Genetic Engineering of Bovine κ-Casein To Improve Its Nutritional Quality

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Caseins are slightly deficient in sulfur amino acids. It was possible to insert three methionine residues into κ-casein by using recombinant DNA technology. κ-Casein cDNA, which encodes the entire mature bovine κ-casein protein, was manipulated to improve the nutritive quality. An oligonucleotide, a 35-mer coding for the three methionine residue insertion, was synthesized. Mutagenesis was performed by using a gapped duplex method. Three methionine residues were inserted between Ala_{167} and Thr_{168} of κ-casein. The mutation was confirmed by DNA sequencing, and there is no mutation in any other region of the κ-casein cDNA. Mutant κ-casein cDNA was cloned into the expression vector pIN-III-$ompA_1$ to express κ-casein in *Escherichia coli* strain AR68, which is protease deficient. Expression of κ-casein in *E. coli* was detected, and its expression level was determined as being 2 mg/L of medium. Most of the expressed κ-casein was guided to the periplasmic space of *E. coli*. These experiments indicate that it is possible to produce milk proteins with a better amino acid balance in terms of nutritive quality by using recombinant DNA technology.

INTRODUCTION

Some food proteins are deficient in one or more of the essential amino acids. Lysine, methionine, and tryptophan are often the limiting essential amino acids. There have been efforts to improve the nutritional quality of food proteins by the covalent attachment of essential amino acids using chemical or enzymatic methods. Soybean proteins, caseins, pea proteins, bean proteins, and wheat gluten are some examples, wherein the protein and amino acid derivative are treated with a carbodiimide coupling reagent (Sheehan et al., 1955; Puigserver et al., 1979; Matheis et al., 1985). *N*-Carboxy anhydrides of amino acids have also been used to covalently attach amino acids to proteins (Puigserver and Gaertner, 1982). Chemical methods, however, may have undesirable side effects of actually reducing the nutritive value of proteins (Feeney, 1977) or generating toxic contaminants. Transglutaminase catalyzes the transfer of carboxyamide groups of glutamine in proteins to amino acid esters and has been used to improve the nutritive value of proteins (Itakura et al., 1981). Plastein-like reactions have been used to couple essential amino acid esters to proteins (Satterlee and Chang, 1982; Watanabe and Arai, 1982). Papain catalyzes the transfer of peptidyl groups into proteins (Watanabe and Arai, 1982). Papain also catalyzes the transfer of the acyl moiety of a thiolester to an amino compound to form a peptide bond (Sung et al., 1983). However, enzyme modification is expensive and sometimes yields products that behave much differently from the starting materials. Transglutaminase does not work well on globular proteins (Bercovici et al., 1987) and, as yet, there is no readily available source of this enzyme.

Recombinant DNA technology might be used to improve the nutritive value of proteins without the foregoing drawbacks. Recently, a novel artificial protein using recombinant DNA technology was designed (Biernat, 1987). Its optimal amino acid composition was based on data from FAO and WHO nutrition reports and on the composition of commercially available products. Caseins are slightly deficient in the sulfur amino acids, having a biological value (0.8) slightly less than the ideal based on a value of 1.0 for whole egg protein (Kon, 1972). Methionine is one of the most deficient essential amino acids in the caseins. The amount of methionine in whole egg is 4.9 g/100 g of protein, whereas amounts of methionine are 2.8 g/100 g of casein and 1.7 g/100 g of κ-casein, respectively (Gordon and Kalan, 1974; Lineweaver and Klose, 1955). Thus, methionine is the most deficient essential amino acid in the caseins or κ-casein when compared to whole egg. It is possible to replace, delete, or insert specific amino acids into a target protein by using recombinant DNA technology. The objective of this study is to enhance the nutritional quality of κ-casein by inserting three methionine residues between Ala_{167} and Thr_{168} of κ-casein using recombinant DNA technology.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *Escherichia coli* strain JM107 was purchased from Amersham Corp., Arlington Heights, IL, and the *E. coli* strain AR68 was a generous gift from Dr. Shatzman, Smith Kline and French Laboratories. Plasmids M13mp18 RF (replicative form) and M13mp19 RF were obtained from Boeringer Mannheim, Indianapolis, IN, and the pIN-III-$ompA_1$ was obtained from Dr. Inouye, New Jersey Medical College, Newark, NJ.

Single-Stranded Template Preparation. Bovine cDNA for κ-casein has been cloned and expressed (Kang and Richardson, 1988). The plasmid, pKR76, contains a full-length cDNA fragment that codes for mature bovine κ-casein. The pKR76 was digested with *Msp*I endonuclease. The resulting 716 base pair (bp) DNA fragment from an *Msp*I digest of pKR76 was purified by electrophoresis in low-temperature agarose gel and by eluting the DNA band from the agarose using an Elutip-d minicolumn (Schleicher and Schuell, Keene, NH). The DNA fragment was subsequently digested with *Nla*IV endonuclease, and the resulting blunt-sticky ended fragment was purified. The purified *Msp*I-NlaIV fragment was inserted between *Sma*I and *Acc*I sites of bacteriophage M13mp18 RF. The ligation, transformation, and screening of recombinant M13 bacteriophage plaques were performed according to the protocol described by Messing (1983). From the selected phage plaques, single-stranded template was prepared (Messing, 1983).

Mutagenesis Reaction. An oligonucleotide (35-mer) which induces the insertion of three methionine residues between Ala_{167} and Thr_{168} of κ-casein was prepared by J. Pressley at the Protein Structure Research Laboratories, University of California at Davis. The gapped duplex method (Figure 1) was used for mutagenesis (Kramer et al., 1984). Five hundred nanograms of M13mp18 RF was double digested with *Hin*dIII and *Eco*RI en-

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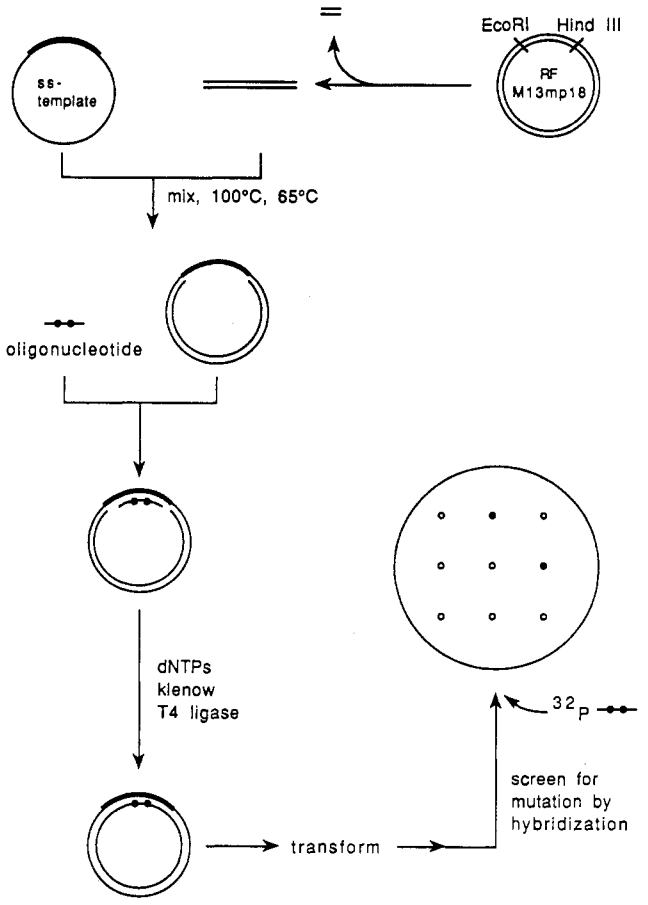

Figure 1. Schematic diagram of site-directed mutagenesis of κ-casein. A heterogapped duplex is formed after single-stranded (ss) template is mixed with and replicative form (RF) of M13mp18 cleaved with *Eco*RI and *Hin*dIII endonucleases. The oligonucleotide is annealed on the specific site that induces a change of bases. The gapped duplex with the oligonucleotide is then closed by polymerization and ligation. Screening of mutants is performed by oligonucleotide hybridization using the ^{32}P-labeled oligonucleotide after transformation.

donucleases and purified by using an Elutip-d. Ten nanograms of phosphorylated oligonucleotide, 4 μL of ligase buffer, and 100 ng of single-stranded template with the κ-casein cDNA insert was added to the mix, and H_2O was added with mixing to a final volume of 40 μL. This solution was heated to 95 °C for 10 min, cooled to room temperature for 30 min, and then kept on ice for 10 min. To this solution were added 1 μL of 2.5 mM dNTPs, 40 units of T4 DNA ligase, 1 unit of Klenow fragment, 1 μL of ligase buffer, 2 μL of 10 mM ATP, and 4 μL of H_2O, and the solution was incubated at 4 °C for 30 min, at 12 °C for 1 h, and, finally, at 37 °C for 30 min.

Ten microliters of the above solution was mixed with 0.3 mL of $CaCl_2$-treated competent *E. coli* JM107 cells, and the culture was kept on ice for 40 min. This mixture was heat-shocked at 42 °C for 3 min. Two hundred microliters of exponentially growing fresh JM107 cells and 10 mL of 2X YT media were mixed together with the heat-shocked competent cells in a 50-mL flask. This mixture was incubated at 37 °C overnight. The cell culture was transferred to Eppendorf tubes and centrifuged for 5 min in a microfuge. A series of dilutions (10^1–10^{10}) of the cell culture supernatant fluid were prepared. The diluted supernatant portions were added to 3.5 mL of H top agar containing 300 μL of fresh JM107 cells, 10 μL of 200 mM isopropyl β-D-thiogalactopyranoside (IPTG), and 50 μL of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (20 mg/mL in dimethylformamide). This mix was gently shaken and poured onto LB plates (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 ml of 1 N NaOH, and 15 g of agar per liter). The LB plates were kept at room temperature for 15 min and incubated at 37 °C for 8 h.

Screening of Mutants. *Plaque Hybridization.* Zoller and Smith's method (1984) was used for plaque hybridization. Plates that contained 100–400 plaques were chosen for plaque lifts. The moist nitrocellulose filters were placed on the LB plates for 1 min to obtain the first replica and 3 min for the second replica. These nitrocellulose filters were then baked in an 80 °C vacuum oven for 2 h after brief air drying. The filters were placed in prehybridization solution (6× SSC, 10× Denhardt's buffer, 0.2% SDS), incubated for 1 h at 65 °C with rocking, and then washed with 200 mL of 6× SSC for 5 min at room temperature. The filters were then placed in hybridization solution (6× SSC, 10× Denhardt's buffer, ^{32}P-labeled oligonucleotide) and incubated for 1 h at room temperature with rocking. The filters were washed three times (5 min each wash) in 200 mL of 6× SSC at room temperature. The filters were dried slightly, covered with Saran Wrap, and then exposed to X-ray film (Kodak X-Omat AR) with the aid of an intensifying screen (Du Pont, Lightning) overnight.

Mismatched primers were removed from the filters by using 55 and 65 °C washings in 6× SSC for 10 min. The washing temperature was determined according to the method of Suggs et al. (1981).

Restriction Mapping. M13 RF DNA was prepared by using the alkaline lysis method (Birnholm and Doly, 1979). The DNA was digested with appropriate restriction enzymes. The restriction enzymes were obtained from New England Biolabs and used according to the manufacturer's specifications. Digests of RF DNA were analyzed on agarose gels as described by Maniatis et al. (1982).

Sequencing of Mutagenized κ-Casein cDNA. The mutagenized κ-casein cDNA was subcloned into M13mp18 bacteriophage and sequenced by using the dideoxy chain termination method (Sanger et al., 1977).

Labeling the Oligonucleotide with [γ-^{32}P]ATP. The following conditions were used for labeling the oligonucleotide used for screening using polynucleotide kinase. Two hundred nanograms of oligonucleotide, 10 μL of 50 mM $MgCl_2$, 5 μL of 1 M Tris-HCl, pH 7.6, 5 μL of 200 mM β-mercaptoethanol, 100 μCi [γ-^{32}P]ATP, and 10 units of T4 polynucleotide kinase in a final volume of 50 μL were incubated at 37 °C for 1 h. The reaction was stopped by incubating the mixture at 65 °C for 10 min.

Construction of the Expression Vector pKOR-Met. Mutant κ-casein cDNA was purified by using a CsCl–EtBr gradient (Maniatis, 1982). The expression vector pIN-III-ompA$_1$ (Ghrayeb et al., 1984) was used to obtain expression of mutant κ-casein with three methionine residues in *E. coli* strain AR68, which is protease deficient.

Five micrograms of the plasmid pIN-III-ompA$_1$ in 100 μL of buffer was completely cleaved with *Eco*RI endonuclease. The restriction enzyme was inactivated by heating for 10 min at 65 °C. The linearized DNA fragment was isolated by using an Elutip-d column after low-temperature agarose gel electrophoresis. The linearized DNA was again cleaved by using *Hin*dIII endonuclease and purified by using an Elutip-d column. The κ-casein cDNA insert of pKC was isolated after digestion of the plasmid with *Eco*RI and *Hin*dIII endonucleases. Five micrograms of pKC was cleaved with *Eco*RI, and the linearized DNA was precipitated by using ethanol. The linearized DNA was redissolved in water and then cleaved with *Hin*dIII endonuclease. The κ-casein cDNA insert was isolated by using an Elutip-d column after low-temperature agarose gel electrophoresis and then dissolved in 100 μL of H_2O. After ligation, the digested DNA was transformed into competent *E. coli* JM107 and AR68 cells. Selection of the transformants was accomplished by using the ampicillin resistance marker of the expression vector. From the individual transformants, the plasmid was purified by using the miniscreen procedure. Purified DNA was digested with *Pst*I endonuclease and subjected to agarose gel electrophoresis to verify the insertion of κ-casein cDNA.

Detection of κ-Casein Expressed in *E. coli*. Transformant cells harboring pKOR-Met were selected and grown in 1 mL of LB–ampicillin broth to detect the expression of κ-casein in *E. coli* by SDS–PAGE, Western blot, and immunochemical staining. The cell pellets from 1 mL of the overnight culture derived from colonies containing expression plasmids were collected and resuspended in 100 μL of SDS sample buffer. They were boiled for 5 min, and 50-μL aliquots for each sample were subjected to 10% SDS–PAGE. The proteins were blotted on nitrocellulose

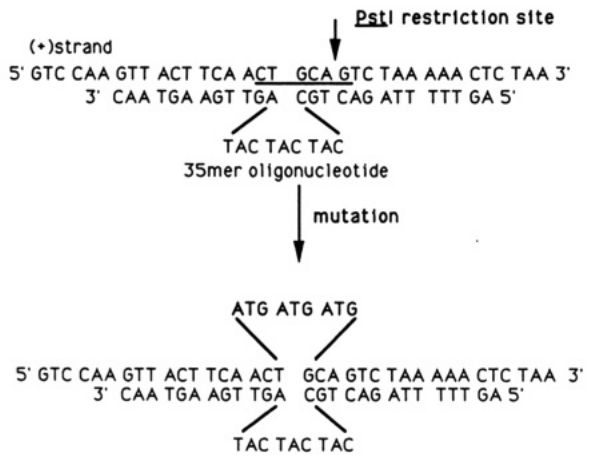

Figure 2. Synthetic oligonucleotide (35-mer) used to insert three methionine residues between Ala_{167} and Thr_{168} of κ-casein.

membranes and tested for the presence of κ-casein as described by Hawkes (1982) with antibodies against bovine κ-casein.

Subcellular Localization. The method used for osmotic shock of the *E. coli* host cells was adapted from Libby et al. (1987). *E. coli* strain AR 68 harboring the pKOR-Met vector was grown at 37 °C in LB containing ampicillin (50 mg/mL) to an OD_{600} of 0.25. Cultures were induced with IPTG and grown for 4 h. Cells were harvested after centrifugation for 2 min in an Eppendorf microfuge at 4 °C. The resulting pellets were resuspended in 150 μL of ice-cold 10 mM Tris-HCl, pH 7.5, containing 20% (w/v) sucrose. After 5 min at 0 °C, 5 mL of 0.5 M EDTA was added, and the mixture was incubated an additional 15 min at 0 °C and then centrifuged at 4 °C in the microfuge. The resulting pellets were redissolved in 150 μL of ice-cold water and incubated 15 min at 0 °C. After centrifugation in the microfuge, one-tenth volume of trichloroacetic acid was added to the supernatant. The precipitate was collected by centrifugation, washed two times with 1.0 mL of ice-cold 70% ethanol, dried, resuspended in 100 μL of SDS sample buffer, and saved as the periplasmic fraction. The pellet from the above separation was used to prepare cytoplasmic and membrane fractions. Pellets were resuspended in 100 μL of 50 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), and 5 μL of lysozyme (2 mg/mL). The suspension was incubated at 37 °C for 15 min, freeze-thawed three times in a dry ice–ethanol bath, and passed through a 26-gauge needle three times to shear the DNA. The supernatant fluid containing the cytoplasmic fraction was recovered after centrifugation for 2 h in a Beckman ultracentrifuge using a 50 VTi rotor (150000*g*). The pellet containing the membrane-associated proteins was resuspended in 100 μL of SDS sample buffer and heated to 100 °C for 3 min to solubilize the membrane proteins. Samples (50 μL) were subjected to electrophoresis in 10% SDS–polyacrylamide gels. Western blots and immunochemical staining methods were used to detect κ-casein.

RESULTS AND DISCUSSION

Template Preparation. The *Msp*I fragment of pKR76 (Kang and Richardson, 1988), which contains whole mature κ-casein cDNA, was further cleaved with *Nla*IV endonuclease. This *Msp*I and *Nla*IV enconuclease cleaved fragment, which has a blunt end at one end and a sticky end at the other end, was purified by using low-temperature agarose gel electrophoresis coupled with the use of an Elutip-d minicolumn. *Sma*I–*Acc*I-cleaved RF of bacteriophage M13mp18 will yield the right fragment for the forced cloning of *Msp*I–*Nla*IV-cleaved fragment of pKR76. Both *Sma*I and *Nla*IV endonuclease generate blunt cells. *Msp*I and *Acc*I produce cohesive ends which are complementary to each other so that they can readily be ligated in a forced cloning mode. Therefore, the *Msp*I–*Nla*IV-cleaved fragment of pKR76 can be ligated to *Sma*I–*Acc*I-cleaved RF of M13mp18 bacteriophage in one orientation.

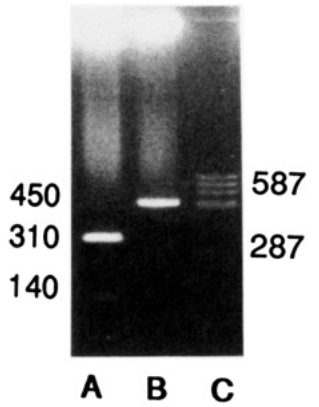

Figure 3. Agarose gel electrophoresis of pKC-Met digested with *Pst*I endonuclease. (Lane A) Pattern from digestion of pKC1 (wild type) with *Pst*I endonuclease. Three fragments are shown, 140, 310, and 7410 bp. (Lane B) Pattern from digestion of pKC-Met with *Pst*I endonuclease. Two fragments are shown, 450 and 7410 bp. (Lane C) Molecular weight markers, pBR322 digested with *Hae*III endonuclease.

After ligation, the ligation mixture was used to transform the competent cells of *E. coli* JM107. Each colorless plaque was transformed into a tube of fresh JM107 cells. After growing for 8 h, the supernatant fluids were used to purify single-stranded DNA. From the same tubes, pellets were used to purify RF of the recombinant bacteriophage. The purified RF was analyzed by using restriction enzyme patterns. The recombinant plasmid pKC contains a κ-casein cDNA insert. *Eco*RI–*Hin*dIII-cleaved pKC revealed a 660-bp band on 1.2% agarose gel electrophoresis. The presence of κ-casein cDNA insert cleaved by *Pst*I endonuclease was shown on 1.2% agarose gel electrophoresis. Ten RF preparations of 10 white plaques showed that they all have the insert. The RF DNA of recombinant phage was prepared in large scale by using a CsCl–EtBr method for later use. For the preparation of single-stranded recombinant phage DNA, recombinant RF was transformed into competent *E. coli* JM107, and the cells were grown for 8 h. Each colorless plaque was transferred into a tube of fresh *E. coli* JM107. After growing 8 h, the supernatant fluids containing single-stranded phage were used for the preparation of single-stranded recombinant phage DNA (Messing, 1983).

Synthesis and Purification of Oligonucleotide. The oligonucleotide (35-mer) which would induce the insertion of three methionine residues between Ala_{167} and Thr_{168} of κ-casein was synthesized at the Protein Structure Research Laboratories of the University of California at Davis. The sequence of the oligonucleotide was based on the (+) strand of the κ-casein cDNA insert sequence. The oligonucleotide was designed so that the mismatches are located near the middle of the molecule because the oligonucleotide would be used as a probe to screen for mutants. Placement of the mismatches in the middle yields the greatest binding differential between a perfectly matched duplex and a mismatched duplex. Another consideration concerns the protection of the mismatches from exonuclease activity of DNA polymerase "large fragment" which has a 3′ to 5′ exonuclease activity. With these considerations in mind, an oligonucleotide was prepared (Figure 2).

The oligonucleotide mixture was purified by using electrophoresis in 20% polyacrylamide gel [19:1 acrylamide/bis(acrylamide)] containing 7 M urea and 1× TBE buffer (90 mM Tris, 65 mM boric acid, 2.5 mM EDTA, pH 8.3). Material containing 30 mg of synthetic oligonucleotide was applied to one lane (2 cm wide × 1.5 mm thick) and

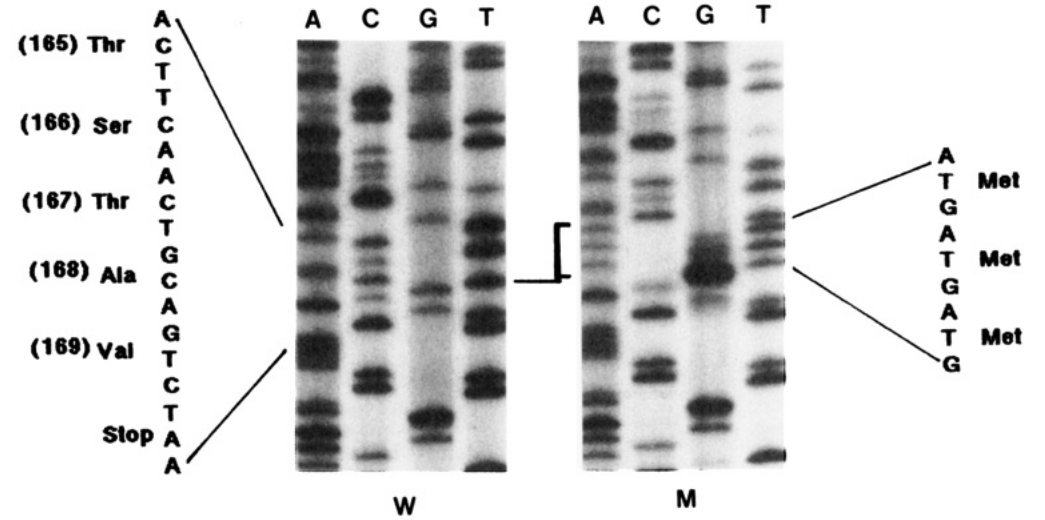

Figure 4. Sequences of relevant fragments of wild-type, W (pKC1), and mutant, M (pKC-met), DNA. (W) DNA sequences of COOH-terminal region of κ-casein cDNA; (M) DNA sequence of COOH-terminal region of κ-casein cDNA with insertion of three ATGs.

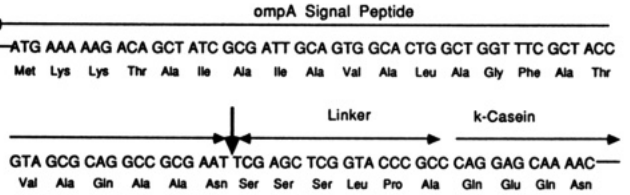

Figure 5. DNA sequence of ompA signal peptide, linker, and κ-casein cDNA in the 5′ region. The thick solid arrow denotes the signal peptide cleavage site. There are 21 amino acids from the ompA signal peptide, 6 amino acids from the linkers, and 1 amino acid from the signal peptide of κ-casein (last amino acid of κ-casein signal peptide) and the mature κ-casein.

the gel subjected to 400 V until the bromophenol blue migrated to the bottom of the gel (12 cm). The gel slab was covered with Saran Wrap and placed on a thin-layer chromatography (TLC) plate containing a fluorescent indicator. The gel was illuminated by using a hand-held long-wave UV lamp (Ultraviolet Products Inc., San Gabriel, CA) to reveal the gel pattern.

The slowest moving band was excised from the gel. This gel fragment was diced, and the DNA was eluted by diffusion at 37 °C overnight into Maxam–Gilbert gel elution buffer [0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1% (w/v) SDS, and 0.1 mM EDTA] (Maxam and Gilbert, 1980). Desalting of the solution was carried out with a Sep-Pak C_{18} minicolumn (Waters Inc., Milford, MA) (Palmenberg et al., 1983). The Sep-Pak minicolumn was equilibrated with 5 mL of TE buffer (pH 7.9). The sample was loaded, and the Sep-Pak minicolumn was washed with 5 mL of TE buffer. The oligomer was eluted with 40% acetonitrile in water. The first 0.5 mL was discarded, and the next 1.5 mL of eluant was collected. The oligomer solution was dried under vacuum.

Oligonucleotide-Directed Mutagenesis. RF DNA of M13mp18 bacteriophage was cleaved with *Hin*dIII and *Eco*RI endonucleases. The lineared large double-stranded DNA fragment was purified by using an Elutip-d minicolumn after low-temperature agarose gel electrophoresis.

Single-stranded template DNA was mixed with the linearized, double-cleaved RF DNA of M13mp18 bacteriophage, resulting in the gapped heteroduplex (Figure 1). The mutagenic oligonucleotide was annealed onto the single-stranded DNA of the gapped duplex and extended by DNA polymerase large fragment. After transformation into competent cells of JM107, this mixture was plated onto an LB plate.

The mutagenic oligonucleotide (35-mer) contains 14 nucleotides flanking the 5′ side of the three ATG insertion and 12 nucleotides flanking the 3′ side and is designed to insert the three ATGs from the complementary lower (−) strand near the COOH-terminal region of κ-casein. The site of insertion of the three methionine residues in κ-casein was chosen between Ala_{167} and Thr_{168} because of the convenience of screening mutants by using *Pst*I endonuclease and the potential alteration in the functionality of the mutant κ-casein. The 35-mer oligonucleotide was shown to be unique, by computer search, in the recombinant DNA target. Whole mixtures of oligonucleotide, without purification of the 35-mer oligonucleotide, were used for mutagenesis.

After plaque lift and prehybridization of the nitrocellulose membrane, the probe solution was added to the membrane. The membrane was washed, and then autoradiography was carried out after each wash for 10 min at 55 and 65 °C. Twenty-three plaques exhibited strong positive signals after the 65 °C wash. The efficiency of mutagenesis was about 1%. Restriction endonuclease digest was done to confirm the site of mutagenesis. When three ATGs were successfully inserted, the *Pst*I site (CTGCAG) is changed and is no longer available. Phages from two of the suspected mutants were plated, and double-stranded DNA from 12 individual plaques was isolated and rescreened for the mutation by restriction digestion. There are two *Pst*I sites in κ-casein cDNA insert and one site in M13mp18 bacteriophage. The *Pst*I site at position 6666 is no longer cleaved by *Pst*I endonuclease after successful insertion of three ATGs. As a consequence, fragments of 140 and 310 bp disappeared in the lane of the mutant, and these fragments were substituted by a 450-bp fragment after agarose electrophoresis. While the wild-type DNA showed three bands, 7400, 310, and 140 bp, the mutant DNA showed two distinct bands, 7.4 kb and 450 bp, indicating the *Pst*I site at 6666 had been changed (Figure 3).

As a last step to confirm the insertion of three ATGs, sequencing was performed by using the dideoxy termination method. Double-stranded DNA of the mutant was prepared from one mutant phage and digested with *Pst*I and *Eco*RI. The 450-bp fragment was subcloned into RF of M13mp18 bacteriophage cleaved by *Pst*I endonuclease. The 230-bp fragment resulting from *Eco*RI and *Pst*I double

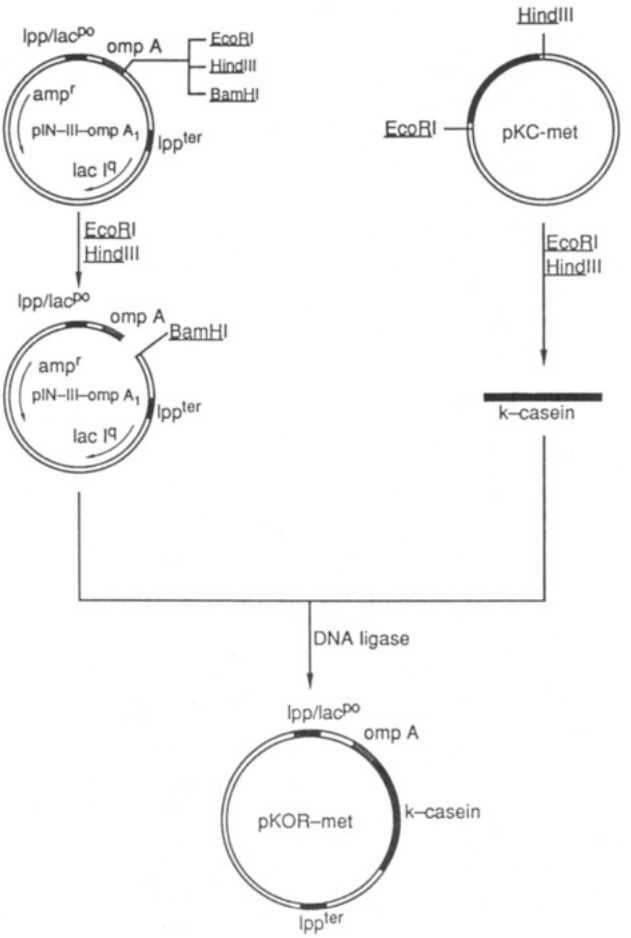

Figure 6. Schematic diagram for the construction of pKOR-Met. Both κ-casein cDNA and linearized pIN-III-ompA$_1$ were purified by double digestion with *Eco*RI and *Hin*dIII endonucleases. Two fragments were ligated by using T4 DNA ligase resulting in a plasmid to express mutant κ-casein in *E. coli*, pKOR-Met.

digestion of the construct was subcloned into *Eco*RI and *Pst*I endonuclease double-cleaved M13mp18 RF. Sequencing of the subcloned fragments resulted in the complete sequence for κ-casein. Universal primer (Pharmacia, Piscataway, NJ) and synthesized nucleotide were used as the primer for sequencing. The three methionine residues inserted between Ala$_{167}$ and Thr$_{168}$ of κ-casein are shown in Figure 4. There is no mutation in any other region of the treated κ-casein cDNA.

Construction of pKOR-Met. The expression vector, pIN-III-ompA$_1$, employs the efficient lipophosphoprotein (lpp) gene promoter (Nakamura and Inouye, 1982) to promote expression. Downstream of lpp, the lac UV5 promoter operator (lac^{op}) fragment was placed to regulate expression. Downstream of this tandem promoter region is a multiple restriction site linker. Cloning κ-casein gene with a compatible reading frame, therefore, resulted in the usage of the lipoprotein Shine–Dalgano sequence, initiation codon, leader sequence in addition to a termination codon and a ρ-independent efficient transcription termination signal.

The *Eco*RI and *Hin*dIII double-digested fragment of pKC encodes for seven additional amino acids at the amino terminus of the κ-casein. The 5′-terminal end of cloned cDNA has the correct reading frame with respect to the leader sequence and the linker provided in the vector, pIN-III-ompA$_1$. The resulting recombinant plasmid would have all the leader sequence of the omp gene (63 bp), the first amino acid sequence of the mature outer membrane

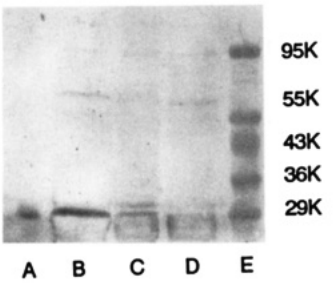

Figure 7. Localization of expressed κ-casein in *E. coli* AR68. *E. coli* AR68 harboring pKOR-Met was grown in LB medium. After induction with IPTG, bacteria were lysed osmotically. Periplasmic fraction was obtained from the supernatant fluid. Pellets from the osmotic lysis were subsequently fractionated into cytoplasmic and membrane fractions. Expressed κ-casein was detected by using immunochemical staining. (Lane A) Standard bovine κ-casein. (Lane B) Periplasmic fraction. (Lane C) Cytoplasmic fraction. (Lane D) membrane fraction. (Lane E) Molecular weight markers.

protein (omp) (3 bp), a part of the multicloning sites of M13mp18 bacteriophage (18 bp), the last amino acid of the bovine κ-casein signal peptide (3 bp), and the mature bovine κ-casein sequence (504 bp) (Figure 5).

As shown in Figure 6, *Hin*dIII and *Eco*RI endonuclease digested κ-casein cDNA fragment was force-cloned into the *Hin*dIII–*Eco*RI endonuclease cleaved expression vector, pIN-III-ompA$_1$. Therefore, κ-casein cDNA fragments were ligated to the expression vector, pIN-III-ompA$_1$, in only one orientation, which enables the expression of κ-casein under the control of the vector. The recombinant plasmid, pKOR-Met, was confirmed by restriction mapping. Methionine content of κ-casein was increased from 1.7 to 4.3 g/100 g by inserting three methionine residues between Ala$_{167}$ and Thr$_{168}$ of κ-casein using recombinant DNA technology.

Quantitation of Expressed κ-Casein. Expressed κ-casein in *E. coli* AR68 harboring pKOR-Met was quantitated by using laser densitometry after SDS–PAGE, Western blot, and immunochemical staining. A series of increasing amounts of standard κ-casein were used to prepare a standard curve. Cell extracts from 1 mL of culture after induction with IPTG was loaded in SDS–polyacrylamide gels. From the standard curve, the expressed κ-casein was determined to be 2 mg/L of medium.

Localization of Recombinant κ-Casein in *E. coli*. It has previously been demonstrated that the mature portion of β-lactamase (Ghrayeb et al., 1984), staphylococcal nuclease A (Takahara et al., 1985), or human granulocyte-macrophage colony-stimulating factor (Libby et al., 1987) are expressed with an ompA signal peptide fused to their amino termini. The nuclease and β-lactamase are efficiently guided to the periplasmic space, whereas the remaining product was membrane associated. Thus, an ompA signal peptide fusion protein can be guided to either the periplasmic space or the outer membrane. The expression vector pIN-III-ompA$_1$ was chosen because it has proven to effect secretion of expressed proteins out of the cytoplasm of *E. coli*, which may allow fewer steps in purifying the expressed protein. Also, the pIN-III-ompA$_1$ expression vector was available at the time these experiments were performed. However, in our hands, the expressed κ-casein accumulated in the periplasmic space. The use of a secretion vector in yeast was considered for secretion of the expressed protein into the medium, but a suitable vector was not available to us at that time.

E. coli AR68 harboring pKOR-Met was grown in LB medium. After induction for 4 h with IPTG, bacteria were

lysed osmotically according to the procedure of Libby et al. (1987). Pellets from the wash were subsequently fractionated into cytoplasmic and membrane fractions. Most of the κ-casein was detected in the periplasmic space, and less than 15% of the κ-casein was detected in the cytoplasm (Figure 7). No κ-casein band was detected in the membrane according to SDS–PAGE. Interestingly, there are two bands with similar intensity in the cytoplasm; one possesses a similar mobility to κ-casein secreted into the periplasmic space, and the other has less mobility. The band detected with less mobility may result from a lack of signal peptide processing. The ompA signal peptide appears to guide κ-casein out of the cytoplasm, where protease activity is generally high, and into the periplasmic space, where protein stability is enhanced.

For experiments on the nutritional quality, the bioavailability, and the functionality changes of the altered κ-casein, it will probably be necessary to improve the expression level and to further purify the expressed κ-casein mutant.

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