USE OF CHITOSAN FOR SELECTIVE REMOVAL OF β-LACTOGLOBULIN FROM WHEY

Casal, E., Montilla, A., Corzo, N.* and Olano A.
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 Ext. 307
Fax number: (34) 91 564 48 53
e-mail: ncorzo@ifi.csic.es
ABSTRACT

A method for selective removal of β-lactoglobulin from cheese whey based on interactions between whey proteins and chitosan is described. Whey was previously clarified at pH 4.5 with addition of chitosan (25 mg/100ml) and then selective removal of β-lactoglobulin was studied in the pH interval 5.4-6.5, above the isoelectric point of the main whey proteins. Addition of chitosan caused selective precipitation of β-lactoglobulin which increased with pH. The content of β-lactoglobulin in whey decreased as the amount of chitosan added increased. At pH 6.2 addition of 2.5 mg/ml of chitosan led to substantially complete removal of β-lactoglobulin whereas most of the rest of whey proteins remained in solution.

Key words: chitosan, β-lactoglobulin, cheese whey fractionation
INTRODUCTION

Whey, the main by-product generated during cheese and caseinate manufacture, contains about 55% of the milk nutrients including most of the salts, lactose and water-soluble proteins. The use of microfiltration and ultrafiltration / diafiltration for removing lactose and salts enable the industry to manufacture whey protein concentrate (WPC) which has many uses in the food industry (1). The heterogeneous nature of WPC preparations is typical of many commercial products and the observed functionality is the sum of the functionality of individual proteins (2). The development of new processes for the recovery of protein from whey has resulted in considerable increase of the number of whey protein products available increasing their applications as food ingredients.

Whey contains a heterogeneous mixture of proteins with a wide range of functional properties not only for the food industry but also for chemical, pharmaceutical and cosmetic industries. The main whey protein fraction consists of a mixture of β-lactoglobulin, α-lactalbumin, immunoglobulins, serum albumin, and lactoferrin. The development of new processes for the isolation of individual whey protein species with constant and well-defined properties allows new and expanded uses of whey. For instance, the principal protein constituent of human milk is α-lactalbumin, which represents approximately 30% of the total protein in this milk. Moreover, human milk contains negligible quantities of β-lactoglobulin. Under these circumstances, removal of β-lactoglobulin from cheese whey would find immediate application as the primary protein constituent of infant formulas with protein composition that are more similar to that of human milk (3-5). On the other hand, the β-lactoglobulin fraction has excellent functional properties such as gelation and foaming (3) and should find immediately applications in manufactured food products.

Several procedures have been proposed for separation of individual whey proteins including salting-out (6), selective precipitation (7), chromatographic techniques (8-15) and membrane filtration (16-17). 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 the extremes of heat, pH or salt used during the process (16, 18).
Chitosan, a polysaccharide comprising copolymers of glucosamine and N-acetyl-glucosamine has great potential in food industry and biotechnology applications because of its unique cationic character (19). Chitosan has been shown to be an effective coagulating agent in wastewater treatment and recovery of lipids and proteins from plant processing food wastes (20-22) including dairy wastewater (23) however, no selective recovery of individual whey proteins have been achieved by the use of chitosan. Whey proteins show different structures and physical and chemical properties therefore chitosan should be an efficient selective coagulant for individual whey proteins under adequate conditions.

The aim of this work was to find fractionation conditions suitable for selective removal of β-lactoglobulin from whey using chitosan as a coagulant.

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).

Rennet whey preparation. Pasteurized 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 half 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 clarified whey. Precipitation of lipids and suspended solids by chitosan was as follows: different aliquots of chitosan solution (1% solution in 10% acetic acid) were added to cheese whey to obtain solutions of 0.2, 0.25 and 0.3 mg chitosan/mL. The pH was adjusted to 4.5 and the mixture was vigorously stirred for 1 min to reach a complete interaction and coagulation. Then, the different samples were allowed to stand for 10, 20 and 30 min and centrifuged at 5000 g for 10 min to separate the pellets from supernatants. Reduction of turbidity of whey was monitored by measuring the optical
density at 660 nm (DU-70, Beckman). A control, containing no added chitosan, was performed under identical conditions.

Recovery of β-lactoglobulin. Clarified whey, obtained after treatment with 0.25 mg chitosan/mL at pH 4.5, was subjected to treatments with different amounts of chitosan (1% solution in 0.1 M acetic acid) under different pH conditions. Chitosan (0.18-2.5 mg/mL) was added to clarified whey and the pH was adjusted within the range 5.4 - 6.5 with 1M NaOH. The mixtures were vigorously stirred for 1 min to reach a complete interaction and coagulation, then allowed to stand for 10- 30 min and centrifuged at 5000 g for 10 min to separate the pellets from supernatants.

Analytical Methods

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) analysis

Analyses of protein in solution before and after treatment with chitosan were carried out by RP-HPLC following the method described by Moreno et al. (24) with several modifications. RP-HPLC separations were carried out in a C18 Nucleosil column (300 Å, 250 x 4.6 mm i.d., 5 μm particle size) (Hichrom, Reading). Operating conditions were as follows: flow rate, 1 mL/min; solvent A, 1% trifluoroacetic acid (TFA) in double-distilled water; solvent B, 1% TFA in HPLC grade acetonitrile (Scharlau Chemie). The elution was performed with a linear gradient by increasing the concentration of solvent B as follows: 0-35 min, 21.6-35.6%; 35-43 min, 35.6- 42%; 43-58 min, 42-52%; 58-59 min, 52-100%. Absorbance was recorded at 214 nm using a Beckman 166 UV detector (Beckman Instruments). Contents of protein in chitosan-treated solutions were expressed as a percentage of initial content. All treatments were performed in duplicate.

SDS-PAGE

Samples for SDS-PAGE were prepared by heating (100 ºC, for 10 min) whey with 10 mM Tris-HCl buffer, pH 8.0 containing 2.5 % SDS, 10 mM EDTA, and 5 % β-mercaptoethanol. SDS-PAGE was conducted with the Phast-System Electrophoresis apparatus, precast PhastGels Homogeneous 20 %, and PhastGel SDS buffer strips (Pharmacia, Uppsala, Sweden). Electrophoretic conditions and silver staining was
performed following the procedures of the manufacturer. Protein standards, β-lactoglobulin α-lactalbumin, Immunoglobulin G, serum albumin and lactoferrin were purchased from Sigma (St. Louis, MO).

RESULTS

A pre-treatment of whey at pH 4.5 with chitosan was performed to reduce turbidity prior to selective recovery of β-lactoglobulin. The turbidity of raw whey is mainly due to the presence of lipids derived from milk fat globule membrane fragments, which remain dispersed in stable colloidal form due to their high negative charge. The mixture of whey and chitosan was incubated for 10, 20 and 30 min. Maximum turbidity reduction was achieved after 10 min of incubation and did not increase with longer periods of incubation. Table 1 shows the reduction of turbidity followed by measuring optical density at 660 nm. Addition of 20 mg chitosan /100 mL of whey removed more than 95% of the initial turbidity from whey.

To determine whether loss of whey proteins occurred during treatment with chitosan at pH 4.5, the supernatant, obtained after precipitation, was analysed by RP-HPLC. The chromatographic profile of clarified whey was compared with untreated whey and they were identical, indicating that major whey proteins remain in whey after clarification. The absence of proteins in the precipitate was confirmed by SDS-PAGE whereas untreated whey and supernatant obtained after treatment with chitosans (0.25 mg/mL) had similar protein profiles (results not shown). Since the major whey proteins are either positively charged or electrically neutral at pH 4.5, they did not interact with the positively charged chitosan. These results are in agreement with previous studies (25).

The effect of treatment of previously clarified whey with chitosan 1.05 mg/mL at different pHs is showed in figure 1. The studied pH range 5.4-6.5 is above the isoelectric point of the main whey proteins therefore; all these proteins are negatively charged and are susceptible to form complexes with positively charged chitosan. β-lactoglobulin complexes stronger than α-lactalbumin and precipitation of β-lactoglobulin increased considerably with pH whereas a slight precipitation of α-lactalbumin was observed. Previous studies on polyanions complexing with whey proteins indicate that for α-lactalbumin significatively more of its positively charged
groups are part of large charge patches than for β-lactoglobulin. Furthermore, α-
lactalbumin was found to have one particularly large charge patch, consisting of a
cluster of six positively charged groups (26). The presence of large positively charged
patch may give rise to a strong electrostatic repulsion between α-lactalbumin and
polycationic chitosan. Although not only electrostatic forces contribute to the observed
complexation behaviour, the differences in the distribution of charge patches over the
surfaces of both proteins may play a role in the observed interaction between chitosan
and whey proteins.

The effect of the addition of different amount of chitosan on the removal of β-
lactoglobulin from whey at pH 6.2 was investigated by RP-HPLC analysis of
supernatants. Addition of 1.4 mg chitosan/ml gave rise to selective removal of β-
lactoglobulin as it is shown in figure 2. The content of β-lactoglobulin in solution
decreased as the amount of chitosan added increased (see Figure 3). Addition of 2.5 mg
chitosan/ml led to substantially complete removal of β-lactoglobulin whereas most of
the rest of whey proteins remained in solution.

This work shows the feasibility of using chitosan to reduce initial β-
lactoglobulin present in whey. Chitosan gave good results with the additional advantage
that no denaturation of proteins occurs. Since human milk contains negligible quantities
of β-lactoglobulin, the removal of β- lactoglobulin from whey would find immediate
application as the primary protein constituent of infant formulas with protein
composition that are more similar to that of human milk (4-5). Moreover, the procedure
allows the isolation of undenatured β-lactoglobulin that should find immediately
applications in manufactured food products.

ACKNOWLEDGEMENTS

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LITERATURE CITED.


Table 1. Turbidity change (optical density at 660 nm) of whey treated (10 min) with various amounts of chitosan.

<table>
<thead>
<tr>
<th>Chitosan concentration (mg/100 mL whey)</th>
<th>Optical Density (660 nm)*</th>
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<tr>
<td>0</td>
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<tr>
<td>20</td>
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<tr>
<td>25</td>
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<td>0.008</td>
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* Results are average of three experiments.
FIGURE CAPTIONS

Figure 1. Effect of pH on the precipitation of proteins from clarified whey by addition of chitosan (1.05mg/ml). β-LG: β-lactoglobulin; α-LA: α-lactalbumin; CMP: caseinmacropeptide; SA: serum albumin.

Figure 2. RP-HPLC chromatograms of the untreated (a) and treated whey (b) with chitosans (1.4 mg/mL) at pH 6.2.

Figure 3. Effect of the amount of chitosan added to whey on the precipitation of proteins at pH 6.2. β-LG: β-lactoglobulin; α-LA: α-lactalbumin; CMP: caseinmacropeptide, SA: serum albumin.
Figure 1
Figure 2
Figure 3