Affinity Chromatography Matrices for Depletion and Purification of Casein Glycomacropeptide from Bovine Whey

María F. Baieli
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956 (1113) Buenos Aires, Argentina

Instituto de Nanobiotecnología (NANOBIOTEC), UBA, CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

Nicolás Urtasun
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956 (1113) Buenos Aires, Argentina

Instituto de Nanobiotecnología (NANOBIOTEC), UBA, CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

María J. Martinez
Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Industrias, Ciudad Universitaria (1428), Buenos Aires, Argentina

Daniela B. Hirsch
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956 (1113) Buenos Aires, Argentina

Instituto de Nanobiotecnología (NANOBIOTEC), UBA, CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

Ana M. R. Pilosof
Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Industrias, Ciudad Universitaria (1428), Buenos Aires, Argentina

María V. Miranda
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956 (1113) Buenos Aires, Argentina

Instituto de Nanobiotecnología (NANOBIOTEC), UBA, CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

Osvaldo Cascone
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956 (1113) Buenos Aires, Argentina

Instituto de Nanobiotecnología (NANOBIOTEC), UBA, CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

Federico J. Wolman
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956 (1113) Buenos Aires, Argentina

Instituto de Nanobiotecnología (NANOBIOTEC), UBA, CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

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Casein glycomacropeptide (CMP) is a 64- amino acid peptide found in cheese whey, which is released after κ-casein specific cleavage by chymosin. CMP lacks aromatic amino acids, a characteristic that makes it usable as a nutritional supplement for people with phenylketonuria. CMP consists of two nonglycosylated isoforms (aCMP A and aCMP B) and its different glycosylated forms (gCMP A and gCMP B). The most predominant carbohydrate of gCMP is N*-acetylneuraminic acid (sialic acid). Here, we developed a CMP purification process based on the affinity of sialic acid for wheat germ agglutinin (WGA). After formation of*

Correspondence concerning this article should be addressed to F. J. Wolman atfwolman@ffyb.uba.ar

chitosan beads and adsorption of WGA, the agglutinin was covalently attached with glutaraldehyde. Two matrices with different WGA density were assayed for CMP adsorption. Maximum adsorption capacities were calculated according to the Langmuir model from adsorption isotherms developed at pH 7.0, being 137.0 mg/g for the matrix with the best performance. In CMP reduction from whey, maximum removal percentage was 79% (specifically 33.7% of gCMP A and B, 75.8% of aCMP A, and 93.9% of aCMP B). The CMP was recovered as an aggregate with an overall yield of 64%. Therefore, the matrices developed are promising for CMP purification from cheese whey. *Biotechnol. Prog.*, 33:171–180, 2017
Keywords: chitosan, wheat germ agglutinin, chromatography matrix, casein glycomacropeptide, bovine whey

Introduction

Sweet whey is obtained from milk coagulation with chymosin in the manufacture of cheese. Chymosin hydrolyzes κ-casein, generating two fragments: casein glycomacropeptide (CMP) (a hydrophilic acid moiety of 64 amino acid residues, which remains in whey) and para-κ-casein (a hydrophobic basic moiety of 105 amino acid residues, which is largely retained in the coagulum).[1,2] Glycosylated forms of CMP (gCMP) represent about 50% of total CMP.[3] The glycosidic moiety of CMP consists of *N*-acetylneuraminic acid (NeuNAc, sialic acid), galactose and *N*-acetylgalactosamine. The different gCMP forms result from two nonglycosylated isoforms (aCMP A and B). These genetic variants of aCMP have molecular masses of 6.79 and 6.76 kDa, respectively, whereas the most highly glycosylated CMP monomer has a molecular mass of up to 9.63 kDa.[4] CMP, which is the principal whey protein containing sialic acid, is devoid of aromatic amino acids[2,5] and rich in threonine residues. Thus, removing CMP from whey leaves lower levels of threonine, which is important because high levels of this amino acid in milk formulae bring about an inadequate brain development in infants.[6,7] Moreover, it is known that the amino acid profile of CMP-free whey is similar to that of human milk.[8] Thus, CMP-free whey can be used for different infant formulations.

Chitosan, the deacetylated derivative of chitin, is a linear copolymer of 2-acetamide-2-dioxy-β-D-glucose (D-GlcNAc) and 2-amino-2-dioxy-β-D-glucose (D-GlcN) residues randomly distributed,[9–12] which has been used as a chromatographic matrix because it presents the advantages of low cost, high availability and biodegradability, biocompatibility, and nontoxicity.[9,11,13,14] It is soluble in acid media and insoluble in basic media and has high resistance to chemical and biological degradation.

Wheat germ agglutinin (WGA) is a 35 kDa lectin formed by two identical subunits of 17 kDa (117 residues each),[15] with high specificity for sialic acid and two binding sites for D-GlcNAc per subunit.[10,15–17] For these reasons, WGA can be used as a ligand to purify glycoconjugates by affinity chromatography.[18]

CMP has been previously purified by various techniques[1] such as ultrafiltration,[19,20] size exclusion chromatography[21] and ion exchange chromatography.[5,22] However, affinity chromatography is a good alternative, when prejudices about its high cost are overcome, due to its greater selectivity and the possibility of using affinity matrices when the starting material has a low-concentration of the target protein.

Since gCMP forms contain 7–8% of sialic acid[23] and WGA has affinity for sialic acid, it would be possible to use WGA as a ligand for gCMP purification by affinity chromatography. In this case, adsorption only of the sialylated fraction to the matrix would be expected; however, previous studies using dynamic light scattering (DLS) have reported the self-assembly of different fractions of CMP.[24] On the basis of this, in this work, we studied the possible adsorption of aCMP to gCMP previously bound to the matrix, to allow the almost complete removal of the CMP from whey. Thus, the aim of this work was to generate and characterize an affinity chromatography matrix able to purify CMP and obtain CMP-free whey. Such matrix consisted of chitosan mini-spheres with immobilized WGA. The process used to purify WGA from wheat germ extract using chitosan mini-spheres was characterized and developed in a previous work.[25]

Materials and Methods

Materials

Chitosan (low viscosity, acetylation degree 41%) and 25% glutaraldehyde were from Sigma-Aldrich (St. Louis, MO, USA) and epichlorohydrin was from Fluka Analytical (Buchs SG, Switzerland). Wheat germ was purchased at a local health food store and lectin of *Triticum vulgaris* (WGA) was from Megazyme (Bray Wicklow, Ireland). CMP was kindly donated by Davisco Food International (Eden Prairie, MN). Hollow Fiber Cartridge UFP-10-C-4MA (10,000 NMWCO) and Sephadex G-25 columns (PD10) were from GE Healthcare (Piscataway, NJ, USA). Bovine cheese whey was kindly donated by Cooperativa Lechera Gualeguaychú (Gualeguaychú, Entre Ríos, Argentina). All other reagents were analytical reagent grade.

Preparation of the support matrix

The support matrix was synthesized as previously.[25] A 2% chitosan solution was prepared by dissolution in acetic acid (2.0%). The solutions were centrifuged at 17,212 *g* for 25 min and dripped on 2 *M* NaOH through a 15G needle.[26] For the crosslinking reaction, a 250 m*M* epichlorohydrin solution was used as previously.[25] The support matrix generated is named hereafter CH.

Characterization of the matrix

Size and Porosity of the Support Matrix. The size of the matrix generated was measured in quintuplicate with a Vernier sensitive to 0.01 mm, whereas its porosity was calculated according to Roh and Kwon[27] by the liquid displacement technique with a freeze-dried matrix.

Scanning Electron Microscopy (SEM). SEM (Zeiss Supra 40 microscope) was used to analyze the morphology of the chitosan support matrix. Prior to the analysis, the support matrix was lyophilized and metalized with gold (gold sputtering).

To characterize possible changes in the matrix structure and morphology during the process, the matrix was analyzed by SEM before and after CMP adsorption from a solution of 2 mg/mL of commercial CMP.

Generation of chitosan mini-spheres with immobilized WGA

For WGA adsorption to the support matrix, we used a WGA extract obtained by the procedure reported in Baieli et al.[25] To adsorb WGA to the chitosan mini-spheres, two ratios of matrix weight (g) to WGA extract volume (mL) were assayed: 1/10 and 1/50. The matrices were incubated with the extract overnight with gentle agitation and then washed with 20 m*M* phosphate buffer, pH 7.0. A part of each matrix was stored without further modification (CH10 and CH50) and another was reacted with 0.05% glutaraldehyde in 20 m*M* phosphate buffer, pH 7.0, to covalently bind WGA to the matrices (CH10G and CH50G). The reaction was carried out with stirring at room temperature for 1 h, maintaining a ratio of 15 mL of glutaraldehyde solution per gram of matrix.[28] The matrices generated are listed in Table 1. The matrices were stored in 20 m*M* phosphate buffer, pH 7.0, with 150 m*M* NaCl, 20 m*M* D-GlcNAc and 0.08% sodium azide.

The amount of WGA in each matrix was measured by RP-HPLC.[25]

Preliminary test for CMP adsorption

For the preliminary studies, 25 mg of each matrix (Table 1) was gently stirred overnight with 1 mL of 1 mg/mL commercial CMP solution at pH 4.0, 5.0, 7.0, or 8.5. The adsorption solutions were 20 m*M* acetate buffer, pH 4.0, 20 m*M* acetate buffer, pH 5.0, 20 m*M* phosphate buffer, pH 7.0 and 20 m*M* borate buffer, pH 8.5. The concentration of CMP in the supernatants was determined spectrophotometrically at 230 nm.[29] The equilibrium concentration of CMP bound to the matrix per unit amount of matrix was calculated by the difference between the concentration of CMP at the beginning of the experiment and that remaining in the soluble phase at the end of the experiment.

All the determinations were performed in triplicate and the results expressed as the mean ± standard deviation.

Commercial CMP adsorption isotherms

Adsorption isotherms were performed at pH 7.0 and 8.5. To this end, 25 mg of CH10G and 25 mg of CH50G (Table 1) were soaked with 1 mL commercial CMP solutions (0.125–10 mg/mL in adsorption solution at pH 7.0 or 8.5). All suspensions were gently shaken overnight at room temperature to reach equilibrium. The concentration of CMP in the supernatants was determined spectrophotometrically at 230 nm.[29] Maximum capacities (Q_{max}) and dissociation constants (K_d) were calculated as described by Chase.[30]

All the determinations were performed in triplicate and the results expressed as the mean ± standard deviation.

Table 1. Matrices with Wheat Germ Agglutinin (WGA) Immobilized

Matrix	Name
Chitosan matrix	CH
Chitosan matrix incubated with WGA extract in a 1/10 ratio*	CH10
Matrix CH10 with WGA covalently attached	CH10G
Chitosan matrix incubated with WGA extract in a 1/50 ratio*	CH50
Matrix CH50 with WGA covalently attached	CH50G

*Chitosan beads (g) to WGA extract volume (mL) ratio.

Elution studies

For elution studies, 25 mg of CH10G and 25 mg of CH50G (Table 1) were incubated for 16 h at 25°C with 1 mL of a solution of CMP 1 mg/mL in adsorption solution at pH 7.0. After three washes with adsorption solution, the matrices were incubated for 16 h with gentle shaking in 1 mL of the eluting solution. Fifteen elution solutions were tested: (1) 0.5 *M* D-GlcNAc, pH 7.0; (2) 1 *M* D-GlcNAc, pH 7.0; (3) 0.5 *M* D-GlcNAc, pH 5.0; (4) 1 *M* D-GlcNAc, pH 5.0; (5) 0.5 *M* D-GlcNAc, pH 3.0; (6) 1 *M* D-GlcNAc, pH 3.0; (7) 1 *M* acetic acid; (8) 2 *M* NaCl, pH 7.0; (9) 2 *M* NaCl, pH 3.0; (10) 4 *M* urea, pH 7.0; (11) 4 *M* urea, pH 5.0; (12) 4 *M* urea, pH 3.0; (13) 1 *M* D-GlcN, pH 7.0; (14) 1 *M* D-GlcN, pH 5.0; and (15) 1 *M* D-GlcN, pH 3.0. The concentration of eluted CMP was determined spectrophotometrically at 230 nm.

All the determinations were performed in triplicate and the results expressed as the mean ± standard deviation.

Dynamic light scattering (DLS)

DLS measurements were carried out in a Zetasizer Nano-Zs (Malvern Instruments, Malvern, UK) with a measurement range of 0.6–6 μm and a fixed scattering angle of 173°, provided with a He-Ne laser (633 nm) and a digital correlator, Model ZEN3600. Samples were contained in a disposable polystyrene cell.

To obtain the percentile distribution of particle/aggregate sizes, Contin's algorithm was used as described elsewhere.[31]

The samples for DLS were filtered through 0.45, 0.22, and 0.02 μm microfilters (Whatman International, Maidstone, England) before measurements. The assay was performed in triplicate. The concentration of the CMP solution was 2 mg/mL (similar to that in whey) and that of the WGA solution was 1 mg/mL, pH 7.0.

CMP depletion from sweet whey

CMP adsorption was performed in batch by incubating 25 mg of CH10G and 25 mg of CH50G with 1 mL of whey overnight with stirring at room temperature. Whey and supernatants were monitored by measurement of CMP concentration with RP-HPLC and by sialic acid determination.

All the determinations were performed in triplicate and the results expressed as the mean ± standard deviation.

CMP purification from sweet whey

Once CMP adsorbed to the matrices, the matrices were washed three times with adsorption buffer for 1 h each and treated with 1 mL of 1 *M* D-GlcN, pH 7.0 for 16 h under stirring. The eluates were monitored by measuring total nitrogen by the Kjeldahl method with prior removal of D-

GlcN using PD10 columns.[23] The determination was made by dry combustion in an autoanalyzer LECO Truspec CHNS. The eluates were also analyzed by SDS-PAGE 15% and MALDI-TOF-MS.

All the determinations were performed in triplicate and the results expressed as the mean ± standard deviation.

Analytical assays

RP-HPLC. The analytical assays were performed on a Shimadzu LC-20AT System according to Thomä et al.[23] A 218TP54 Vydac column (4.6 mm × 25 cm, Separations Group, Hesperia, CA, USA) was used to analyze all the samples from the purification process. Prior to the run, the samples were pretreated according to Thomä et al.[23] Briefly, samples were exposed to 2% perchloric acid (PCA) as final concentration and incubated for 30 min at 25°C for the precipitation of residual casein and whey protein. The flow rate was 1 mL/min, the injection volume was 20 μL and the effluent was monitored by its absorbance at 214 and 280 nm. The solvents were 0.065% TFA in water (A) and 0.05% TFA in acetonitrile (B). The elution gradient was: 0–5 min, 15% B; 5–25 min, 15–70% B; 25–26 min, 70–100% B; and 26–31 min, 100% B. The peak heights were converted into concentrations by using a calibration curve constructed using a CMP standard at a concentration range of 0.0625–4 mg/mL. The calibration curve obtained was: peak height (cm) = 120.33 [mg CMP/mL] – 1.2951 ($R^2 = 1$). It is important to point out that this approximation was done with the main peak of CMP. Also, for a more exhaustive analysis, all peaks, corresponding to the various forms of CMP (gCMP and aCMP), were analyzed.[23]

Sialic Acid Determination. Initially, residual casein and whey proteins were precipitated using a 2% PCA solution. To this end, a 100 μL aliquot of each sample was incubated with 100 μL of a 4% PCA solution for 30 min at 25°C. Samples were then centrifuged at 2000*g* for 20 min at 4°C. To measure sialic acid, we followed the protocol reported by Fukuda et al.[32] with some modifications. Briefly, 300 μL of distilled water, 500 μL of glacial acetic acid, and 500 μL of acidic ninhydrin solution (0.25 g ninhydrin, 4 mL hydrochloric acid and 6 mL glacial acetic acid) were added to 200 μL of samples. After mixing, samples were incubated in a boiling water bath for exactly 10 min. The samples were cooled on ice and the absorbance was measured at 470 nm.

MALDI-TOF-MS. Spectra were recorded on a 4700 Proteomics Analyzer Instrument (Applied Biosystems, Foster City, CA, USA) at the facility of LANAIS-PROEM, Buenos Aires, Argentina. Briefly, the samples were loaded onto a stainless steel target with sinapinic acid as the matrix in 30% acetonitrile in 0.1% trifluoroacetic acid.

SDS-PAGE. Whey was diluted ten times before 15% SDS–PAGE. Gels were stained with Coomassie Blue under standard conditions.

Statistical analysis

All the experiments were performed at least in duplicate. Data were subjected to analysis of variance (ANOVA) ($p < 0.05$) using the statistical program Statgraphics Centurion XV (Statgraphics, Warrenton, VA, USA).

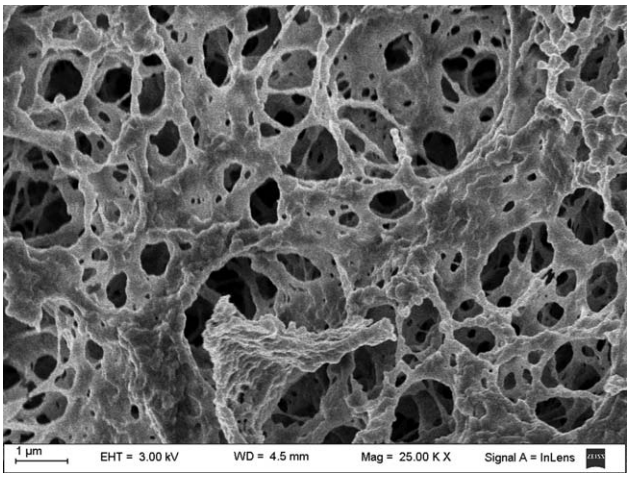

Figure 1. SEM image of matrix CH.

Results and Discussion

Characterization of the support matrix

It has been previously reported that the porous structure and the size of the chitosan beads are affected by changes in the viscosity of the chitosan solution and this effect is mediated by the exchange rate between the solvent (acetic acid) and nonsolvent solution (NaOH) as well as by the concentration of the chitosan solution.[27]

Chitosan concentrations below 1% produced brittle matrices, while chitosan concentrations greater than 2% yielded solutions with extremely high viscosity, which were inadequate to obtain beads of 1–2 mm diameter by dripping.[27] Therefore, a chitosan concentration of 2% was used in this work. The support matrix showed a size of 1.9 ± 0.1 mm and a porosity of 23.8 ± 1.2%. The porosity was determined on lyophilized matrix and, after the process, a reduction in the size of beads was observed. The porosity degree calculated was well correlated with the structure observed in the SEM images. Images were also made with the freeze-dried matrix (Figure 1). Dissolving chitosan in 4% acetic acid generated matrices that took longer times for the formation of the mini-spheres and led to no improvement in the process in comparison to those generated by dissolving chitosan in 2% acetic acid (data not shown).

Generation of chitosan-WGA matrices

Chitosan matrices with immobilized WGA as a ligand were generated and named according to Table 1. Two ligand densities were tested. The amount of immobilized WGA was 7.9 ± 0.3 mg WGA adsorbed/g matrix for matrices CH10 and CH10G, and 39.3 ± 2.2 mg WGA adsorbed/g matrix for matrices CH50 and CH50G. The matrix with highest adsorption capacity for WGA was that in which the amount/extract volume ratio was 1/50.

Preliminary test of CMP adsorption

An arbitrary concentration of 1 mg/mL commercial CMP was used for the preliminary adsorption assay. The CMP adsorption performance at different pH values is shown in Table 2. At pH values of 7.0 and 8.5, all the matrices where the immobilized WGA was covalently attached to mini-spheres showed a CMP adsorption capacity similar to that of

Table 2. Influence of pH Values on CMP Adsorption

	CMP adsorbed/matrix (mg/g)*			
Matrix	pH 4.0	pH 5.0	pH 7.0	pH 8.5
CH	11.9 ± 0.6^{e}	31.0 ± 0.3^{c}	11.6 ± 4.4*	0.0 ± 0.0*
CH10	4.0 ± 0.5*^{b,c}	16.9 ± 1.8*^{b}	13.4 ± 2.7*	$19.4 \pm 0.3^{c,d}$
CH10G	6.8 ± 0.3^{d}	14.4 ± 1.7*	15.9 ± 0.8*^{b}	$18.9 \pm 0.3^{c,d}$
CH50	2.2 ± 0.5*	16.4 ± 0.4*^{b}	12.5 ± 4.6*	15.9 ± 0.7^{b}
CH50G	$4.4 \pm 0.5^{b,c}$	14.7 ± 3.1*	14.9 ± 1.6*^{b}	19.9 ± 0.6^{d}

*Mean values in column with different letter were significantly different ($p < 0.05$).

the matrices without crosslinking. This suggests that crosslinking stabilizes the WGA immobilization without decreasing the interaction degree between WGA and CMP. At both pH values, adsorption was similar for all the matrices with crosslinked WGA. The adsorption was in the range of 15–20 mg CMP per g matrix. Another interesting fact is that—in the range studied—ligand density did not affect the CMP adsorption capacity of the matrices. At pH 8.5, matrices without bounded WGA showed no adsorption of CMP.

On the other hand, at pH 4.0 and 5.0, CMP adsorption was highest for matrices without bounded WGA, thus suggesting that, at these pH values, binding is mediated by ion exchange between chitosan and CMP rather than by affinity. This can be explained since the chitosan pKa is 6.5[33] and the isoelectric point (pI) of CMP is ~3.5 (between 3.15, the pI of gCMP and 4.1, the pI of aCMP)[34]; therefore, at pH values around 6.5 or below, ion exchange occurs between chitosan and CMP. The electrostatic nature of the interaction was evidenced by the adsorption impairment with the increase in the ionic strength of the CMP solution (data not shown).

For further studies, matrices CH10G and CH50G and pH 7.0 and 8.5 were chosen because CMP adsorption by these matrices was similar to or higher than that by other matrices.

Commercial CMP adsorption isotherms

To characterize the matrices and choose the best pH for CMP adsorption, adsorption isotherms at pH 7.0 and 8.5 were performed. All the results were analyzed according to the Langmuir model ($R^2 \geq 0.97$) and the thermodynamic parameters were calculated. The maximum adsorption capacity for CMP obtained with matrices CH10G and CH50G at pH 7.0 was 137.0 ± 19.3 and 96.6 ± 12.1 mg/g, respectively, whereas that at pH 8.5 was 149.4 ± 24.7 and 95.5 ± 14.2 mg/g, respectively. The adsorption capacity of matrices CH10G and CH50G did not change significantly with the pH value. The adsorption capacities of CH10G were higher than those of CH50G at both pH values. This means that these systems show no linear correlation between ligand density and adsorption capacity, suggesting that the CMP-WGA interaction is affected by steric factors.

The K_d values of matrices CH10G and CH50G at pH 7.0 were 10.0 ± 2.4 and 6.0 ± 1.4 mg/mL, respectively, whereas those at pH 8.5 were 8.6 ± 2.5 and 4.9 ± 1.6 mg/mL, respectively. The lowest K_d values—meaning highest affinity—were those for matrix CH50G.

Elution studies

To study the impact of different elution conditions, a salt, a chaotropic agent and a sugar were tested under various pH values. The efficiencies of the eluents tested are shown in Table 3. The best eluents for CMP were 1 *M* D-GlcN at pH 7.0 and 4 *M* urea at pH 5.0. A specific interaction of D-GlcNAc with WGA has been reported[35] and D-GlcNAc is the eluent used by commercial suppliers of matrices with immobilized WGA as ligand.[36] Thus, different elution conditions using D-GlcNAc were tested; however, no significant elution was obtained (data not shown).

Therefore, the eluent selected for the purification process was 1 *M* D-GlcN, pH 7.0, because it showed the best elution for both matrices.

Since the two matrices tested showed similar adsorption and elution behavior under the best condition, neither was discarded for subsequent experiments.

Table 3. CMP Elution Performance

	Elution (%)*	
Eluents	CH10G	CH50G
1*M* acetic acid	2.0 ± 0.0*^{,b}	3.2 ± 0.0*^{,b}
2*M* NaCl, pH 7.0	9.4 ± 4.2*^{,b}	7.1 ± 2.3*^{,b}
2*M* NaCl, pH 3.0	14.1 ± 0.2^{b}	12.8 ± 0.4^{b}
4*M* urea, pH 7.0	27.7 ± 12.6^{c}	35.8 ± 6.2^{c}
4*M* urea, pH 5.0	$57.1 \pm 0.0^{d,e}$	60.7 ± 19.4^{d}
4*M* urea, pH 3.0	2.6 ± 0.5*^{,b}	2.9 ± 0.4*^{,b}
1*M* D-GlcN, pH 7.0	70.2 ± 12.9^{e}	81.7 ± 4.1^{e}
1*M* D-GlcN, pH 5.0	52.3 ± 5.1^{d}	26.9 ± 0.0^{c}
1*M* D-GlcN, pH 3.0	29.6 ± 10.8^{c}	37.8 ± 2.3^{c}

*Mean values in column with different letter were significantly different ($p < 0.05$).

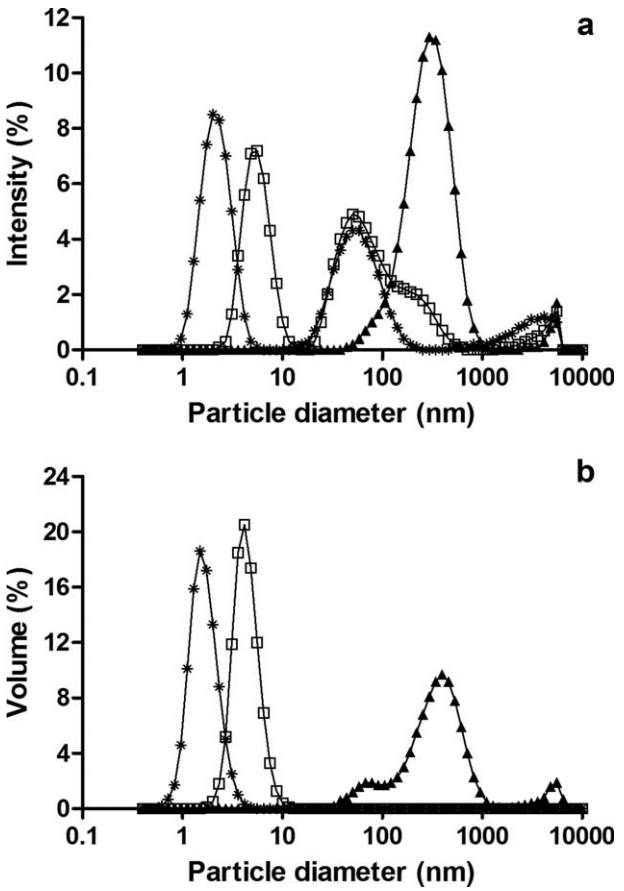

Figure 2. (A) Intensity and (B) volume size distribution of WGA 1 mg/mL (□), CMP 2 mg/mL (*) and WGA 1 mg/mL + CMP 2 mg/mL (▲). Temperature: 25°C.

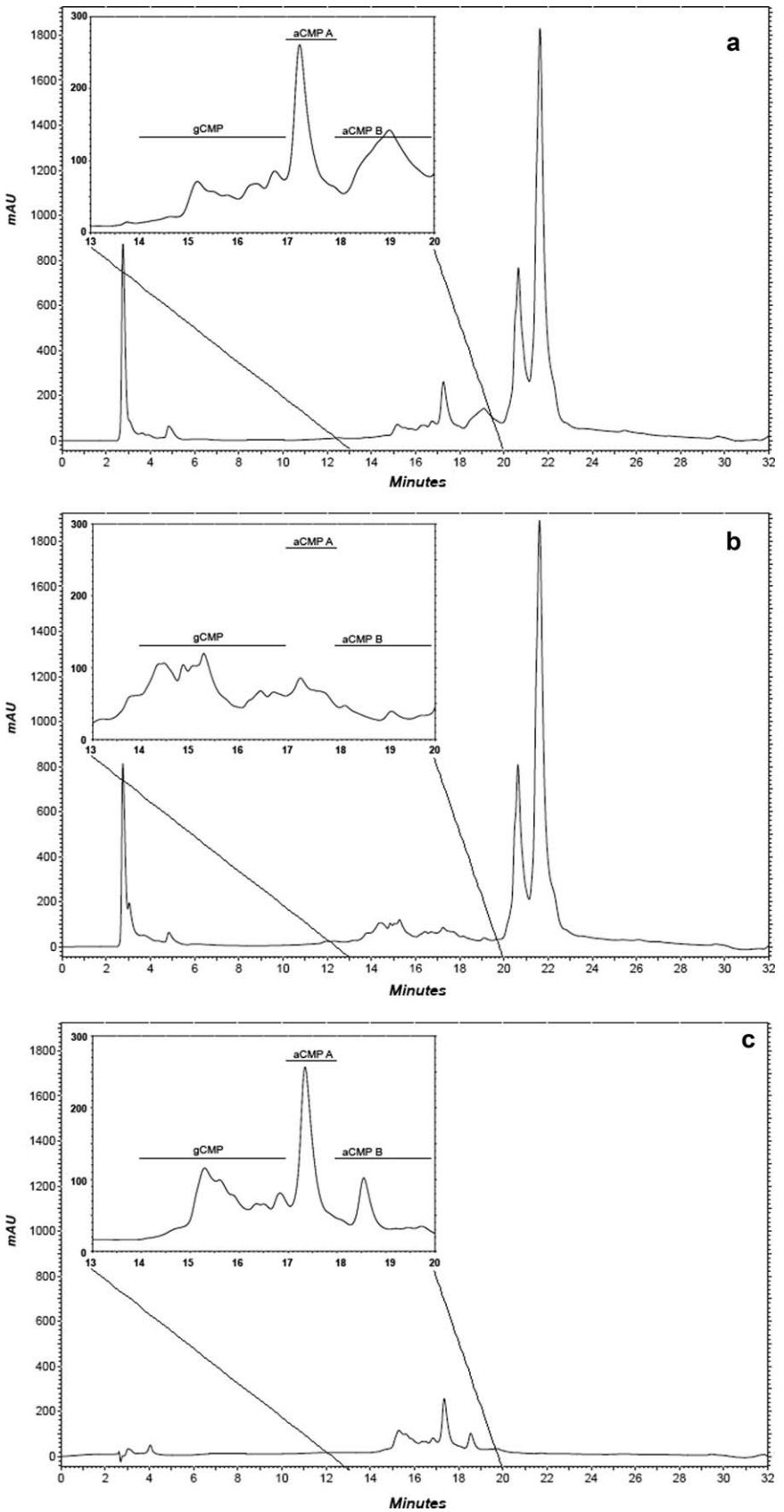

Figure 3. RP-HPLC patterns of samples from the CMP depletion process using matrix CH10G: (A) sweet whey, (B) sweet whey after CMP depletion, (C) commercial CMP 2 mg/mL.

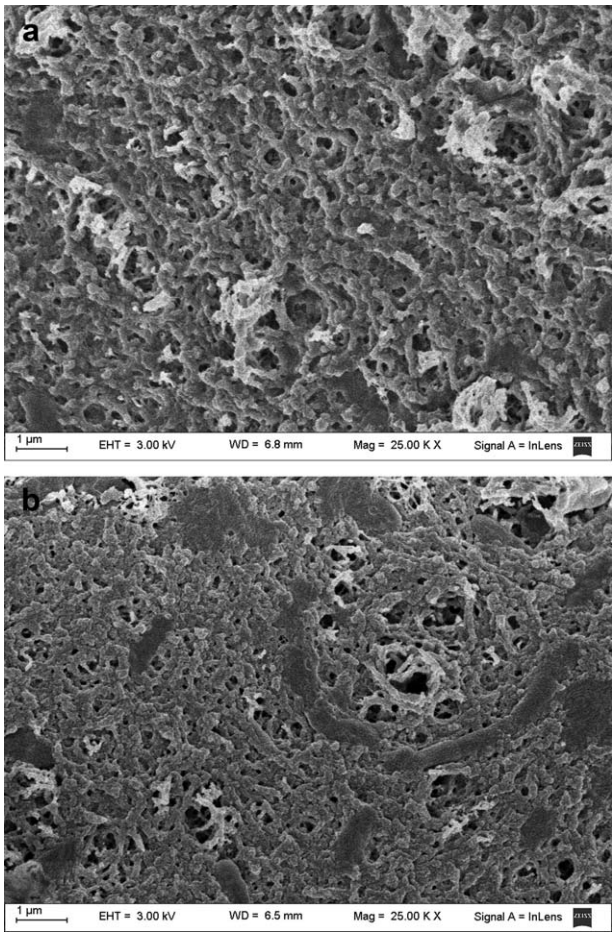

Figure 4. (A) SEM images of matrix CH10G and (B) matrix CH10G with 8.1 mg CMP adsorbed/g matrix.

Adsorption analysis of different CMP forms

On the basis of the work of Thomä et al.[23], where the different forms of CMP were analyzed by RP-HPLC, we studied the supernatants remaining after adsorption of commercial CMP solution. We observed the disappearance not only of the peaks corresponding to gCMP (time retention between 12 and 16.5 min) but also of those corresponding to aCMP (time retention between 17 and 19 min) (data not shown). A possible explanation of this phenomenon is that CMP adsorption to the matrices is a consequence of an initial interaction between WGA and gCMP followed by an interaction of the gCMP bound to the matrices with gCMP and/or aCMP, depending on the pH. Since aCMP and gCMP can also interact in solution, total CMP may disappear from the solution by binding the aCMP/gCMP complex to the mini-spheres. To corroborate this hypothesis, the interaction between WGA and CMP was studied by DLS.

The intensity size distribution of a WGA sample showed two populations (Figure 2A); however, from the volume size distribution, the population with higher sizes can be negligible (Figure 2B). The hydrodynamic diameter of the predominant lower size peak was found close to 5.6 nm. A tool of the Zetasizer Nano-Zs software, used to estimate the molecular mass related to this predominant peak, showed that it is equivalent to 37 kDa for globular proteins, which is in agreement with the molecular mass of the monomer of WGA. The intensity and volume size distribution of CMP

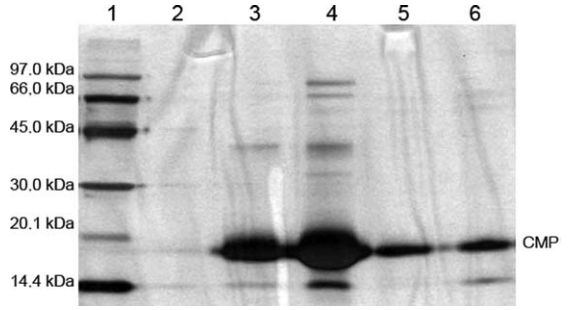

Figure 5. SDS–PAGE 15% of the CMP purification process. Lanes: (1) molecular weight marker, (2) empty, (3) commercial CMP 2 mg/mL, (4) whey diluted 1/10, and (5, 6) material eluted using matrices CH10G and CH50G, respectively.

(Figure 2) indicate that it was in its monomeric form, as it has been extensively explained in a previous work.[24]

Finally, in the same figure, we plotted the size distribution of the CMP-WGA mixture and found no peaks corresponding to CMP or WGA individually, but observed a peak at higher size (between 40 and 1000 nm with a maximum value at 300 nm) corresponding to a larger molecular mass size than expected for the CMP-WGA complex.

It should be mentioned that, to correlate the behavior observed in solution (DLS) with the heterogeneous phase experiments (purification process), we used equivalent amounts of the WGA present in the matrices generated (ligand density of 7 mg/g) and of the CMP in the whey samples. We observed that, by mixing WGA and CMP, the latter was associated with the lectin, generating a larger complex.

CMP depletion from sweet whey

We next tested the performance of matrices CH10G and CH50G for CMP depletion from sweet whey. All the results were calculated from the RP-HPLC patterns. The maximum adsorption degree was obtained with matrix CH10G, which suggests that these systems showed no linear correlation between ligand density and adsorption capacity. The same result was obtained from adsorption isotherms. So, matrix CH10G showed better depletion (93.4%) than CH50G (64.7%), leaving the remaining whey with 6.6% and 35.3% CMP, respectively. The highest binding of CMP to a matrix with lower ligand density may be due to steric effects that result from the formation of a high molecular weight aggregate of CMP on the matrix.

Figure 3 shows the RP-HPLC patterns of the depletion process using matrix CH10G (Figures 3A,3B) and the pattern of commercial CMP (Figure 3C). The chromatograms clearly show the CMP depletion from sweet whey as judged by the decrease of peaks corresponding to CMP in the supernatant (Figure 3B). Figure 3 also shows no differences in the pattern of the peaks corresponding to other proteins present in sweet whey, thus evidencing the specificity of the interaction between CMP and the matrix.

In the CMP depletion process, both peaks corresponding to gCMP and aCMP decreased (Figure 3). Therefore, we analyzed the CMP fractions more thoroughly. Thomä et al.[23] analyzed the CMP content and its different fractions in cheese whey by RP-HPLC and found that all CMP fractions eluted between 12 and 20 min. These authors specifically observed gCMP A and B between 12 and 16.5 min, aCMP

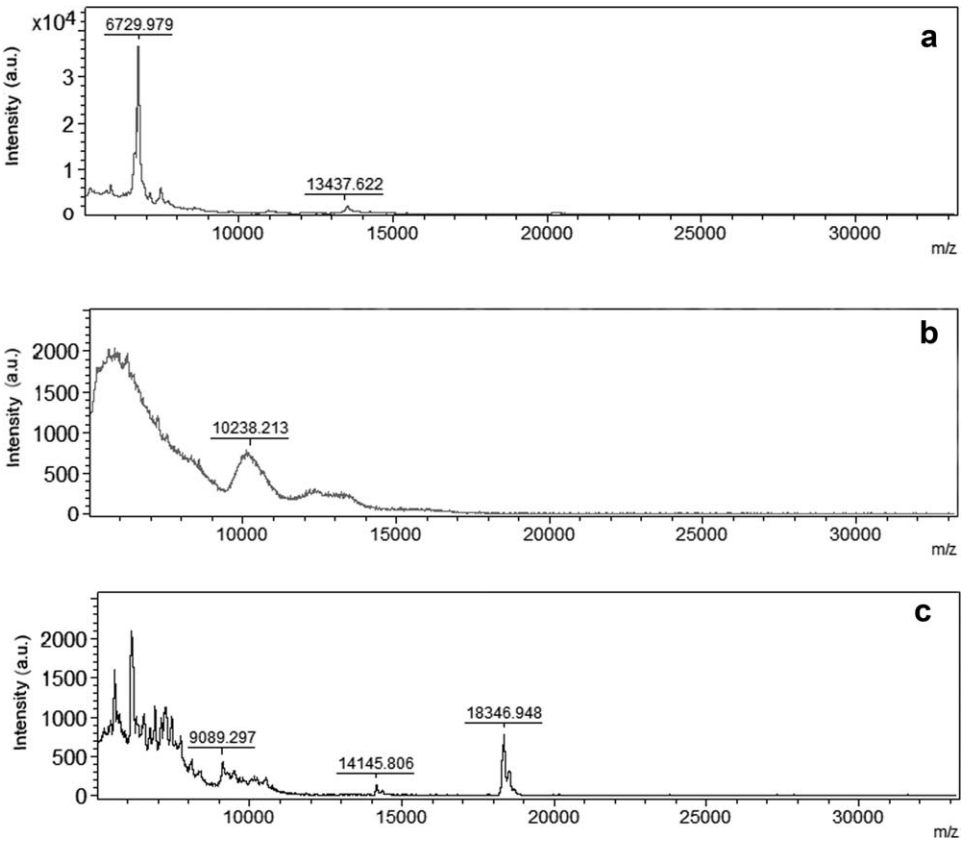

Figure 6. MALDI-TOF mass spectrum of samples from the purification process.

(A) Commercial CMP 2 mg/mL, (B) eluate using matrix CH10G, and (C) whey.

A at 17 min, and aCMP B at 19 min. In the present work, when analyzing the different fractions of the CMP present in cheese whey, in the supernatant after adsorption, and in commercial CMP solution, we observed the same pattern of peaks. Specifically, we observed gCMP A and B between 14 and 17 min, aCMP A between 17 and 18 min, and aCMP B between 18 and 20 min (Figure 3). After CMP depletion, we observed an adsorption of 79.0% of total CMP, specifically 33.7% of gCMP A and B, 75.8% of aCMP A, and 93.9% of aCMP B. It should be mentioned that when we analyzed adsorption considering only the height of the highest peak (93.4%) rather than the area of each fraction, the difference was only 15.4%. Therefore, this rapid analysis by the height of the highest peak can be used to make preliminary tests to study these processes.

Finally, we measured the content of the sialic acid present in cheese whey (0.114 mg/mL) and in the supernatants after CMP depletion (0.076 mg/mL), and found a decrease of about 33.3% of the sialic acid present in cheese whey. This result agrees with that obtained by analyzing the RP-HPLC chromatograms of the CMP depletion process, where the matrix adsorbed 33.7% of the gCMP A and B present in cheese whey.

To characterize possible changes in the matrix structure and morphology before and after the CMP depletion process, matrix CH10G was analyzed by SEM (Figure 4). The image of the support matrix shows a structure with open pores (Figure 1), whereas the image of matrix CH10G shows more closed pores (Figure 4A), probably due to the immobilized WGA. The image of the matrix with adsorbed CMP (Figure 4B) shows a closed structure and saturation.

CMP purified from sweet whey

CMP elution could not be tested by RP-HPLC due to the large size of the eluted aggregates. Therefore, the Kjeldahl method was used to measure total protein. To analyze the eluate, we considered a 6.38 protein factor conversion of nitrogen content to protein for milk samples.[23,36] As a result, 4.88 ± 0.14 mg of eluted CMP was obtained from 5 mL of whey, representing an overall process yield of 63.8%.

The eluates were analyzed by SDS-PAGE 15% (Figure 5). As previously reported,[24] CMP presented a broad and irregular band between 23 and 28 kDa, corresponding to aggregated forms of CMP and a band between 6.5 and 14.4 kDa corresponding to the monomer. When analyzing the SDS-PAGE of the eluates from the process (Figure 5), we observed a major band of about 20 kDa, which can be attributed to an aggregated form by hydrophobic interactions of aCMP and/or gCMP, as previously reported by Galindo-Amaya et al. (2006).[37] These authors suggested the formation of CMP aggregates of about 20.8 kDa that could not be dissociated even in the presence of SDS. Commercial CMP presented the same band pattern as the eluates obtained in the CMP purification process.[24]

In addition, cheese whey and the eluate of the purification process using matrix CH10G were analyzed by MALDI-TOF-MS (Figure 6). The spectrum obtained for the eluate can be assigned to a form of high-molecular weight aggregate.

The eluate obtained using matrix CH10G was analyzed by DLS (Figure 7). The size distribution in intensity and volume of the process eluate showed a large peak that may

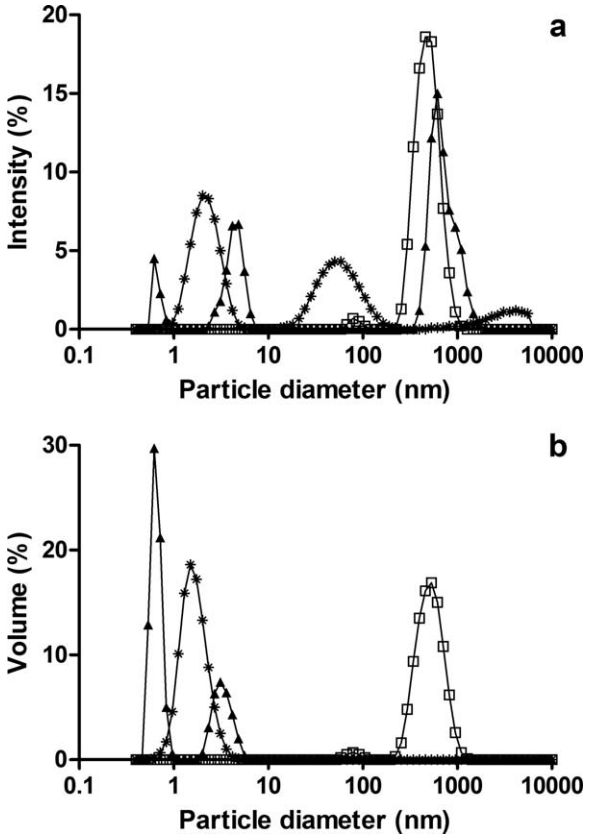

Figure 7. (A) Size distribution in intensity and (B) volume of commercial CMP 2 mg/mL (*) eluate using matrix CH10G (□) and solution composed of WGA 1 mg/mL + CMP 2 mg/mL + 1 M D-GlcN (▲). Temperature: 25°C.

correspond to an aggregated CMP. The commercial CMP size distribution in intensity and volume (Figure 7) showed a peak near 2.5 nm, indicating the CMP monomer. Finally, when analyzing the solution composed of WGA 1 mg/mL + CMP 2 mg/mL + 1 *M* D-GlcN, we observed three populations: a smaller peak corresponding to the eluent used (D-GlcN), an intermediate peak with a hydrodynamic diameter size about 5.6 nm corresponding to the monomer of WGA, and a larger peak, which may correspond to a CMP aggregate.

Matrix CH10G was used for five consecutive purification cycles to assess its reuse (data not shown). After the elution step, the matrix was washed with adsorption buffer before a new purification cycle. After five cycles of use, the CMP adsorption capacity, the elution percentage and the yield did not change significantly.

Different methods have been published for CMP purification from whey.[2,38] The method described by Tanimoto et al.[39] based on membrane filtration of whey at variable pH values allows obtaining an aggregated CMP with low yield. Other methods combine two operation processes such as ultrafiltration and ion exchange chromatography. Based on the latter, Kawasaki et al.[40] proposed CMP purification by an adjustment of the pH value of whey, its passage through a cation exchanger under nonadsorptive conditions, and subsequent purification of the CMP fraction by pH adjustment and ultrafiltration. The purity thus obtained for the CMP was between 80 and 88%. However, the reported recoveries were very low. Finally, there are methods based on ion exchange chromatography. In the method reported by Outinen et al.,[41] whey was clarified by microfiltration and the pH value was adjusted to pass through an anion exchange resin, obtaining CMP with 70–80% of purity and a process with a final yield of 70%. In another method published by Nakano and Ozimek,[5] dialyzed whey was used to purify CMP using anion exchange chromatography because when nondialyzed whey was used only highly sialylated CMP was adsorbed. Another variant process using a strong anion exchanger prior to pH value adjustment followed by metal affinity chromatography allows obtaining a substantially pure k-casein macropeptide from whey.[42]

As mentioned, CMP has been purified from whey by using various techniques. However, in all these techniques the whey must be preconditioned by dialysis/ultrafiltration, pH adjustment, or precipitation of contaminants, among others. As opposed to this, the proposed approach has the advantage of selective capture of CMP from cheese whey without the need of any pretreatment or a previous chromatography, allowing further commercialization of whey reduced in CMP. This is very advantageous from the industrial point of view because no extra steps are required for purification and whey can follow its usual trade route because it has not been substantially modified. Moreover, due to their size and density, the affinity matrices are easily recovered after CMP adsorption from whey by simple filtration without the need of centrifugation and without matrix loss in between cycles.

Conclusion

The results obtained in this study are encouraging because we were able to synthesize chromatographic matrices based on chitosan crosslinked with epichlorohydrin. After adsorption and covalent immobilization of WGA as affinity ligand, these matrices showed adequate CMP adsorption and elution capacities. Interestingly, the performance of the matrix with lowest ligand density (CH10G) was similar to that of the matrix with highest ligand density (CH50G). Furthermore, the matrices generated adsorbed CMP from cheese whey without preconditioning step, leaving the remaining whey reduced in CMP. This would allow using this CMP-reduced whey to manufacture foods for infants. Moreover, the matrices developed have promising application for CMP purification from cheese whey. They are easy to manufacture, manipulate, and recover by simple filtration through a strainer after adsorption, washing and elution, without any loss of matrix. Moreover, these matrices have adequate mechanical resistance to ensure their reutilization up to five cycles.

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