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Reversed-phase high-performance liquid chromatographic separation of bovine κ-casein macropeptide and characterization of isolated fractions

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Abstract

From complex mixtures of non-glycosylated and differently glycosylated caseinomacropeptides (CMP; κ-casein fragment 106–169; $M_r \approx 7000$) various fractions were isolated and further purified by reversed-phase HPLC. The fractions were characterized by mass determination and composition analysis and also used in gel-permeation chromatography and NMR studies to investigate their molecular size behaviour as a function of pH, ionic strength, peptide concentration and degree of glycosylation. No evidence was found for association of any CMP fraction as a function of the experimental conditions applied, which is in contrast with suggestions made in the literature. The increased molecular size (apparent molecular mass approx. 30–45 kDa) is rather explained by a large voluminosity of the molecular species due to internal electrostatic and steric repulsion. Furthermore, the susceptibility of some non-glycosylated and glycosylated CMP fractions to enzymic attack by the Glu-specific endopeptidase from *Staphylococcus aureus* V8 was studied. Initial rates of proteolysis by this enzyme were independent of the degree of glycosylation. Only in the case of highly glycosylated CMP was further hydrolysis to smaller fragments inhibited. Hydrolytic products were identified by electrospray ionization and fast-atom bombardment mass spectrometry.

Keywords: Glycosylation; *Staphylococcus aureus*; Caseins; Caseinomacropeptide; Endopeptidase; Enzymes; Peptides

1. Introduction

Caseinomacropeptide (CMP) is the C-terminal fragment of κ-casein (κ-CN), i.e., residues 106–169 (numbering of the bovine protein [1]), which has a nominal molecular mass of ca. 7000 Da. During the initial stage of the cheesemaking process this fragment is released from the casein micelle via specific cleavage of the κ-CN component by the milk-clotting enzyme chymosin [2].

CMP is heterogeneous. Three genetic variants have been structurally determined, originating from the precursor κ-CNs A, B and E, respectively [3]. The A and B forms are the most common κ-CN variants in bovine milk. Moreover, κ-CN occurs in differently glycosylated and phosphorylated forms (for a review see Ref. [4]), posttranslational modifications that are exclusively situated in the CMP part of the molecule. κ-CN from cow's milk has been reported to contain five kinds of carbohydrate

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moieties: a monosaccharide, i.e., GalNAc (N-acetylgalactosamine); a disaccharide containing GalNAc and Gal (galactose); two kinds of trisaccharides containing GalNAc, Gal and NeuAc (N-acetylneuraminic acid); and a tetrasaccharide containing GalNAc, Gal and two NeuAc residues [5,6]. They may be attached to six (in the A variant) or five (in the B variant) different Thr residues [7] and probably also to one Ser residue [8,9], although the latter has been contradicted in Ref. [7]. κ-CN can contain up to nine or ten NeuAc residues [10] and up to three phosphate residues [10,11] per molecule.

CMP or fragments thereof are reported to show various biological activities such as regulation of the action of the digestive tract and immunomodulating, antithrombotic or antihypertensive activity. In several cases bioactivity of CMP or of fragments thereof has been associated with the nature and content of the attached carbohydrate moieties (for a review see Ref. [12]).

Under certain conditions of pH and ionic strength CMP also revealed heterogeneity in apparent molecular mass, as measured by gel-permeation chromatography (GPC) [13–16], which has been attributed to polymerization occurring at pH>4 [15,16].

Reversed-phase (RP-) HPLC was applied to separate CMP or its fragments [7,11,17]. Mollé and Léonil [11] have recently used RP-HPLC with online electrospray-ionization mass spectrometry (ESI-MS) for the separation and identification of different glycoforms of whole CMP A. Their fractions were heterogeneous, but ESI-MS allowed the accurate identification of individual components. RP-HPLC also offers a relatively simple procedure for the preparative isolation of samples. Application of a volatile buffer system furthermore allows the purification of samples without the need for dialysis.

In this paper we present the comparative characterization of CMP fractions, separated via preparative RP-HPLC, by sugar analysis, high-performance gel-permeation chromatography (HPGPC), nuclear magnetic resonance (NMR), mass spectrometry and determination of the susceptibility to enzymic digestion. With HPGPC and NMR the molecular association behaviour of CMP was studied as a function of the degree of glycosylation, pH, ionic strength and peptide concentration. Mass spectrometry was also used for the characterization of enzymic degradation products of differently glycosylated CMP fractions.

2. Materials and methods

2.1. Materials

κ-CN A and B fractions with different carbohydrate content prepared by anion-exchange chromatography [10] were gifts from Dr H. J. Vreeman. For the preparation of CMPs five fractions of κ-CN were used: non-glycosylated κ-CN A, non-glycosylated κ-CN B, slightly glycosylated κ-CN A, highly glycosylated κ-CN A and highly glycosylated κ-CN B.

Chymosin (EC 3.4.23.4) was a purified preparation as described in Ref. [10]. Glu-specific endopeptidase from *Staphylococcus aureus* V8 (EC 3.4.21.19) was a product of Boehringer-Mannheim (endoproteinase Glu-C; cat. No. 791156).

2.2. κ-CN cleavage by chymosin

κ-CN samples were dissolved in 0.05 *M* ammonium acetate buffer, pH 6.6, to a concentration of approx. 2.5 mg ml^{-1}. Then 4 μl of a stock solution of chymosin (0.1 mg ml^{-1} in the buffer described above) per ml of κ-CN solution were added. Incubations were carried out at room temperature (19–21°C). The reaction times needed for complete hydrolysis of particular κ-CN fractions were as follows: 60 min for non-glycosylated κ-CN B, 120 min for slightly glycosylated κ-CN A and highly glycosylated forms of both genetic variants, and 240 min for non-glycosylated κ-CN A. The relatively long incubation time required for complete hydrolysis of non-glycosylated κ-CN A was due to coprecipitation of this fraction with the para-κ-CN (fragment 1–105) formed. κ-CN particles in the coprecipitate were less accessible to chymosin than those in solution. The reaction was stopped by inactivation of the chymosin through raising the pH to 8–8.5 via addition of 0.1 *M* ammonia. Samples were then centrifuged (4000 *g*, 10 min). Supernatants were lyophilized. The samples were redissolved in a

small amount of ammoniacal water at pH 7.5 and lyophilized again.

2.3. Reversed-phase high-performance liquid chromatography

Analytical RP-HPLC was carried out using two M 6000A pumps in combination with a high-sensitivity accessory block (Waters), an ISS-100 injector (Perkin-Elmer), a Kratos 783 UV detector and a Waters Model 680 gradient controller. A 250 mm×4.6 mm Widepore C_{18} column (Bio-Rad) was used with a C_{18} cartridge (Bio-Rad) as a guard column. The equipment used for preparative RP-HPLC consisted of two pumps (Waters Model 510), an automatic sample injector (Gilson, Model 231), a UV detector (Separation Analytical Instruments) and a Waters Model 680 gradient controller. A Widepore C_{18} 250 mm×20 mm column (Bio-Rad) was used with a C_{18} cartridge (Bio-Rad) as a guard column. Both pieces of equipment were linked to a data acquisition and processing system (Turbochrom, Perkin-Elmer).

Solvent A was a mixture of acetonitrile–water–trifluoroacetic acid (TFA) (100:900:1, v/v/v). Solvent B contained the same components (900:100:0.8, v/v/v). Gradients applied for separation of CMP and CMP fragments are presented in Table 1. Each gradient program was started immediately after injection.

Analytical separations were carried out at 30°C and at a flow-rate of 0.8 ml min^{-1}. Peaks were detected at 220 nm. Sample concentrations were approx. 0.4 mg ml^{-1} and injection volumes 50 μl. Samples collected for monitoring κ-CN cleavage (containing CMP, κ-CN and para-κ-CN) were reduced during 60 min at room temperature in 0.1 *M* 1, 3 - Bis[tris(hydroxymethyl) - methylamino]propane (Bis-Tris) containing 4 *M* urea and 0.3% (v/v) 2-mercaptoethanol (protein concentration 2 mg ml^{-1}), then diluted fivefold with solvent A containing 6 *M* urea and adjusted to pH 2.2 by addition of TFA. Samples containing CMP only were directly dissolved in solvent A (pH 2.2) containing 6 *M* urea, without reduction. Samples of products of CMP hydrolysis were dissolved in solvent A (pH 2.2) without prior reduction or addition of urea.

Preparative CMP separations were carried out at 30°C and at a flow-rate of 11 ml min^{-1}. The injection volume was 800 μl (CMP concentration 10 mg ml^{-1} in solvent A containing 6 *M* urea, pH 2.2) and peak detection was at 220 nm. CMP fractions were collected and stored at −20°C before evaporation. After evaporation in vacuo at 30°C, the fractions were further dried by lyophilization for 30 min to remove residual TFA. They were then dissolved in water as described in Section 2.2 and lyophilized again.

The separation of products of CMP hydrolysis was

Table 1
Gradient programs applied for the separation and purification of CMP fractions[a]

Sample	Gradient of solvent B in A[b]
CMPs (analytical runs)	Starting with 15% of solvent B; 15–28% over 13 min, 28–32% over 22 min
Non-glycosylated CMP B (preparative runs)	Starting with 16% of solvent B; 16–20% over 1 min, 20–34% over 30 min
Non-glycosylated and slightly glycosylated CMP A (preparative runs)	Starting with 16% of solvent B; 16% for 1 min, 16–30% over 30 min
Highly glycosylated CMP B (preparative runs)	Starting with 16% of solvent B; 16% for 1 min, 16–25% over 30 min
Highly glycosylated CMP A (preparative runs)	Starting with 12% of solvent B; 12% for 1 min, 12–22% over 30 min
CMP digestion products (analytical and preparative runs)	Starting with 5% of solvent B; 5–10% for 6 min, 10–30% over 34 min

[a] For composition of solvents, see Section 2.3.
[b] Linear gradients were used. After completion of the gradients, the concentration of solvent B was increased to 70% over 3 min and kept constant for 5 min before returning to the initial conditions. For further experimental conditions, see Section 2.3.

performed with the equipment used for analytical separations at 30°C and using a flow-rate of 0.8 ml min^{-1}. The sample concentrations were 0.4 and 0.75 mg ml^{-1} in solvent A and injection volumes were 50 and 150 μl for analytical and preparative separations, respectively. Samples from preparative separations were collected into Eppendorf vials. The acetonitrile was removed from samples by evaporation in a vacuum centrifuge (SpeedVac, Savant). Samples were then freeze-dried.

2.4. Determination of monosaccharide and phosphate content

The contents of GalNAc, Gal and NeuAc were determined according to methods described in [18], as applied using the Perkin-Elmer kits for monosaccharide analysis [19] and for sialic acid composition analysis [20], respectively. When the recommended protocols in [18] and [19,20] differed, we followed the latter references. Both kits include a special CHO C_{18} column for RP-HPLC. The HPLC equipment was the same as for analytical RP-HPLC (see Section 2.3). The NeuAc content was also determined as described by Warren [21] after hydrolysis in 0.1 *M* H_2SO_4 at 80°C for 1 h.

Phosphate contents were derived from mass spectrometric results.

2.5. High-performance gel-permeation chromatography

A Superdex-75 HR 10/30 column (Pharmacia Biotech) was used in combination with the equipment and data acquisition system used for preparative RP-HPLC. The composition of buffers was as follows: 0.025 *M* ammonium acetate–0.095 *M* Na_2SO_4, pH 3, ionic strength 0.3 *M*; 0.1 *M* Tris·H_2SO_4–0.05 *M* Na_2SO_4, pH 7, ionic strength 0.3 *M*; 0.1 *M* Tris·H_2SO_4–0.5 *M* Na_2SO_4, pH 7, ionic strength 1.7 *M*. All experiments were carried out at 20°C and using a flow-rate of 0.44 ml min^{-1}. The concentration of macropeptide in the samples was 0.5 mg ml^{-1}. Non-glycosylated and highly glycosylated CMP A were also chromatographed using concentrations of 3 mg ml^{-1} (pH 3 and 7) and 7 mg ml^{-1} (pH 7). Non-glycosylated CMPs A and B were not readily soluble in elution buffer of pH 3. They were, therefore, dissolved in 0.1 *M* ammonium acetate buffer (pH 3.5). The following molecular-mass standards were applied: bovine serum albumin (67 kDa), bovine β-lactoglobulin A (36 kDa), soya trypsin inhibitor (21 kDa), aprotinin (6.5 kDa) and bacitracin (1.4 kDa) [22]. The concentration of standards in the elution buffers was 0.1–0.2 mg ml^{-1}.

2.6. Nuclear magnetic resonance

^{1}H-NMR spectra were obtained with a Bruker AM400 spectrometer operating at 400.13 MHz, connected to an Aspect 3000 computer. WINNMR software (Bruker) was used for data processing. The NMR experiments were carried out at 25°C. The CMP concentrations were approx. 10 mg ml^{-1} and 1 mg ml^{-1} in 0.1 *M* NaCl solutions in ^2H_2O. The pH meter reading was adjusted to 7 or 3 using 1 and 0.1 *M* NaO^2H or ^2HCl solutions in ^2H_2O, respectively. The CMP content in samples was calculated from the total intensity of resonances of protons from $-CH_3$ groups of Val, Leu and Ile (Fig. 4). [23]. For concentration determination [2,2,3,3-$^2H_4$]3-(trimethylsilyl)propionic acid (TSP) (Aldrich), 2,2,2-trifluoroethanol and methanol were used as standards. NMR spectra were referenced to external TSP.

2.7. Mass spectrometry

ESI-MS analysis was performed on a VG Platform quadrupole mass spectrometer. Samples were dissolved in a mixture of acetonitrile–water–formic acid (500:500:1, v/v/v) and injected into a flow of acetonitrile–water (1:1, v/v, 5 μl min^{-1}). The capillary tip was maintained at 3.2 kV, the cone voltage was 30 V. Nitrogen was used as nebulizing and drying gas. Calibration was performed using horse heart myoglobin.

Positive-ion FAB mass spectra were recorded on a Jeol JMS-SX/SX102A four-sector instrument of B_1E_1-B_2E_2 geometry operating at an accelerating voltage of 10 kV. Xenon was used as FAB gas; the FAB gun was operated at 6 kV and a 10 mA emission current. The magnet was scanned from 10–2000 amu in 30 s and resolution was 1000.

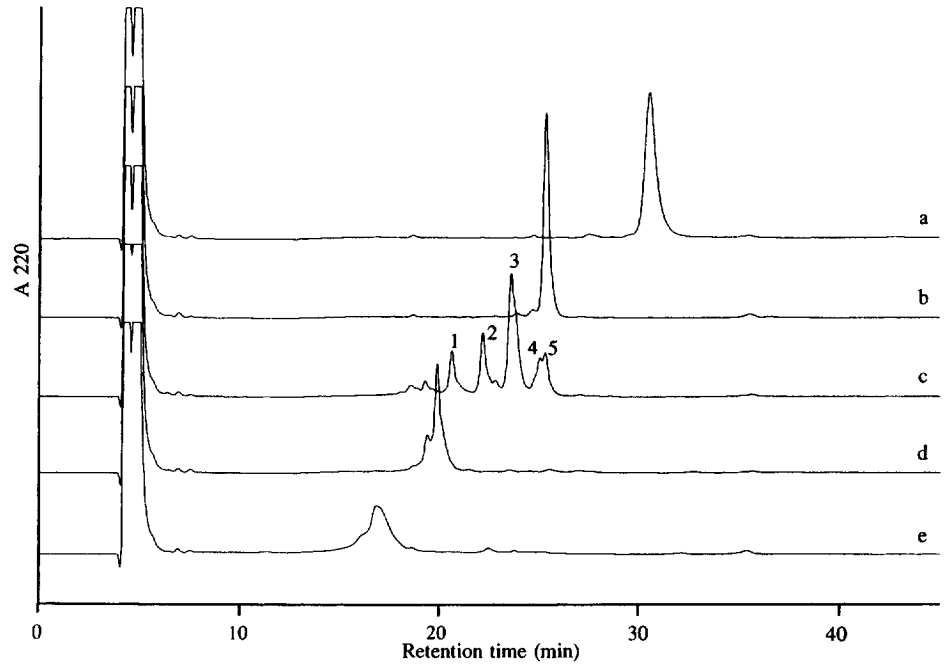

Fig. 1. Analytical chromatograms of CMP fractions. (a) Non-glycosylated CMP B; (b) non-glycosylated CMP A; (c): slightly glycosylated CMP A (individual fractions indicated by numbers 1–5); (d) highly glycosylated CMP B; (e) highly glycosylated CMP A.

Samples were dissolved in methanol–water (1:1, v/v) and loaded into a glycerol matrix.

2.8. Enzymic digestion of CMP and analysis of products

Three CMP fractions, a non-glycosylated CMP A, a slightly glycosylated CMP A, and a highly glycosylated CMP A, were subjected to hydrolysis by Glu-specific endopeptidase from *Staphylococcus aureus* V8 [24,25]. The conditions of digestion on an analytical scale were as follows: CMP concentration 1 mg ml^{-1} in 0.05 *M* ammonium acetate buffer (pH 4); incubation temperature 25°C; enzyme concentration 4.5 $\mu g\ ml^{-1}$; incubation times 15 min, 30 min, 1 h, 2 h and 3 h. The reaction was stopped by bringing the pH down to below 3 via addition of acetic acid.

Preparative digestions of a non-glycosylated CMP A and of a slightly glycosylated CMP A were performed in Eppendorf vials at 37°C for 6 h. CMP and enzyme concentrations were 1.5 mg ml^{-1} and 15 $\mu g\ ml^{-1}$, respectively.

Enzymic degradation products were analysed by analytical RP-HPLC and identified by FAB-MS and/or ESI-MS.

3. Results and discussion

3.1. Isolation, purification and composition analysis of CMP

Analytical chromatograms of CMP fractions are shown in Fig. 1. The (different) gradients used in the preparative RP-HPLC runs with these fractions (Table 1; chromatograms not shown) gave better resolution between individual peaks than the ones applied in the analytical runs. Analytical data of isolated components are presented in Table 2 and Fig. 2. Five fractions of the slightly glycosylated CMP A were collected. Sugar analysis and ESI-MS revealed that fraction 5 represented the regular non-glycosylated CMP A containing one phosphate group. Fraction 4 was also found to be non-glycosylated, but it contained two phosphate residues per

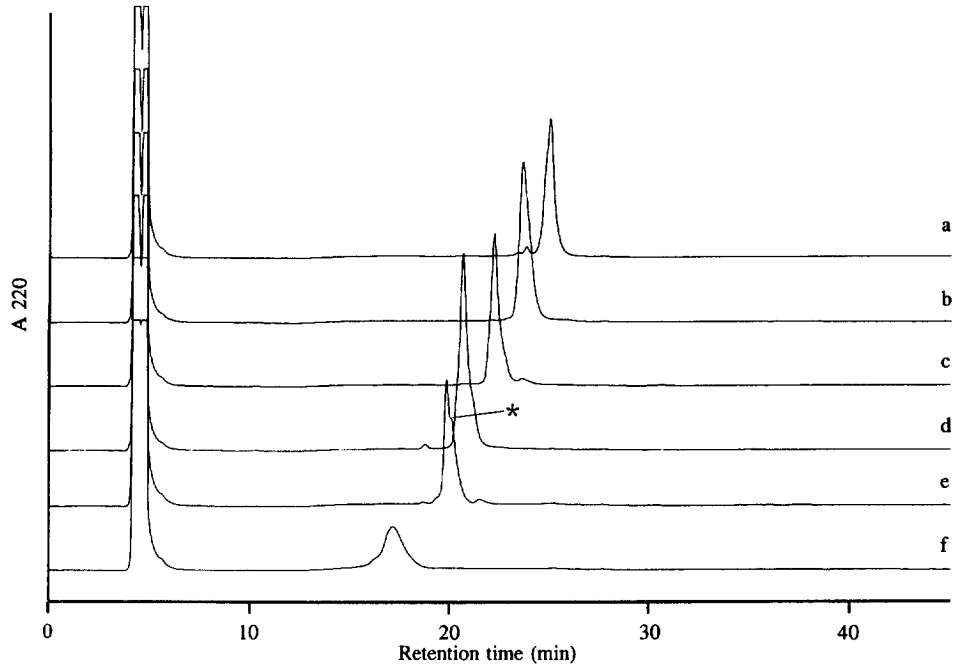

Fig. 2. Analytical chromatograms of glycosylated CMP fractions (Fig. 1) after their purification by preparative RP-HPLC. (a—d) Fractions 4, 3, 2 and 1, respectively, of slightly glycosylated CMP A; (e) highly glycosylated CMP B; (f) highly glycosylated CMP A. The asterisk indicates a shoulder that appeared after preparative chromatography.

molecule. The remaining fractions in Table 2 contained one phosphate group per molecule. Highly glycosylated CMP B and A (Fig. 1 d and e) were collected without the preceding shoulders. The composition of the entire sugar moieties could be derived from the ESI-MS analytical data (Table 2). The compositions of several CMP fractions corresponded to those of peak material identified after RP-HPLC on-line with ESI-MS by Mollé and Léonil [11]. Like these authors we found components with identical mass in different peaks of the chromatogram. One explanation may be the attachment of sugar moieties of identical composition to different glycosylation sites of the same peptide. Another possibility could be partial conversion of Gln and/or Asn residues to Glu and/or Asp, respectively, leading to mass differences within the experimental error. In fraction 1 of the slightly glycosylated CMP A (Fig. 2d, Table 2) the occurrence of a minor component of 7281 Da could be explained by the attachment of a sugar moiety consisting of only GalNAc and NeuAc (1:1). Such a sugar composition has not been reported previously for any κ-CN species. Partial degradation of an existing sugar moiety or incomplete in-vivo glycosylation of κ-CN could form the basis for such an unusual sugar composition. The former possibility seems less likely, because the release of Gal requires more drastic conditions than that of NeuAc [18]. Sugar contents (Table 2, second column) were determined by taking into account the peptide contents of the various fractions, as established by NMR analysis. GalNAc/Gal ratios obtained from chemical analysis were nearly the same as those obtained from ESI-MS. Generally, absolute amounts of the three sugars were underestimated, which was probably due to systematic errors such as differences in behaviour between standards and samples during the hydrolysis procedure. The method of NeuAc determination using derivatization by thiobarbituric acid [21] following acidic hydrolysis generally leads to underestimated contents of this component, as was pointed out by Takeuchi et al. [26]. Altogether, mass spec-

Table 2
Data sheet of CMP fractions isolated by preparative RP-HPLC

Fraction	Content of individual sugars (mol mol^{-1})[a]	Experimental molecular mass[b,c]	Calculated molecular mass	Phosphate content and composition of sugar moieties (mol mol^{-1})[d,e]
Non-glycosylated CMP B	No sugar	6753.5	6755.47	No sugar, 1P
Non-glycosylated CMP A	No sugar	6785.9	6787.43	No sugar, 1P
Slightly glycosylated CMP A - fr. 5	No sugar	6787.2	6787.43	No sugar, 1P
Slightly glycosylated CMP A - fr. 4	No sugar	6866.2	6867.41	No sugar, 2P
Slightly glycosylated	0.8GalNAc, 0.7Gal,	7443.7	7444.02	1GalNAc, 1Gal, 1NeuAc, 1P
CMP A - fr. 3	1.0NeuAc (0.8NeuAc)	7735.4	7735.28	1GalNAc, 1Gal, 2NeuAc, 1P
Slightly glycosylated	0.9GalNAc, 0.9Gal,	7443.9	7444.02	1GalNAc, 1Gal, 1NeuAc, 1P
CMP A - fr. 2	1.5NeuAc (1.3NeuAc)	7735.4	7735.28	1GalNAc, 1Gal, 2NeuAc, 1P
Slightly glycosylated	1.3GalNAc, 1.4Gal,	7280.7	7281.88	1GalNAc, 1NeuAc, 1P
CMP A - fr. 1	1.8NeuAc (1.7NeuAc)	7443.7	7444.02	1GalNAc, 1Gal, 1NeuAc, 1P
		7735.4	7735.28	1GalNAc, 1Gal, 2NeuAc, 1P
Highly glycosylated	1.1GalNAc, 1.0Gal,	8068.0	8068.67	2GalNAc, 2Gal, 2NeuAc, 1P
CMP B	2.2NeuAc (3.4NeuAc)	8359.5	8359.93	2GalNAc, 2Gal, 3NeuAc, 1P
		8650.2	8651.18	2GalNAc, 2Gal, 4NeuAc, 1P
Highly glycosylated	2.2GalNAc, 1.9Gal,	9048.3	9048.48	3GalNac, 3Gal, 4NeuAc, 1P
CMP A	3.9NeuAc (4.3NeuAc)	9339.6	9339.74	3GalNAc, 3Gal, 5NeuAc, 1P
		9626.6	9631.00	3GalNAc, 3Gal, 6NeuAc, 1P

[a] Determined according to Refs. [18–20]; NeuAc values in parentheses were obtained according to Ref. [21] (see Section 2.4).
[b] Average molecular mass as determined by ESI-MS.
[c] The experimental error was within 0.02% ($n=3$), except for the components of highly glycosylated CMP A, for which an error of ca. 0.2% ($n=7$) was found.
[d] Derived from the ESI-MS results.
[e] For some glycoforms different structures of glycosidic moieties are possible [11].

trometry appeared to be the most useful tool for obtaining reliable information about the composition of sugar moieties and identification of individual CMP glycoforms.

No contamination of CMP by other peptides was observed. The presence of such contaminating peptides was reported in CMP obtained from whole casein [17,27].

The retention times of glycosylated forms of CMP A and B overlap, as was also reported by Beucher et al. [17]. Therefore, RP-HPLC preparation of individual CMPs can be best achieved by starting from purified individual genetic variants of κ-CN.

We found no spontaneous degradation of CMP to small peptides as reported by Pisano et al. [7] in solutions containing 20–30% of acetonitrile stored at −20°C for up to three weeks. Some minor changes in CMP, such as a (partial) release of sugar residues, may have occurred during evaporation. For instance, the shoulder appearing after preparative RP-HPLC of highly glycosylated CMP B (Fig. 2e) might be due to co-elution of the main component with the ones containing 3 and 2 NeuAc moieties (see Table 2), resulting from a release of 1 or 2 NeuAc residues from CMP containing 4 NeuAc. In the literature, heating with dilute TFA at 80°C [7] or at pH 3 and 100°C [28] is recommended as a method for the removal of NeuAc. Minor degradation of glycosidic chains may also occur at lower temperature in concentrated TFA. In our case, CMP samples were briefly exposed to concentrated TFA at the end of the evaporation procedure. On the other hand, it should be realized that coelution of different glycoforms of CMP has been found using RP-HPLC on-line with

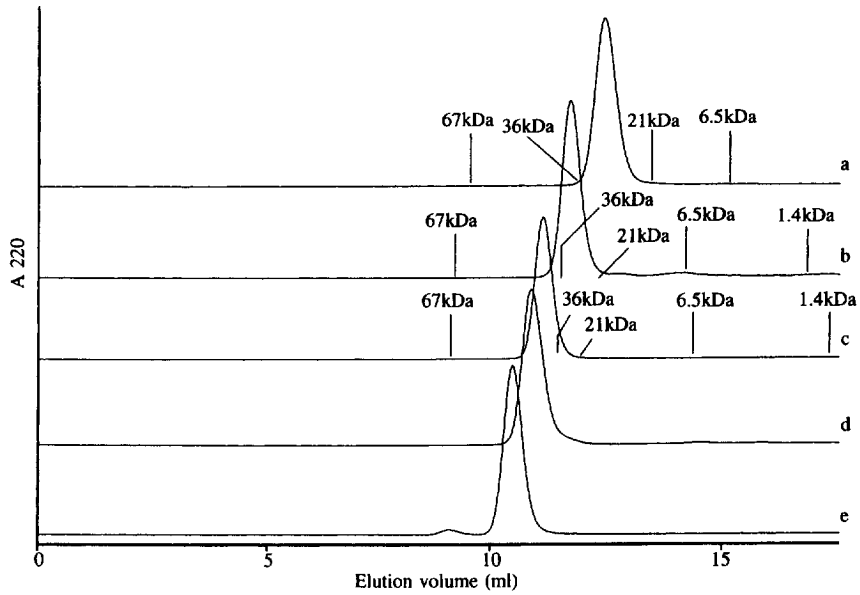

Fig. 3. HPGPC chromatograms of CMP. (a) Non-glycosylated CMP A in high-ionic-strength buffer, pH 7; (b) idem in low-ionic-strength buffer, pH 3; (c) idem in low-ionic-strength buffer, pH 7; (d) non-fractionated, slightly glycosylated CMP A in low-ionic-strength buffer, pH 7; (e) highly glycosylated CMP A in low-ionic-strength buffer, pH 7 (see Section 2.5). The elution volumes (dependent on pH and ionic strength) and molecular masses of standards are indicated in chromatograms a, b and c. Elution volumes of standards in chromatograms c, d and e (obtained under the same conditions) are the same. The elution volume of bacitracin (1.4 kDa) in high-ionic-strength buffer, pH 7, was 22.7 ml (not shown). kDa=kilodalton.

ESI-MS without intermediate evaporation [11], which for our case would make TFA-induced modification less probable.

3.2. High-performance gel-permeation chromatography

Typical HPGPC chromatograms of CMP fractions are presented in Fig. 3. All fractions gave single peaks at pH 3 and 7. The apparent molecular masses of individual CMP fractions varied within the range 30 000–45 000. Elution volumes (V_e) did not vary with CMP concentrations in the range 0.5–7 mg ml^{-1} (data not shown). However, the glycosylation degree and also the pH and the ionic strength influenced V_e (Fig. 3). Obviously, the presence of hydrophilic and ionizable glycosidic moieties enlarges the voluminosity of CMP due to enhanced internal steric and electrostatic repulsion. Further, a decrease of pH (Fig. 3b,c) or increase of ionic strength (Fig. 3a,c) may lead to a decrease in voluminosity of CMP particles due to suppression of

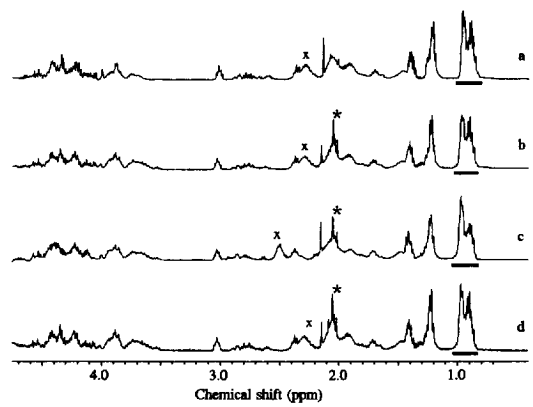

Fig. 4. NMR spectra of CMP. (a) Non-glycosylated CMP A, 10 mg ml^{-1}, pH 7; (b) lightly glycosylated CMP A, fraction 3, 10 mg ml^{-1}, pH 7; (c) fraction 3, 10 mg ml^{-1}, pH 3; (d) fraction 3, 1 mg ml^{-1}, pH 7. Bars indicate resonances used for the determination of CMP concentration. The asterisk indicates a peak which may be tentatively assigned as resonance of acetamide protons from GalNAc and NeuAc. x: resonances of γ-CH_2 protons from glutamic acid.

electrostatic repulsion between carboxylic groups of acidic amino acids and NeuAc. Different interaction with the column material as a function of pH and ionic strength may also play a role.

HPGPC patterns of all fractions at pH 7 are similar to those obtained earlier by others [15,29,30]. All of these authors found a single peak of whole CMP at this pH. Our patterns at pH 3 are different from those obtained by Kawasaki et al. [15], who observed several peaks with significantly different elution volumes using a Superose 6 column at pH 3.5. We have found a much weaker dependence of V_e on the glycosylation degree of CMP than reported by Varsanovich et al. [13,14]. They found that slightly glycosylated CMP had a two times larger V_e on Bio-Gel P-30 at pH 5.65 than the highly glycosylated CMP. A possible explanation for these results may be non-steric exclusion effects by interactions with the column material.

Our results do not confirm the finding that, dependent on the pH of the medium, CMP can form aggregates [13–16] (for a review, see Ref. [12]). The differences between apparent molecular masses of CMP observed by us were too small to be interpreted as a result of association. We suggest that CMP instead exists in the monomeric form throughout and that its relatively high apparent molecular mass may be due to an open structure with large molecular voluminosity.

3.3. Nuclear magnetic resonance

Typical examples of ^{1}H-NMR spectra of CMP fractions are presented in Fig. 4. Spectra of glycosylated CMP showed a peak at 2.05 ppm, which was absent in the spectra of non-glycosylated CMP. The presence of such a peak in the spectrum of κ-CN was also reported by Rollema et al. [31]. Peaks in the region between 2.03 and 2.06 ppm correspond to protons from acetamide -CH_3 groups of GalNAc and NeuAc [5]. Spectra of glycosylated CMP obtained at pH 7 were similar to the spectrum of whole CMP obtained by Griffin and Roberts [32] at pH 6.2. At pH 3 the chemical shift of the resonance of the γ-CH_2 protons from Glu residues increases due to protonation of carboxylic groups.

In the pH 3–7 region the NMR spectra taken at the concentrations of 1 and 10 mg ml^{-1} also did not show any effects that could be ascribed to association of CMP, such as line broadening due to immobilization of particular groups. The NMR data thus confirmed the results obtained by HPGPC.

3.4. Digestion of CMP by Glu-specific endopeptidase and identification of products

The chromatographic patterns of products of CMP proteolysis by Glu-specific endopeptidase after 3 h at 25°C and after 6 h at 37°C are shown in Fig. 5. The identification of CMP degradation products by FAB-MS or ESI-MS has been summarized in Table 3. The rate of initial proteolysis of CMP (primary cleavage), indicated by a reduction of the peak intensity of intact CMP, was approximately the same for all fractions subjected to proteolysis (data not shown). The highly glycosylated CMP appeared to be more resistant to secondary cleavage (hydrolysis of smaller fragments) than the non-glycosylated and slightly glycosylated fractions. Peaks with short retention times (e.g., peaks 2 and 3; for composition see Table 3), which appeared to be final products of cleavage of the non-glycosylated as well as of the slightly glycosylated CMP fraction (Fig. 5a,b,d and e), are absent in the chromatogram of the hydrolysate of highly glycosylated CMP (Fig. 5c). Obviously, in CMP containing three glycosidic moieties some peptide bonds formed by Glu carboxylic groups seem to be protected against enzymic attack by the Glu-specific endopeptidase. Highly glycosylated CMP has also been reported to be resistant to hydrolysis by subtilisin [9]. We were not able to separate the various components of the highly glycosylated CMP-fragments by the RP-HPLC gradient program used (Fig. 5c). Further hydrolysis and separation of such fragments may be possible after partial degradation of the sugar moieties [7,9].

On the basis of the known amino acid sequence and the possible posttranslational modifications of CMP, MS allowed us to identify CMP digestion products including peptides containing phosphate, glycosidic moieties and methionine sulfoxide (see Table 3). In one case (i.e., peak 11 from the digest of slightly glycosylated CMP A) the observed molecular mass could be assigned to different κ-CN fragments. Therefore, unambiguous identification of that peptide would require further analysis. We have not found

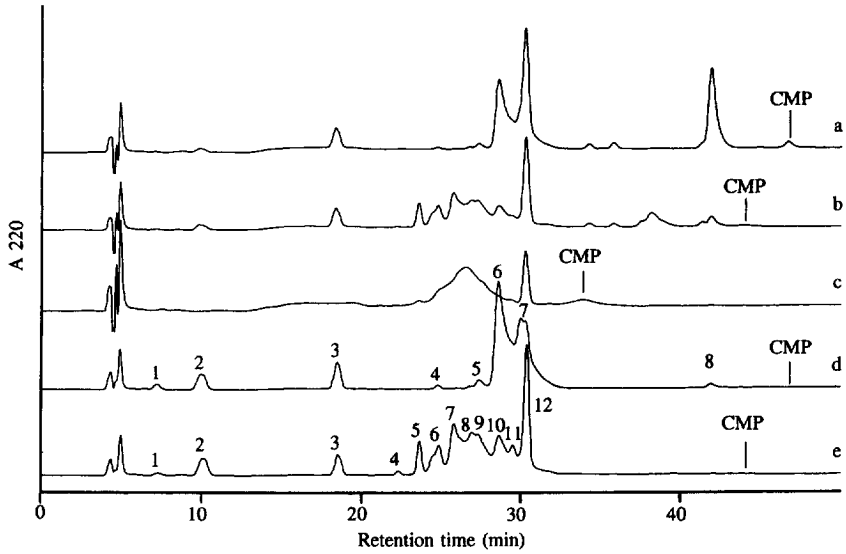

Fig. 5. RP-HPLC chromatograms of products of CMP hydrolysis by Glu-specific endopeptidase from *Staphylococcus aureus*. (a) Non-glycosylated CMP A, 3 h, 25°C; (b) slightly glycosylated CMP A, fraction 3, 3 h, 25°C; (c) highly glycosylated CMP A, 3 h, 25°C; (d) non-glycosylated CMP A, 6 h, 37°C; (e) slightly glycosylated CMP A, fraction 3, 6 h, 37°C. Numbers correspond to fractions subjected to MS analysis (see Table 3). CMP: position of the corresponding intact CMP.

peptides containing three or four amino acid residues. Such peptides were found in a digest of non-glycosylated CMP by Miranda et al. [3]. Probably they were not detected because of their low absorbance at 220 nm. Another possibility is that formation of short peptides requires an incubation time longer than 6 h, since Miranda et al. [3] used overnight hydrolysis.

As follows from Table 3, we have found evidence for cleavage of only four bonds in non-glycosylated CMP A (Glu_{137}-Ala_{138}, Glu_{140}-Ser_{141}, Glu_{147}-Asp_{148} and Glu_{154}-Ser_{155}) and five bonds in fraction 3 of slightly glycosylated CMP A (Glu_{137}-Ala_{138}, Glu_{140}-Ser_{141}, Glu_{147}-Asp_{148}, Glu_{151}-Val_{152} and Glu_{154}-Ser_{155}) (see also Fig. 6). Glu-Ala and Glu-Ser positions are hydrolysed easily, which is in agreement with earlier data obtained using CMP [3] and other proteins [24,25,33,34]. Glu-Pro (as occurring at position 129-130) is resistant to the action of Glu-specific endopeptidase, as reported previously [33]. We also found no hydrolysis of Glu-Ile linkages. One such linkage (Glu_{158}-Ile_{159}) was also reported as not hydrolysed by Miranda et al. [3]. This finding confirms the conclusion of Houmard and Drapeau [24] that bonds between Glu and strongly hydrophobic amino acids are not readily hydrolysed by Glu-specific endopeptidase. Glu_{147}-Asp_{148} is one of the most easily hydrolysed bonds. The peptide containing residues 106–147 of κ-CN (peak 8 of the non-glycosylated CMP hydrolysate) is an intermediate product formed at short incubation times (cf., Fig. 5a and d). The susceptibility of peptide bonds to cleavage by the staphylococcal peptidase strongly depends on the kind of protein in which they are located (for a review see Ref. [34]). In our case, the presence of one glycosidic moiety did not suppress proteolysis of CMP by the peptidase.

Glycosidic moieties appeared to be attached to the fragments 106–137, 148–169, 152–169 and possibly also 141–151 (see Table 3). The region between residues 131 and 142 has been reported to contain most of the glycosidic residues [7,9] (Fig. 6). Pisano et al. [7] have recently reported that Thr_{121} and Thr_{165} can also be glycosylated. Therefore, our present results are in accordance with these data. Taking into account that the above fragments were obtained from CMP A fraction 3, containing only one glycosidic moiety per molecule (see Table 2 and

Table 3
Products of hydrolysis of CMP by Glu-specific endopeptidase from *Staphylococcus aureus* (peak numbers refer to Fig. 5)

Sample	Observed molecular mass[a]	Calculated molecular mass	Fragment of κ-CN[b]	Modifications
Non-glycosylated CMP A				
Peak 1	867.2	867.33	148–154	1 P
Peak 2	719.4	719.38	141–147	
Peak 3	1018.6	1018.53	138–147	
Peak 4	1541.9	1541.80	155–169	
Peak 5	3426.8	3427.84	106–137	1 methionine sulfoxide
Peak 6	3410.7	3411.84	106–137	
Peak 7	3410.7	3411.84	106–137	
	3710.0	3711.17	106–140	
Peak 8	4412.2	4412.95	106–147	
Slightly glycosylated CMP A, fraction 3				
Peak 1	867.2	867.33	148–154	1 P
Peak 2	719.2	719.38	141–147	
Peak 3	1018.5	1018.53	138–147	
Peak 4	2540.6	2540.72	152–169	1GalNAc, 1Gal, 1NeuAc
	2831.5	2831.98	152–169	1GalNAc, 1Gal, 2NeuAc
Peak 5	3339.6	3340.36	148–169[c]	1GalNAc, 1Gal, 2NeuAc, 1P
Peak 6	1541.8	1541.80	155–169	
Peak 7	4067.7	4068.44	106–137	1GalNAc, 1Gal, 1NeuAc
	4359.3	4359.70	106–137	1GalNAc, 1Gal, 2NeuAc
Peak 8	4067.8	4068.44	106–137	1GalNAc, 1Gal, 1NeuAc
	4360.6	4359.70	106–137	1GalNAc, 1Gal, 2NeuAc
	4658.3	4659.03	106–140	1GalNAc, 1Gal, 2NeuAc
Peak 9	3427.3	3427.84	106–137	1 methionine sulfoxide
Peak 10	3411.3	3411.84	106–137	
Peak 11	1884.2	1884.12	152–169 and/or	
		1884.78	141–151	1GalNAc, 1Gal, 1NeuAc, 1P
	3411.7	3411.84	106–137	
Peak 12	3710.7	3711.17	106–140	

[a] ESI-MS and FAB-MS results, converted to uncharged masses, are given. For masses >1600 Da (ESI-MS) average mass values of unresolved isotopic ion clusters are shown. Mono-isotopic masses are given for masses <1600 Da (FAB-MS). The experimental error was within 0.02% ($n=3$).

[b] Numbering of residues according to positions in bovine κ-CN [1,4].

[c] The observed mass also accounts for fragment 130–154 containing 1GalNAc, 1Gal, 1NeuAc, 1P, but assignment to this fragment is very unlikely on the basis of resistance of bond 129–130 (Glu-Pro) to proteolytic attack [33,34].

Section 1), the presence of glycosidic moieties attached to different CMP fragments indicates that this fraction contained components with different glycosylation sites.

In both fractions subjected to preparative hydrolysis only the fragments containing Ser_{149} were found to be phosphorylated. This position is known as a main phosphorylation site [35].

The occurrence of glycosylated 106–137 fragments in both the (neighbouring) peaks 7 and 8 of the slightly glycosylated CMP A (see Table 3 and Fig. 5e) should probably be ascribed to poor chromatographic separation. A similar contamination of peak 9 with components of peak 8 was observed (not shown in Table 3).

Some of the methionine-containing CMP fragments occurred in forms with and forms without oxidized methionine. Oxidation of methionine often takes place during isolation and separation of proteins and peptides (for a review see Ref. [36]). The

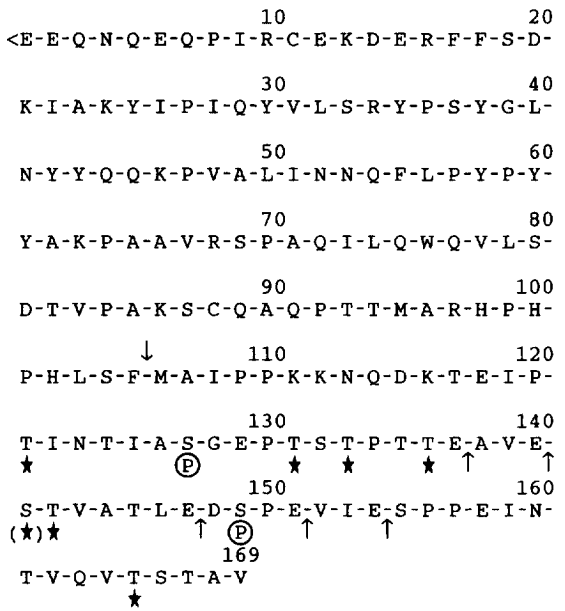

Fig. 6. Amino acid sequence of bovine κ-CN A [1]. In genetic variant B residues 136 and 148 are Ile and Ala, respectively. Fragment 106–169 represents the CMP released by chymosin action (↓). Encircled P, identified phosphorylation sites [35]; ★, identified glycosylation sites [7–9]; ↑, positions found in the present study to be cleaved in CMP by Glu-specific endopeptidase; <E, pyroglutamic acid.

oxidation could occur at any step of an experimental procedure or during storage under aerobic conditions.

4. Conclusions

RP-HPLC forms an effective tool for the analytical and preparative separation of the complex mixture of glycosylated and non-glycosylated CMPs. Within the glycosylated fractions co-elution of different components remains a problem (see also Ref. [11]). For the preparation of separate A and B forms one can best start from single variants, since overlap of glycosylated fractions of the two variants occurs in the RP-HPLC patterns.

In contrast to what has been reported in the literature [13–16], no association takes place between CMP molecules, either non-glycosylated or glycosylated, as a function of the experimental conditions, as judged by HPGPC and NMR analysis. The high negative charge and hydrophilicity rather leads to internal electrostatic and steric repulsion, resulting in a larger voluminosity and therefore in GPC patterns in which an increase in apparent molecular size is shown.

ESI-MS can be successfully applied to the mass determination of isolated CMP fractions. For mass determination of smaller fragments, obtained after enzymic digestion of CMPs, both ESI-MS and FAB-MS are very useful techniques.

The initial rate of hydrolysis by Glu-specific endopeptidase is almost equal for differently glycosylated CMP A fractions. Only in the case of the highly glycosylated CMP A is further hydrolysis of the primary products inhibited. Digestion of the various CMP fractions by this enzyme may be useful for obtaining suitable peptide fragments having some sort of biological activity [12].

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