Expression and Bioactivity Analysis of Recombinant Beta-CPP Dimer

Y. J. Jiang, Q. Z. Li, H. B. Yan, and L. J. Geng

Life Science and Biotechnology Research Center,
Northeast Agricultural University,
Harbin, Heilongjiang, 150030 China

ABSTRACT

Beta-casein phosphopeptide (beta-CPP) is a bioactive peptide that carries different minerals, especially calcium. To investigate more effects of beta-CPP, eukaryotic expression vector of beta-CPP dimer was constructed and transfected into Chinese hamster ovary (CHO) cells. After selection, the cell lines stably expressing beta-CPP dimer were obtained, and the recombinant product was identified and purified. Activity assay of recombinant protein indicated that the recombinant beta-CPP dimer could improve Ca^{2+} uptake of sperm, stimulate the proliferation of spleen cells, and induce apoptosis of some malignant tumor cells.

(**Key words:** beta-casein phosphopeptide, dimer, eukaryotic expression, bioactivity)

Abbreviation key: $[\text{Ca}^{2+}]_i$ = intracellular free Ca^{2+} concentration, **CHO** = Chinese hamster ovary, **CNBr** = cyanogen bromide, **Con A** = concanavalin A, **CPP** = casein phosphopeptide, **FBS** = fetal bovine serum.

INTRODUCTION

Bioactive peptides derived from milk directly influence numerous biological processes evoking antimicrobial (Zucht et al., 1995; Lahov and Regelson, 1996; Kuwata et al., 1998), antihypertensive (Karaki et al., 1990; Abubakar et al., 1998; Anne et al., 2000), antithrombotic (Beatrice et al., 1995; Bal et al., 1996), immunological (Siemion et al., 1995; Lahov and Regelson, 1996; Harsharnjit et al., 2000), neurological (Kampa et al., 1996; Jinsmaa and Yoshikawa 1999; Chaud et al., 2002; Teschemacher, 2003), and ion-binding (Sato et al., 1986; Park et al., 1998; Matsui et al., 2002) response. Herein, bioactive peptides derived from milk may function as health care products, providing therapeutic value for either treatment or prevention of disease. Especially,

casein phosphopeptides (**CPP**) are currently used both as dietary supplements and as pharmaceutical reagents (Clare and Swaisgood, 2000; Meisel and FitzGerald, 2003; Otani et al., 2003).

Most CPP contain the same pattern: 3 serine phosphate clusters followed by 2 glutamic acid residues [Ser(P)-Ser(P)-Ser(P)-Glu-Glu], functioning as carriers for different ions, especially calcium (David and Yuan, 1992; Gerd et al., 2000). Among the CPP, beta-CPP has received the most attention. The secondary structure of beta-CPP was examined through X-ray, Fourier transform infrared spectroscopy, and circular dichroism spectroscopy. All of these methods predict a common structure for this peptide, which includes polyproline II elements as well as beta-extended sheet and turn-like elements. This structure makes beta-CPP highly stable from 5 to 70°C (Cross et al., 2001, Farrell et al., 2002). For the remainder of phospho-serine, strong intermolecular and intramolecular interactions may give rise to a stable secondary and quaternary structure of closely packed polypeptide chains. Because of its structure, beta-CPP is able to block the growth process of dicalcium phosphate at the earliest stages (Holt et al., 1996, 1998). Because beta-CPP can be used to enhance the absorption of calcium and prevent the risk of pathological calcification, it has very extensive pharmacological effects.

However, currently most CPP were obtained by proteinase hydrolysis (Adamson and Reynolds, 1995; Park et al., 1998). There was no information about CPP obtained through DNA recombination technology. Furthermore, most CPP derived from casein hydrolysate are a mixture of various phosphopeptides in length, and this mixture can also limit pharmacological application. Thereby, DNA recombination technology is a valuable method to obtain pure CPP. However, the work to express short peptide, such as CPP, by DNA recombination technology has certain difficulties. In the study, our aims were 1) to express beta-CPP dimer in Chinese hamster ovary (**CHO**) cells through DNA recombinant technology and 2) to study the effects of this recombinant protein on Ca^{2+} uptake, proliferation, and apoptosis to different cell lines. Finally, through this method, the cell lines stably expressing recombinant

Received January 20, 2004.

Accepted March 19, 2004.

Corresponding author: Q. Z. Li; e-mail: qingzhangli@hotmail.com.

P1 =

Materials

The male rabbit and BALB/c mice were purchased from Harbin Veterinary Research Institute (Harbin, China).

To get the gene of beta-CPP dimer, 2 nucleotide acid sequences were designed. The 2 nucleotide acid sequences were partially complementary in their 3' terminals. The sequences included sites of restriction endonucleases for *Kpn* I, *Xba* I, *Nhe* I, and *Xho* I (Sambrook and Russell, 2001). At the same time, some ATG (adenine-thymine-guanine) were designed in 2 nucleotide acid sequences, which would be translated into Met that could be cleaved by cyanogen bromide (**CNBr**) so that the monomer of beta-CPP would be obtained when necessary. The 2 nucleotide acid sequences were

and $P2 =$

The pcDNA 3.1/His A vector and recombinant plasmid T-beta-CPP₂ were cleaved by *Kpn* I and *Xho* I, respectively, then the recovered vector and fragment, including gene of beta-CPP dimer, were ligated and

transformed into *E. coli* JM109. After identification by cleaving and sequencing, we obtained the recombinant eukaryotic expression vector named beta-CPP₂-His A. Strategy of constructing the beta-CPP₂-His A recombinant expression vector is illustrated in Figure 1.

Establishment of Stable Cell Lines Expressing Beta-CPP Dimer

The CHO cells were maintained at 37°C with 5% CO₂ in a 24-well cell culture plate (Corning Inc.) with DMEM/F12 medium (supplemented with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL streptomycin). One day before transfection, cells were plated into growth medium (without antibiotics). When the cells were 90% confluent, the DNA-lipofectamine complexes (with 0.8 µg of DNA in 50 µL of DMEM and 2.0 µL of lipofectamine in 50 µL of DMEM) were added into the well, and the cells were incubated at 37°C in 5% CO₂ for 24 h. Then, the cells were passaged at a 1:10 dilution into fresh growth medium. The transfected cells were selected by G418 during the following days. The pcDNA 3.1/His A vector without gene of beta-CPP dimer is the control of transfection and selection.

When the cell clone formed, the clone was expanded, and the total RNA of the cells was extracted by TRIzol reagent. Then, reverse transcription was performed to synthesize first-strand cDNA with the total RNA as template and P2 (synthesized nucleotide acid sequence) as gene-specific primer. The PCR extension reactions were carried out for 3 cycles at 94°C for 0.5 min, 42°C for 0.5 min, and 72°C for 1 min and then another 30 cycles at 94°C for 0.5 min, 60°C for 0.5 min, and 72°C for 1 min. The products of reverse transcription PCR were analyzed by electrophoresis on 1.5% agarose gel.

Recombinant Protein Purification and SDS-PAGE Analysis

The stable, transfected CHO cells lines were expanded and harvested by trypsinization. The cells were resuspended in native binding buffer (50 mmol/L NaPO₄, 0.5 mol/L NaCl, 10 mmol/L imidazole; pH 8.0) with addition of leupeptin to inhibit the protease. Then, the cells were lysed by 2 freeze-thaw cycles using liquid nitrogen and 42°C water bath. The lysate was centrifuged at 3000 × *g* for 15 min (Avanti 30 centrifuge; Beckman) to pellet the cellular debris, and the supernatant was collected. The supernatant was added into a prepared purification column with Ni²⁺ ion resin and native binding buffer. To keep the resin suspended in the lysate solution, the mixture was incubated for 1 h to bind by gentle agitation. Following these steps, the purification column was centrifuged at a low speed (800

× *g*), and the supernatant was aspirated carefully. Then, the column was washed with native wash buffer (50 mmol/L NaPO₄, 0.5 mol/L NaCl, 20 mmol/L imidazole; pH 6.0), and the resin was settled by low speed centrifugation (800 × *g*). This step was repeated 3 times. Finally, the recombinant protein was eluted with native elution buffer (45 mmol/L NaPO₄, 0.5 mol/L NaCl, 250 mmol/L imidazole; pH 6.0). The eluted fractions were collected and analyzed with SDS-PAGE.

The SDS-PAGE analysis was performed according to the protocol described by Sambrook and Russell (2001). The concentrations of separating gel and stacking gel were 15 and 5%, respectively. After 1 h of electrophoresis at a voltage of 80 V to stacking gel and 2.5 h of electrophoresis at a voltage of 140 V to separating gel (PowerPac 300, Bio-RAD), the gel was stained with staining solution (1 g of Coomassie Brilliant Blue R-250 was dissolved in a 1-L mixture of ethanol:glacial acetic acid:distilled water = 9:1:9) for 4 h and then decolorized with decoloring solution (methanol:glacial acetic acid:distilled water = 1:1:8) until the background was clear.

Western Blotting Detection

Samples were subjected to SDS-PAGE analysis as described previously, and the gel was then transferred to a nitrocellulose membrane at a constant current of 100 mA for 1 h (Trans-Blot Semi-Dry Electrophoretic Transfer Cell, Bio-Rad). After washing and blocking, the membrane was incubated for 1 h with horseradish peroxidase-labeled Anti-His antibody (Anti-His antibody can tightly bind to the 6 × His tagged protein, thus giving it a sensitive detection), followed by washing, staining, and photographing (white/ultraviolet transilluminator; UVP Inc.).

Dephosphorylation of Part of Recombinant Protein

Part of the purified recombinant protein was dephosphorylated by digestion of acid phosphatase at 30°C for 10 min in the digestion buffer (40 mmol/L piperazine bisethanesulfonic acid, 1 mmol/L dithiothreitol, 20 µg/mL aprotinin, 20 µmol/L leupeptin; pH 6.0) (Ausubel et al., 1995). The final concentration of acid phosphatase was 100 µg/mL, and the digestion was terminated by sodium phosphate (100 mmol/L).

Measurement of the Intracellular Free Ca²⁺ Concentration in Rabbit Sperm Treated by Recombinant Protein

The rabbit sperm cells were collected and prepared by routine method. The sperm cells with >90% viability

Figure 1. Strategy for constructing the beta-CPP₂-His A recombinant expression vector. CPP = Casein phosphopeptide.

assessed by the trypan blue dye exclusion test were used for analyses. Then, the sperm cells were incubated with CPP, recombinant beta-CPP dimer, dephosphorylated beta-CPP dimer, and Hank's buffer in the dark for 1 h at 38°C, respectively. The final concentrations of all the 3 kinds of test substances were 1 µg/mL.

The intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of rabbit sperm was measured with a fluorescent Fura2/AM (Mori et al., 1996). Briefly, the cells were resuspended in Hank's buffer containing 1 mmol/L Fura2/AM to a final concentration of 2×10^6 cells/mL and then incubated at 38°C for 1 h. After the loading of Fura2/AM, 3 mL of a cell sample was transferred to a quartz cuvette, and fluorescence was measured with a fluorescence spectrophotometer (RF-1501 Fluorescence Spectrophotometer, Shimadzu, Japan). The $[\text{Ca}^{2+}]_i$ was calculated by the following formula (Gryniewicz et al., 1985):

$$[\text{Ca}^{2+}]_i = kd(F - F_{\min})/(F_{\max} - F), \quad kd = 224 \text{ nmol/L}.$$

Assay of Proliferative Activity of Recombinant Protein

Male mice were killed by vertebral dislocation, and their spleens were removed aseptically. The spleen cell suspensions were prepared by gentle manipulation of the tissues in DMEM medium (supplemented with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL streptomycin). The cells were washed 3 times in DMEM and resuspended at a concentration of 6×10^6 cells/mL (Otani et al., 2001).

The spleen cells were seeded in a 96-well cell culture plate with test substance. The final concentrations of test substance were CPP, 1 µg/mL; recombinant beta-CPP dimer, 1 µg/mL; dephosphorylated recombinant beta-CPP dimer, 1 µg/mL; concanavalin A (**Con A**), 0.5 µg/mL; and LPS, 50 µg/mL. The mixture was incubated at 37°C with 5% CO_2 for 48 h.

At the end of the incubation period, the proliferation of spleen cells was measured using the colorimetric MTT assay (Mosmann, 1983). The absorbance was measured spectrophotometrically at 570 nm by a microplate reader (Bio-Rad model 450, Bio-Rad).

Assessment of Apoptosis-Inducing Activity of Recombinant Protein

Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragments as well as single-strand breaks. Therefore, apoptotic cells could be stained and determined using a photometric immunoassay, which is based on the identification of

Figure 2. Restriction map of pcDNA 3.1/His A and beta-CPP₂-His A. CPP = casein phosphopeptide. Lane 1: DNA marker λ -EcoT14 (19,329, 7743, 6223, 4254, 3472, 2690, and 1882 bp); Lane 2: pcDNA 3.1/His A digested by *Bam*H I, 5500 bp; and Lane 3: beta-CPP₂-His A digested by *Bam*H I, 5716 bp.

these labeled DNA fragments to the free 3'-OH terminal through enzymatic reaction.

To assess the apoptosis-inducing activity of recombinant beta-CPP dimer, HT-29 cells and PC12 cells were selected as the cell models. HT-29 cells were grown in a 96-well cell culture plate at 37°C with 5% CO_2 in DMEM medium (supplemented with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL streptomycin). PC12 cells were grown in a 96-well cell culture plate at 37°C with 5% CO_2 in RPMI 1640 medium (supplemented with 10% equine serum, 5% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin). The 2 types of cells were passaged every 3 to 4 d routinely.

Figure 3. Determination of beta-CPP dimer RNA by reverse transcription-PCR. CPP = Casein phosphopeptide. Lane 1: DNA marker DL2000 (2000, 1000, 750, 500, 250, and 100 bp) and Lane 2: Identification of reverse transcription-PCR production of beta-CPP dimer (108 and 216 bp).

After seeding the HT-29 cells and PC12 cells in a 96-well plate for 10 h, the cells were incubated with N6-(2-isopentenyl)-adenosine and recombinant beta-CPP dimer for another 36 h at the final concentrations of 0.2 and 1 $\mu\text{g/mL}$, respectively. Then, the cells were fixed and blocked on slides, labeled with modified nucleotide at the free 3'-OH terminal of their DNA strand breaks by terminal deoxynucleotidyl transferase. Using antibody from sheep conjugated with horseradish peroxidase and substrate solution (300 mg of diaminobenzidine was dissolved in 1 L of PBS mixed with 0.1% H_2O_2) to stain the apoptotic cells, the stained dead cells were analyzed under light microscope followed by photographing (Hartmann and Meisel, 2002).

Statistical Analysis

Results are reported as means ($\pm\text{SD}$). Student's *t*-test was used to determine significant differences between 2 mean values.

RESULTS

Identification of Recombinant Eukaryotic Expression Vector Beta-CPP₂-His A

Figure 2 shows that the DNA fragment of beta-CPP dimer has ligated into the pcDNA 3.1/His A success-

Figure 4. The SDS-PAGE analysis of expression of recombinant protein. Lanes 1 and 2: protein of transfected Chinese hamster ovary (CHO) cells with gene of beta-CPP dimer; Lane 3: purified recombinant protein; Lane 4: protein of nontransfected CHO cells (negative control); and Lane 5: protein marker (66, 45, 36, 29, 24, 20, and 14.2 Kda).

fully. The DNA fragment of beta-CPP dimer cleaved from T-beta-CPP₂ was recovered and ligated into pcDNA 3.1/His A between the *Kpn* I/*Xho* I restriction sites by T4DNA ligase. The fragment of recombinant expression vector beta-CPP₂-His A cleaved by *Bam*H I was longer than pcDNA 3.1/His A without the DNA of beta-CPP dimer. The result of restriction identification was verified by sequencing. According to the sequencing result, the structure of expressing production from CHO cells was predicted by software. The prediction showed the structural comparability of the location of helix and strand segments between beta-CPP dimer and natural beta-CPP.

Figure 5. Western blot of recombinant protein. Lane 1: protein of nontransfected Chinese hamster ovary cells (negative control); Lane 2: purified recombinant protein; and Lane 3: protein marker (66, 45, 36, 29, 24, 20, 14.2, and 6.5 Kda).

Analysis of the Transfected CHO Cell Lines by RT-PCR

Figure 3 shows that mRNA directing the synthesizing of amino acid of beta-CPP dimer has existed in the transfected CHO cells. The agarose electrophoresis pic-

ture illustrates that the resulting fragment of reverse transcription-PCR has 2 bands with lengths about 108 and 216 bp, the characteristic sign of beta-CPP dimer.

Expression of the Recombinant Protein

Figure 4 shows the expression profile of the CHO cells. According to SDS-PAGE, the recombinant beta-CPP dimer was successfully expressed. No expression production was observed in nontransfected cells with gene of beta-CPP dimer.

To identify whether or not the expressed protein was the desired beta-CPP dimer, we performed Western blotting as shown in Figure 5. The desired beta-CPP dimer, which was purified by the method described previously, was clearly detected.

Effect of Recombinant Protein on Ca^{2+} Uptake of Rabbit Sperm

In the rabbit sperm samples treated with CPP and recombinant beta-CPP dimer, $[\text{Ca}^{2+}]_i$ increased significantly ($P < 0.05$) compared with the untreated group (Table 1). In contrast, in the samples treated with dephosphorylate recombinant beta-CPP dimer and Hank's buffer, the intracellular Ca^{2+} concentration remained constant.

Effect of Recombinant Protein on Proliferation of Mouse Spleen Cells

Mouse spleen cells were cultured with test substances with or without commercial mitogens, such as LPS and Con A. After a 4-h incubation with MTT, the absorbance of the yellow-colored formazon, which was converted from MTT by the metabolic activity of viable cells, was measured spectrophotometrically at 570 nm.

As shown in Table 2, recombinant beta-CPP dimer displayed mitogenic activity toward spleen cells, as well as the CPP, by itself in the absence of the commercial mitogens. When LPS was present, recombinant beta-CPP dimer showed significant mitogenic activity compared with DMEM, although the MTT value of the DMEM group increased stimulated by the same mitogen. After adding Con A, recombinant beta-CPP dimer induced proliferation that was very significantly different from DMEM treated with Con A. The recombinant beta-CPP dimer and CPP displayed no difference in their promotive effect to proliferation with all 3 different treatments. However, the dephosphorylated recombinant beta-CPP dimer could not display the proliferation-promoting activity without commercial mitogens.

Table 1. Effect of the recombinant protein to rabbit sperm intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) ($\pm$ SD).

Sample	$[\text{Ca}^{2+}]_i$	
	Untreated	Treated
	(nmol/L)	
CPP ¹ (positive control)	167.4 $\pm$ 22.3	210.6 $\pm$ 16.2*
Recombinant beta-CPP dimer	174.2 $\pm$ 18.5	219.1 $\pm$ 20.8*
Recombinant beta-CPP dimer (dephosphorylated)	166.3 $\pm$ 12.7	152.8 $\pm$ 21.6
Hank's buffer (negative control)	159.0 $\pm$ 19.8	175.3 $\pm$ 14.2

¹CPP = Casein phosphopeptide.

* $P < 0.05$ compared with the untreated group ($n = 5$).

Table 2. Effect of recombinant protein on proliferative responses of mouse spleen cells ($\pm$ SD).

Sample	MTT value		
	Mitogen-free	LPS	Con A ¹
	$(A_{570\text{ nm}})^2$		
CPP ³ (positive control)	0.381 $\pm$ 0.016*	0.583 $\pm$ 0.007*	0.864 $\pm$ 0.008**
Recombinant beta-CPP dimer	0.396 $\pm$ 0.008*	0.575 $\pm$ 0.004*	0.902 $\pm$ 0.015**
Recombinant beta-CPP dimer (dephosphorylated)	0.276 $\pm$ 0.017	0.574 $\pm$ 0.009*	0.861 $\pm$ 0.012**
DMEM (negative control)	0.255 $\pm$ 0.022	0.436 $\pm$ 0.014	0.652 $\pm$ 0.016

¹Con A = Concanavalin A.²Measured in units of absorbance at 570 nm.³CPP = Casein phosphopeptide.* $P < 0.05$ compared with the untreated group ($n = 6$).** $P < 0.01$ compared with the untreated group ($n = 6$).

Effect of Recombinant Protein on Apoptosis of HT-29 Cells and PC12 Cells

Current knowledge indicates that the key mechanism of apoptosis is endonuclease activation, leading to internucleosomal double-stranded DNA breaks. In the test to assess the apoptosis-inducing activity of recombinant protein, the breaks were detected and stained with diaminobenzidine. As shown in Figure 6, the cells treated with recombinant protein displayed brown as the positive control.

DISCUSSION

Numerous bioactive peptides have been isolated and characterized. Some of them, such as lactoferricin and CPP, have caught the eyes of many investigators interested in discovering new drugs (Ait-Oukhatar et al., 2002; Eliassen et al., 2002; Wakabayashi et al., 2003). However, the constituents of products obtained from enzymatic hydrolyzation of milk proteins are neither unique nor certain. The non-uniqueness and uncertainty limit the pharmacological application of these bioactive peptides. Accordingly, the purpose of this study was to establish a new way to produce CPP by DNA recombinant technology. It is well known that the full biological activity of some proteins requires many kinds of posttranslational modification, for example glycosylation, phosphorylation, and/or other forms of posttranslational modification. These proteins may best be expressed in a eukaryotic host, especially in mammalian cells (Weaver, 1999). Because CPP are phosphorylated peptides, this study determined the pcDNA 3.1/His A-CHO as the most appropriate vector-host system to express beta-CPP dimer.

In this study, expressing beta-CPP dimer was based upon two points: 1) it is difficult to express short peptides such as beta-CPP just 25 amino acids directly by DNA recombinant technology; and 2) the predicted

result of product expressed by CHO cells suggested that the location of helix and strand segments of beta-CPP dimer were consistent with beta-CPP monomer, by which we presumed that the beta-CPP dimer might have similar functions as natural beta-CPP.

To obtain the tandem gene of beta-CPP, *Nhe* I and *Xba* I, a couple of isocaudamers, were set in the 2 designed nucleotide acid sequences (Shi et al., 2001). At the same time, ATG (a coding sequence to be translated into Met) were designed in the 2 nucleotide acid sequences so that the beta-CPP monomer could be obtained by cleaving with CNBr after the beta-CPP dimer had been expressed by CHO cells. pcDNA 3.1/His A was a mammalian cell expression vector with 6 $\times$ His tag that could tightly bind to nickel-chelating resin to purify the protein expressed by CHO cells. Because few natural proteins could bind to nickel-chelating resin, 6 $\times$ His-labeled proteins generated by recombinant techniques can be purified in a single step by nickel chelate affinity chromatography. Nickel chelate chromatography has become popular because it is highly effective to purify the recombinant protein, and the 6 $\times$ His tag could not interfere with the proper folding and function of target protein (Sambrook and Russell, 2001).

In this study, the recombinant beta-CPP dimer with 6 $\times$ His tag was expressed and purified. In the test of bioactivity assessment, the recombinant proteins show the same cytomodulative effect as the commercial CPP. The beta-CPP dimer can increase both Ca^{2+} uptake of rabbit sperm and proliferation of mouse spleen cells. However, the dephosphorylated beta-CPP dimer has no effect on $[\text{Ca}^{2+}]_i$ and proliferation of cell samples. These results indicate that the "strategic zone" of the recombinant protein, that is, the Ser-Ser-Ser sequence, should be phosphorylated so that the beta-CPP dimer can have biological activity. This result also proved indirectly that the recombinant protein had been phosphorylated in CHO cells.

Figure 6. Analysis of HT-29 cells treated with recombinant protein by light microscopy (400 fold) (left-hand column): A) N6-(2-isopentenyl)-adenosine (positive control), B) recombinant protein, and C) DMEM (negative control). Analysis of PC12 cells treated with recombinant protein by light microscopy (200 fold) (right-hand column): D) N6-(2-isopentenyl)-adenosine (positive control), E) recombinant protein, and F) RPMI 1640 (negative control).

PC12 cells and HT-29 cells manifested typical tumor properties (Ferraretto et al., 2001). Therefore, the 2 cell lines are suitable models for the detection of an apoptosis-inducing effect of recombinant beta-CPP dimer. The results in the test showed that the beta-CPP dimer induced apoptosis of the 2 types of malignant cells. The primary reason for the results was that the increased $[Ca^{2+}]_i$ induced by beta-CPP dimer resulted in the increasing of endonuclease activation. Subsequently, the endonuclease cleaved genomic DNA into fragments followed by cell apoptosis (Emad and Gerald, 1990). However, when the apoptosis-inducing effects of beta-CPP dimer on CHO cells and mouse spleen cells were evaluated, the same bioactivity could not be found in either cell model (results not shown). The probable reason was the complexity of apoptosis mechanism. Apoptosis involves intracellular pH, $[Ca^{2+}]_i$, and the distributing of endonuclease, depending on the types of cell (Shemtov et al., 1995). However, in this study, the result that malignant cells were more responsive to apoptotic stimulation of beta-CPP dimer than non-malignant cells suggests that beta-CPP dimer has potential pharmacological application.

The main goals of our study were accomplished, and the cells lines stably expressing recombinant beta-CPP dimer in the scale of microgram were established for further use and study. However, there are two problems: 1) Could the recombinant beta-CPP dimer with $6 \times \text{His}$ be cleaved into beta-CPP monomer by CNBr as we designed? 2) Does the beta-CPP monomer have the same or even more effects? These problems are being addressed through research in progress as we try to discover more clues to its cytomodulatory function.

ACKNOWLEDGMENTS

This study was supported by the Grant for Promotion of Science and Technology from the Government of Heilongjiang Province (No. WB02202).

REFERENCES

- Abubakar, A., T. Saito, and K. Haruki. 1998. Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *J. Dairy Sci.* 81:3131–3138.
- Adamson, N. J., and E. C. Reynolds. 1995. Characterization of multiply phosphorylated peptides selectively precipitated from a pancreatic casein digest. *J. Dairy Sci.* 78:2653–2659.
- Ait-Oukhatir, N., J. M. Peres, S. Bouhallab, D. Neuville, F. Bureau, G. Bouvard, P. Arhan, and D. Bougle. 2002. Bioavailability of caseinophosphopeptide-bound iron. *J. Lab. Clin. Med.* 140:290–294.
- Anne, P. L., P. Koskinen, and K. Piilola. 2000. Angiotensin I-converting enzyme inhibitory properties of whey protein digests: Concentration and characterization of active peptides. *J. Dairy Res.* 67:53–64.
- Ausubel, F. M., R. Brent, and R. E. Kingston. 1995. *Short Protocols in Molecular Biology*. 3rd ed. John Wiley & Sons, Inc., Hoboken, NJ.
- Bal, D. C. Sollier, L. Drouet, G. Pignaud, C. Chevallier, J. Caen, A. M. Fiat, C. Izquierdo, and P. Jolles. 1996. Effect of kappa-casein split peptides on platelet aggregation and on thrombus formation in the guinea-pig. *Thromb. Res.* 81:427–37.
- Beatrice, C., P. Jolles, and C. Izquierdo. 1995. Characterization of an antithrombotic peptide from casein in newborn plasma after milk ingestion. *Br. J. Nutr.* 73:583–590.
- Chaud, M. V., C. Izumi, Z. Nahaal, T. Shuhama, M. L. Bianchi, and O. Freitas. 2002. Iron derivatives from casein hydrolysates as a potential source in the treatment of iron deficiency. *J. Agric. Food Chem.* 50:871–877.
- Clare, D. A. and H. E. Swaisgood. 2000. Bioactive milk peptides: A prospectus. *J. Dairy Sci.* 83:1187–1195.
- Cross, K. J., N. L. Hug, W. Bicknel, and E. C. Reynolds. 2001. Cation-dependent structural features of b-casein-(1–25). *Biochem. J.* 356:277–286.
- David, D. K., and Y. V. Yuan. 1992. Caseinophosphopeptides and calcium bioavailability. *Trends Food Sci. Technol.* 3:31–35.
- Eliassen, L. T., G. Berge, B. Sveinbjornsson, J. S. Svendsen, L. H. Vorland, and O. Rekdal. 2002. Evidence for a direct antitumor mechanism of action of bovine lactoferricin. *Anticancer Res.* 22:2703–2710.
- Emad, S., and L. Gerald. 1990. Activation of internucleosomal DNA cleavage in human CEM lymphocytes by glucocorticoid and novobiocin. *J. Biol. Chem.* 265:17323–17333.
- Farrell, H. M. Jr., P. X. Qi, E. D. Wickham, and J. J. Unruh. 2002. Secondary structural studies of bovine caseins: Structure and temperature dependence of beta-casein phosphopeptide (1–25) as analyzed by circular dichroism, FTIR spectroscopy, and analytical ultracentrifugation. *J. Protein Chem.* 21:307–321.
- Ferraretto, A., A. Signorile, C. Gravaghi, A. Fiorilli, and G. Tettamanti. 2001. Casein phosphopeptides influence calcium uptake by cultured human intestinal HT-29 tumor cells. *J. Nutr.* 131:1655–1661.
- Gerd, E. V., T. Langsrud, and C. Svenning. 2000. Mineral-binding milk proteins and peptides: Occurrence, biochemical and technological characteristics. *Br. J. Nutr.* 84 (Suppl. 1):S91–S98.
- Gryniewicz, G., M. Poneie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicator with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.
- Harsharnjit, S. G., F. Doull, and K. J. Rutherford. 2000. Immunoregulatory peptides in bovine milk. *Br. J. Nutr.* 84 (Suppl. 1):s111–s117.
- Hartmann, R., and H. Meisel. 2002. Cytochemical assessment of phosphopeptides derived from casein as potential ingredients for functional food. *Nahrung.* 46:427–431.
- Holt, C., P. A. Timmins, N. Errington, and J. Leaver. 1998. A core-shell model of calcium phosphate nanoclusters stabilized by beta-casein phosphopeptides, derived from sedimentation equilibrium and small-angle X-ray and neutron-scattering measurements. *Eur. J. Biochem.* 252:73–78.
- Holt, C., N. M. Wahlgren, and T. Drakenberg. 1996. Ability of a beta-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters. *Biochem. J.* 314:1035–1039.
- Jinsmaa, Y., and M. Yoshikawa. 1999. Enzymatic release of neocasomorphin and beta-casomorphin from bovine beta-casein. *Peptides* 20:957–962.
- Kampa, M., S. Loukas, A. Hatzoglou, P. Martin, P. M. Martin, and E. Castanas. 1996. Identification of a novel opioid peptide (Tyr-Val-Pro-Phe-Pro) derived from human alpha S1 casein (alpha S1-casomorphin, and alpha S1-casomorphin amide). *Biochem. J.* 319:903–908.
- Karaki, H., K. Doi, and S. Sugano. 1990. Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. *Comp. Biochem. Physiol.* 2:367–371.
- Kuwata, H., T. T. Yip, M. Tomita, and T. W. Hutchens. 1998. Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. *Biochem. Biophys. Acta* 1429:129–141.

- Lahov, E., and W. Regelson. 1996. Antibacterial and immunostimulating casein-derived substances from milk: Casocidin, isracidin peptides. *Food Chem. Toxicol.* 34:131–145.
- Liang, G. D. 2001. *Current Molecular Biology Laboratory Manual*. Science Press, Beijing, China.
- Matsui, T., H. Okumura, and H. Yano. 2002. Absorption of zinc from dietary casein phosphopeptide complex with zinc in rats given a soybean protein-based diet. *J. Nutr. Sci. Vitaminol.* 48:247–250.
- Meisel, H., and R. J. FitzGerald. 2003. Biofunctional peptides from milk proteins: Mineral binding and cytomodulatory effects. *Curr. Pharm. Des.* 9:1289–1295.
- Mori, T., M. Hirayama, K. Suzuki, H. Shimizu, and T. Nagai. 1996. Effect of casein phosphopeptides and Ca^{2+} on penetration of boar spermatozoa into pig oocytes matured in vitro. *Biol. Reprod.* 55:364–369.
- Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65:55–63.
- Otani, H., K. Nakano, and T. Kawahara. Casein phosphopeptide enhances mucosal IgA response to LPS. *Drug Weekly* July 11, 2003:356–357.
- Otani, H., T. Watanabe, and Y. Tashiro. 2001. Effects of bovine beta-casein (1-28) and its chemically synthesized partial fragments on proliferative responses and immunoglobulin production in mouse spleen cell cultures. *Biosci. Biotechnol. Biochem.* 65:2489–2495.
- Park, O., H. E. Swaisgood, and J. C. Allen. 1998. Calcium binding of phosphopeptides derived from hydrolysis of alpha s-casein or beta-casein using immobilized trypsin. *J. Dairy. Sci.* 81:2850–2857.
- Sambrook, J., and D. W. Russell. 2001. *Molecular Cloning: A Manual*. 3rd ed. Cold Spring Harbor Laboratory Press, New York.
- Sato, R., T. Naguchi, and H. Naito. 1986. Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestines. *J. Nutr. Sci. Vitaminol.* 32:67–76.
- Shemtov, M. M., D. L. Cheng, L. Kong, W. P. Shu, M. Sassaroli, M. J. Droller, and B. C. Liu. 1995. LAK cell mediated apoptosis of human bladder cancer cells involves a pH dependent endonuclease system in the cancer cell: Possible mechanism of BCG therapy. *J. Urol.* 154:269–274.
- Shi, J.H., Y. Q. Zhang, and Y. T. Zhao. 2001. Cloning, expression of thymosin alpha 1 gene in *E.coli* and its purification and characterization. *Chin. J. Biochem. Mol. Biol.* 17:344–349.
- Siemion, I. Z., J. Slon, and Z. Wiczorek. 1995. The immunosuppressive mini-domain of human lactoferrin. *J. Pept. Sci.* 1:295–302.
- Teschemacher, H. 2003. Opioid receptor ligands derived from food proteins. *Curr. Pharm. Des.* 9:1331–1344.
- Wakabayashi, H., M. Takase, and M. Tomita. 2003. Lactoferricin derived from milk protein lactoferrin. *Curr. Pharm.* 9:1277–1287.
- Weaver, R. F. 1999. *Molecular Biology*. McGraw-Hill Companies, Inc., New York.
- Zucht, H. D., M. Raida, K. Adermann, H. J. Magert, and W. G. Forssman. 1995. Casocidin-I: A casein-alpha s2 derived peptide exhibits antibacterial activity. *FEBS Lett.* 372:185–188.