC-FID analysis.
127 Prior to derivatisation and GC analysis, 1 mL of filtered hydrolysates and 0.2 mL of
128 a solution of 0.01% (w:v) phenyl-β-D-glucoside in methanol/water (70:30, v/v) (internal

129 standard) were mixed and dried at 38-40ºC in a rotavapor. Then, 100 μL of pyridine and
130 100 μL of N-trimethylsilylimidazole were added to sylilate the carbohydrates and the
131 derivatisation reaction was completed in 30 minutes at 70ºC. The reaction was stopped by
132 cooling and the sylilated carbohydrates extracted with 0.1 mL of hexane and 0.2 mL of
133 water. Volumes in the range of 1-2 μL of the organic phase containing silyl derivatives
134 were injected onto the column. The trimethylsilyl ethers were separated using a commercial
135 8 m x 0.25 mm inside diameter, 0.25 μm film fused silica capillary column CP-SIL 5 CB
136 (100% dimethylsiloxane, non polar) (Chrompack, Middelburg, The Netherlands). The
137 temperatures of injector and detector were 300ºC, the oven temperature was programmed
138 from 140ºC to 180ºC at 5ºCmin^{-1}, then at 50ºCmin^{-1} to 250ºC and keeping this temperature
139 for 5 min, followed for an increase up to 300ºC at rate of 50ºCmin^{-1}, and finally keeping
140 this temperature for 7 min. Injections were made in the split mode (1:10). Data acquisition
141 and integration was done using HP ChemStations software (Hewlett-Packard, Wilmington,
142 USA).
143 GC analysis of glucosamine gave rise to two anomers as previously shown
144 (Karkkainen and Vihko, 1969). The quantitation of glucosamine was performed with the
145 more retained anomer as it did not overlap with other peaks. This anomer represented
146 39.3% of the glucosamine. GC analyses were performed in quadruplicate. Linear
147 correlation analyses between glucosamine and hydrolysed chitosan contents were
148 performed using the MS Excel programme.
149
150 ***NMR analysis of β-lg.*** NMR was used to compare the spectra of the chitosan-purified β-lg
151 and a commercial and native β-lg (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 D_2O 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 ºC, 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 ^{1}H 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 t_2 and t_1 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%

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

205 Once achieved a high recovery of the precipitated β-lg, we proceed to estimate the
206 protein purity of the supernatant by RP-HPLC analysis. The supernatant comprised of
207 94.5% β-lg, 3% of α-lactalbumin, 2% of caseinmacropeptide and 0.3% of BSA (Fig. 3).
208 The minor presence of some whey proteins might be attributed to an incomplete phase
209 separation occurred during the precipitation of β-lg with chitosan and, therefore, some
210 remnants of other whey proteins could be absorbed on the pellet. Unsuccessful attempts
211 were made to obtain a better phase separation between the complexed β-lg with chitosan
212 and the rest of the whey proteins remaining in solution by increasing the centrifugation
213 speed from 5,000 to 10,000 *g* and/or performing several rinses of the pellet with water and
214 sodium hydroxide prior to the solubilization of β-lg (results not shown).

215

216 ***Determination of chitosan by GC-FID following acid hydrolysis***

217

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

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

228 The concentration of glucosamine found in the supernatants of the solubilized β-lg
229 was 0.25 ng / mL supernatant (n=10) which is equivalent to 4 μg of chitosan per mL of
230 supernatant. To elucidate the origin of the glucosamine, i.e. whether it comes from the
231 chitosan or from whey, β-lg was precipitated by decreasing the pH with acetic acid down to
232 4.5, followed by heating at 80ºC for one hour, and then was redissolved with 0.04% sodium
233 hydroxide (w:v). This sample showed a content of 0.54 ng of glucosamine / mL supernatant
234 (n=2) thus, it can be concluded that the low levels of glucosamine detected in the
235 supernatant of the resuspended β-lg were not derived from the chitosan.

236

237 ***NMR analysis of the recovered β-lactoglobulin***

238

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

244 a commercial source. The 1D and COSY spectra of the chitosan-purified and the native β-
245 lg were very similar, regarding intensity and chemical shift of resonances (results not
246 shown). This indicates that the chitosan-purified protein presented a similar structure to that
247 of the native β-lg. In addition, no line broadening was observed, consistent with a non-
248 aggregated protein. Finally, NOESY spectra cross-peaks, that represent the "structural
249 fingerprint" of the protein, were also very similar to the native protein (Fig. 5). It has to be
250 considered that the isolated protein was not tested under the same conditions that it was
251 recovered, i. e. 0.1M sodium acetate. However, if lowering the pH and removing the salts
252 led to a protein with a structure very close to that of native β-lg; it can be deduced that the
253 structure of the sodium acetate extracted protein was also able to reverse any
254 conformational changes induced by the alkaline pH or the presence of salts. This also opens
255 up the possibility of obtaining a native-like β-lg by removal of solvent co-solutes.
256
257 **CONCLUSIONS**
258
259 In summary, from these results it can be inferred the key role of electrostatic
260 interactions on the complexation of β-lg and chitosan. This can explain the easy
261 solubilization, and consequently high yield of β-lg just by a simple extraction step with a
262 solution of sodium acetate. To conclude, these findings point out that the precipitation of β-
263 lg with chitosan and subsequent solubilization may be an economical and feasible method
264 for the recovery of β-lg present in whey. Moreover, the reversibility of the β-lg
265 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.

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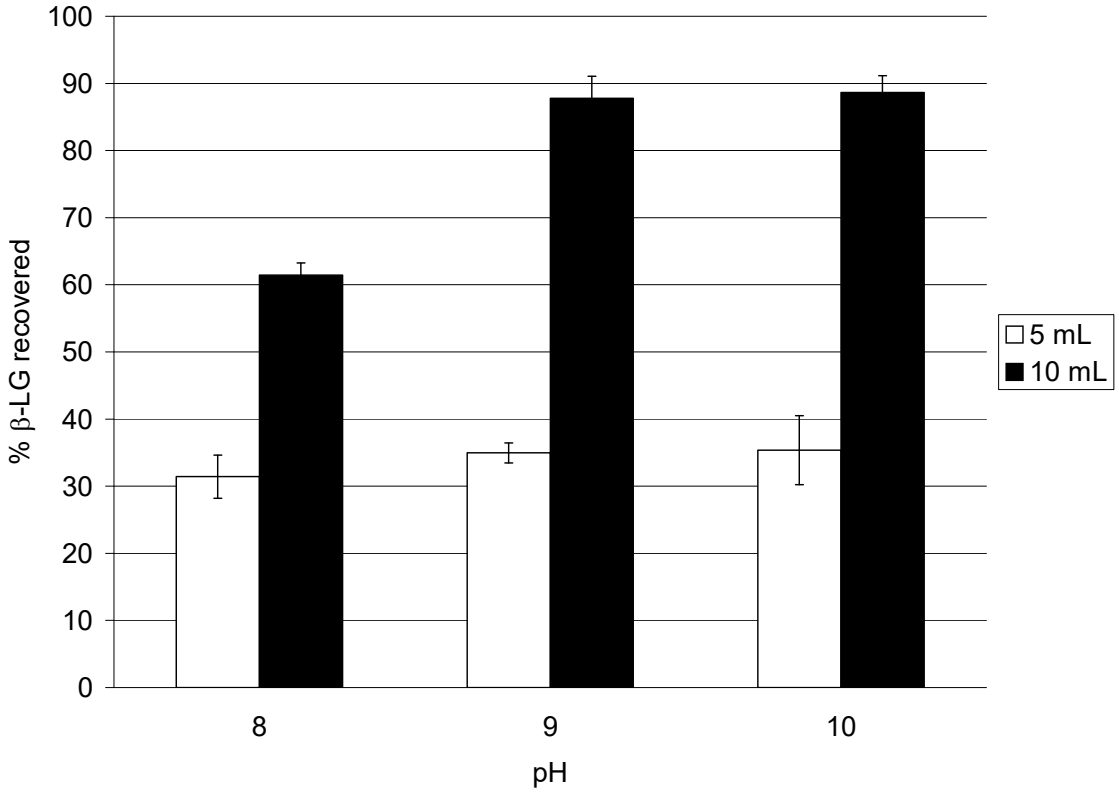
FIGURE CAPTIONS

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,

380 respectively. β-lg was analysed by RP-HPLC following the method described in Materials
381 and Methods section. Vertical bars represent standard deviation values (*n*=3).
382
383 **Figure 2.** β-lactoglobulin (β-lg) solubilized from chitosan complexes following a single
384 extraction step with 5 or 10 mL of 0.08, 0.1, 0.15, 0.2 and 0.3M sodium acetate solutions at
385 pH 9. β-lg was analysed by RP-HPLC following the method described in Materials and
386 Methods section. Vertical bars represent standard deviation values (*n*=3).
387
388 **Figure 3.** RP-HPLC profile of the β-lactoglobulin (β-lg) solubilized from chitosan
389 complexes following a single extraction step with 10 mL of 0.1M sodium acetate solution,
390 pH=9. α-LA: α-lactalbumin; CMP: caseinomacropeptide.
391
392 **Figure 4.** Linear correlation between (A) chitosan hydrolyzed with 3N HCl and
393 glucosamine determined by GC-FID, and (B) known amounts of standard chitosan added to
394 the supernatant containing the solubilized β-lactoglobulin and chitosan concentration
395 determined by GC-FID.
396
397 **Figure 5.** Partial view of NOESY spectra (100 ms mixing time) of (A) chitosan purified β-
398 lactoglobulin (β-lg) and (B) commercial β-lg in D_2O, at pH 2 and 35 ºC. For more details,
399 see Materials and Methods section.
400

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



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405 **Figure 2. Montilla et al.**

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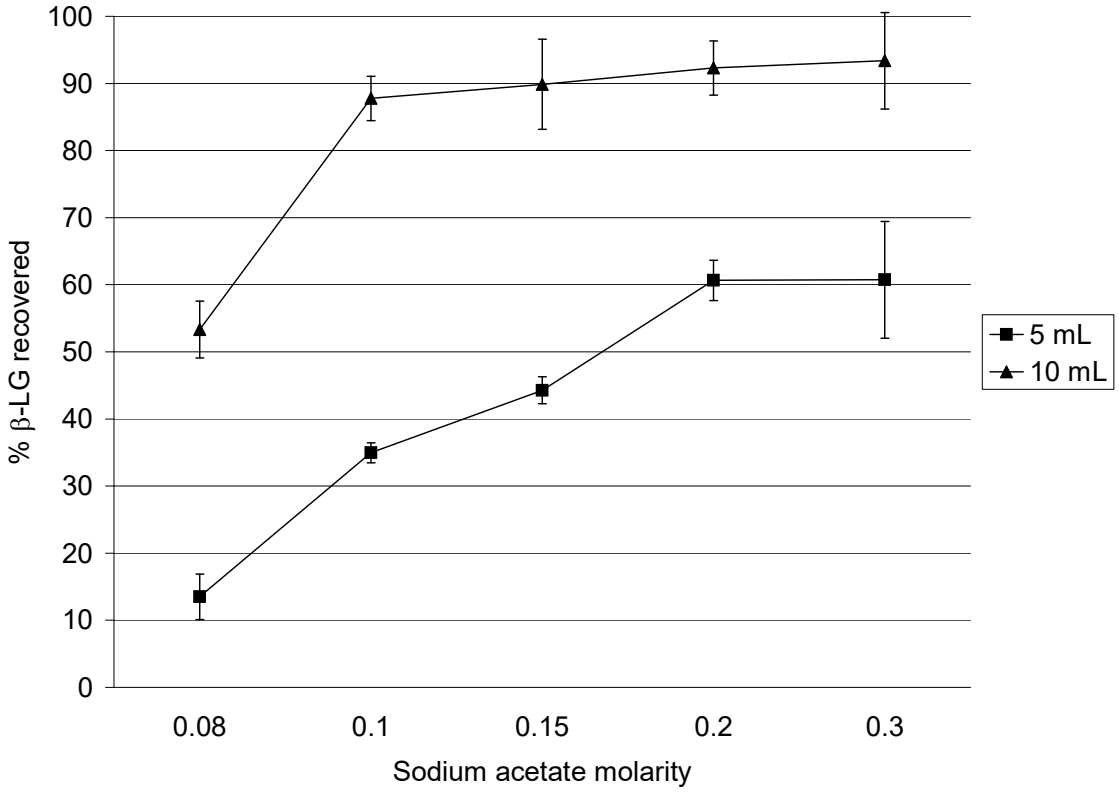

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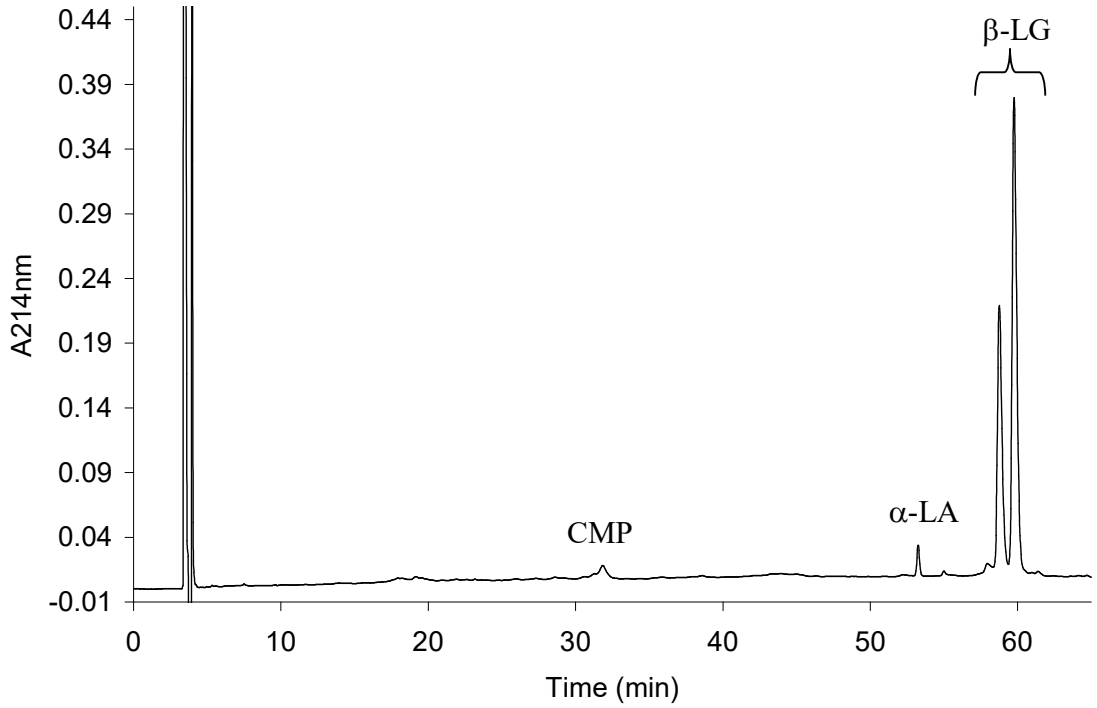
Figure 3. Montilla et al.



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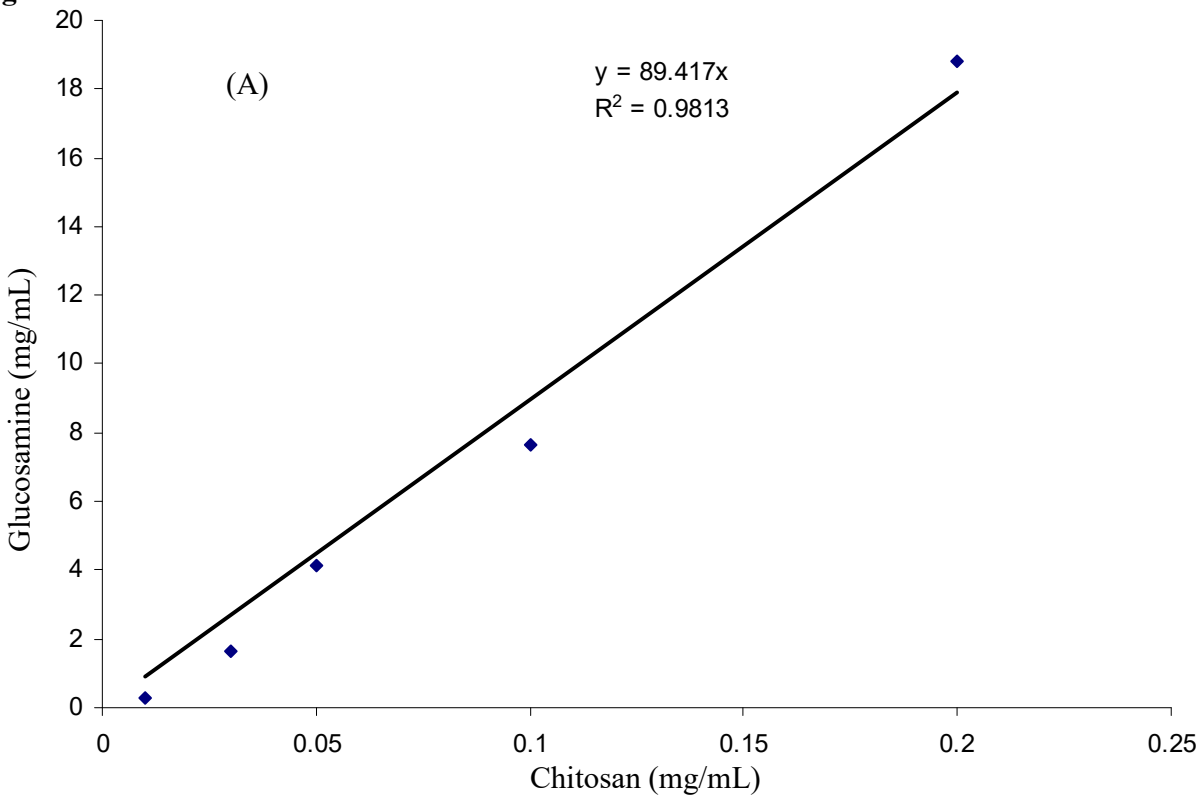

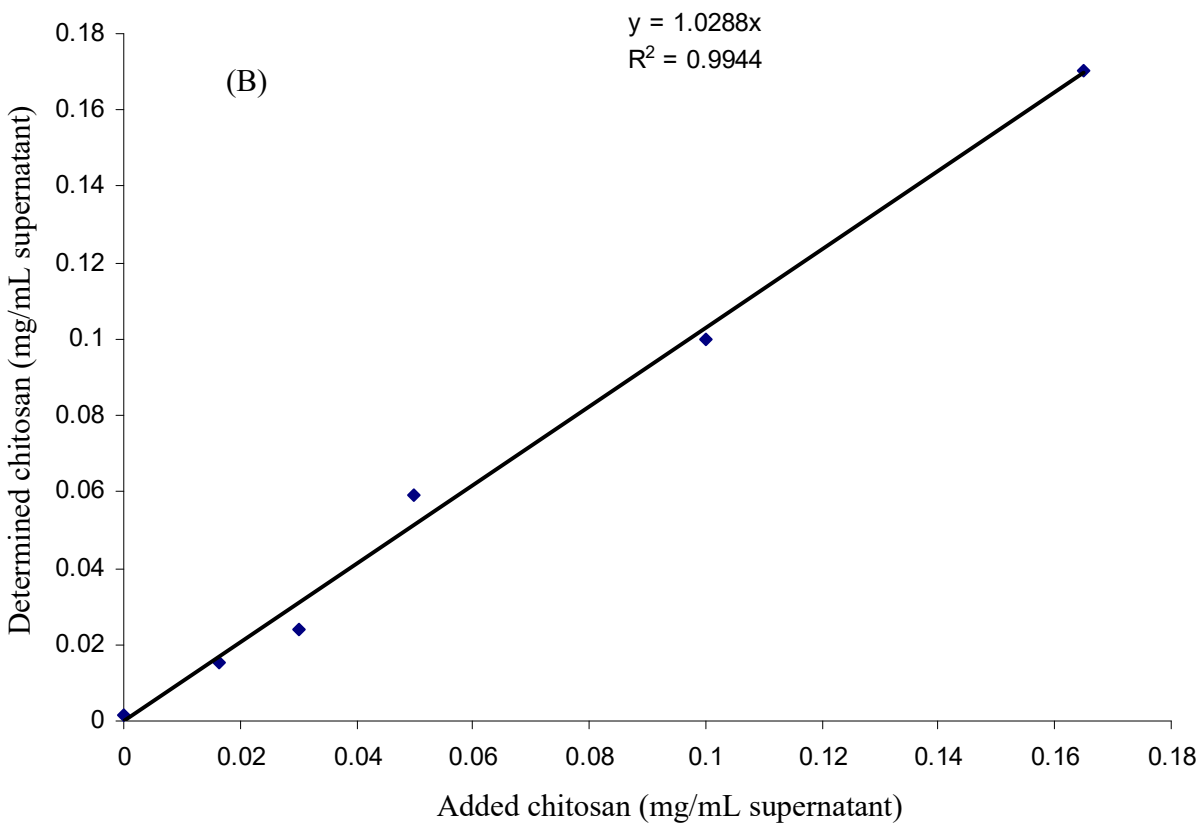

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458 **Figure 5. Montilla et al.**

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