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Selective Recovery of Glycosylated Caseinmacropeptide with Chitosan

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The use of chitosan, a partially deacetylated chitin, to fractionate aqueous solutions of caseinmacropeptides (CMPs) was studied. The polycationic character of chitosan at acidic pH values allows the formation of complexes with negatively charged CMP molecules, inducing their flocculation. Glycosylated CMP (GMP) has higher affinity for chitosan than nonglycosylated forms (NGMP). The carboxylic groups in the carbohydrate moiety of the GMP increase the negative charge of the molecule and may play a role in the selective precipitation. At pH 5.0, 0.08 mg/mL of chitosan completely removed the GMP whereas 70% of NGMP remained in solution. As the pH increased, the amount of chitosan to ensure complete removal of GMP increased up to 0.19 and 0.34 mg/mL for pH 6.0 and 6.6, respectively.

KEYWORDS: Glycosylated CMP; fractionation; chitosan treatment; caseinmacropeptide

INTRODUCTION

Caseinmacropeptide (CMP) is the C-terminal fragment of κ-casein released by the action of chymosin during the milk-clotting process in cheesemaking. As its precursor κ-casein, CMP is a heterogeneous mixture due to genetic variations as well as to differently glycosylated and phosphorylated forms (*1*). It has lately received much attention as a functional ingredient due to its nutritional and biological properties (*2*). Preparation of CMP in the laboratory is usually carried out by enzymatic digestion of casein or pure κ-casein followed by acid precipitation (*3*, *4*). In developed methods for large-scale industrial production, CMP is obtained from cheese whey or whey protein concentrates (WPC) using anion-exchange resins (*5*) or ultrafiltration (UF) based on the pH-dependent changes of apparent molecular weight (MW) of CMP (*6*). The CMP obtained consists of a mixture of carbohydrate-free macropeptide (NGMP) and glycomacropeptide (GMP). Most of the biological properties of CMP have been attributed to the GMP fraction since sugar residues are carriers of information necessary in specific interactions with different cells in organisms, bacteria, viruses, and toxins (*7*).

Chitosan, obtained by partial deacetylation of chitin, is a polysaccharide comprising copolymers of glucosamine and *N*-acetyl-glucosamine. Chitosan has great potential in food industry and biotechnology applications because of its unique cationic character (*8*). Chitosan has been shown to be an effective coagulating agent in wastewater treatment and recovery of lipids and proteins from food-processing plants (*9*, *10*). It has also been described as acting as a dietary fiber with hypocholesterolemic effect (*11*), and it has also been shown to possess antimicrobial properties (*12*). Protein−polysaccharide conjugates have been proposed to be useful as new functional biopolymers (*13*, *14*), and extensive work has been done on the chitosan−protein interactions and their contribution to the formulation of stable products for food and nonfood uses (*14*−*16*).

The aim of the present work was to study the interactions between chitosan and CMP with a view to the formation of CMP−chitosan aggregates and to the possible use of chitosan for the recovery of glycosylated CMP from the total CMP fraction.

MATERIALS AND METHODS

Obtention of CMP. Bulk bovine milk (Holstein−Friesian breed) was provided by a local dairy farm, from the central region of Spain. CMP was obtained following the method of Moreno et al. (*17*). Whole casein was prepared by precipitation from bovine skim milk by adjusting the pH to 4.6 with 1 M HCl, followed by centrifugation at 4500*g* and 5 °C for 15 min. The casein precipitate was washed three times with 1 M sodium acetate−acetic acid buffer, pH 4.6, thoroughly dialyzed against water and lyophilized. Commercial rennet powder containing 85% chymosin (EC3.4.23.4) and 15% bovine pepsine (EC 3.4.23.1) was obtained from Chr. Hansen's Laboratorium (DK-1250 Copenhagen, Denmark). Rennet solution (1 mL, 4 mg/mL) was added to bovine casein solution (25 g/L) in 0.1 M sodium phosphate buffer, pH 6.5 (100 mL), and the mixture was incubated at 35 °C for 20 min. To inactivate chymosin, 0.2 M NaOH was added to pH 9.0−9.5, followed by heating at 60 °C for 15 min (*3*). The sample was adjusted to pH 4.6 with 1 M HCl and centrifuged at 4500*g* and 5 °C for 15 min. The supernatant was filtered through glass wool, subjected to exhaustive dialysis against water at 4 °C, and finally lyophilized.

CMP−Chitosan Treatments. High-molecular-weight chitosan (HMWC), 643 kDa, was supplied by Idebio S. L. (Spain) (*18*); medium MW chitosan (MMWC), average 400 kDa, and low MW chitosan (LMWC), average 120 kDa, were supplied by Aldrich (Milwaukee, WI). The deacetylation degree of all chitosan samples was about 85%.

Stock solutions of chitosans were prepared by their dissolution in 5.0 mL of 0.2 M acetic acid and adjusted to the desired pH with 0.2 M NaOH, followed by dilution to 10 mL with Milli-Q water. In this way were obtained solutions of 0.1, 0.2, 0.3, 0.5, 0.7, and 0.9 mg chitosan/mL.

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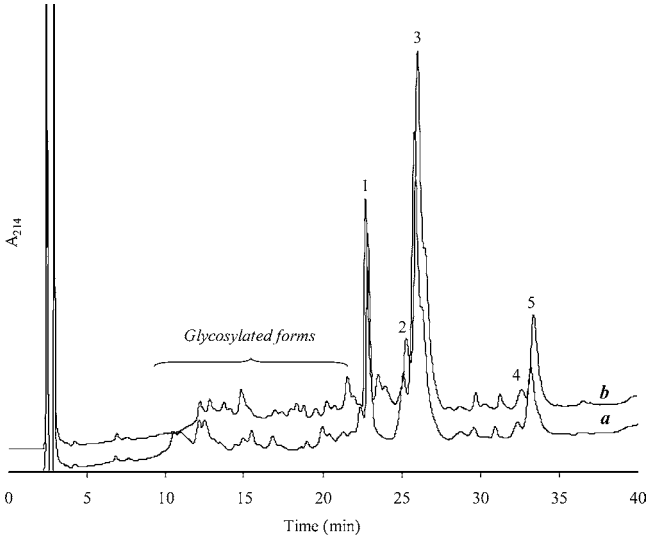

Figure 1. RP-HPLC chromatograms of the CMP fraction obtained by the action of chymosin on whole casein: (**a**) before neuraminidase treatment; (**b**) after neuraminidase treatment. Peaks with retention times of 9–22 min, glycosylated forms (GMP); peak 1, α_{s1}-casein fragment; peaks 2–5, nonglycosylated macropeptides (NGMP).

Aqueous solutions of CMP were prepared by dissolving 60 mg of CMP in 100 mL of Milli-Q water. All solutions were freshly prepared in our laboratory prior to use. After that, 0.5 mL of CMP solution was mixed with 0.3 mL of chitosan solution and stirred for 1 min to reach a complete interaction. The mixture was allowed to stand at 20 °C for 15 min and then centrifuged at 5000*g* for 10 min to separate the sediment from the supernatant. Previous assays at 15 and 30 min of treatment showed similar results. Final pH values of mixture chitosan and CMP solutions were in the range of 4.6−6.6.

Removal of any remaining chitosan in solution was achieved by addition of 0.1 M NaOH until pH 7.5 was reached. The solution was allowed to stand for 15 min at 20 °C before centrifugation (5000*g* for 15 min). The supernatant was then filtered through a 0.22 μm filter (Millex GV).

CMP−Neuraminidase Treatment. CMP (6.5 mg) in 1 mL of 50 mM sodium acetate was incubated with neuraminidase (EC 3.2.1.18 from *Clostridium perfringens*, Boehringer) according to Coolbear et al. (*19*). After 24 h, the enzyme was inactivated completely by heating for 10 min at 80 °C and free neuraminic acid was removed by ultrafiltration through a centrifugal filter (Centricon YM-3, 3000 Da cutoff, Millipore). A solution of CMP incubated with heat-inactivated neuraminidase was used as the reference sample.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) Analysis of CMP. Analyses of CMP before and after treatment with chitosan were carried out by RP-HPLC following the method described by Moreno et al. (*17*) with several modifications. RP-HPLC separations were carried out in a C18 Nucleosil column (300 Å, 250 × 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

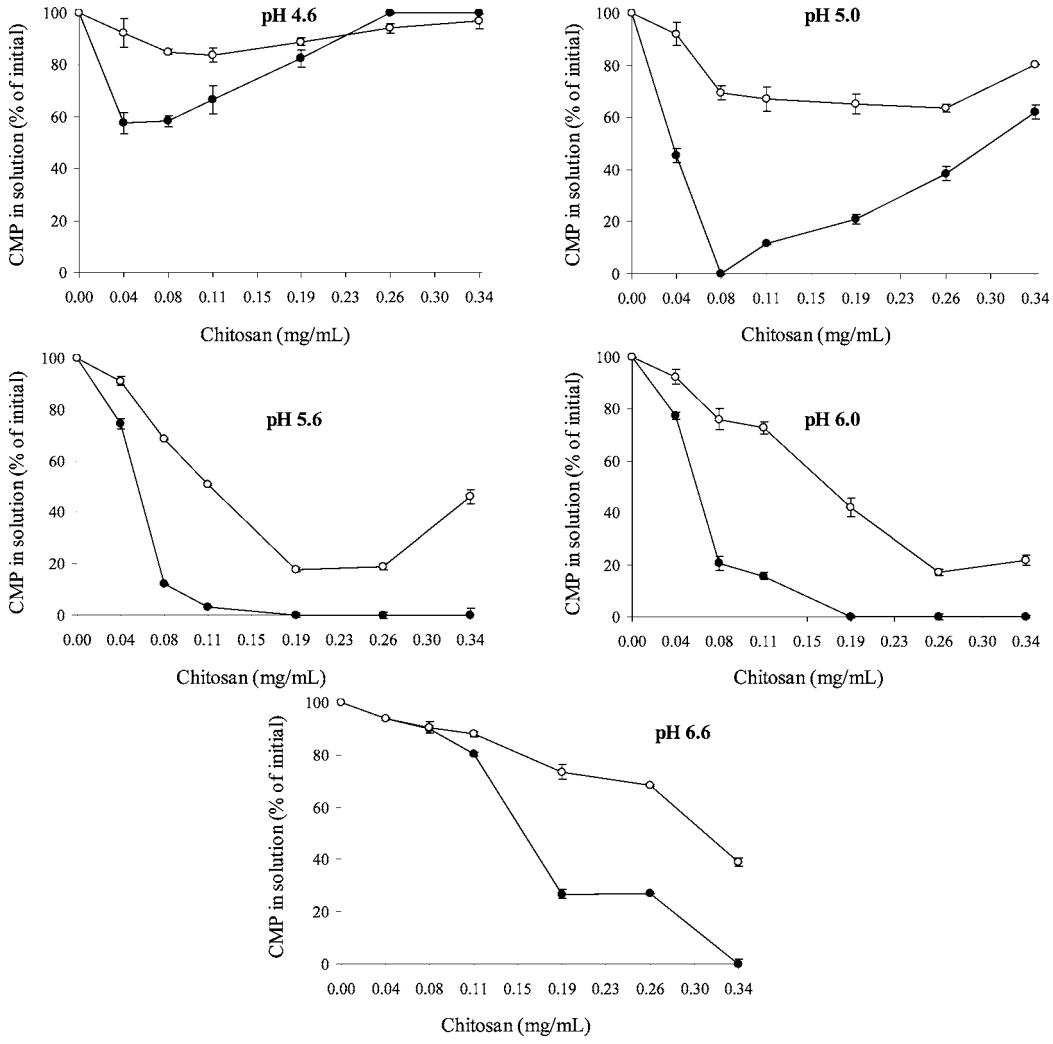

Figure 2. Effect of pH and addition of 120 kDa chitosan on the precipitation of glycosylated (●) (GMP) and nonglycosylated (○) (NGMP) forms of caseinmacropeptide.

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 CMP in chitosan-treated solutions were expressed as a percentage of initial content. All treatments were performed in quadruplicate.

RESULTS

The CMP fraction obtained by action of chymosin on whole casein was analyzed with reverse-phase HPLC, and the eluted peptides were monitored at 214 nm; **Figure 1a** shows the RP-HPLC chromatogram obtained. The identity of the CMP peaks was assigned by comparison of their retention times with those of previous studies (*7*, *17*, *20*). Two mean peaks (peaks 3 and 5) with retention times of 26.4 and 33.6 min correspond to the nonglycosylated macropeptides (NGMP); peak 1 at 23.7 min corresponds to fragment 1-23 of α_{s1}-casein (*21*). Earlier eluted peaks (retention time of 9−22 min) were assigned to the glycosylated forms (GMP). The chromatographic repeatability was evaluated by repeated injections ($n = 5$) of the same sample on different days, and the repeatability of the precipitation procedure was determined by analyzing five different solutions obtained after treatment of CMP with chitosan under the same experimental conditions. In all cases, the relative standard deviation (RSD) was lower than 10%.

When chitosan solutions were mixed with CMP solutions, a precipitate was formed and the resultant solution always had lower CMP content than the original solution, due to the formation of insoluble CMP−chitosan aggregates. Analysis of CMP solutions treated with chitosan showed no aditional removal of CMP beyond 15 min of treatment regardless of molecular weight of chitosan, concentration, and pH. The polycationic character of chitosan at acidic pH values allows the formation of complexes with negatively charged molecules, inducing their flocculation. The chemical composition of CMP with 10 acidic amino acids makes this compound sensitive to interactions with chitosan.

Formation of CMP−chitosan complexes was evaluated through determination of the glycosylated (GMP) and nonglycosylated (NGMP) forms in the supernatant obtained after chitosan treatment in different conditions. **Figure 2** shows the effects of pH and chitosan concentration used. As can be observed, chitosan addition to CMP solution resulted in precipitation of the CMP−chitosan complex in every test performed in the pH interval of 4.6−6.6. Using low concentrations of chitosan, the decrease of the glycosylated form in solution was higher than that of the nonglycosylated form, indicating that GMP has higher affinity for chitosan than NGMP.

The loss of CMP in the solution increases as chitosan concentration does, until a concentration level is reached above which the percentage of precipitated CMP decreases. Previous studies on protein−polysaccharide interactions showed that at low concentrations of polysaccharide, an insufficient amount is present to cover completely all the particle surface and some of the polysaccharide molecules become attached simultaneously to more than one particle giving rise to the bridging flocculation. At higher polysaccharide concentrations, particles are completely covered and bridge formation is prevented (*13*, *22*). Furthermore, the necessary amount of chitosan to attain maximum complex formation varies widely with the different pH conditions assayed. This fact could be explained by the changes in charge density with pH of both reactants. Regarding GMP interaction with chitosan, at pH 4.6 analysis showed that maximum insoluble

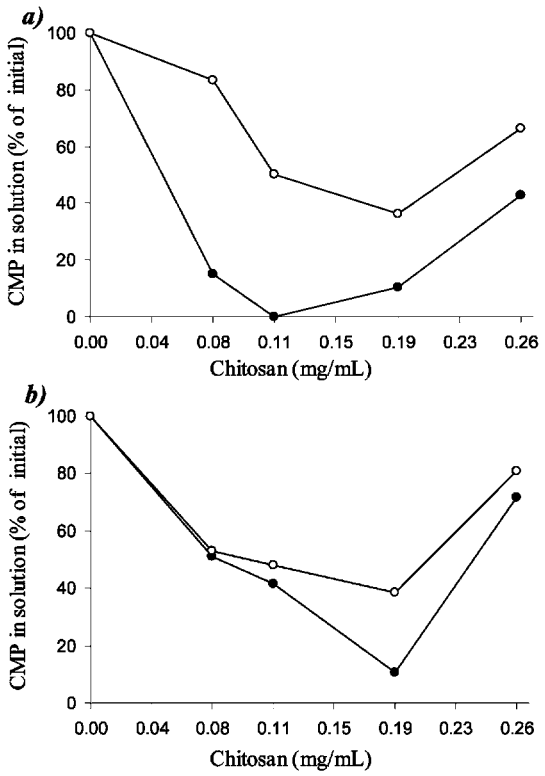

Figure 3. Recovery of CMP by precipitation with 120 kDa chitosan from solutions of (**a**) CMP and (**b**) neuraminidase-treated CMP: (●) glycosylated (GMP); (○) nonglycosylated (NGMP).

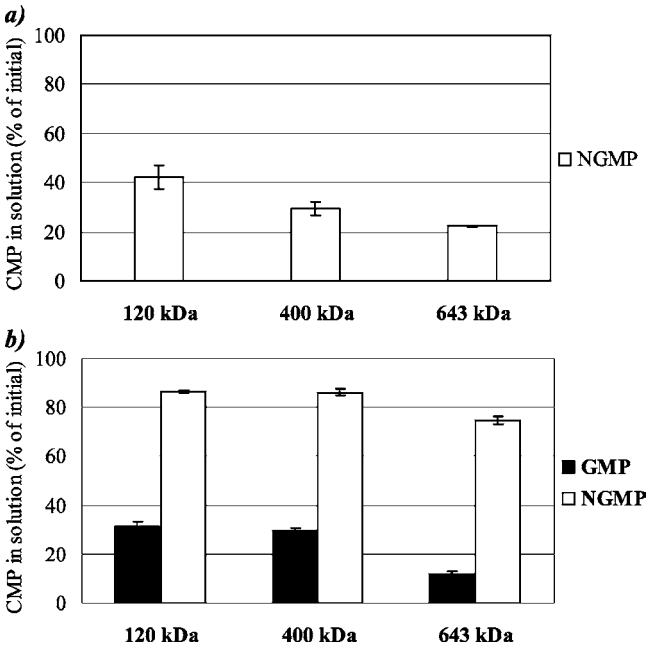

Figure 4. Effect of molecular weight of chitosan on the recovery of CMP from solutions: (**a**) addition of 0.19 mg chitosan/mL at pH 6.0 and (**b**) addition of 0.08 mg chitosan/mL at pH 5.6. Bars represent the standard deviation (treatments were performed in quadruplicate).

chitosan−GMP complex formation occurred in samples with a chitosan concentration of 0.04 mg/mL and did not exceed 45%. Precipitation of GMP increased with rising pH, and the necessary amount of chitosan to reach maximun precipitation also increased with pH. At pH 5.0, addition of 0.08 mg/mL of chitosan completely removed the GMP whereas 70% of NGMP remained in solution. As pH increases, the amount to ensure complete re-

moval of GMP increases up to 0.19 and 0.34 mg/mL of chitosan for pH 6.0 and 6.6, respectively. With regard to NGMP, the maximum precipitation increased with increase in pH from 4.6 to 6.0 and then decreased rapidly with further increase in pH.

Possible reasons for behavioral differences between the NGMP and GMP observed during treatment with chitosan may be attributed to the carbohydrate moiety of the GMP. Treatment of CMP with neuraminidase results in some changes in the HPLC pattern of the GMP peaks (see **Figure 1b**). The loss of neuraminic acid, always located on the outer part of the oligosaccharidic chains, may give rise to changes in the retention times of the resulting asialo-GMP forms. **Figure 3** shows the removal of CMP from solution before and after treatment of CMP with neuraminidase. As can be observed, original GMP forms were more selectively removed from solution than asialo-GMP forms. This indicates that acid residues of neuraminic acid actively participate in the chitosan−CMP interactions. The presence of neuraminic acid increases the negative charge of the molecule and may play a role in the selective precipitation of glycosylated forms.

The effect of the molecular weight of chitosan on the interaction with CMP is shown in **Figure 4**. As expected, higher affinity for GMP was observed in all assays since the mechanism of interaction between chitosan and CMP is independent of the molecular weight of chitosan. However, precipitation of CMP seems to increase with molecular weight. This may be attributed to lower solubility properties of higher molecular weight complexes. Polymer molecular weight influences the destabilization mechanism, and some studies have shown the existence of a preferential molecular weight, at which coagulation reaches its maximum. This fact seems logical since the size of aggregates increases as the molecular weight of chitosans increases (*23*).

In conclusion, the present results demonstrated the efficacy of chitosan in the formation of CMP−chitosan complexes and the selective recovery of GMP from the total CMP fraction. These results open up new perspectives concerning the possible applications of chitosan and CMP in food technology.

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