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# Effect of Trichloroacetic Acid on the Solubility of Caseinomacropeptide

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## Abstract

**Crude caseinomacropeptide (CMP) was prepared from Na-caseinate using a commercial renneting enzyme. Most of the crude CMP was released from the Na-caseinate by hydrolyzing with the enzyme for 40 min. The hydrolysis of the *k*-casein with carbohydrate was slower than that of the *k*-casein without carbohydrate, as shown by the analyses of the sialic acid content and the tricine-SDS-polyacrylamide gel electrophoresis. The yield of crude CMP from Na-caseinate was 3.7%. Cation exchange chromatography showed that the crude CMP consisted of 40.5% CMP and 59.5% caseinogylcomacropetide (CGP). The effect of the TCA concentration on the solubility of CMP and CGP was determined by using crude CMP. The amounts of crude CMP and sialic acid decreased in the proportion to the increase of trichloroacetic acid (TCA) concentration from 2 to 12%, suggesting that the CGP containing carbohydrate, as well as the CMP having no carbohydrate, was precipitated in a range of 4 to 12%, depending on the TCA concentration. This result supports the hypothesis that the different non-glycosylated and glycosylated forms of CMP have different sensitivities to TCA precipitation.**

**Key words:** crude caseinomacropeptide (CMP), caseinomacropeptide, caseinogylcomacropetide (CGP), solubility, trichloroacetic acid (TCA) precipitation

## INTRODUCTION

Caseinomacropeptide (CMP) is a hydrophilic peptide which is released from bovine *k*-casein by the action of the milk clotting enzyme, chymosin, at the $Phe^{105}$-$Met^{106}$ peptide bond. The CMP represents the C-terminal part of *k*-casein from residue 106 (Met) to the C-terminal 169 (Val). CMP is a heterogeneous peptide having the same peptide chain except variable carbohydrate and phosphorous contents. The CMP without carbohydrate constitutes a large part of the CMP fraction. It is low in sulfur amino acids and devoid of aromatic amino acids such as Phe, Tyr and Trp, His and Arg. The CMP shows no absorption at 280 nm and can be detected at 205~217 nm. Differences in UV absorption at 214 / 280 nm are frequently used to characterize the purity of CMP (1)

It has been reported that CMP has many biological and physiological functions, including being a growth-promoting activity for *Bifidobacteria* (2), a suppression of gastric secretion (3,4), an inhibition of the aggregation of ADP-treated platelets (5,6), an inhibition of oral Actinomyces adhesion to cell membranes (7,8), an inhibition of the binding of Cholera toxin to its receptor (9), an inhibition of influnza virus hemagglutinin (10), an effect of the growth of lactic acid bacteria (11,12) and an inhibition of the mitogen-induced proliferation of mouse spleen lymphocytes (13). Therefore, it has been considered to be an ingredient to neutraceutical and pharmaceutical.

CMP is industrially prepared from cheese whey or whey protein concentrate using ultrafiltration (14,15), alcohol precipitation and large-scale ion exchange chromatography (16). Estimates of CMP have been determined by high performance liquid chromatography (HPLC) or fast protein liquid chromatography (FPLC) on a gel filtration (17,18), an ion exchange (19) and a reversed-phase column (20, 21). These methods rely on pretreatment with trichloroacetic acid (TCA) to remove residual components. However, there have been some doubts about this TCA pretreatment because the different non-glycosylated and glycosylated forms of CMP may have different sensitivities to TCA precipitation, according to the level of glycosylation. The objective of this study is to investigate the effect of TCA on the solubility of CMP.

## MATERIALS AND METHODS

### Materials

Sodium caseinate was purchased from New Zealand Milk Products (Wellington, N.Z.) and rennet powder was from Chr. Hansen Laboratory (Horsholm, Denmark). Calcium chloride, thiobarbituric acid, N-acetylneuraminic acid (sialic acid), N,N-methylene bisacrylamide, N,N,N,N-te-

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tramethylenediamine, ammonium persulfate, sulfosalicylic acid, urea, trichloroacetic acid (TCA) were obtained from Sigma Chemical Co. (St. Louis, USA). Coomassie brilliant blue, bromophenol blue, tricine, sodium dodecyl sulfate (SDS), Tris, and polypeptide SDS-PAGE molecular weight standards from Bio-Rad Co. (Hercules, USA) were used.

**Hydrolysis of Na-caseinate by renneting enzyme**

Fifty milliliters of 2.5% sodium caseinate were dissolved in distilled water containing 0.9 mM $CaCl_2$. Seventy five microliters of rennet solution (1 mg/mL) was added to the caseinate solution which was incubated at 37°C for 30 min. After hydrolysis for a certain period time, the caseinate solution was incubated at 75°C for 30 min to inactivate the renneting enzyme and cooled to 30°C. The solution was lowered to pH of 4.6 with HCL to precipitate proteins other than the crude CMP and centrifuged at 3,000×g for 10 min at 10°C. The supernatant was filtered through Whatman No. 1 filter paper, measured at 217 nm spectrophotometrically, dialyzed against deionized water for one day, freeze dried and stored at -20°C for analysis.

**Preparation of crude CMP by ultrafiltration**

One thousand and five hundred grams of sodium caseinate was dissolved in 60 L of deionized water containing 0.9 mM $CaCL_2$ (2.5% w/v). The caseinate solution was heated to 37°C in a water bath. Ninty milliliters of rennet powder solution at a concentration of 1 mg/mL was added to the sodium caseinate solution and the temperature was maintained at 37°C during hydrolysis. After incubation for 60 min, the solution was heated to 75°C for 30 min to inactivate the rennet and then cooled to 30°C. The solution was lowered to pH of 4.6 with 1 N HCL to precipitate proteins other than CMP, and then centrifuged at the rate of 3,000×g for 10 min at 10°C to remove the precipitated proteins. The supernatant was filtered through Durapore 0.45 micron PVDF, and concentrated and diafiltrated through PLBC 3K regenerated cellulose in Pellicon ultrafiltration system (Millipore Co., Bedford, USA) (15). The diafiltrated solution was freeze-dried and stored at -20°C for analysis.

**Tricine-SDS-polyacryamide gel electrophoresis**

The protein samples were incubated for 30 min at 40°C in 4% SDS, 12% glycerol (W/V), 50 mM Tris, 2% mercaptoethanol (V/V), and 0.01% Coomassie brilliliant blue G-250 adjusted with HCl to pH of 6.8. Ten microliters of a protein sample was laid under the cathode buffer using a microliter syringe. Discontinuous gel electrophoresis, consisting of 4% stacking gel, 10% spacer gel and 16.5% separating gel was used (22). A 0.2 M Tris (pH 6.8) buffer and 0.1 M Tris / 0.1 M Tricine buffer (pH 8.25) were used as an anode and cathode buffer, respectively. An Electrophoresis was run using a minigel electrophoresis system (Hoefer Scientific Instruments, San Francisco, USA) for 2 hours and 30 min at 100 V and 30 mA. Gel was fixed in 50% methanol and 10% acetic acid for 30 min, stained in 0.03% Coomassie brilliliant blue G-250 in 10% acetic acid for one hour and destained in 10% acetic acid.

**Ion exchange chromatography**

A Q-Sepharose fast flow (Pharmacia Biotech Co., Uppsala, Sweden) was packed in a Spectra / Chrom LC column (2.5×35 cm) and installed in a ProteamTM LC System 210 (Isco Co., Lincoln, USA) composed of a Foxy Jr. fraction collector, a Tris pump, a UA-6 detector-optical unit, and a model 160 gradient former. Eight milliliters of crude CMP solution containing 0.1 g protein was loaded into a Q-Sepharose fast flow which was equilibrated with 0.01 M Tris-HCl buffer, pH of 7.5. The CMP was eluted with a gradient of NaCl from 0.0 to 1.0 M at the flow rate of 1 mL/min and collected in 7 mL quantities. The absorbance of each fraction was measured at 214 using a UA-160A spectrophotometer (Shimadzu Co., Kyoto, Japan).

**Solubilization of crude CMP in different TCA concentrations**

A 0.2 gram of crude CMP was dissolved in 200 mL of a 2, 4, 5, 8 and 12% TCA solution by stirring with a magnetic bar for one hour. The crude CMP solution was centrifuged at 15,000×g for 60 min at 10°C to remove the precipitated proteins. The supernatant was filtered through Whatman No. 1, dialyzed against deionized water and freeze-dried for storage. The weight of sialic acid of freeze-dried supernatant was analyzed.

**Measurement of sialic acid**

The content of sialic acid was determined by the colorimetric periodic acid-thiobarbituric assay which is specific for sialic acid (23). Free sialic acid was released from the sample by the treatment with 0.1 N $H_2SO_4$ at 80°C for 45 min. The chromophore formed was extracted using cyclohexane and measured in triplicate at 549 nm by a UA-160A spectrophotometer using sialic acid as a standard.

## RESULTS AND DISCUSSION

**Production of crude CMP from Na-caseinate**

*k*-Casein exists as two different types, which are, a glycosylated and a non-glycosylated type, according to the presence of a carbohydrate moiety (1). When *k*-casein is hydrolyzed by the renneting enzyme, CMP without carbohydrate (CMP) and CMP with carbohydrate (caseinoglycomacroprptide, CGP) are released into the whey. In

this paper to avoid confusion arising from disagreement over the terminology definitions in literature, the following terminology has been used: CMP and CGP indicate a CMP without carbohydrate and a CMP with carbohydrate, respectively. Therefore, the total CMP is the sum of the CMP and the CGP. The crude CMP contains the total CMP and other contaminating proteins.

Crude CMP consisting of pure CMP and CGP as major components was produced from Na-caseinate using a commercial renneting enzyme. During the rennet hydrolysis of Na-caseinate by the renneting enzyme, the time-dependent release of crude CMP was characterized by measuring its absorbance at 217 nm and the amount of crude CMP. According to Fig. 1 and 2, most of the crude CMP was released from Na-caseinate by the renneting enzyme which hydrolyzes the $Phe^{105}$-$Met^{106}$ peptide bond of *k*-casein into para *k*-casein and caseinomacropeptide (CMP) in 40 min. This result is in good agreement with that of Lieske and Konrad (24) who used a spectrophotometric approach at 217 nm for studying the enzymatic release of CMP and CGP from whole casein. The analyses of the sialic acid content and the tricine-SDS-polyacrylamide gel electrophoresis were done to monitor the enzymatic release of CMP with carbohydrate (CGP) from whole casein (Fig 3 and 4). Fig. 3 showed a slightly different hydrolysis pattern from Fig. 1 and 2, indicating that there might be a slight difference in the sensitivity of the $Phe^{105}$-$Met^{106}$ peptide bond between *k*-casein without carbohydrate and *k*-casein with carbohydrate. During the initial stage of hydrolysis, the enzymatic release of CGP was slower than that of CMP, due to the late breakdown of the $Phe^{105}$-$Met^{106}$ peptide bond of the *k*-casein with carbohydrate the by renneting enzyme. The later hydrolysis of the renneting enzyme on the *k*-casein with carbohydrate than on the *k*-casein without carbohydrate, may be explained by the unfavorable accessibility of the $Phe^{105}$-$Met^{106}$ peptide bond to the renneting enzyme due to the structural arrangement resulting from the existence of the hydrophilic carbohydrate moiety in the *k*-molecule.

The yield of crude CMP from Na-caseinate by ultra-

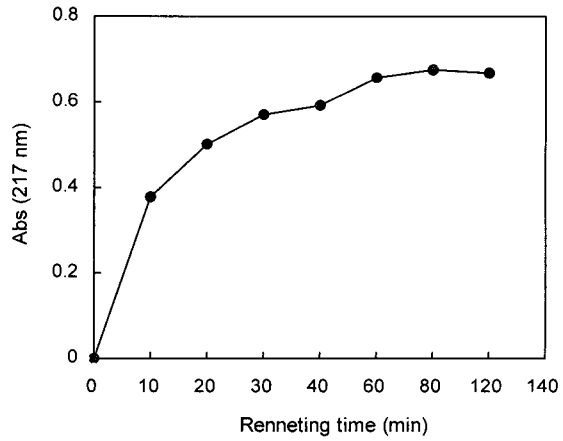


**Fig. 1.** Time-dependent release of crude CMP during renneting of sodium caseinate solution.

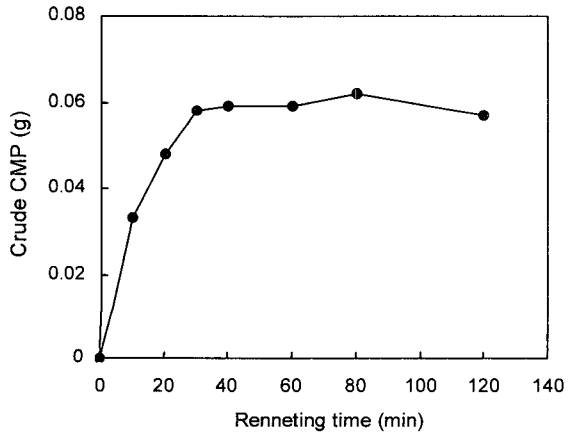


**Fig. 2.** Changes of crude CMP with renneting time.

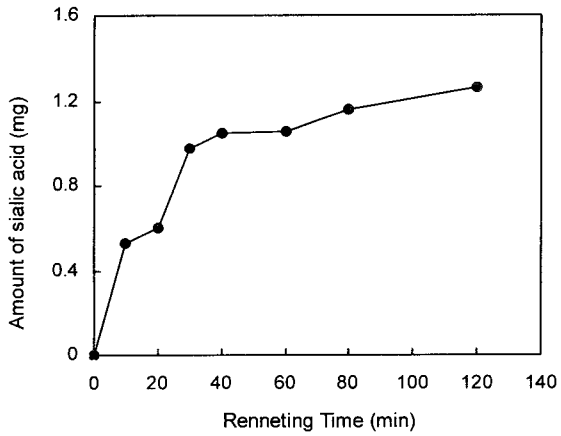


**Fig. 3.** Changes of sialic acid with renneting time.

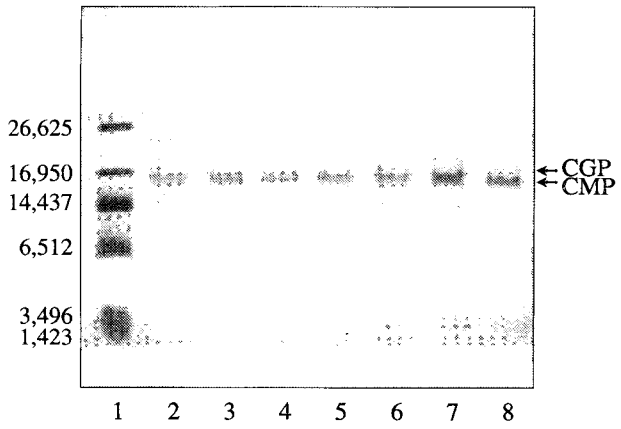


**Fig. 4.** Electrophoretic diagram of crude CMP with renneting time. Lane 1, Molecular weight standard; Lane 2, 10 min; Lane 3, 20 min; Lane 4, 30 min; Lane 5, 40 min; Lane 6, 60 min; Lane 7, 80 min; Lane 8, 120 min.

filtration was 3.7%. the CMP and CGP of crude CMP prepared in this study seem to exist as dimers, considering that their bands appeared at a position close to 17,000 Da (Fig. 4) and that the molecular weight of CMP without carbohydrate is 6,754 Da (24). The crude CMP consisted of 40.5% CMP and 59.5% CGP, as determined by cation exchange chromatography (Fig. 5 and 6).

**Effect of TCA concentration on the solubility of CMP and CGP**

TCA pretreatment has been frequently used to determine the amount of or to isolate CMP and CGP from various sources. According to this approach, CMP which is soluble in 2% TCA reflects the total CMP content, whereas CMP which is soluble in 12% TCA contains the whole carbohydrate of *k*-casein and is called CGP. The difference between total CMP and CGP is the CMP (1). In other words, CMP is soluble in 2% TCA but insoluble in 12% TCA, whereas CGP is soluble in 12% TCA, as well as 2% TCA. However, there are some questions about the sensitivity of non-glycosylated and glycosylated forms of CMP to TCA precipitation (1,9,19).

The effect of TCA concentration on the solubility of CMP was studied using crude CMP prepared by ultrafiltration. The amount and sialic acid content of the CMP

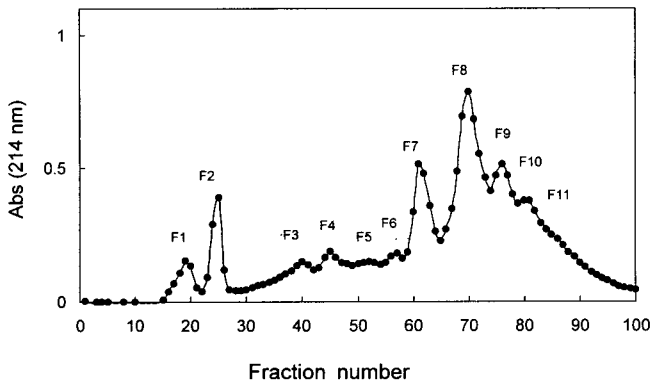


**Fig. 5.** Chromatogram of crude CMP on Q-Sepharose fast flow.

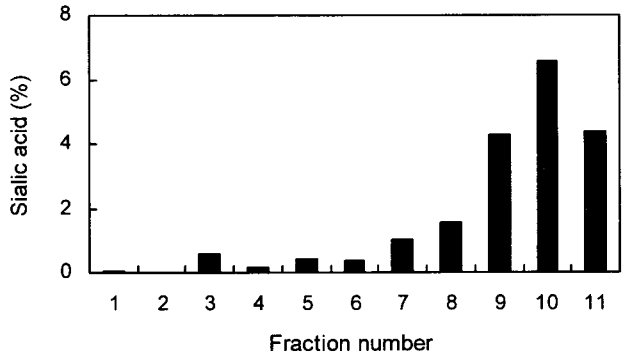


**Fig. 6.** Amounts of sialic acid in fractions isolated by Q-Sepharose fast flow. CMP was the sum of F1, F2, F3, F4, F5 and F6. and CGP was the sum of F7, F8, F9, F10 and F11.

which was soluble in different TCA concentrations were estimated (Fig. 7). The protein components of CMP which were soluble in different TCA concentrations are shown on the tricine-SDS-polyacrylamide gel electrophoretic diagram (Fig. 8). The amounts of crude CMP and sialic acid decreased in the proportion to the increase of TCA concentration from 2 to 12%, suggesting that CGP containing carbohydrate as well as CMP without carbohydrate was precipitated in a range of 4 to 12%, depending on the TCA concentration. The 12% TCA-soluble CMP contains only CGP having the carbohydrate moiety.

This result is consistent with the findings of other researchers that different non-glycosylated and glycosylated forms of CMP, have different sensitivities to TCA precipitations (9,19). Because a certain amount of CMP with carbohydrate (CGP) could be precipitated in TCA concentration from 4% to 12%, CGP obtained with 12% TCA

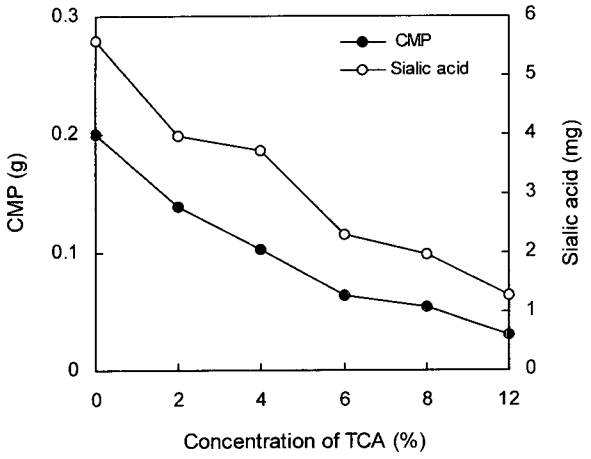


**Fig. 7.** Changes in the amount of CMP and sialic acid with changes in TCA concentration.

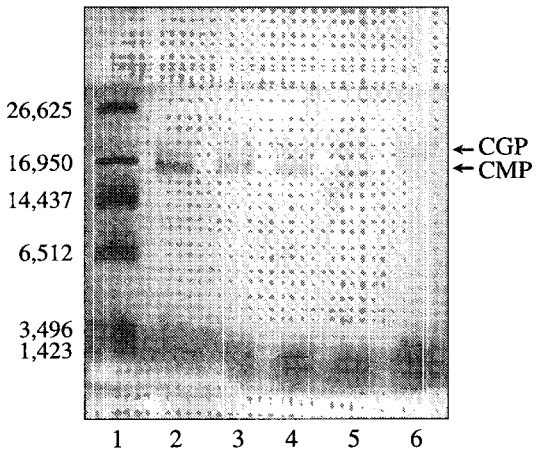


**Fig. 8.** Electrophoretic diagram of soluble CMP and CGP in different TCA concentrations. Lane 1, Molecular weight standard; Lane 2, 2% TCA; Lane 3, 4% TCA; Lane 4, 6% TCA; Lane 5, 8% TCA; Lane 6, 12% TCA.

precipitation might underestimate the actual amount of CGP having a carbohydrate moiety, even if it did not contain CMP without carbohydrate. Consequently, the experimental conditions used for the determination and separation of CMP or CGP by TCA precipitation must be taken into consideration if one is to precisely analyze the data available in the literature.

## ACKNOWLEDGEMENTS

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