Detection of the Cholera Toxin-binding Activity of $\kappa$-Casein Macropptide and Optimization of Its Production by the Response Surface Methodology

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The cholera toxin (CT)-binding activity of purified $\kappa$-casein macropptide (CMP) from bovine $\kappa$-casein was detected. In addition, a statistical model was developed to optimize the production of CMP. CMP was prepared by chymosin hydrolysis of $\kappa$-casein and a subsequent 3% trichloroacetic acid treatment. CMP was further fractionated in an ion-exchange column by FPLC. CT binding activity was eluted at 0.18 M NaCl and was a single 8.9 kDa peptide without tryptosine and arginine residues. The CT binding activity was rapidly lost by a carbohydrate treatment. The conditions for CMP production with chymosin were optimized by using the response surface methodology (RSM). The estimated optimum levels of the factors were as follows: reaction temperature, 38.5°C; pH, 6.44; and time, 35.9 min. A validation experiment was performed in which CMP was prepared under the predicted parameters, and it was ascertained that the estimated optimum conditions gave better production of CMP than any other conditions.

Key words: $\kappa$-casein macropptide; cholera toxin; response surface methodology

$\kappa$-Casein is a unique milk protein that contains such carbohydrates as galactose, N-acetylgalactosamine (GalNAc), and $N$-acetylneuraminic acid (NeuAc). $\kappa$-Casein is discretely heterogeneous in that all of the components are closely related and are efficient micelle stabilizers. $\kappa$-Casein has approximately five carbohydrate binding sites in its C-terminus area and has been separated in to several fractions depending on the type and content of carbohydrates. Chymosin cleaves the peptide bond between the methionine (105) and phenylalanine (106) residues in $\kappa$-casein, resulting in the separation of para-$\kappa$-casein (1-105 residues) and macropetide (106-169 residues). $\kappa$-Casein macropetide (CMP) consist of two parts, a carbohydrate-containing macropetide and a carbohydrate-free macropetide. For this reason, CMP is referred to as a glycomacropetide due to its carbohydrate content. The macropetides containing carbohydrate moieties have the same peptide chain, but variable carbohydrate and phosphorus contents. Several biological and physiological functions have been reported for $\kappa$-casein and its hydrolysate: opioid effects, a calcium absorption promoting effect, immunoinactivating effect, inhibition of the angiotensin-converting enzyme, and bifidus factors.

*Vibrio cholerae* excretes a potent enterotoxin (cholera toxin, CT) that is responsible for profuse watery diarrhea commonly associated with this intoxication. CT is composed of one A subunit that is surrounded by five B subunits with a molecular weight of 11 kDa per subunit. The B subunits are responsible for binding the toxin molecule to ganglioside $G_{M1}$ which is present on mucosal enterocytes. Since CMP has a similar carbohydrate structure to that of $G_{M1}$, it has been reported to exhibit the ability to bind CT and inhibit the toxin. Kawasaki et al. have shown that macropetides from cheese whey inhibited the binding of CT to gangliosides. However, no report has been published on which of the fractions among CMP bind to CT.

In order to produce the biologically active peptides by enzymatic hydrolysis on an industrial scale, it is necessary to optimize the conditions. Improved reaction conditions for enzymatic hydrolysis can be identified by a multi-variate analysis in which the combined effects of various factors are considered as simultaneous responses. The factors affecting the enzymatic activity are the concentration of the enzyme, substrate, pH, temperature, and the presence of inhibitors or promoters. Foltman has reported that the amount of CMP released by the action of
chymosin was affected by the ionic strength, pH and temperature.
This study evaluates the inhibitory activity of CMP on the binding of the cholera toxin in vitro, and the conditions that would maximize the production of CMP.

Materials and Methods

Preparation of \( \kappa \)-casein. Acid casein was first prepared by adjusting the pH value of fresh raw skim milk to 4.6 with 1 N HCl and centrifuging at 10,000×g for 20 min. A gel permeation column (Superose 6HR 10/30; Pharmacia, Sweden) was equilibrated with a 5 mM tris-6 M urea buffer (pH 8.0) at a flow rate of 0.3 ml/min, and the retention time of a \( \kappa \)-casein standard (Sigma, U.S.A.) was measured. The acid casein was then applied to the column, and \( \kappa \)-casein was collected at its retention time.

Chymosin reaction. One hundred mg of purified \( \kappa \)-casein was dissolved in 15 ml of a 50 mM sodium citrate buffer (pH 6.6). The \( \kappa \)-casein solution was digested with chymosin (20 units; Sigma, U.S.A.) for 15 min at 35°C. To terminate the reaction, the mixture was cooled to 4°C, and a 12% TCA solution was added to give a final concentration of 3%. The mixture was then centrifuged, and the supernatant was assayed by the TNBS (2,4,6-trinitrobenzensulphonic acid) procedure according to Samples et al.\(^{11}\)

Detection of the cholera toxin-binding activity. The \( \kappa \)-casein macropeptide, which was fractionated as illustrated in Fig. 1,\(^{10}\) was dialyzed against water (MW cut-off of 1 kDa) at 4°C and then lyophilized. One \( \mu \)g of the sample was resuspended in 100 \( \mu \)l of 50 mM phosphate-buffered saline (PBS, pH 7.0) and then loaded in to a 96-well microtiter plate, which was kept overnight at 40°C. Horseradish peroxidase-conjugated cholera toxin B subunit (Sigma, U.S.A.) was diluted from 1:100 to 1:12,800 with the PBS buffer, and 100 \( \mu \)l of the dilution was added to each well, before incubating at 37°C for 2 h. After the incubation, each plate was washed with PBS, and 100 \( \mu \)l of the substrate solution (o-phenylenediamine dihydrochloride, 0.02% \( \text{H}_2\text{O}_2 \)) was added per well. After 15 min, the reaction was stopped with 0.5 M \( \text{H}_2\text{SO}_4 \) (50 \( \mu \)l per well). The absorbance was measured at 490 nm with an ELISA reader (Biotek L311, Pharmacia Co., U.S.A.).

Tricine-SDS-PAGE and amino acid analysis. The molecular weight of the active fraction was estimated by tricine-SDS-PAGE (pH 8.0).\(^{13}\) The electrophoresis was performed at 125 V (constant) for 1 h, and the gel was stained with Coomassie brilliant blue R250 (Bio-Rad, U.S.A.) and destained with an aqueous solution of 40% (v/v) methanol and 10% (v/v) acetic acid. The molecular weight of the pro-
tein was determined by comparing its relative mobility to that of a molecular standard (Bio-Rad, U.S.A). To analyze the amino acid contents, the active fraction was hydrolyzed with 6 N HCl at 100°C for 24 hrs, the resulting hydrolysate then being analyzed with an amino acid analyzer (LKB-4150, England).

**Optimization of CMP production.** The effects of the reaction temperature, pH, and time on the production of CMP were investigated by using the response surface methodology (RSM).<sup>7</sup> A central composite design in two blocks was used to assign treatments in this experiment that was conducted for a period of two days. The first block, implying the first day of the experiment, contained the 2<sup>3</sup> factorial runs and four center runs. The second block contained the second day of the experiment, 6 axial runs with α = 1.682 and four center runs (Table 1). A polynomial second-order model was adapted for estimating the production of CMP in terms of the values for temperature, pH, and time that correspond to that point.

**Statistical analysis.** A statistical analysis was carried out by using procedures of SAS-STAT,<sup>8,9</sup> and three-dimensional plots of the predicted values of the response variables were generated by SAS-GRAPH.<sup>8,10</sup>

### Results and Discussion

**Cholera toxin-binding activity**

The κ-casein macropeptide (CMP) fractions were assayed for their binding activity to the cholera toxin B-subunit. As shown in Fig. 2, one fraction (peak III) showed a linear relationship with the concentration of the cholera toxin, whereas the other fractions revealed non-specific binding characteristics, except for peak II which showed much lower activity only at higher concentrations. The active fraction accounted for 10.2% of total protein in the macropeptide, whereas the major fractions without any activity accounted for 46.7%.

To further characterize the active fraction, the fraction was treated with N-acetylneuraminidase and β-galactosidase. Figure 3 shows reduced CT-binding activity with the enzyme treatments, indicating the involvement of carbohydrates in the binding activity of CMP. In particular, NeuAc appeared to be most important for the activity. Kawasaki et al.<sup>7</sup> have reported that the glycomacropeptide inhibited the binding of CT to animal cells, and that the sialic acid moiety in CMP was responsible, at least in part, for the inhibitory activity. Shida et al.<sup>11</sup> have also shown the involvement of a carbohydrate moiety in the CT binding activity of the glycopeptide. CT binds to glycolipid G<sub>3m</sub> on the surface of eucaryotic cells. The affinity between G<sub>3m</sub> and the B-subunit of the cholera toxin is driven by the hydrogen bond.
interaction with terminal galactose and NeuAc.\(^6\) Idota et al.\(^{10}\) have reported that neuraminylactose in human and bovine milk bound to CT because of the structural similarity between \(\text{G}_{\text{M}2}\) and neuraminylactose. However, this report showed that neither lactose nor NeuAc bound individually to CT, which suggests the structural incorporation of the carbohydrate moiety in order to be active.

**Molecular mass and amino acid composition**

The CMP fraction showing CT-binding activity yielded a single band by tricine-SDS-PAGE with a molecular mass of ca. 8.9 kDa, which is slightly higher than the theoretical value of 8 kDa (Fig. 4).

The bulky nature of the carbohydrate moiety associated with CMP may be associated with the size discrepancy of CMP. Morr and Seo\(^{73}\) and Sharma et al.\(^{18}\) have all found that the estimated molecular weight of CMP by gel filtration was higher than the theoretical value for CMP. Another report has also shown some discrepancies in the molecular weight estimation of CMP, the apparent molecular weight at pH 7.0 ranging from 20 to 50 kDa, but at pH 3.5, it ranged from 10 to 30 kDa.\(^{19}\)

Table 2 shows an amino acid analysis of the CT-binding fraction. Compared to the primary structure of \(\kappa\)-casein B,\(^{20}\) the result shows certain similarities and some differences. The fraction did not contain...
tyrosine, tryptophan, and arginine residues. However, the contents of leucine and phenylalanine residues from the active fraction were higher, whereas the content of the hydrophobic isoleucine residue was lower than that reported for the complete structure of $\kappa$-casein. Interestingly, cysteine and phenylalanine residues were found in the CMP fraction, which disagrees with the reported $\kappa$-casein structure. Tanimoto et al.\textsuperscript{23} have shown that differences existed in the contents of amino acid residues between the CMP fraction obtained by purification with FPLC and those of the reported $\kappa$-casein structure. They also showed the presence of phenylalanine in the CMP fraction. The importance of certain amino acid residues in the CT binding activity has yet to be elucidated.

**Optimization of CMP production**

RSM was used to optimize the conditions for CMP production by the enzyme treatment from $\kappa$-casein. Table 3 shows that the second-order model fitted for the production of CMP was highly significant ($P = 0.0001$), while the result of the lack-of-fit test measured the failure of the model to represent data ($P = 0.041$). Significant lack of fit may indicate the inadequacy of the regression equation, and this test may not have been appropriate in the present study.\textsuperscript{22}

Therefore, high-order terms were used to develop a better model. Since each factor has five levels, up to quartic terms could be included in our model. The best-fit model is represented in Table 4. This model allowed a better reconstruction of the experimental data: it has a larger $R^2$ value (0.987 > 0.944), smaller coefficient of variation (11.42 < 5.491), the lack of fit being insignificant ($P = 0.9531$), and all variables being significant at the 5% or lower level. The data calculated by the better model were found to be 38.5°C, pH 6.44, and 35.9 min.

The partial effect plot for the production of CMP is shown in Fig. 5. CMP production was affected by all the variables. Above all, the reaction time was found to be the most influential factor for macro-peptide production over the pH and temperature. A three-dimensional plot of CMP production according to the effect of the various factors is shown in Figs. 6, 7 and 8. The response surface plot for CMP production (Fig. 6) indicates that, at pH 6.44, as the reaction time was increased from 8 to 36 minutes, CMP production increased, but that it tended to decrease above 36 minutes. Relationships were compared between the CMP production, reaction time and temperature. CMP production increased with increasing time from 4 to 36 min, and the pH had less of an influence on CMP production than the reaction time, although it was the maximum at a reaction temperature of 38.5°C (Fig. 7). CMP production increased as the reaction temperature in-

### Table 3. Analysis of Variance for Evaluating the Second-order Model

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F-value</th>
<th>P-value</th>
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<td>Total</td>
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$R^2 = 0.944$, coefficient of variation $= 11.42\%$.

### Table 4. Analysis of Variance and Coefficient Estimates in the Regression Model Selected through Variable Selection

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<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
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<th>F-value</th>
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<td>Total</td>
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<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient estimate</th>
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$R^2 = 0.987$, coefficient of variation $= 5.491\%$. 

![Fig. 5. Partial-effect Plot of Temperature (●), pH (○) and Time (△) on CMP Production.](image-url)
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Fig. 6. Response Surface for the Effects of Reaction Time and pH on the Production of CMP at a Reaction Temperature of 38.5°C.

Fig. 7. Response Surface for the Effects of Reaction Time and Temperature on the Production of CMP at pH 6.44.

Fig. 8. Response Surface for the Effects of Reaction Temperature and pH on the Production of CMP at a Reaction time of 35.9 min.

Fig. 9. Release of CMP by Chymosin under Three Different Conditions. Optimum conditions from the improved RSM model (●), second-order model (●), and center point (▲).

creased to 38.5°C and up to a pH value of 6.44. However, production tended to decrease at higher pH values and at temperatures above 38.5°C (Fig. 8).

A validation experiment was carried out to confirm the optimum conditions for the factors established in this study, three different conditions being compared. The highest CMP production was achieved at the optimum conditions from the improved RSM model, while the center point and optimal conditions obtained by conventional RSM resulted in lower production of CMP (Fig. 9).

References


