Study of Putative Glycosylation Sites in Bovine β-Casein Introduced by PCR-Based Site-Directed Mutagenesis

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The bovine β-casein gene, A^2 genetic variant, has been mutated at positions 70 and 71 for the introduction of a glycosylation signal (**Asn-X-Ser**). These mutants have been constructed to study the functionality of β-casein glycosylated exclusively at Asn_{68}. The mutation was generated using PCR-based site-directed mutagenesis, and it was derived from bovine β-casein cDNA. The mutant cDNAs including the wild-type β-casein gene have been subcloned into the yeast *Pichia pastoris* expression vector pHIL-D2, which contains the methanol-inducible alcohol oxidase (*AOX1*) promoter. Three expression vectors were constructed and designated pCJ-βWT (wild-type bovine β-casein gene), pCJ-β68 (substitution of Ser_{70} for Leu_{70}), and pCJ-β6873 (Ser_{70}-Ser_{71} for Leu_{70}-Pro_{71}). Bovine β-casein produced in yeast was found to contain a sugar moiety on Asn_{68} (N-linked glycosylated) when produced from a strain containing the pCJ-β6873 construct in its chromosome. N-Glycosylation of bovine β-casein at position 68 was completely inhibited in transformants carrying vector pCJ-β68 with the single substitution of Ser_{70} for Leu_{70}. The concentration of bovine β-casein in this expression system was in the range of 0.7−1.0 g/L.

Keywords: *Bovine β-casein; mutagenesis; Pichia pastoris*

INTRODUCTION

β-Casein is the second most abundant of the bovine caseins and a single polypeptide consisting of 209 amino acids (Walstra and Jenness, 1984). Its structure has been well characterized (Swaisgood, 1992) and presents important features that can be linked to its nutritional and calcium-transporting function. For example, the hydrophilic N-terminal region of bovine β-casein has a phosphoserine cluster in amino acid residues 15, 17, 18, and 19. Comparison of the reported cDNA sequences of β-casein in different species demonstrates that the N-terminal region is more conserved than the rest of the molecule, suggesting that this region is important for its biological function. In contrast, its C-terminal region is less conserved among species, very hydrophobic, and rich in proline residues, which limits the solubility of the protein. The amphiphilic nature of β-casein and high content of proline make it a good functional ingredient in processed foods. However, the functional properties of β-casein can be improved if the hydrophobic/hydrophilic contrast of the molecule is enhanced or if its solubility is improved. None of the β-casein genetic variants identified to date, nor their counterparts in domestic animals, are glycosylated (Ribadeau-Dumas et al., 1972; Grosclaude et al., 1972; Eigel et al., 1984).

Courthaudon et al. (1989) have demonstrated that chemical glycosylation of casein increased its solubility and changed viscosity. In addition, there are several examples where chemical modifications of casein have shown improved functionality by modifying functional groups of the protein (Chobert et al., 1987; Cayot et al., 1991; Kato et al., 1992). On the basis of these chemical studies, the addition of sugar residues to the N-terminal region of β-casein should increase its solubility and modify its functional properties. However, chemical modification of proteins is hard to control since it occurs randomly among functional groups of the protein. In contrast, genetic modification makes it possible to modify very specific amino acids by rational design of the protein. Thus, the objective of this work is to change the hydrophilic/hydrophobic balance at the N-terminal of the protein without disrupting the phosphoseryl cluster. We have designed putative N-linked glycosylation signals (**Asn-X-Ser**) to increase the hydrophilicity of the N-terminal region of bovine β-casein. Roitsch and Lehle (1989) have reported that proline residues at the C-terminal of the sequon Asn-X-Ser do not favor oligasaccharide transfer. Thus, the putative sites include the change of positions in one mutant, pCJ-β68, carrying the substitution of Ser_{70} for Leu_{70} and in the second mutant, pCJ-β6873, carrying Ser_{70}-Ser_{71} for Leu_{70}-Pro_{71}. These putative glycosylation signals are post- or cotranslationally modified by corresponding enzymes only in eukaryotes.

Structure modification of the protein using genetic modification and the study of properties of the novel protein require an efficient expression system. We have selected yeast cells as our expression system because it performs post-translational modifications similar to those of higher eukaryotes (King et al., 1989). Whereas a bacterial system such as *Escherichia coli* produces large amounts of heterologous proteins, it cannot perform post-translational modifications, especially glycosylation (Marston et al., 1986). The yeast *Pichia pastoris* has been successfully used for the production of heterologous proteins with high expression levels and can modify proteins post-translationally in a manner very similar to that of mammalian cells (Cregg et al., 1993). In this work, the yeast *P. pastoris* proved to be very efficient as a host strain for high-level production of mutated and wild-type bovine β-caseins.

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MATERIALS AND METHODS

Strains and Plasmids. *E. coli* XL1-blue (*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [F′ *proAB, lacI*^{q}*ZΔM15,* Tn*10* (tet^r)]^c (Stratagene, La Jolla, CA) was used for all plasmid construction and propagation. The *P. pastoris* GS115 (*his4*) was used as a host strain for transformation and for the production of altered bovine β-caseins. pCR II vector and pHIL-D2 were purchased from Invitrogen (San Diego, CA). The pCR II vector contains the *lacZ*α complementation fragment for blue-white color screening, ampicillin and kanamycin resistance genes for selection, and a polylinker sequence. pHIL-D2 is the *E. coli*−*P. pastoris* shuttle and integrating vector. It carries the methanol-inducible alcohol oxidase (*AOX1*) promoter and *HIS4* selectable marker. pJR37 (Jiménez-Flores et al., 1990) carries the wild-type bovine β-casein cDNA (A^2 genetic variant) lacking the ATG codon.

Site-Directed Mutagenesis. Site-directed mutagenesis using the polymerase chain reaction (PCR) was performed to alter specific nucleotides within a structural gene, the A^2 genetic variant of bovine β-casein (Jiménez-Flores et al., 1990). The mutagenic primers are complementary to the *Apa*I site at exon 7 of bovine β-casein gene (primer **N68**, 5′ CCC TTC CCT GGG CCC ATC CCT AAC AGC **TCC** CCA CAA AAC ATC CC 3′; primer **N6873**, 5′ CCT TTC CCT GGG CCC ATC CCT AAC AGC **TCC TCA** CAA AAC ATC CC 3′). Another primer in the opposite direction of the gene is used for PCR amplification of all the mutants (5′ AGC CGG ATC CTC TTA GAC AAT AAT AGG G 3′). PCR was performed using Vent (exo-) DNA polymerase (New England BioLabs, Beverly, MA). Amplification was carried out as follows: 100 ng of template DNA, 10 mM KCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100 (pH 8.8, 25 °C), 20 μM each of dNTP, 50 μM of each primer, and 2.5 units of Vent (exo-) DNA polymerase in a final volume of 100 μL. These samples were overlaid with 100 μL of mineral oil (Sigma Chemical Co., St. Louis, MO) and subjected to 1 cycle of 97 °C for 2 min, 55 °C for 1 min, and 72 °C for 1 min and then linked to 30 cycles of denaturation (97 °C, 1 min), annealing (55 °C, 1 min), and extension (72 °C, 1 min) using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). Additional extension (72 °C, 2 min) was done to ensure that the final extension step was complete. The PCR products were analyzed on an agarose gel containing 2% Nusieve, 1% SeaKem agarose (FMC, Rockland, ME), and 0.5 μg of ethidium bromide per milliliter in Tris−acetate buffer (pH 8.0) (40 mM Tris−acetate, 1 mM EDTA). Two mutant β-casein fragments (464 bp) from PCR amplification were subcloned into pCR II vector with single 3′ T-overhangs at the insertion site and confirmed by DNA sequencing prior to plasmid construction for yeast expression.

DNA Sequence Analysis. The presence of mutated sites was confirmed by both *Alu*I restriction enzyme digestion and DNA sequencing (Applied Biosystems Inc.). Sequencing was performed by using the Taq DyeDeoxy terminator cycle sequencing kit (University of Illinois, Sequencing Lab).

Plasmid Construction. DNA manipulation was performed according to standard techniques (Sambrook et al., 1989; Ausubel et al., 1990). As described in Figure 1, *Eco*RI fragments digested from pCR II vectors containing desired mutations were subcloned into pUC19 to generate the *Eco*RI site at the 3′ end of bovine β-casein cDNA. Then the resulting construct (pCJ1) was digested with *Apa*I and *Hin*dIII and the *Apa*I−*Hin*dIII fragment was substituted for the corresponding *Apa*I−*Hin*dIII fragment in pJR37-1, which carries wild-type bovine β-casein cDNA containing native bovine β-casein signal sequence. pJR37-1 was constructed from pJR37 lacking the ATG codon by generating the ATG codon in its signal sequence of bovine β-casein cDNA. The resulting pCJ137 carries bovine β-casein cDNA flanked by *Eco*RI, and finally the *Eco*RI fragment of pCJ137 was cloned into pHIL-D2 using the unique *Eco*RI site. For wild-type bovine β-casein, the *Apa*I−*Stu*I fragment of pJR37 was cloned into completed yeast expression vector containing mutant bovine β-casein cDNA. Therefore, wild-type and mutant bovine β-casein cDNAs are placed under the control of alcohol oxidase (*AOX1*) promoter, designated pCJ-βWT, pCJ-β68, and pCJ-β6873, respectively.

DNA Transformation. Vectors used for plasmid construction in *E. coli* were transformed by electroporation or calcium-induced competent cell methods (Sambrook et al., 1989). For yeast cell transformation, the yeast expression vectors containing wild-type and mutant bovine β-casein cDNA (pCJ-βWT, pCJ-β68, and pCJ-β6873) were digested with *Not*I restriction enzyme for induction of a homologous recombination between the *P. pastoris* vector and the alcohol oxidase gene (*AOX1*) in the host genome. *Not*I-linealized constructs (10 μg) were introduced into *P. pastoris* GS115 (*his*) by spheroplast transformation according to the protocol provided by the *Pichia* expression kit (Invitrogen, San Diego, CA). The resulting *Pichia* transformants with high expression level were designated WT (wild-type bovine β-casein producer), M68 (mutant β-casein producer carrying Ser_{70}), and M6873 (mutant β-casein producer carrying Ser_{70}-Ser_{71}).

Culture Conditions. For protein expression, transformants were grown to OD_{600} = 1.2−2.0 at 30 °C in minimal glycerol medium (MGY), which contained yeast nitrogen base without amino acids (13.4 g), biotin (400 μg), and glycerol (100 mL) per liter and then medium was replaced with minimal methanol medium (MM)−yeast nitrogen base without amino acids (13.4 g), biotin (400 μg), and methanol (5 mL) per liter, followed by an additional 4−6 day incubation with vigorous shaking (>250 rpm). The methanol concentration in the medium was maintained at 0.5% (v/v) for optimal induction during the entire expression period.

Preparation of Yeast Crude Cell Extract. Yeast cells were washed once in an equal volume of ice-cold breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, and 5% glycerol), and then the cell pellet was resuspended in ice-cold breaking buffer at an OD_{600} of 50. An equal volume of acid-washed glass beads (size 0.5 mm) was added. The cells were disrupted in cycles of 30 s of vortexing using a bead beater (Biospec Products, Bartlesville, OK), followed by 30 s of incubation on ice. This cycle was repeated eight times. The sample was centrifuged at 4 °C for 10 min at 10 000 rpm, and the clear supernatant lysate was used for SDS−polyacrylamide gel electrophoresis analysis.

Gel Electrophoresis and Western Immunoblotting. Yeast crude cell extracts were separated on 12% SDS−PAGE reducing gels (Laemmli, 1970) and were Western blotted according to the method of Jiménez-Flores et al. (1990). The transferred PVDF membrane was incubated with a rabbit anti-bovine β-casein antibody (Dr. Bruce Larson, Urbana, IL) at a 1:3000 dilution, followed by incubation with a 1:5000 dilution of horseradish peroxidase-conjugated mouse anti-rabbit IgG. The results were visualized by the developing solution of 3,3′-diaminobenzidine (Sigma).

Deglycosylation. Deglycosylation of the proteins was performed according to the instructions from Oxford GlycoSystem (Rosedale, NY). For PNGase F treatment, yeast crude cell extract was denatured by heating at 100 °C for 2 min in 10 μL of buffer (phosphate:EDTA, pH 7.5−8, containing 0.5% SDS, 5% β-mercaptoethanol). After cooling, 10% Triton X-100 was added at 5 times the concentration of the SDS prior to the addition of 2 units of peptide-*N*-glycosidase F (Oxford GlycoSystem) and incubated for 18 h at 25 °C. For *O*-glycanase treatment, yeast crude cell extract was denatured by heating at 100 °C for 2 min in 10 μL of buffer (100 mM sodium citrate−phosphate, pH 6, containing 0.1% SDS and 5% β-mercaptoethanol). After cooling, 10% Triton X-100 was added at greater than 10 times the concentration of the SDS prior to the addition of 3 milliunits of *O*-glycanase (Oxford GlycoSystem) and incubated for 20 h at 37 °C. Controls were prepared in the absence of enzyme for both enzyme reactions. Treated samples were analyzed using SDS-PAGE followed by Western immunobloting.

RESULTS AND DISCUSSION

Introduction of N-Linked Glycosylation Sequence into Bovine β-Casein cDNA. N-Linked glycosylation sequences (**Asn-X-Ser**) were introduced into

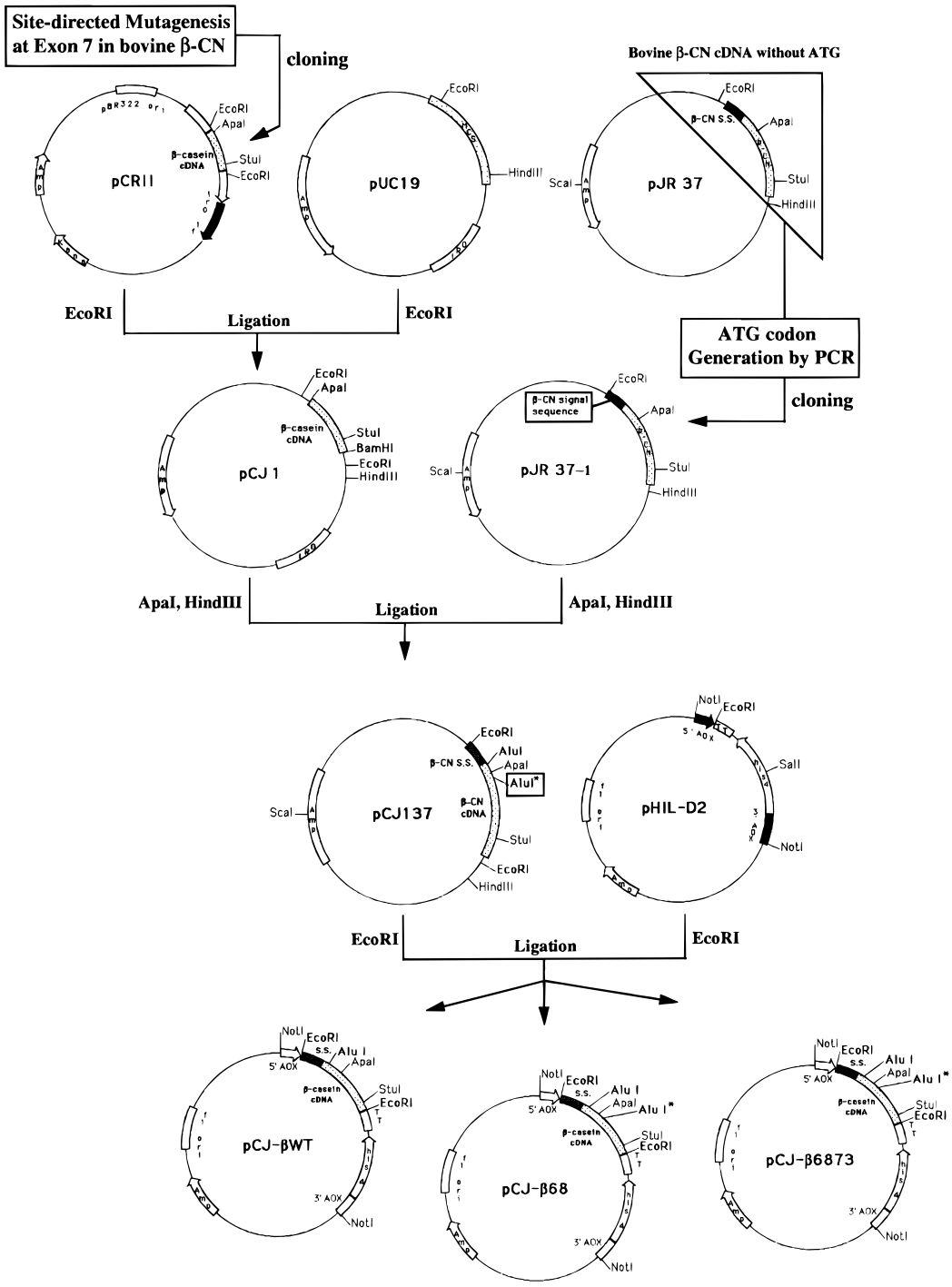

Figure 1. Construction of expression vectors for the production of wild-type and mutant bovine β-casein in the yeast *P. pastoris* (see Materials and Methods). *Alu*I* was generated by mutation in pCJ-β68 and pCJ-b6873 constructs.

bovine β-casein cDNA (A^2 genetic variant) by site-directed mutagenesis using the polymerase chain reaction. There are five Asn residues to select as targets for mutagenesis in β-casein. Asn_{132} was not considered as a possibility due to the very hydrophobic nature of the region around it and because at that position a glycosylation site would not enhance the hydrophylic/hydrophobic contrast of the molecule. Asn residues 7 and 27 correspond to very short gene exons that make mutagenesis in the structural gene very difficult and are very close to the cluster of phosphorylation sites. This proximity would be likely to interfere with the normal phosphorylation of the molecule. Thus, Asn_{68} was selected for this study over Asn_{73} (Choi et al., 1995). The triplet recognition sequence, **Asn-X-Ser**, for enzymatic N-linked glycosylation was generated by a substitution of $\mathbf{Ser}_{70}$ for $\mathbf{Leu}_{70}$ or a substitution of $\mathbf{Ser}_{70}$-$\mathbf{Ser}_{71}$ for $\mathbf{Leu}_{70}$-$\mathbf{Pro}_{71}$ in mature bovine β-casein as shown in Table 1. After site-directed mutagenesis, the resulting PCR products were subcloned into vector pCRII for DNA sequence analysis. Plasmid constructs containing desired mutations were screened using *Alu*I enzyme, which differentiates pCJ-β68 and pCJ-β6873 from the wild-type gene. Wild-type and mutant bovine β-casein cDNAs were cloned into the unique *Eco*RI site in the yeast expression vector, pHIL-D2, where expression of bovine β-casein is controlled by the methanol-inducible *AOX1* promoter. The strategy used for plasmid construction of vectors pCJ-βWT, pCJ-β68, and pCJ-β6873 is described in Figure 1.

Table 1. Mutation Sites of Bovine β-Casein cDNA Carrying Two Different Mutations[a]

		66 Ile	67 Pro	68 Asn	69 Ser	70 **Leu**	71 **Pro**	72 Gln	73 Asn	74 Ile	75 Pro	
pCJ-βWT (wild type)	5′	ATC	CCT	AAC	AGC	CTC	CCA	CAA	AAC	ATC	CCT	3′
pCJ-β68	5′	ATC	CCT	AAC	AGC	**TCC**	CCA	CAA	AAC	ATC	CCT	3′
pCJ-β6873	5′	ATC	CCT	AAC	AGC	**TCC**	**TCA**	CAA	AAC	ATC	CCT	3′

[a] Numbers indicate amino acid positions in bovine β-casein.

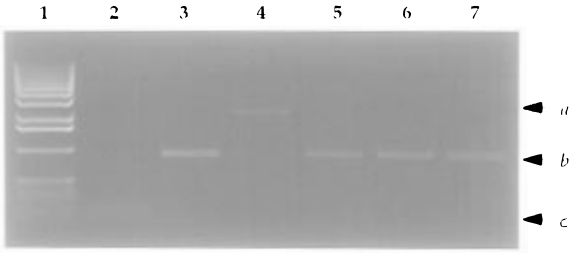

Figure 2. PCR analysis of bovine β-casein integrants on chromosomal DNA in *P. pastoris*: lane 1, 1 kb DNA ladder; lane 2, pHIL-D2 vector; lane 3, pHIL-D2 carrying bovine β-casein cDNA; lane 4, genomic DNA of GS115 (yeast host strain); lanes 5, 6, and 7, genomic DNAs of His^+, Mut^s transformants; WT, M68, and M6873, respectively. Arrow *a* indicates *AOX1*, arrow *b* indicates amplified bovine β-casein cDNA flanked by *AOX1* promoter, and arrow *c* indicates *AOX1* promoter.

PCR Analysis of β-Casein cDNA Integrant on *Pichia* Chromosomal DNA. *Not*I-linealized pHIL-D2 favors homologous recombination between the 5′ and 3′ *AOX1* sequences in pHIL-D2 (see pHIL-D2 in Figure 1) and those in the *Pichia* genome at the *AOX1* locus (Cregg and Madden, 1987; Cregg et al., 1989). Therefore, transformants carrying integrants at the *AOX1* locus on their chromosomal DNA cannot efficiently utilize methanol as a carbon source, showing no or slow growth on MM medium (mut^- phenotype), because of the displacement of the wild-type *AOX1* structural gene by foreign DNA construct.

Integration of wild-type and mutant bovine β-casein constructs into the *AOX1* locus on *Pichia* genome was confirmed by phenotype selection and PCR analysis. Colonies with His^+ Mut^s phenotype (His^+, ability to grow on histidine-deficient medium; Mut^s, slow methanol utilization) were selected, and their genomic DNAs were isolated, as well as from yeast host strain (GS115). These samples of chromosomal DNA were analyzed by PCR. As shown in Figure 2, pHIL-D2 vector (no insert) generated a band of 159 bp that corresponds to the *AOX1* primers (lane 2). In contrast, pHIL-D2 carrying bovine β-casein cDNA (one of the β-casein constructs, pCJ-βWT) resulted in a 987 bp band, composed of 828 bp from the β-casein insert and 159 bp from the *AOX1* promoter (lane 3). The resulting band from GS 115 indicated the absence of the β-casein gene and showed the wild-type alcohol oxidase gene, 2.2 kb (lane 4). Thus, the three transformants (WT, M68, and M6873), showing a band of 987 bp, demonstrate the presence of the β-casein gene on their genomic DNAs (lanes 5−7). The absence of wild-type *AOX1* (2.2 kb) after this PCR analysis and the His^+ Mut^s phenotype in WT, M68, and M6873 indicate that the integration mode of β-casein cDNA into *Pichia* genome is a gene replacement (omega insertion) at the *AOX1* locus (Rothstein, 1983; Struhl, 1983). This insertion is caused by a double crossover between the 5′ *AOX1* promoter and the 3′ *AOX1* regions of the vector and yeast genome. This kind of integration was consistent with His^+ Mut^s phenotype. If the insertion at *AOX1* had been caused by a single crossover, the phenotype of transformants would be His^+ Mut^+ (Mut^+, methanol utilization) because wild-type *AOX1* remains intact on their chromosomal DNA; thus, this kind of transformant can utilize methanol as a carbon source for growth. In addition, two different PCR bands from the wild-type *AOX1* (2.2 kb) and the *AOX1* promoter (159 bp) of integrated expression vector should have been generated by PCR. Our results demonstrate that the mode of integration in *Pichia* transformants carrying bovine β-casein integrants is a gene replacement at the *AOX1* locus.

Production of Mutant Bovine β-Casein in *P. pastoris*. Spheroplast transformation of *P. pastoris* GS115 with 10 μg of two *Not*I-digested constructs including pCJ-βWT (wild-type β-casein gene) resulted in about 25−30% of methanol-slow grown colonies (i.e., impaired growth on medium containing methanol as the sole carbon source), presumably due to the replacement of the *AOX1* structural gene with the expression cassette carrying bovine β-casein cDNA. The reason for slow or no growth on MM has been attributed to the *AOX1* deletion, where *AOX1* represents most of the alcohol oxidase activity (Cregg et al., 1993).

In general, high copy number integrants have expressed foreign proteins at high levels (Cregg et al., 1993). To screen high expression transformants, SDS−PAGE was run with yeast cell extract prepared from methanol-slow transformants (His^+ Mut^-). The total number of strains isolated that were found to be high producers of β-casein were as follows: two from pCJ-βWT, one strain from pCJ-β68, and two from pCJ-β6873. These strains represented 1−2% of the His^+ transformants isolated initially.

As seen in Figure 3A, bovine β-caseins were expressed at high levels in WT (carrying integrant of pCJ-βWT construct, lane 3), M68 (pCJ-β68, lane 4), and M6873 (pCJ-β6873, lane 5) as compared with GS115 (lane 2), and their mobilities appeared to be the same as that of the β-casein standard (lane 1) on SDS−PAGE. The amounts of β-casein produced intracellularly in *P. pastoris* were estimated using Collage image analysis (Fotodyne, New Berlin, WI) after Coomassie Blue staining of SDS−PAGE. The production of β-casein was in the range of 15−18% of total soluble proteins, and the estimated β-casein was approximately 0.7−1.0 g/L.

The presence of glycosylated β-casein in cell extract was identified using Western immunoblotting (Figure 3B). Glycosylated proteins are generally hard to visualize due to poor staining with Coomassie Blue, and this glycosylated casein was not apparent on our SDS−PAGE. The antibodies used did not cross-react with any of the yeast proteins. β-Casein or any other bands were not detected in GS115 control yeast cell extract (without β-casein gene, lane 2), whereas WT, M68, and M6873 transformants produced bovine β-casein. However, only M6873 (lane 5) had an extra band of slower mobility than the normal β-casein, presumably glycosylated bovine β-casein (lane 3).

Roitsch and Lehle (1987) and Bause (1983) have demonstrated that a Pro residue at the C-terminal region from a recognition sequence inhibits N-linked

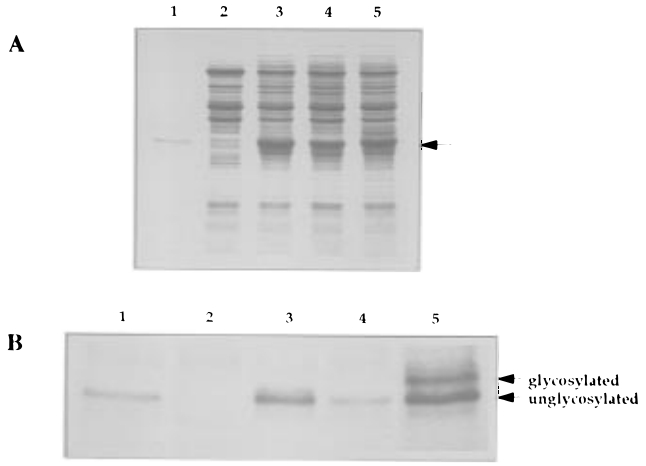

Figure 3. (A) SDS−polyacrylamide gel electrophoresis of crude cell extracts of *Pichia* transformants. After disrupting yeast cells, the supernatants of crude cell extracts were subjected to 12% SDS−PAGE and stained with Coomassie Blue: lane 1, bovine β-casein marker; lane 2, control yeast cell extract from GS115 without bovine β-casein gene; lane 3, WT transformant (wild-type bovine β-casein); lane 4, M68 transformant (mutant bovine β-casein carrying Ser_{70} in place of Leu_{70}); lane 5, M6873 (mutant bovine β-casein carrying Ser_{70}-Ser_{71} in place of Leu_{70}-Pro_{71}). Arrow indicates bovine β-casein produced by *Pichia* transformants (B) Western immunoblot of crude cell extracts of *Pichia* transformants. After a 12% SDS−PAGE was run, proteins on a gel were transferred to PVDF membrane (Millipore, Bedford, MA), followed by immunostain with bovine β-casein antibodies. Lanes are the same as above.

glycosylation at Asn residue in the triplet sequence. This evidence supports the theory N-glycosylation of Asn_{68} in M68 may be inhibited by Pro_{71} at the C-terminal region (Figure 3B, lane 4). That is, Pro_{71} in M68 completely prevented N-glycosylation of Asn_{68} even though there are the structural requirement as an acceptor for N-glycosylation, -Pro_{67}-**Asn**$_{68}$-**Ser**$_{69}$-**Ser**$_{70}$-Pro_{71}-. However, in the case of mutant β-casein M6873 (-Pro_{67}-**Asn**$_{68}$-**Ser**$_{69}$-**Ser**$_{70}$-**Ser**$_{71}$-, Asn_{68} was N-glycosylated (Figure 3B, lane 5). This indicates that the proline residue at the C-terminal side of the triplet sequence plays a significant role as a structural inhibitor for glycosylation.

Transformant M6873 produced glycosylated β-casein in addition to the unglycosylated form. The production of unglycosylated bovine β-casein in M6873 could be due to any of the many factors affecting glycosylation, for example, the accessibility of *N*-glycosyltransferases to the Asn residue in the triplet sequon due to local environment around glycosylation signals (Baenziger and Kornfeld, 1974; Rosner et al., 1980; Ward et al., 1980). Also, the processing enzymes show a difference in sensitivity to glycosylation between Asn-X-Ser and Asn-X-Thr (Bause, 1984; Kaplan et al., 1987; Gavel and Von Heijin, 1990; James et al., 1995). Another possibility is that the positioning of the sequon on the molecule may be exposed to the surface only partially in this very flexible molecule (Richardson et al., 1992).

Relative quantities of glycosylated to total β-casein produced by M6873 cannot be accurately determined by immunological methods since it is uncertain if the affinity of the antibody for the glycosylated form of β-casein is the same as that for the wild type. In fact, in our experience glyco-β-casein has been shown to be less sensitive to bovine β-casein antibody than nonglycosylated β-casein. Further studies include purification of each of the modified β-casein fractions for the study

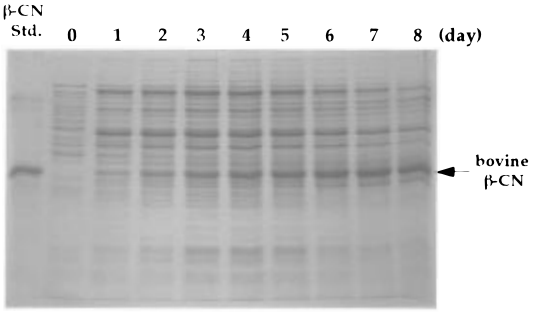

Figure 4. Production of mutant bovine β-casein by time course in *Pichia* transformant (M6873). The transformant was propagated in MGY, and then medium was replaced with MM for the induction of mutant bovine β-casein. The inducer, methanol, was maintained at the level of 0.5% in MM during culture. The production of mutant bovine β-casein was monitored from day 0 through day 8. Yeast cell extracts were analyzed using 12% SDS−PAGE.

of their properties. Additionally, purified glycosylated β-casein is necessary for the determination of its physical and chemical properties and identification of the sugar structure in this protein.

Production of recombinant β-casein was studied over culture growth time and showed that both wild-type and mutant β-casein increased in concentration with time after replacement of MGY with MM (Figure 4). This suggests that intracellularly produced β-casein is relatively stable in yeast, even though partially degraded β-casein was observed on SDS−PAGE and Western immunoblot after disrupting yeast cells. Proteolytic resistance of glycosylated β-casein in yeast (M6873) was observed comparable to wild-type β-casein (data not shown). This proteolytic resistance is in good agreement with results from Jiménez-Flores et al. (1990) and is presumably to be a property conferred to the protein by the putative sugar residue.

Localization of Mutant Bovine β-Casein Produced in *P. pastoris*. Most of the bovine β-casein was found in yeast cell extracts after disrupting cells. That is, wild-type and mutant bovine β-casein were largely produced intracellularly in *P. pastoris* and secreted into media at very low levels (1/20000 of the intracellular), even though the native bovine β-casein signal sequence was linked to β-casein cDNA. This indicates the lack of efficiency of the secretory signal of bovine β-casein in *P. pastoris*. Previous works have studied the localization of heterologous bovine β-casein in the yeast *Saccharomyces cerevisiae* and found it to be accumulated primarily in the cell's periplasmic space. Chung et al. (1991) reported that bovine β-casein containing its native signal sequence or the yeast invertase leader sequence was mainly secreted into the culture broth at level of 50 μg/L. However, Jiménez-Flores et al. (1990) reported that only intracellular expression of bovine β-casein was observed using a yeast-derived signal sequence.

Verification of N-Linked Glycosylation of Mutant β-Casein. As shown in Figure 5, only M6873 has been glycosylated. To prove that the sugar is attached via N-linked glycosylation, yeast cell extracts were treated with PNGase F, which cleaves between Asn and GlucNAc in N-linked oligosaccharides. After PNGase F treatment, samples were blotted to detect bovine β-casein using a rabbit anti-bovine β-casein antibody. The glycosylated band (Figure 5, lane 2) was deglycosylated by the removal of oligosaccharides, and after the treatment, it appeared at the same mobility as normal

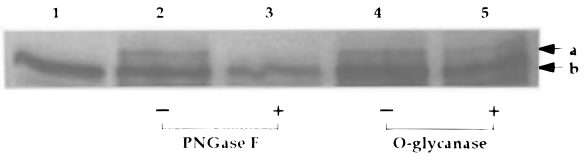

Figure 5. Western immunoblot of deglycosylated yeast cell extracts (M6873). Samples were treated with peptide-*N*-glycosidase F and *O*-glycanase (Oxford GlycoSystem), which cleave only N- and O-linked oligosaccharides, respectively. After 12% SDS−PAGE run, transferred samples on PVDF membrane were immunostained with bovine β-casein antibodies: lane 1, bovine β-casein marker; lanes 2 and 3, M6873 without and with PNGase F; lanes 4 and 5, M6873 without and with *O*-glycanase. Arrows a and b indicate glyco-β-casein and nonglycosylated bovine β-casein, respectively.

β-casein (Figure 5, lane 3). Therefore, this deglycosylation treatment showed that the higher molecular weight band observed over the authentic β-casein one was due to the sugar moiety on Asn_{68}.

In yeast, many proteins are O-mannosylated by attaching mannose residues to serine or threonine, whereas in mammalian cells O-glycosylation of proteins show a different core structure (Tanner and Lehle, 1987; Herscovics and Orlean, 1993). *O*-Glycanase was used to test if any sugars, except mannose, were linked to serine or threonine residues because *P. pastoris* mimics the glycoprotein structure found in higher eukaryotes. *O*-Glycanase cleaves between Galβ1-3GalNAc and serine or threonine alone. This enzyme treatment did not result in any protein band shifts (Figure 5, lanes 4 and 5), thus confirming N-glycosylation of the bovine β-casein along with the results of PNGase F treatment.

Yeasts are known to perform N- or O-linked glycosylation of proteins, and the mode of glycosylation and oligosaccharide structure are highly species dependent (Tanner and Lehle, 1987). Jiménez-Flores et al. (1990) have reported that wild-type bovine β-casein produced by *S. cerevisiae* was O-glycosylated. *P. pastoris* predominantly glycosylates proteins at the asparagine residues and rarely does so on serine (Cregg et al., 1993). Therefore, the use of *P. pastoris* allowed us to generate a more homogeneous protein that would yield more consistent results for structure/function studies of bovine β-casein than *S. cerevisiae*.

According to Western immunoblot analysis, N-glycosylated bovine β-casein, both from yeast and from transgenic mouse milk from another study, showed similar molecular weights (data not shown). Unlike hyperglycosylation of mannose in *S. cerevisiae*, *P. pastoris* glycosylated bovine β-casein in a pattern similar to that of higher eukaryotes in terms of the molecular weight of the oligosaccharide attached to Asn.

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