Expression and Purification of Glycosylated Bovine β-Casein (L70S/P71S) in *Pichia pastoris*

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Post-translational glycosylation of bovine β-casein (L70S/P71S) that results in Asn_{68}-linked glycan on the protein was obtained in up to 30% of total β-casein expressed in the methylotrophic yeast *Pichia pastoris*. Among the growth/induction media used, buffered minimal glycerol (BMG)/buffered minimal methanol (BMM) media were best for the production of glycosylated bovine β-casein, indicating pH-dependent glycosylation. Glycosylated bovine β-casein (L70S/P71S) expressed in *P. pastoris* was purified to homogeneity by the combination of ammonium sulfate fractionation, Concanavalin A−Sepharose affinity column, and Mono Q anion-exchange FPLC. The purified glycosylated bovine β-casein was specific only to Concanavalin A, and the oligosaccharide structure of glycosylated β-casein was of high-mannose type. Unlike the hyperglycosylation that occurred in yeast, the majority of bovine β-casein was not hyperglycosylated in *P. pastoris*, and its molecular weight was estimated to be 33.6 kDa. Glycosylated bovine β-casein was normally phosphorylated to the same degree as native bovine β-casein.

Keywords: *Glycosylated bovine β-casein; Con A column; Mono Q FPLC; Pichia pastoris*

INTRODUCTION

Many heterologous proteins have been produced using a variety of expression systems such as prokaryotic, eukaryotic, insect or mammalian cells, and, ultimately, transgenic animals to meet specific demands. High-value enzymes or pharmaceuticals have been produced in such systems. The main challenge in biotechnology is to produce these bioactive proteins in high yield and with high specific activity. In contrast, food proteins have not been extensively introduced in heterologous systems because of their abundance, wide variety of sources, and easy availability. However, the demand for novel food proteins with specific functionality has increased along with the necessity for specific functions of proteins for use in formulated foods. As a result, in an attempt to create a valuable model for structure−function study of caseins we have modified the β-casein gene and introduced it in the yeast *Pichia pastoris*. Subsequently, in this line of study, genetically engineered proteins must be purified from the expression systems for their evaluation and assessment.

For the purpose of the biochemical characterization or quantification, a number of techniques have been used to separate target proteins from a variety of protein sources. Whole casein is a complex mixture containing a number of fractions. Traditionally, fractionation of casein is achieved by low-pressure column techniques such as DEAE-cellulose (*1*, *2*) and hydroxyapatite (*3*, *4*). High-performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) technology have made it possible to achieve efficient separation of protein mixtures in a relatively short time. Thus, FPLC has become a useful tool in the dairy field. For instance, bovine β-casein genetic variants A^1, A^2, and B have been separated using cation-exchange chromatography from bovine casein (*5*) and from transgenic mouse milk (*6*). In addition, κ-caseins A and B and β-caseins A^1 and C were separated using anion-exchange chromatography (*7*, *8*). Besides ion-exchange chromatography, size exclusion (*9*−*11*), reversed-phase (*12*, *13*), and hydrophobic interaction chromatographies (*14*) have been used to fractionate each casein.

Bovine β-casein has been genetically modified to β-casein (L70S/P71S) and expressed in the methylotrophic yeast *P. pastoris*. Previous studies (*15*) detected bovine β-casein (L70S/P71S) on gels and suggested that a portion of the protein produced was N-glycosylated in *P. pastoris*. In this study the engineered glycosylated bovine β-casein produced in *P. pastoris* was purified and partially characterized.

MATERIALS AND METHODS

Strains, Media, and Production of Glycosylated Bovine β-Casein (L70S/P71S). The growth of *P. pastoris* GS115 carrying bovine β-casein (L70S/P71S) with an N-linked glycosylation sequence integrated into chromosomal DNA (strain M6873, His^+/Mut^s) and its induction were performed as described previously (*15*). Briefly, strain M6873 was grown to an OD_{600} of 1.5−2.0 at 30 °C in buffered minimal glycerol (BMG) containing 100 mM potassium phosphate, pH 6.0, yeast nitrogen base without amino acid (YNB) (13.4 g/L), biotin (400 μg/L), and 1% glycerol. The medium was replaced with buffered minimal methanol (BMM)−100 mM potassium phosphate, yeast nitrogen base without amino acid (13.4 g/L), biotin (400 μg/L), and 0.5% methanol, followed by an additional 5-day incubation with vigorous shaking (250 rpm). The methanol

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10.1021/jf001298f CCC: $20.00
Published on Web 03/22/2001

Table 1. Purification of Recombinant Bovine β-Casein Expressed in *P. pastoris*

purifn step	total protein (mg)	glyco-β-CN (mg)	purity (%)	fold purifn	yield (%)
cell lysate	4711.5	245.0	5.2	1.0	100.0
ammonium sulfate precipitate	2579.0	208.9	8.1	1.5	85.2
Con A column	137.0	115.1	84.0	16.1	46.9
Mono Q	99.3	96.0	96.6	18.5	39.1

concentration in the medium was maintained at 0.5% (v/v) for optimal induction during the entire expression period. In addition, the following media were used: (1) minimal glycerol medium (MGY)−yeast nitrogen base without amino acids, biotin, and 1% glycerol per liter; (2) minimal methanol medium (MM)−yeast nitrogen base without amino acid, biotin (400 μg/L), and 0.5% methanol; (3) buffered methanol−complex medium (BMMY)−yeast extract (10 g/L), peptone (20 g/L), 100 mM potassium phosphate, pH 6.0, YNB (13.4 g/L), biotin (400 μg/L), and 0.5% methanol; and (4) buffered glycerol−complex medium (BMGY)−yeast extract (10 g/L), peptone (20 g/L), 100 mM potassium phosphate, pH 6.0, YNB (13.4 g/L), biotin (400 μg/L), and 1% glycerol.

Purification of Glycosylated Bovine β-Casein. Glycosylated bovine β-casein was purified according to the following procedure, with all operations carried out at 4 °C, unless otherwise stated. A 5-day culture grown in BMM medium (1 L) as an induction medium for glycoslyated bovine β-casein production was harvested and resuspended in breaking buffer [0.1 M Tris, pH 8.0, 1 mM PMSF, 1 mM EDTA, and leupeptin (50 μg/mL)]. Cells were disrupted using a bead beater (Biospec Products, Barlesville, OK) with 0.5 μm glass beads. A clear cell lysate was obtained by centrifugation at 12000*g* for 30 min. Proteins in the supernatant were fractionated by the addition of solid ammonium sulfate, at which the supernatant was brought to 30% final concentration (w/v). The resulting precipitate was resuspended with binding buffer (20 mM Tris-Cl, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) for Concanavalin A (Con A)−Sepharose affinity chromatography (Pharmacia, Uppsala, Sweden) and dialyzed in the same buffer with three buffer changes. The dialyzed sample was centrifuged at 12000 rpm for 1 h and loaded at a rate of 1 mL/min onto the Con A−Sepharose column equilibrated in Con A binding buffer. The Con A−Sepharose column (1.0 × 10 cm bed size) was operated by the Econo system (Bio-Rad Laboratories, Hercules, CA). The column was washed with Con A binding buffer containing 5 mM α-methyl glucopyranoside until unbound proteins were not detected. Bound proteins were eluted with 0.2 M α-methyl glucopyranoside containing 20 mM Tris-Cl, pH 7.4, and 0.15 M NaCl. The fractions corresponding to elution peak were pooled and dialyzed against buffer A (25 mM Bis-Tris propane, pH 6.8) for Mono Q HR 5/5 anion-exchange column (Pharmacia) for further purification. Prior to application to the Mono Q HR 5/5 column, the dialyzed fraction was filtered through a 0.22 μm membrane (Nalge Co., Rochester, NY). The Mono Q column was operated by an HPLC system (Rainin Instruments Co., Woburn, MA), and the column was equilibrated in buffer A (pH 6.8). The elution was achieved using an increasing NaCl gradient in buffer B (25 mM Bis-Tris propane, pH 6.8, 0.5 M NaCl) at a flow rate of 1 mL/min. The elution of protein was monitored by its absorbance at 280 nm. The purification steps and final recovery of purified glycosylated bovine β-casein are outlined in Table 1.

Gel Electrophoresis and Western Immunoblotting. The samples were separated on 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (*16*) and were subjected to Western blotting according to the method of Jiménez-Flores et al. (*17*). The proteins in gels were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Millipore Co., Bedford, MA), and the membrane was incubated with a rabbit anti-bovine β-casein antibody (Dr. Bruce Larson, Urbana, IL), followed by incubation of horseradish peroxidase-conjugated mouse anti-rabbit IgG. The blot was visualized using a developing solution of 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO). Silver staining of SDS-PAGE was performed according to the manufacturer's instructions (Bio-Rad Laboratories), and the purity of glycosylated bovine β-casein from Mono Q fractions was determined using densitometry with NIH Image software (DCRT, National Institutes of Health, Bethesda, MD) after silver staining of SDS-PAGE and protein assay (*18*).

Con A Binding Analysis. The samples purified from yeast were separated by 12% SDS-PAGE and transferred to a PVDF membrane. The blotted proteins were analyzed using horseradish peroxidase-conjugated Con A (Con A, specific for N-linked high mannose) (Sigma Chemical Co.). The blot was visualized using a developing solution of 3,3′-diaminobenzidine (Sigma Chemical Co.).

Phosphorylation Analysis. Phosphorylation analysis was performed using calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim, Indianapolis, IN). The sample of purified protein (500 ng) was resuspended in 20 μL of alkaline phosphatase buffer (20 mM Tris, pH 8.0, 1 mM $MgCl_2$, and 1 mM $MnCl_2$) and 0.1 unit of CIP was added. The reaction mixture was incubated at 37°C for 30 min and analyzed using urea-PAGE (*19*).

Deglycosylation. Deglycosylation of the proteins was performed according to the instructions from Oxford GlycoSystem (Rosedale, NY). For peptide-*N*-glycosidase F (PNGase F) treatment, purified glycosylated bovine β-casein (60 μg) 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 and 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 PNGase F and incubated for 18 h at 25 °C. Controls were prepared in the absence of PNGase F. For the subsequent dephosphorylation of treated samples, the reaction buffer was exchanged with CIP buffer by using a Microcon 10 ultrafiltration device (Amicon, Beverley, MA). Dephosphorylation was performed as described above.

RESULTS AND DISCUSSION

Production of Bovine β-Casein (L70S/p71S) with N-Linked Glycan. Bovine β-casein (L70S/P71S) has been produced in *P. pastoris*, which is capable of producing recombinant β-casein at high levels (0.7−1.0 g/L) (*15*). On the basis of several time course experiments, the incubation time for the maximum production of bovine β-casein (L70S/P71S) was optimized to 120 h. There was no significant increase of bovine β-casein (L70S/P71S) and little change in glycosylation pattern over 120 h of incubation that was monitored by Con A binding assay (data not shown). In general, the glycosylation pattern is affected by a number of factors. The cell culture environment has influenced protein glycosylation in yeast (*20*). Therefore, to investigate the production efficiency of glycosylated β-casein (L70S/P71S), several growth and induction media were tried. As shown in Figure 1, glycosylated β-casein (L70S/P71S) was more abundantly produced in BMGY/BMMY (lane 2) and BMG/BMM (lane 3) than in MGY/MM (lane 4) and MGY/BMM media (lane 5). This implies that the buffered media (pH 6.0) such as BMGY/BMMY and BMG/BMM are suitable for the induction of more glycosylated β-casein (L70S/P71S) even though *P. pastoris* is capable of growing across a relatively broad range of pH from 3.0 to 7.0. In nonbuffered media (initial pH 5.5) such as MGY/MM, the pH of the culture medium at the end of induction dropped to 3.0, but the pH of the buffered medium was maintained at ∼6.0 during the induction period. In addition, there was no significant difference in the glycosylation patterns of bovine β-casein from nonbuffered MGY/MM (lane 4) and

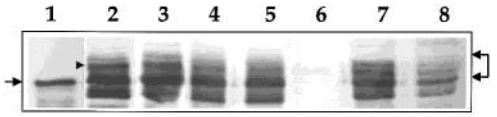

Figure 1. Effect of culture media on glycosylated bovine β-casein production. Yeast cell extracts were separated on 12% SDS-PAGE and then transferred to a PVDF membrane. Bovine β-casein on the membrane was visualized with a rabbit anti-bovine β-casein antibody. Lane 1, bovine β-casein standard; lanes 2−8, yeast cell extracts (strain M6873) [left arrow indicates bovine β-casein (nonglycosylated); arrowhead, glycosylated β-casein (33.6 kDa); and right arrows variously glycosylated β-casein (diffuse bands)]. The strain M6873 was grown, and bovine β-casein was induced in the following media (each medium composition was described under Materials and Methods): lane 2, BMGY/BMMY (pH 6.0); lane 3, BMG/BMM (pH 6.0); lane 4, MGY/MM (pH 3.0); lane 5, MGY/BMM (pH 6.0); lane 6, MGY/MM + 0.5% glycerol (pH 3.0); lane 7, MGY/MM + 2% peptone (pH 3.0); lane 8, MGY/MM + 1% casamino acid (pH 3.0). The pH indicates a final culture pH measured before cells were harvested.

from MGY/BMM (nonbuffered growth, but buffered induction medium) cultures (lane 5), suggesting that the pH of the growth medium should be maintained at pH 6.0 for more efficient glycosylation of β-casein (L70S/P71S). As shown in the effect of the pH of the culture media in the expression level and glycosylation of recombinant protein (*21*), it seems that the production of glycosylated β-casein (L70S/P71S) in *P. pastoris* is also affected by the pH of the growth medium. Neither bovine β-casein nor glycosylated β-casein (L70S/P71S) was produced in MM medium containing both methanol and glycerol in which they were maintained at 0.5 and 1% levels, respectively, during the induction period (lane 6). This indicates that despite the presence of methanol as an inducer, the addition of glycerol switched off bovine β-casein (L70S/P71S) expression that is driven by alcohol oxidase (*AOX1*) promoter. That is, *P. pastoris* is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source by alcohol oxidase (*AOX1*) that is tightly regulated and induced by methanol. Taken together, it is suggested that glycerol tightly represses the *AOX1* promoter by utilizing glycerol preferably rather than methanol as a carbon source. Barr et al. (*22*) have reported that peptone or casamino acids as amino acid-rich supplements were used to reduce protein degradation by yeast proteases in case the target proteins were secreted into media. Bovine β-casein has been produced intracellularly in *P. pastoris* using bovine β-casein signal sequence (*15*) and in *S. cerevisiae* using a yeast-based signal sequence (HXK1) (*17*). It has been reported as being secreted into culture media at very low levels (50 μg/L) using the yeast invertase leader sequence (*23*). On the basis of previous studies in bovine β-casein production with its native or yeast-based signal sequences, it is not likely to secrete bovine β-casein into culture media even if there have been many heterologous proteins secreted successfully using the α-factor prepro leader sequence in *P. pastoris*. However, the inclusion of 2% peptone in MM increased glycosylated β-casein (L70S/P71S) production slightly (lane 7), compared to that in MGY/MM, whereas 1% casamino acids in MM resulted in a decrease in overall glycosylated β-casein (L70S/P71S) production (lane 8).

The heterogeneity in the molecular mass of the expressed β-casein (33.6 kDa and diffuse bands) was due to the diversity of oligosaccharide structure attached to the protein (Figure 1). The resulting glycosylation pattern has been verified by PNGase F treatment in a previous study during which enzymatic deglycosylation resulted in only one band at 30 kDa (*15*).

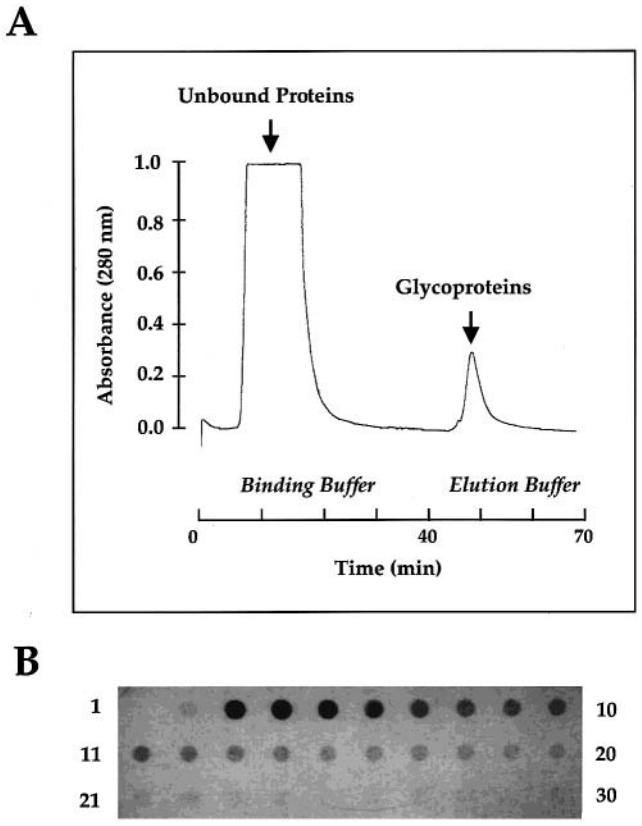

Figure 2. Con A−Sepharose affinity chromatography. (A) Yeast cell extract (M6873) fractionated with 30% ammonium sulfate (w/v) was applied to the Con A−Sepharose column, and then the unbound proteins were washed with Con A binding buffer containing 5 mM α-methyl glucopyranoside. Total glycoproteins were eluted for 30 min using elution buffer containing 0.2 M α-methyl glucopyranoside. Thirty fractions (1 mL/min) were collected from 41 min through 70 min. (B) is a dot blot of fractions eluted from the Con A−Sepharose column. Twenty-five microliters of each fraction (1−30) was spotted on the membrane. Bovine β-casein was immunostained using a rabbit anti-bovine β-casein antibody.

Purification of Glycosylated Bovine β-Casein (L70S/P71S). The proteins in the disrupted cell lysate were fractionated by the addition of ammonium sulfate at which most of the β-casein (L70S/P71S) was precipitated at 30% (final concentration). The precipitate was used for the subsequent purification. First, glycoproteins were isolated from the precipitate using Con A−Sepharose affinity column (Figure 2A). To reduce contaminants, the stepwise elution was performed with 5 mM/0.2 M α-methyl glucopyranoside. That is, after the column had been washed out with 5 mM α-methyl glucopyranoside, where the eluted solution contained degraded β-casein and other minor contaminants from yeast glycoproteins, the glycosylated β-casein (L70S/P71S) was eluted with 0.2 M α-methyl glucopyranoside because uniformly glycosylated β-caseins were observed in the eluates using 0.2 M α-methyl glucopyranoside. Pooled fractions (from 2 through 22) (Figure 2B) corresponding to the glycoprotein elution peak were further purified using Mono Q anion-exchange column (Figure 3A), by which homogeneous glycosylated bovine β-casein (Figure 3B,C) was obtained using a linear NaCl gradient between 0 and 0.15 M.

Con A binds to unsubstituted nonreducing α-D-glucose and α-D-mannose residues (*24*), but weakly. The basic

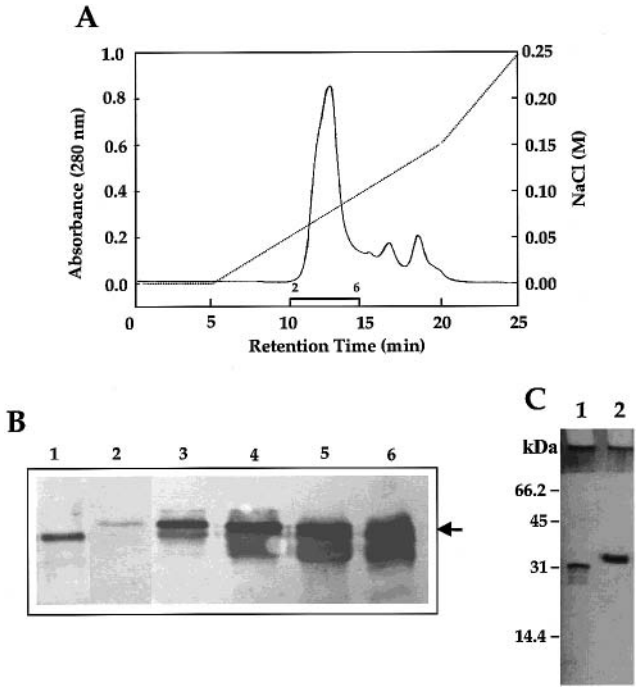

Figure 3. Mono Q anion-exchange chromatography. (A) The glycoprotein fractions eluted from the Con A−Sepharose column were loaded onto a Mono Q anion-exchange column. The elution (1 mL/min) of glycosylated β-casein was achieved using an increasing NaCl gradient: (—), absorbance (A_{280}); (- - -), NaCl gradient. (B) is a Western immunoblot of Mono Q fractions. Lane1, bovine β-casein standard; lanes 2−6, Mono Q fractions 2−6 in chromatogram (arrow indicates glycosylated bovine β-casein). (C) is a silver-stained SDS-PAGE of the Mono Q fraction. Lane 1, bovine β-casein standard; lane 2, purified glycosylated bovine β-casein.

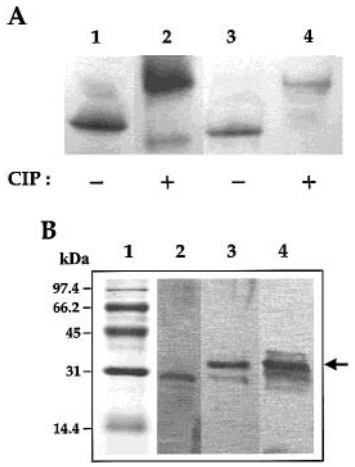

Figure 4. (A) Western immunoblot of dephosphorylated recombinant bovine β-casein. Prior to dephosphorylation of glycosylated β-casein, it was deglycosylated with PNGase F. Bovine β-casein standard and glycosylated β-casein were treated with CIP and then separated on urea-PAGE, followed by Western immunoblot. Lanes 1 and 2, bovine β-casein standard; lanes 3 and 4, recombinant bovine β-casein. (B) is a Western immunoblot and Con A blot of the Mono Q fraction. Lane 1, protein low molecular weight marker (Coomassie blue-stained); lane 2, bovine β-casein standard; lanes 3 and 4, recombinant bovine β-casein. Lanes 2 and 3 were immunostained with rabbit anti-bovine β-casein antibodies, and lane 4 was visualized with horseradish peroxidase-conjugated Con A (arrow indicates glycosylated bovine β-casein).

requirement is that hydroxyls at C-3, C-4, and C-6 must be available where the trimannosyl core structure, Manα1−6(Manα1−3)Man, specifically binds at high affinity to oligosaccharides (*25*, *26*). Con A has adifferent binding strengths to oligomannose structures ranging from mono- to penta-anntenary. That is, Con A is known to have a strong affinity to biantennary oligosaccharide structure but has no affinity to triantennary type of complex glycosyl chains (*27*, *28*). Thus, the Con A−Sepharose column was effectively used for the separation of high-mannose type glycoproteins from the non-glycoprotein mixture in yeast cell extract; the fractions from the Con A−Sepharose column mainly consisted of glycosylated β-casein with minor contamination of yeast glycoproteins (data not shown). In addition, Con A has a strong affinity to N-linked oligosaccharides, whereas it has no or very weak affinity to O-linked oligosaccharide (*29*). This affinity characteristic allows the separation of N-linked glycoproteins from O-linked glycoproteins by using the differential elution. As a further purification step, a Mono Q anion-exchange column was found to be an efficient way to separate glycosylated β-casein (L70S/P71S) from other yeast glycoprotein contaminants present in the Con A−Sepharose column fraction. The purity of isolated glycosylated β-casein (L70S/P71S) was estimated to be >96% (Figure 3C).

Bovine β-casein (L70S/P71S) was relatively stable to degradation in intracellular expression of *P. pastoris*, but it was considerably degraded during purification steps, especially dialysis against Con A binding buffer in the absence of protease inhibitors. This degradation is considered to be due to residual yeast proteases. Thus, the use of a protease-deficient yeast strain (*30*, *31*) or secretion of recombinant protein into the culture medium could be an alternative to reduce the degradation of the target proteins expressed in yeast.

Phosphorylation State of Glycosylated β-Casein (L70S/P71S). Phosphorylation of casein is a very important post-translational modification. It allows casein micelle formation and calcium transport and provides phosphate for the developing neonate. Bovine β-casein (A^2 genetic variant) is a phosphoprotein that contains five phosphoseryl groups at its N-terminal region. These phosphorylation sites have been conserved among species. These phosphates are involved in the formation of casein micelle structure by calcium phosphate bridges and are considered to be important for carrying mineral ions such as Ca^{2+} and Zn^{2+} (*32*). Glycosylated bovine β-casein (L70S/P71S) produced from *P. pastoris* was analyzed using CIP and urea-PAGE to determine their phosphorylation states. As shown in Figure 4A, the deglycosylated β-casein treated with CIP (lane 4) showed a slower migration than untreated deglycosylated β-casein (lane 3) on urea-PAGE because the removal of phosphates from proteins resulted in a change of the native charge of the proteins. On the basis of authentic bovine β-casein migration with or without CIP treatment on urea-PAGE (Figure 4A, lanes 1 and 2), this indicates that glycosylated β-caseins were phosphorylated to the same degree as authentic bovine β-casein. In some cases, it has been reported that mannose in core N-glycan structure or outer mannose chain were phosphorylated in yeast (*33*, *34*). This phosphomannose can change protein migration on urea-PAGE. However, glycosylated bovine β-casein has shown the same protein migration as native bovine β-casein (nonglycosylated but phosphorylated) on urea-PAGE, which is the evidence of absence of phosphomannose (data not shown).

The attachment of oligosaccharides at Asn_{68} in bovine β-casein (L70S/P71S) did not interrupt phosphorylation

of the protein, which is an important factor in the functional properties of this protein.

Glycosylation of Bovine β-Casein (L70S/P71S). The oligosaccharide of glycosylated bovine β-casein (L70S/P71S) produced in yeast *P. pastoris* was analyzed using lectin binding assays. Lectin blot analysis of glycosylated β-casein showed its specificity to Con A, confirming that the attached sugar is a high-mannose type oligosacchride structure and is similar to glycoproteins normally found in yeast (Figure 4B, lane 4).

On the basis of the relative mobility of glycosylated β-casein (L70S/P71S) on SDS-PAGE, its molecular weight was estimated to be ~33.6 kDa. The molecular weight of bovine β-casein (A^2) calculated from amino acid sequence is 23573 Da, but it migrates at ~30 kDa on SDS-PAGE. It has been proposed that the abnormal migration of bovine β-casein is due to the uneven distribution of negative charge along the polypeptide chain (*35*) and excessive binding of SDS to β-casein (3.4 g of SDS/g), compared to other proteins (1.4 g/g of protein) (*36*). Bovine β-casein (the native cDNA) produced in *Saccharomyces cerevisiae* was glycosylated despite the absence of any N-glycosylation site. The glycosylated form of casein had a relative molecular weight of 36 kDa and was determined to have an O-linked sugar moiety (*17*). In contrast, the native β-casein was not glycosylated in *P. pastoris*. This contrast between the two yeast species is unexplained because O-mannosylation has been found in some recombinant proteins expressed in *P. pastoris* (*37*−*39*).

On the bais of the size of the N-linked oligosaccharide structure of the glycoprotein produced in *P. pastoris*, the majority of oligosaccharide structure consists of Man8−11 (*40*). The addition of ~3.6 kDa of glycan to β-casein (L70S/P71S) suggests that the size of N-glycan is in the range of that of Man8−14. Unlike the hyperglycosylation (≥Man50) of proteins found in *S. cerevisiae* (*41*, *42*), *P. pastoris* did not hyperglycosylate bovine β-casein (L70S/P71S); a very interesting fact is that this protein seemed to have a molecular weight similar to that of glycosylated β-casein (L70S/P71S) produced in transgenic mice (*43*, *44*).

The amphiphilic nature of bovine β-casein has been correlated with emulsifying, foaming, and gelling characteristics. Because of the chemical glycosylation of bovine casein (*45*), the post-translational addition of sugar at the asparagine residue 68 would make more polar the N terminus of β-casein (L70S/P71S), increasing its solubility and water holding capacity with a concomitant increase of its amphiphilic characteristics. This novel functional protein would have high potentials for food applications that need its beneficial properties. This purified protein is further being studied for its altered functional properties; preliminary experiments have shown dramatically increased solubility and interfacial properties.

ABBREVIATIONS USED

Glyco-β-CN, glycosylated β-casein (L70S/P71S); PMSF, phenylmethanesulfonyl fluoride; Asn, asparagine; Man, mannose; PVDF, poly(vinylidene difluoride); HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; BMM, buffered minimal medium; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CIP, calf intestinal alkaline phosphatase; MGY, minimal glycerol medium; MM, minimal methanol medium; BMMY, buffered methanol-complex medium; BMGY, buffered glycerol-complex medium; BMM, buffered minimal methanol medium; BMG, buffered minimal glycerol; EDTA, ethylenediaminetetraacetate.

ACKNOWLEDGMENT

We acknowledge the work of the Cal Poly DPTC staff. We especially thank K.J.S. for help with the manuscript.

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Received for review October 31, 2000. Revised manuscript received February 14, 2001. Accepted February 14, 2001. We acknowledge support for this work from DMI and CDRF.

JF001298F