Liberation of tryptic fragments from caseinomacropeptide of bovine κ -casein involved in platelet function

Kinetic study

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Carbohydrate-free caseinomacropeptide (CMP) was purified from rennet-hydrolysed caseinate by trichloroacetic acid precipitation and DEAE-TSK Fractogel-650 ion-exchange chromatography. To study the liberation of 106–112, 106–116 and 113–116 fragments from carbohydrate-free CMP involved in platelet function, a quantitative study was made on the rate of hydrolysis of the three peptidic bonds that are susceptible to the action of trypsin. Data were obtained from reverse-phase (Ultrasorb column) and cationic-exchange (Mono S column) h.p.l.c. On the basis of the disappearance of substrate, k_{cat} and K_m were respectively 3.95 s^{-1} and 0.2 mM . The two 111–112 and 112–113 bonds were split according to similar kinetic parameters ($k_{cat} = 1.97\text{ s}^{-1}$, $K_m = 0.2\text{ mM}$) and much faster than the 116–117 bond. The difference in susceptibility of the bonds can probably be attributed to the nature of residues flanking the primary proteolytic sites rather than to their accessibility to the proteinase. On the basis of our results the 106–116 fragment cannot be formed.

INTRODUCTION

Biologically active peptides have been identified as digestion products of several food proteins, especially from milk (Maubois & Léonil, 1989). Jollès *et al.* (1986) have previously shown that an undecapeptide (residues 106–116) derived from bovine κ -casein by chymosin and tryptic digestion inhibited both aggregation and binding of fibrinogen to ADP-treated platelets. The same authors reported that two smaller tryptic peptides (residues 106–112 and 113–116) contained in the undecapeptide and tested at the same concentrations had a much smaller effect on platelet aggregation and did not inhibit fibrinogen binding.

Hydrolysis of bovine κ -casein by chymosin (rennin, EC 3.4.23.4) constitutes the first stage of milk clotting and has been thoroughly investigated. It is known (Delfour *et al.*, 1965) that this aspartic proteinase rapidly hydrolyses only one bond (Phe-105–Met-106) of κ -casein, leading to the release of an *N*-terminal fragment (para- κ -casein; residues 1–105) and a *C*-terminal fragment (caseinomacropeptide, CMP; residues 106–169) from which can be derived tryptic peptides with anti-thrombotic activity. As far as we are aware, a kinetic study of the tryptic hydrolysis of CMP has not hitherto been carried out, though this constitutes a potential economical source of biologically active peptides. Insofar as production can be developed on a large scale (Brulé *et al.*, 1980), we have now studied the kinetics of formation of tryptic peptides from CMP. κ -Casein is heterogeneous with respect to its carbohydrate moiety, which is exclusively linked to the macropeptide part of κ -casein with different degrees of substitution (MacKinlay & Wake, 1965). Therefore a carbohydrate-free CMP was used. It has been reported that the addition of 12% (w/v) trichloroacetic acid (final concentration) to whole CMP (glycosylated and non-glycosylated) led to the precipitation of carbohydrate-free CMP and kept carbohydrate-containing CMP in solution (Alais *et al.*, 1953; MacKinlay & Wake, 1965; Armstrong *et al.*, 1967; Creamer *et al.*, 1973). However, little is known concerning the precise composition of the trichloroacetic acid-insoluble fraction. Using the trichloroacetic acid procedure, we describe methods

for further purification and identification of carbohydrate-free CMP and tryptic fragments derived from it. We also report our results on the susceptibility to trypsin of the three bonds that are split.

MATERIALS AND METHODS

Materials

Uvasol acetonitrile was obtained from E. Merck (Darmstadt, Germany) and trifluoroacetic acid was from Pierce Chemical Co. (Mallet S. A., Roissy Ch. De Gaulle, France). All other products were of analytical grade. Buffers and mobile phase were prepared from h.p.l.c.-grade water (Millipore Corp., Bedford, MA, U.S.A.) and degassed under vacuum before use. Chymosin (EC 3.4.23.4) was purified by the method of Garnot & Mollé (1982). Its proteolytic activity was 40 units/mg as determined spectrophotometrically at 230 nm and 30 °C with the synthetic hexapeptide Leu-Ser-Phe-Nle-Ala-Ile-OMe described by Vreeman *et al.* (1986). *N*-Tosyl-L-Phe-CH₂Cl ('TPCK')-treated trypsin (EC 3.4.21.4) (bovine pancreas; 131 units/mg) from Serva (Heidelberg, Germany) was used. Trypsin activity was determined on the synthetic substrate *N*-tosyl-L-Arg-OMe by the procedure of Hummel (1959). For both enzymes used 1 unit is defined as the amount of enzyme that catalyses the formation of 1 μ mol of product/min. Synthetic peptide Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys corresponding to sequence 106–116 of κ -casein was obtained from the Neosystem Laboratory (Strasbourg, France). DEAE-TSK Fractogel-650 (medium grade), was from E. Merck (Nogent sur Marne, France). Spectrapor dialysis tubing membrane with M_r cut-off 1000 was purchased from Polylabo (Strasbourg, France).

Methods

Preparation of carbohydrate-free CMP. Milk from an individual cow homozygous for κ -casein A-variant was skimmed by centrifugation at 40 °C (3000 *g* for 5 min). Whole casein in which the α_{s1} -, α_{s2} -, β - and κ -casein components occur in the approximate proportions 3:1:3:1 (Swaigood, 1982) was pre-

Abbreviations used: CMP, glycosylated and non-glycosylated caseinomacropeptide; carbohydrate-free CMP, carbohydrate-free caseinomacropeptide.

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pared from skimmed milk by acidification to pH 4.6 with addition of 1 M-HCl. The precipitate was washed three times with distilled water. For the preparation of sodium caseinate, the precipitate was dissolved in distilled water and the pH adjusted to pH 6.5 with 1 M-NaOH.

CMP was prepared by the action of chymosin. In a typical experiment a 2.5% (w/v) sodium caseinate solution was hydrolysed for 1 h at pH 6.3 and 35 °C at an enzyme/substrate ratio of 1:50000 (w/w). The reaction was stopped by adding trichloroacetic acid at a final concentration of 2% (w/v) in order to remove the non-hydrolysed casein fraction. The supernatant contained CMP. Carbohydrate-free CMP was isolated as described by Armstrong *et al.* (1967) by precipitation with 12% (w/v) trichloroacetic acid (final concentration). After centrifugation, the pellet was dissolved in water at about 1.5% (w/v), and dialysed overnight at 4 °C against distilled water and then 20 mM-Tris/HCl buffer, pH 7.5, for 8 h.

The dialysed product (80 ml) was further purified on a DEAE-TSK Fractogel-650 column (12 cm × 3 cm) equilibrated with 20 mM-Tris/HCl buffer, pH 7.5, at a flow rate of 1.5 ml/min. Elution was performed by a salt gradient (0–0.2 M-NaCl) in the same buffer. The fractions containing carbohydrate-free CMP were pooled, dialysed against several changes of distilled water and freeze-dried. The chromatography was followed by reverse-phase h.p.l.c. (Ultrapase column) as described below. The purity of carbohydrate-free CMP was assessed by reverse-phase h.p.l.c. and amino acid composition.

Enzymic assay. Hydrolysis of carbohydrate-free CMP was performed at 40 °C in 10 mM-sodium phosphate buffer, pH 7.5. Digestion time was started by addition of trypsin (0.5 unit/ml of substrate solution). At selected times 300 µl samples were withdrawn and the reaction was stopped by adjusting the solution to pH 2.0 with 20 µl of 5% (v/v) trifluoroacetic acid.

H.p.l.c. Reverse-phase h.p.l.c. (Spectra Physic SP 8800 pump and SP 8490 detector) was on an Ultrapase C₁₈ column (4.5 mm × 250 mm; Société Française Chromato Colonne, Neuilly-Plaisance, France). Operating conditions were: column temperature, 50 °C; flow rate, 0.8 ml/min; solvent A, 0.1% (v/v) trifluoroacetic acid; solvent B, 60% (v/v) acetonitrile and 40% (v/v) solvent A. Elution was obtained with a linear gradient from 0 to 60% (v/v) solvent B in 45 min. Absorbance was recorded at 214 nm.

Cation-exchange h.p.l.c. was carried out on a Mono S column fitted to an f.p.l.c. apparatus (Pharmacia) comprising a model LCC 500 automated gradient controller supplied with two P-500 solvent pumps and a 214 nm-wavelength detector linked to a chart recorder and a Frac 100 collector. Elution was performed at a constant flow rate of 1.0 ml/min. A linear ionic gradient of 0–0.5 M-NaCl in 10 mM-KCl/HCl at pH 2 was used at room temperature.

Both h.p.l.c. systems were fitted with 2600 Chromatography Nelson Analytical software by means of a 900 Series Intelligent Interface (Nelson, CA, U.S.A.).

Peptides (carbohydrate-free CMP and tryptic peptides) was determined quantitatively from the relation between peak areas and injected amounts of each pure peptide whose concentration had been determined by amino acid composition. In each case an excellent correlation (linear-regression analysis) was found when the areas were plotted against the injected amounts.

Amino acid analysis. Amino acid analyses of samples hydrolysed for 24 h in 6 M-HCl at 110 °C under vacuum were performed with a Beckman automatic amino acid analyser.

Determination of kinetic parameters. The Michaelian para-

eters k_{cat} and K_m were determined by using the procedure of Eisenthal & Cornish-Bowden (1974). An M_r of 24000 was used for trypsin. All measurements were performed in triplicate.

Determination of *N*-acetylneuraminic acid. The determination of *N*-acetylneuraminic acid was carried out with the thio-barbituric acid method of Warren (1959) after acid hydrolysis.

RESULTS

Fractionation of 12%-trichloroacetic acid-insoluble material containing carbohydrate-free CMP

Fig. 1(a) shows the reverse-phase h.p.l.c. profile of the 12%-trichloroacetic acid-insoluble fraction on Ultrapase column in the trifluoroacetic acid/acetonitrile gradient. About eight major peaks were obtained. Therefore, this material was further purified by ion-exchange chromatography with DEAE-TSK Fractogel-650. The elution pattern is given in Fig. 2. Unabsorbed fraction A on the anion-exchanger corresponded to peaks 5 and 8 in the reverse-phase h.p.l.c. system. Peak 5 was unidentified, whereas the amino acid composition indicated that peak 8 was the 1–23 sequence of α_{s1} -casein, one of components of whole casein.

Fig. 1. Reverse-phase h.p.l.c. elution profile of 12%-trichloroacetic acid-insoluble CMP on a reverse-phase h.p.l.c., Ultrapase column (250 mm × 4.6 mm; 5 µm particle size) (a) before and (b) after purification by chromatography on DEAE-TSK Fractogel-650

Conditions were as follows: column temperature, 50 °C; solvent A, 0.1% trifluoroacetic acid; solvent B, 60% acetonitrile and 40% solvent A; flow rate, 0.8 ml/min.

Fig. 2. Fractionation of 12%-trichloroacetic acid-insoluble CMP on DEAE-TSK Fractogel-650

Conditions were as follows: column (12 cm $\times$ 3 cm) equilibrated with 20 mM-Tris/HCl buffer and eluted with 0–0.2 M-NaCl gradient. Protein applied was 1 g and the flow rate was 1.5 ml/min. Fractions (10 ml) were collected and pooled in A, B, C, D and E as indicated. Effluent was monitored at 214 nm. Retained fractions were read after 1/100 dilution. Numeration of fractions was begun again from the start of gradient. The inset shows *N*-acetylneuraminic acid (NeuAc) content of fractions B, C, D, E and R where reference R is CMP.

Carbohydrate-free CMP, an acidic polypeptide, was eluted in fraction B (gradient from 30 mM- to 52 mM-NaCl) of the major peak retained on the DEAE-TSK Fractogel-650 column. Carbohydrate-free CMP, purified by an additional step on anion-exchanger, was homogeneous as assessed by the reverse-phase h.p.l.c. profile (Fig. 1b), which shows that it corresponded to peak 7 in Fig. 1(a). Its assignment was established by amino acid composition (results not shown) and confirmed by determination of *N*-acetylneuraminic acid, which is a specific indicator for the presence of carbohydrate-containing caseinomacropeptide (DeKoning *et al.*, 1963).

The *N*-acetylneuraminic acid content of retained fractions on the DEAE-TSK Fractogel-650 column is given in Fig. 2 inset. Among them, only fractions B and C do not contain *N*-acetylneuraminic acid groups. Fraction C was carbohydrate-free CMP but was not considered in the pooling of pure component. The amount of *N*-acetylneuraminic acid groups in carbohydrate-containing fractions can be compared with that of CMP. About 70 % of *N*-acetylneuraminic acid groups were recovered. Indeed, this value was underestimated owing to the presence of impurities such as fragments arising from other caseins in the starting material. For 1 g of 12%-trichloroacetic acid precipitate the yield of pure carbohydrate-free CMP was 267 mg.

Identification of tryptic fragments of carbohydrate-free CMP

Fig. 3 shows the *N*-terminal part of CMP according to Mercier *et al.* (1973). Fig. 4 shows the separation of a tryptic digest of carbohydrate-free CMP by reverse-phase h.p.l.c. (Ultrasbase column) with a solvent system containing 0.1% trifluoroacetic acid in water and acetonitrile. For peak assignments, eluted peptides were collected and then subjected to amino acid analysis. The non-retained peak corresponded to trifluoroacetic acid. Peaks a, b, c, d, e and f were well resolved and identified as corresponding to peptides 112–116, 106–112, 106–111, 112–169, 113–169 and 117–169 respectively. Under these experimental conditions peptide 106–116 was not identified, and therefore a synthetic peptide was used to determine its retention time. The downward arrow in Fig. 4 indicates its elution position when it was injected on to the column. Because of their close elution positions, controls were run that ensured that the 106–116

Fig. 3. Amino acid sequence of *N*-terminal part of CMP and tryptic cleavage sites (indicated by arrows)

Fig. 4. Reverse-phase h.p.l.c. profile on Ultrabase column (250 mm × 4.6 mm; 5 μm particle size) of a tryptic digest of carbohydrate-free CMP

The respective carbohydrate-free CMP and trypsin concentrations were 1.35 M and 4.08 units/ml; hydrolysis time was 4 h. Elution conditions were as follows: column temperature, 50 °C; solvent A, 0.1% trifluoroacetic acid; solvent B, 60% acetonitrile and 40% solvent A; flow rate, 0.8 ml/min. Fragments: a, 112–116 peptide; b, 106–112 peptide; c, 106–111 peptide; d, 112–169 peptide; e, 113–169 peptide; f, 117–169 peptide. The first peak is trifluoroacetic acid. The downward arrow indicates the elution position of the fragment 106–116

Fig. 5. Cation-exchange chromatography on Mono S column (HR5/5 Pharmacia) showing the separation of larger fragments resulting from trypsin action on carbohydrate-free CMP

The respective carbohydrate-free CMP and trypsin concentrations were 0.13 mM and 0.5 unit/ml; hydrolysis time was 8 min. Elution conditions were as follows: gradient, 10 mM-KCl/HCl, pH 2, plus 0.5 M-NaCl; flow rate, 1 ml/min. Fragments: A, trifluoroacetic acid + 117-169 peptide; B, 113-169 peptide; C, 112-169 peptide; D, 106-169 peptide as carbohydrate-free CMP.

peptide was not co-eluted with the 106-112 peptide (peak b). Injection of the latter mixed with the synthetic peptide gave two distinct peaks by reverse-phase h.p.l.c. (results not shown). The 106-111 and 106-112 peptides emerged as two peaks that could be only slightly resolved. The observed peak broadening for these peptides was probably a consequence of a slow *cis-trans* conformational equilibrium of a Pro-Pro bond involved in their sequences (Fig. 3), as previously reported by Gesquière *et al.* (1989). However, reverse-phase h.p.l.c. failed to separate the 112-169 and 113-169 peptides and carbohydrate-free CMP when the latter was present. Therefore cation-exchange chromatography was used for the separation on the basis of differences in net positive charge due to different amounts of lysine residues.

Fig. 5 shows the separation of a tryptic hydrolysate of carbohydrate-free CMP by cation-exchange h.p.l.c. (Mono S column) with a 0-0.5 M-NaCl linear gradient in 10 mM-KCl/HCl at pH 2. Four peaks were observed. By amino acid analysis they were respectively assigned to the sequences: A, 117-169; B, 113-169; C, 112-169; D, 106-169. As the 117-169 peptide contained no basic residue, it was not retained by the exchanger. Under the ionic conditions employed (0-0.5 M-NaCl, pH 2), 106-111 and 106-112 sequences were eluted only at the end of the gradient, but were not easily detected because of their low absorbance.

Kinetic study

The time course of the tryptic hydrolysis of carbohydrate-free CMP was studied under mild reaction conditions (substrate concentration 0.13 mM and enzyme/substrate molar ratio 1:1800). Fig. 6 shows the quantitative appearance of larger fragments separated by cation-exchange h.p.l.c. The 112-169

Fig. 6. Time course of the release of larger fragments involved in the first stages of the action of trypsin on carbohydrate-free CMP (○) and fragments 112-169 (△), 113-169 (●) and 117-169 (■)

and 113-169 fragments appeared rapidly in the very early stages of the tryptic digestion of carbohydrate-free CMP. Appearance of the 117-169 peptide was not significant over the 24 min reaction period. The same pattern was obtained by reverse-phase h.p.l.c. for 106-111 and 106-112 fragments (results not shown). At no time was the 106-111 peptide detected.

Rates of appearance of different peptides were determined at substrate concentrations of 0.04, 0.13, 0.19 and 0.37 mM for a constant enzyme concentration (0.07 μ M). The 112-169 and 113-169 peptides were released at equal rates for each substrate concentration used. Therefore cleavage of both these bonds proceeded with similar K_m and k_{cat} . By using the procedure of Eisenthal & Cornish-Bowden (1974), K_m was determined as 0.2 mM and k_{cat} as 1.97 s^{-1} .

Michaelian parameters obtained from depletion of carbohydrate-free CMP were K_m 0.2 mM and k_{cat} 3.95 s^{-1} . After 8 min, the difference between the quantitative evolution of two peptides, 113-169 and 112-169, indicated that the latter was slowly cleaved with a probable release of the 117-169 fragment. This release was associated with the appearance of a 112-116 peptide (results not shown). In contrast, the 113-116 tetrapeptide was not formed under these mild conditions.

DISCUSSION

CMP contains various amounts of carbohydrate attached in the region residues 127-141 in the form of tri- and tetrasaccharide units of *N*-acetylneuraminic acid, galactose and *N*-acetylgalactosamine: NeuAca(2 $\rightarrow$ 3)Gal β (1 $\rightarrow$ 3)GalNAc β 1-Thr/Ser or Gal β (1 $\rightarrow$ 3)[NeuAca2 $\rightarrow$ 6]GalNAc β 1-Thr/Ser or NeuAca(2 $\rightarrow$ 3)Gal β (1 $\rightarrow$ 3)[NeuAca(2 $\rightarrow$ 6)]GalNAc β 1-Thr/Ser (Van Halbeek *et al.*, 1980). About 34-40% of CMP is not glycosylated (Vreeman *et al.*, 1986). It appears from the results that addition of 12% trichloroacetic acid to CMP does not afford complete separation of carbohydrate-free CMP and carbohydrate-containing CMP. Further purification by one chromatography step was necessary for total elimination of glycosylated forms in the trichloroacetic acid-insoluble part containing carbohydrate-free CMP. Indeed, a major difficulty in the preparation of

carbohydrate-free CMP is that the number of glycosylation sites varies between 0 and 5 (Fiat *et al.*, 1988), which may be extended to 10 according to Vreeman *et al.* (1986). Taking this into account, it is unlikely that this differential solubility of CMP in trichloroacetic acid would be great enough for fine-tuned isolations. As a consequence of our results, methods used to follow the action of chymosin on κ -casein, usually based on the release of glycosylated forms of CMP in the 12% trichloroacetic acid-soluble fraction, were obviously not suitable for quantitative determinations. Indeed, separation of carbohydrate-containing CMP molecules that are soluble or insoluble at a given concentration of trichloroacetic acid was not well characterized. The other difficulty encountered was the digestion of whole casein with chymosin, which gave rise not only to CMP but also to fragments resulting, among others, from degradation of α_{s1} -casein. Although the proteolytic coefficient (k_{cat}/K_m) reported for the Phe-105-Met-106 bond of κ -casein was several times higher than that of Phe-23-Phe-24 bond of α_{s1} -casein (Carles & Ribadeau Dumas, 1985), these kinetic parameters established for individual casein were probably different in milk, where whole casein exists in a micellar state (Swaisgood, 1982).

CMP contains three potential specific cleavage sites by trypsin, i.e. Lys-111, Lys-112 and Lys-116 among its 64 amino acid residues. These three residues are located near the *N*-terminal end of CMP (Fig. 3). Complete action of trypsin results in the release of lysine-112 and three end-peptides corresponding to sequences 106–111, 113–116 and 117–169. However, if it is assumed that the three bonds were split at the same rate, five intermediate products could be expected to appear during the primary stage of proteolysis. They are residues 106–112, 106–116, 112–116, 112–169 and 113–169. Of these, sequences 106–112, 106–116 and an end-peptide (113–116) have been reported to have effect on platelet function. Our aim was to investigate under which conditions these potentially anti-thrombotic fragments were released during the time course of tryptic hydrolysis of CMP.

All peptides resulting from hydrolysis have been separated either by reverse-phase h.p.l.c. (Ultrasbase column) or cation-exchange h.p.l.c. (Mono S column) and identified. From the kinetic results, it was clearly shown in Fig. 6 that, among the three trypsin-susceptible bonds, only two of them (111–112 and 112–113) were rapidly cleaved to a significant extent. Moreover, in the range of substrate concentrations used, both were split at similar rates, leading to fragments 106–111, 112–169, 106–112 and 113–169. Use of cation-exchange h.p.l.c. instead of reverse-phase h.p.l.c. led to a better resolution of carbohydrate-free CMP and large tryptic fragments; it was thus possible to obtain quantitative data. Moreover, this procedure can be scaled up. An identical K_m was found for the splitting of Lys-111–Lys-112 and Lys-112–Asn-113 bonds during tryptic hydrolysis of carbohydrate-free CMP. The same K_m value was also obtained in measuring the disappearance of carbohydrate-free CMP, whereas the K_{cat} value (1.97 s^{-1}) found for the splitting of both lysyl bonds was half that of the substrate. Considering the proximity of both bonds, it is likely that the interaction of enzyme with each is mutually exclusive but occurs with equal probability.

Our results do not agree with those obtained by Jollès *et al.* (1986), who indicate that the 106–116 peptide was released with 37% yield during tryptic digestion of CMP. This peptide was never isolated under our conditions. In addition, at the beginning of hydrolysis, the overall yield of the release of two peptides (112–169 and 113–169) amounted to 100% of the conversion of the carbohydrate-free CMP. This was consistent with the delay in the appearance of the 117–169 peptide, complementary to the 106–116 peptide. Such results were incompatible with the formation of the 106–116 peptide under our conditions. The kinetic

study was in direct agreement with the observations made in the absence of the 106–116 peptide when we identified tryptic hydrolysis products of carbohydrate-free CMP. The discrepancy between our results and those of Jollès *et al.* (1986) could be due to their use of CMP instead of the carbohydrate-free CMP as in our study. Nevertheless, this heterogeneity does not concern the first 20 *N*-terminal residues, and even in tryptic digests of CMP we have never isolated the 106–116 peptide, which has the highest biological activity.

The difference in lability of the 111–112 and 112–113 bonds compared with the 116–117 bond can probably be ascribed to differences in the adjacent amino acid residues rather than to differences in accessibility to the enzyme. According to Ono *et al.* (1987), CMP has little organized structure (i.e. α -helix, β -sheet or β -turn). Although the effect of secondary interaction on the enzymic activity of trypsin is very small in comparison with other serine proteinases (Fruton, 1975), it is known that the cleavage by trypsin takes place more slowly when the basic residue is adjacent in sequence to an acidic residue or to cystine (Smyth, 1967). Our results agree with this since there is an aspartic acid residue at position P_1 of the lysine-116 as shown in Fig. 3. For convenience, the amino acid residues in a peptide substrate are numbered P_1 , P_2 etc. to the left of the bond hydrolysed, and P_1' , P_2' etc. to the right.

Further tryptic hydrolysis of carbohydrate-free CMP leads to the splitting of the 116–117 bond. This bond is more easily cleaved in the 112–169 peptide than in the 113–169 peptide (Fig. 6). Such a difference is most probably related to the variation of the net charge induced by the presence or the absence of the *N*-terminal lysine residue and its effect on the binding site of trypsin. It is only through extensive tryptic hydrolysis of CMP that the release of the 113–116 tetrapeptide is observed.

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