ELSEVIER

Journal of Chromatography A, 708 (1995) 223–230

JOURNAL OF CHROMATOGRAPHY A

Heterogeneity of the bovine κ-casein caseinomacropeptide, resolved by liquid chromatography on-line with electrospray ionization mass spectrometry

Daniel Mollé, Joëlle Léonil*

Institut National de la Recherche Agronomique, Laboratoire de Recherches de Technologie Laitière, 65 rue de St-Brieuc, 35042 Rennes Cedex, France

First received 12 January 1995; revised manuscript received 21 March 1995; accepted 21 March 1995

Abstract

Microheterogeneity occurs in the population of caseinomacropeptides (residues 106–169 of κ-casein) due to variation in the extent and type of oligosaccharide linked to this phosphoglycopeptide. Although caseinomacropeptide A variant (CMP_A) was poorly resolved using reversed-phase high-performance liquid chromatography (RP-HPLC) with spectrophotometric detection, it could be analysed with on-line electrospray-ionization mass spectrometry (ESI-MS). From the already established O-linked glycan chains at least fourteen glycosylated forms of CMP_A were identified, besides the non-glycosylated and multiphosphorylated (1, 2 or 3 phosphate groups) peptides, giving a maximum number of eighteen known forms. Major subcomponents in CMP_A are disialylated species. A maximum of three out of the five potential glycosylation sites were found to be substituted with carbohydrate chains in the most highly glycosylated forms, which may contain up to six N-acetylneuraminic acid residues per molecule. A minor form, diphosphorylated with one disialylated chain, was also detected. From these results, it was shown that the on-line coupling of HPLC with ESI-MS offers a very promising alternative for the analysis of complex mixtures.

1. Introduction

Caseinomacropeptide (CMP) is a polypeptide of 64 amino acid residues (106–169) derived from the C-terminal part of bovine κ-casein. It is released by chymosin (EC 3.4.23.4) action during the primary phase of milk clotting. Some biological functions of CMP have been reported including a growth promoting effect on bifidobacteria [1], suppression of gastric secretion [2,3], depression of platelet aggregation [4], inhibition of oral actinomyces adhesion to cell membranes [5] and inhibition of cholera toxin binding to its receptor [6]. CMP exists as an heterogeneous mixture since it carries both genetic variations (four genetic variants, A, B, C and E are known [7]) as well as carbohydrate and phosphorylation sites responsible for the polymorphism of its precursor. κ-Casein displays mucin-type carbohydrate chains comprising N-acetylneuraminyl (NeuAc), galactosyl (Gal) and N-acetylgalactosamine (GalNAc) residues [8]. A monosaccharide alditol, $GalNAc_{OH}$, and four

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00386-X

oligosaccharide alditols Galβ(1-3)GalNAc$_{OH}$, NeuAcα(2-3)Galβ(1-3)GalNAc$_{OH}$, Galβ(1-3)-[NeuAcα(2-6)]GalNAc$_{OH}$ and NeuAcα(2-3)Gal-β(1-3)[NeuAcα(2-6)]GalNAc$_{OH}$ have been identified in mature bovine κ-casein [9]. Another source of heterogeneity is the variable level of phosphorylation, ranging from 1 to 3 phosphate groups [10–12]. Thus, from 7 to 10 subcomponents have been reported for the bovine κ-casein [10,11]. However, the distribution of carbohydrate units on the κ-casein molecule still remains unclear.

The complete structural analysis of κ-casein requires isolation of the individual constituents. The majority of the studies reported so far have been accomplished using two chromatographic methods, anion exchange and high-performance size exclusion [11,13], in order to separate the subcomponents of κ-casein. These subcomponents are then enzymatically or chemically cleaved for further analysis of their carbohydrate and phosphorus content. However, these methods are time-consuming and limited by the poor chromatographic resolution obtained. Therefore the number of subcomponents, as well as their precise structure are, as yet, not definitively established neither for κ-casein nor, consequently, for CMP. On account of the potential applications of CMP in pharmacology a better knowledge of this peptide is required. Thus a method for identifying the different molecular forms of CMP would be valuable. It has become increasingly clear that the carbohydrate chains play a critical role in the functional properties of these glycoproteins [14]. In a previous publication, we have reported a procedure for isolating CMP from whey, using cation-exchange chromatography and its subsequent analysis by RP-HPLC [15]. Since the separation of glycoproteins is still a challenging task, because of the occurrence of glycoforms with little difference in size or charge, the emergence of a soft ionization technique such as the electrospray method (ESI) has expanded the capability of liquid chromatography–mass spectrometry (LC–ESI-MS). The present work describes the first use of two combined methods, RP-HPLC and ESI-MS, for the analysis and characterization of the CMP_A subcomponents.

2. Experimental

2.1. Purification of caseinomacropeptide A

CMP_A was isolated from the milk of an individual homozygous cow A/A at the locus κ-casein A variant, according to the method previously described [15] based on the hydrolysis of κ-casein by chymosin, followed by chromatography on a S-Sepharose Fast Flow column (Pharmacia Biotech, Saint-Quentin, Yvelines, France). CMP was found to be 97% homogeneous, based on amino acid composition [15].

2.2. RP-HPLC–ESI-MS analyses of caseinomacropeptide A

The three RP-HPLC columns that were used in order to optimize the separation of the different subcomponents of the CMP_A were: Superspher 100 RP-18 (150 × 2.1 mm I.D., 5 μm particle size) (Merk, Darmstadt, Germany); Nucleosil C_{18}-AB (125 × 2.1 mm I.D., 5 μm particle size) (Macherey-Nagel, Strasbourg, France); Delta-Pack C_{18} (300 Å, 150 × 2 mm I.D., 5 μm particle size) (Waters, Milford, MA, USA). The mobile phase was delivered by a Waters 625 LC pump. The gradient elution was performed with acetonitrile as the organic modifier, at 40°C and at a flow-rate of 0.2 ml/min. Solution A was 0.1% TFA dissolved in double-distilled water (v/v) and solution B was 0.1% TFA dissolved in acetonitrile–double distilled water (80:20, v/v). After equilibration of the column with 27% of solution B, samples were applied to the column and eluted by increasing the concentration of solution B as follows: 0–40 min, 27–47%; 40–42 min, 47–80%. Eluted peaks were detected by absorbance at 214 nm using a Waters 990 Series photodiode array detector.

The mass spectrometer API I Sciex (Thornhill, Ont., Canada) was a single-quadrupole mass spectrometer equipped with an atmospheric-pressure ionization ion source. API I Sciex was operated in the positive mode. Multiply-charged protein ions were generated by spraying the sample solution through a fused-silica capillary of 75 μm I.D., introduced into a stainless steel capillary held at high potential. The voltage on the sprayer was usually set between 5 and 5.5 kV. A coaxial air-flow along the sprayer was provided to assist the liquid nebulization; the nebulizer pressure was usually adjusted within the range of 0.3–0.4 MPa. For the infusion experiment, the sample was delivered to the sprayer by a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, MA, USA). The liquid flow-rate was usually set at 5–10 μl/min for sample introduction. For analysis by RP-HPLC coupled with ESI-MS, splitting of the liquid flow was achieved by a low-dead volume connection and the column effluent was diverted partly to the mass spectrometer (15% of the effluent) and partly to the UV detector (85% of the effluent). This arrangement permitted a straightforward correlation of the total-ion current (TIC) trace with the UV trace. The interface between the sprayer and the mass analyser consisted of a small conical orifice of 100 μm diameter. The potential on the orifice was 80 V. A gas curtain, formed by a continuous flow (0.8–1.2 l/min) of nitrogen in the interface region, served to break up any clusters. The instrument m/z scale was calibrated with ammonium adduct ions of poly-(propylene glycol)s. All protein mass spectra were obtained from the signal averaging of multiple scans. HPLC–ESI-MS experiments were performed while scanning the m/z range 800–2400 at a step-size of 0.33 amu and a dwell time of 0.5 ms. UV absorbance was detected simultaneously with the MS signal and registered with Tune 2.0 software Sciex. Molecular masses were determined from the measured m/z values for the protonated molecules. Data were collected on an Apple Macintosh Quadra 900 computer and were processed using the software package Mac Spec 3.2 Sciex. The reconstructed molecular mass profile was obtained by using a deconvolution algorithm (Mac Spec 3.2 Sciex).

3. Results

3.1. Optimization of the separation of caseinomacropeptide by RP-HPLC

Fig. 1 shows the RP-HPLC–UV traces of CMP_A using three different C_{18} columns (Superspher, Nucleosil, Delta-Pack) eluted with the same mobile phase composition, i.e. water–acetonitrile containing TFA. The injected amount (50 μg) and conditions of elution gradient were identical for all three columns. The UV profile provides a series of closely related peaks reflecting the heterogeneity of CMP_A. In order to improve the separation conditions, the gradient slope, the temperature (37, 45, 55°C), the mobile phase system (acetonitrile, acetonitrile–2 propanol, ammonium acetate buffer pH 6.5) as well as ion-pairing (TFA, acetic acid), were all modified. However, despite all efforts it was not possible to separate the different constituents of CMP_A (data not shown). The Delta-Pack column was found to give the best

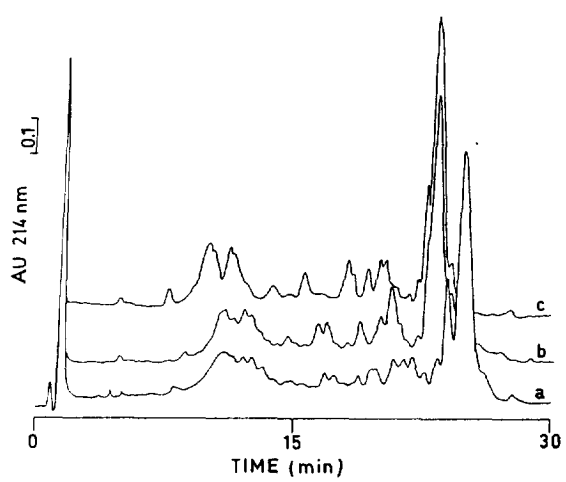

Fig. 1. RP-HPLC elution profile of caseinomacropeptide A variant on (a) Superspher (b) Nucleosil (c) Delta-Pack columns C_{18}. Linear gradient from 21–37% acetonitrile containing 0.1% TFA.

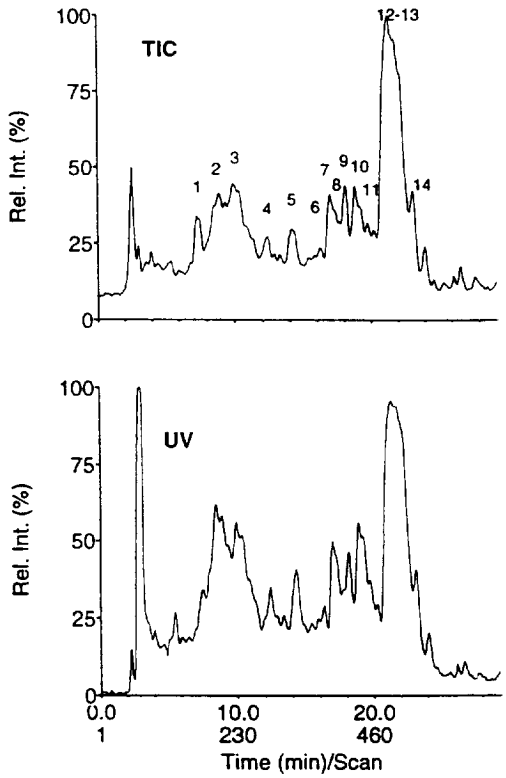

Fig. 2. RP-HPLC spectra of caseinomacropeptide A variant with total ion current (top), as measured by ESI-MS and UV monitoring at 214 nm (bottom).

separation for this complex mixture with an elution acetonitrile gradient containing 0.1% TFA and hence this was used for the coupling with ESI-MS. The major peak, which appeared at a retention time (t_R) of 22 min in Fig. 1c, has been identified as a carbohydrate-free CMP_A [15]. Earlier eluted peaks (t_R 8–20 min) were assigned to glycosylated forms but nothing was known about their carbohydrate content.

3.2. Analysis of caseinomacropeptide A by on-line RP-HPLC–ESI-MS

The reconstructed total ion current (TIC) chromatogram of CMP_A is shown in Fig. 2 (top), for CMP_A injected on the Delta-Pack column. Aliquots of 150 μg were loaded onto the column at a flow-rate of 0.2 ml/min with a split of about 1:7 into the ES source. This chromatogram correlates well with the UV-absorbance profile (Fig. 2, bottom). Peak intensities in TIC and UV absorbance are different because they are based on different physical measurements [16]. The analysis of glycophosphopeptides, and in particular the determination of M_r, was aided by using the two dimensional display or contour plot, in which the individual ions for each scan are plotted as their m/z vs. run time or scan number. Closely related glycoforms are identified by the appearance of a typical diagonal pattern of lines with their respective intensities [17]. This is illustrated in Fig. 3 for the more heavily glycosylated forms observed in the early region of the plot. The M_r of each form can thus be calculated from the contour plot, using the m/z values of any given ion series falling on the same vertical line. These values are presented in

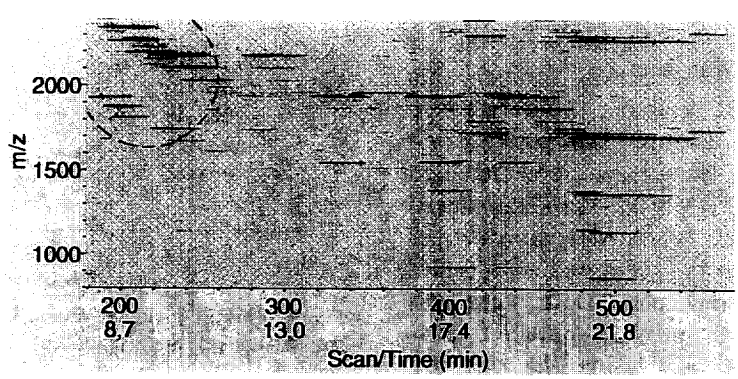

Fig. 3. Two-dimensional display of the ions (contour plot) observed in the HPLC–ESI-MS of caseinomacropeptide A variant. The multiply charged ions of glycoforms are highlighted.

Table 1 (2nd column). As anticipated from our earlier studies, some peaks are not homogeneous and therefore give several masses. As examples, representative spectra of the peaks 2 and 12–13 are shown in Fig. 4A,B respectively. The mass spectrum extracted from each chromatographic peak is characterized by a series of ions having m/z values corresponding to multiple protonated forms of the molecule. Ions from peak 2 were observed at m/z 2336.2, 2262.9, 2191.6, 1927.3, 1868.9, and 1810.8, indicating the coelution of several components. Two series of ions were found, one at m/z 2336.2 and 1868.9 and the other at m/z 2262.9 and 1810.8, which can be related to the $[M+4H]^{4+}$ and $[M+5H]^{5+}$ charged ions. From the measured m/z values for the multiply charged ions and their charge states, the molecular masses were 9339.28 and 9048.88, respectively, for these two components. The low level of the remaining ions in the spectrum

Table 1
Observed molecular masses of the peaks in the UV trace and TIC (Fig. 2) and assignments of different subcomponents of caseinomacropeptide with their calculated molecular masses

Peak No.	Observed M_r	Subcomponent structure of CMP	Calculated M_r
1	3478.40 ± 0.03	–	–
2	9632.82 ± 0.81[a]	CMP_A-3E	9630.92
	9339.28 ± 0.04	CMP_A-2E 1C/D[b]	9339.66
	9048.88 ± 0.04	CMP_A-1E 2C/D[b] or 2E 1B	9048.42
	8759.56 ± 0.70[a]	CMP_A-3C/D[b] or 1E 1C/D 1B	8757.17
3	8684.66 ± 0.74	CMP_A-2E	8683.10
	8392.51 ± 0.42	CMP_A-1E 1C/D[b]	8391.85
	8102.11 ± 0.42	CMP_A-2C/D[b] or 1E 1B	8100.60
4	8683.73 ± 0.74	CMP_A-2E	8683.10
5	7735.48 ± 0.04	CMP_A-1E	7735.27
6	7814.96 ± 1.42[a]	CMP_A (2*P*)-1E	7815.16
7	7736.38 ± 0.69	CMP_A-1E	7735.27
8	2764.56 ± 0.05	–	–
9	6867.88 ± 0.30	CMP_A (2*P*)	6867.33
	7152.97 ± 1.36	CMP_A-1B	7152.85
10	7735.91 ± 0.51	CMP_A-1E	7735.27
	7444.86 ± 0.68[a]	CMP_A-1C/D[b]	7444.10
11	6947.96 ± 0.57[a]	CMP_A (3*P*)	6947.23
12	6868.24 ± 1.08	CMP_A (2*P*)	6867.33
13	6788.04 ± 0.74	CMP_A (1*P*)	6787.42
14	6935.09 ± 1.00[a]	κ-CN (105–169)	6934.60

CMP_A (1*P*) (residue 106–169 of κ-casein) indicates the monophosphorylated carbohydrate-free form in this table. Symbols A, B, C, D, E, are described in Fig. 5.
[a] Determined by infusion.
[b] C and D (C/D), having the same mass, cannot be discriminated by MS analysis.
nd Not determined.

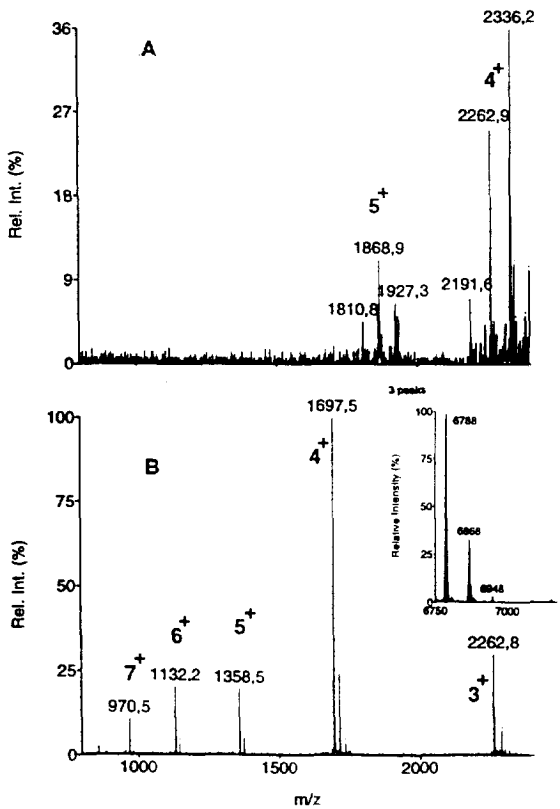

Fig. 4. Mass spectra of peak 2 (A) and peak 12–13 (B) from Fig. 2. Insert in graph B shows the reconstructed molecular spectra. The number of positive charges is indicated at the top of each peak.

precluded their mass determination by Sciex algorithm. However, in the case where only one charge state is observed the m/z difference between related ion signals, as described above for glycoforms, may be used to determine its charge state. For example, only a single charge state is observed for the species detected at m/z 1927.3. The m/z difference of 58.4 between m/z 1927.3 and 1868.9 can be assigned to the mass difference of a NeuAc moiety (M_r NeuAc/5 = 291.6/5) if the charge state is 5. The M_r was determined as 9631.5 ($1927.3 \times 5 - 5$). In order to confirm the M_r of the minor components, higher concentrations of these components were required. Hence each minor peak in RP-HPLC was collected, lyophilized and directly analysed in a carrier solvent by ESI-MS, at a flow-rate of 5 μl/min. The masses of these components are indicated by an asterisk in Table 1. In the spectrum shown in Fig. 4B for peak 13, the predominant M_r measured was 6788.04 ± 0.74, which is in good agreement with the calculated value (6787.42) from the known sequence for the non-glycosylated CMP_A variant [18]. Interestingly, this spectrum reveals the presence of a second distribution pattern of ion peaks (m/z 1145.8, 1374.5, 1717.5, 2290.5) that gives a calculated M_r value of 6867, differing from the non-glycosylated form by 80 amu. These data are consistent with the presence of mono- and di-phosphorylated carbohydrate-free CMPs [11]. In addition, a very minor form with three phosphate groups was also observed in the deconvoluted mass spectrum shown in the Fig. 4B insert. As a linear dependence of the ESI signal upon concentration was observed [19], mono-, di- and triphosphorylated forms occur in the ratios 78%, 20% and 2%, respectively. This implies, of course, that uniform ESI mass spectrometry response of the closely related species was assumed.

Overall, there are fifteen different masses obtained from the CMP analysis by HPLC–ESI-MS within the mass range 6936–9633. Peaks 1 (M_r 3478) and 8 (M_r 2764) were impurities and were not investigated further. The structures of five carbohydrate units reported to be linked to CMP [9] are described in Fig. 5. From these data and mass increments, in excess of the known non-glycosylated peptide mass, the glycan structures shown in Table 1 were assigned to the different peaks separated by HPLC. All masses reported are within 0.02% of those expected for the proposed structures. The same masses recovered along the chromatogram with varying t_R were considered as being different molecular species. Their occurrence is easily attributable to the fact that the same carbohydrate units are linked to different positions in the peptide chain, leading to different t_R values. Peak 18 is a minor component which may correspond to the sequence 105–169 of non-glycosylated κ-casein, κ-CN(105–169), subsequent to the cleavage of the bond Ser104–Phe105. Such a specificity of chymosin has not been reported. However, this kind of cleavage is known for other aspartic

A GalNAc

B Galβ(1-3)GalNAc

C NeuAcα(2-3)Galβ(1-3)GalNAc

D Galβ(1-3)GalNAc
NeuAc
$^{2}_{6}\alpha$

E NeuAcα(2-3)Galβ(1-3)GalNAc
NeuAc
$^{2}_{6}\alpha$

Fig. 5. Structure of the five carbohydrate chains of mature bovine κ-casein [9]. M_r (average): A = 221.2, B = 383.3, C = 674.6, D = 674.6, E = 965.8.

proteinases [20]. At least eighteen different molecular species were identified in CMP_A and consequently in κ-casein A, including non-glycosylated and multiphosphorylated forms.

4. Discussion

We describe here the characterization of a complex mixture of glycophosphopeptides, arising from residues 106–169 of κ-casein, by use of the combination of RP-HPLC and ESI-MS. No single method has been reported to be adequate for the analysis of CMP, but ESI-MS has proved to be a powerful method for the characterization of such mixtures, especially when there is some difficulty in separation by HPLC as seen in Fig. 1. Using this means, the maximum number of subcomponents of CMP_A found in this study was twice as high as that previously reported [11]. The sialylated species were detected as the major subcomponents in CMP_A (Table 1) and the NeuAcα(2–3)Galβ(1–3)[NeuAcα(2–6)]Gal-NAc_{OH} tetrasaccharide containing two NeuAc was shown to be the most dominant sugar chain. This confirms the hypothesis that the ratio of disialylsaccharide chains may be high in CMP subcomponents with a high content of carbohydrate chains [21]. As expected from the reverse-d-phase separation (Fig. 1), these forms are eluted earlier. The average distribution of the different sugar chains A, B, C, D and E was estimated to be 0.8, 6.3, 18.4, 18.5 and 56% respectively, following alkaline borohydride treatment and analysis of the carbohydrates linked on the κ-casein molecule [9]. These data are consistent with our results except for the existence of species containing GalNAc (A), which are not detected under our ESI-MS analysis conditions. This suggests that either these species are present in very small quantities, and consequently at levels that are below the detection limit of the experimental set-up used, or that they do not exist. It cannot be precluded that the monosaccharide A is an artefactual component formed by alkaline β-elimination during the isolation of carbohydrate chains from κ-casein [9]. The points of attachment for carbohydrates are four Thr residues (131, 133, 135 or 136, and 142) and one Ser141 of the peptide chain [11,12]. The maximum number of glycosylation sites deduced from our results was three out of five potential sites. Consequently a maximum of six NeuAc groups can be linked per peptide chain, which is not in agreement with the value of ten obtained for the κ-casein B variant by Vreeman et al. [11]. The latter result was based on the determination of NeuAc from chromatographic fractions. No evidence of such masses was found in MS analysis. This discrepancy may be explained by genetic factors since variant B is known to be more efficiently glycosylated than variant A [22]. From our data, one can conclude that only a part of the potential glycosylation sites are substituted for the variant A κ-casein. There is an ambiguity about the structural assignment of three masses, i.e. those with M_r 9048, 8759 and 8102. For each, two possible distributions of carbohydrate chains can be considered. We cannot discriminate between these two structures with this approach. One potential alternative is to use ESI-MS–MS analysis to this end, as well as to locate the three Thr/Ser residues which are glycosylated. Efficient strategies are based on enzymatic digestion

of the glycoprotein into smaller fragments followed by a selective detection of O-linked glycopeptides by comparison of mass spectral data obtained before and after deglycosylation. Glycopeptides are then chosen for MS–MS analysis in order to locate the attachment sites. The same procedure involving degradation of the large molecule to smaller fragments is, of course, valid for the location of the phosphorylation sites. In this work, we have demonstrated the occurrence of triphosphorylated forms consistent with the literature [11]. However, of the three phosphorylation sites in κ-casein only two of are known. Ser127 and Ser149 are partly and completely phosphorylated, respectively [23]. Therefore, the third phosphate group might occur on either potential acceptor, Thr135 or Thr145, located in a tripeptide recognition site [23]. Another interesting result is the finding of a diphosphorylated form of CMP_A with only one E chain. Such a structure has not been reported previously. The low abundance of di- and triphosphorylated carbohydrate chains indicates that multiphosphorylation drastically inhibits the ability of the κ-casein molecule to be glycosylated. It is noteworthy that all glycosylation and phosphorylation sites are positioned on a short segment of the polypeptide chain involving only 20 amino acid residues.

To conclude, the results presented here demonstrate that the combination of RP-HPLC online with ESI-MS is a powerful method for the precise determination of the masses of a large number of glyco- and phosphopeptides within a heterogeneous mixture. As shown from the example of CMP_A, structural details of different molecular forms can be easily obtained when both the peptide and oligosaccharide chains are known. This procedure is rapid and a great amount of information can be obtained from one chromatographic run. The approach described here should be suitable for solving a wide variety of protein and peptide analytical problems.

Acknowledgement

The authors wish to thank Drs J.C. Mercier and P. Martin for helpful discussions.

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