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Secretion of Glycosylated α-Lactalbumin in Yeast *Pichia pastoris*

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The secretion of *N*-linked glycosylated α-lactalbumin was much higher in the expression system of yeast *Pichia pastoris* carrying goat α-lactalbumin cDNA than in mammalian milk. This is possibly because of the presence of *N*-linked glycosylation signal sequences, Asn[45]-Asp[46]-Ser[47] and Asn[74]-Ile[75]-Ser[76], in wild-type α-lactalbumin. Attempts to elucidate the mechanism of the higher secretion of glycosylated α-lactalbumin in *P. pastoris* were made. Mutant N45D that deleted the *N*-linked glycosylation signal sequence at position 45 predominantly secreted nonglycosylated protein. On the other hand, mutant D46N with another *N*-glycosylation signal site at position 46 only secreted *N*-linked glycosylated α-lactalbumin, *i.e.* not the nonglycosylated protein. The total secreted amount of mutant N45D was greatly enhanced, while the secreted amounts of the wild-type and mutant D46N were very low, suggesting that the increase in the number of glycosylation sites greatly reduced the secretion of α-lactalbumin. It seems likely that the glycosylated α-lactalbumin may be degraded by the quality control system.

Key words: α-lactalbumin, *N*-linked glycosylation, *Pichia pastoris* expression.

It is well known that α-lactalbumin is a major whey protein in the milk of various mammalian species and is involved in the synthesis of lactose. In addition to this function, it has been reported that α-lactalbumin induces the apoptosis of mammary cancer (*1, 2*), and that folding variants exhibit antimicrobial activity (*3, 4*). Although a small part of α-lactalbumin occurs in the glycosylated form in fresh milk, not much attention has been paid to the glycosylated form, because most α-lactalbumins are secreted in the nonglycosylated form in mammalian milk. The carbohydrate moiety does not seem to be involved in lactose synthesis (*5*). However, it is noteworthy that the consensus signal sequence for *N*-linked glycosylation (Asn-X-Ser/Thr) is conserved in human, bovine and goat α-lactalbumins at positions 45 and 74. What is the physiological significance of glycosylated α-lactalbumin? It might be related to functions such as the apoptosis and antimicrobial actions. In order to elucidate the structural and functional relationship of glycosylated α-lactalbumin, we investigated the expression of α-lactalbumin in yeast *Pichia pastoris*. The expression of proteins in yeast has the advantage of protein folding in a similar manner to as in eukaryotes. In addition, yeast can attach the *N*-linked carbohydrate chain at the amide nitrogen of an asparagine residue in a protein that has the consensus signal sequence Asn-X-Thr/Ser for *N*-glycosylation as well as mammalian cells can.

It is striking that goat α-lactalbumin was obtained in the glycosylated form in a considerable secreted amounts in yeast *P. pastoris*, unlike in mammalian milk. Solution of this contradiction may shed light on the fate of the glycosylated α-lactalbumin in yeast and mammalian cells.

This paper describes the expression and secretion of wild-type α-lactalbumin and the mutants with decreased or increased *N*-linked glycosylation sites in yeast *P. pastoris*, and discusses the possible fate of glycosylated α-lactalbumins in the quality control system in ER.

MATERIALS AND METHODS

Materials—Restriction endonucleases, T4 DNA ligase and *Dpn*I were purchased from Takara Shuzo (Kyoto). The DNA sequencing kit was also purchased from Takara Shuzo. Synthetic oligonucleotides were purchased from Amersham Japan. DNA sequencing was carried out with a Big Dye™ Terminator Cycle Sequencing from Ap-plied Biosystems (USA).

An EasySelect™ Pichia Expression Kit and YNB (Yeast Nitrogen Base) were purchased from Invitrogen (Carlsbad, CA). The pT7 Blue T-vector was purchased from Novagen (USA). Endo-β-*N*-acetylglucosaminidase (Endo-H) was purchased from Bio Rad (New England). The DEAE-Sepharose FF resin used for purification of α-lactalbumin was purchased from Pharmacia (Sweden). The bovine α-lactalbumin used as a control was purchased from Sigma Chemical (USA). Goat α-lactalbumin cDNA was prepared from mammary gland tissue by RT-PCR. The rabbit anti–bovine α-lactalbumin antiserum for Western blotting was purchased from Cosmo Bio (Tokyo). An ECL+Plus kit (Western blotting detection reagents) was purchased from Amersham Pharmacia Biotech. (UK). All other chemicals were of analytical grade for biochemical use.

Strains—*Escherichia coli* XL-1 blue (*lac* [F′*pro*AB, *lac*I^{q}ZDM15, Tn*10*(Tet^R)] *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1) and Top10F′ (F′[*pro*AB, *lac*I^q ZΔM15, Tn*10*(Tet^R)] *mcr*A, Δ(*mrrhsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, λ*ara*D139, Δ(*ara*-

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Abbreviations: RT-PCR, reverse transcriptase–polymerase chain reaction; ER, endoplasmic reticulum; Endo-H, endo-β-*N*-acetylglucosaminidase; Asn, asparagine; Glu, glutamic acid; Ser, serine; Ile, isoleucine; Thr, threonine; Man, mannose; NAG, *N*-acetyl glucosamine.

leu)7697, *gal*U, *gal*K, *rps*L(Str^R), *end*A1, *nup*G) were used as host cells for cloning experiments. *P. pastoris* X-33 (wild-type, Mut$^+$) was purchased from Invitrogen as a yeast expression strain for α-lactalbumins.

Plasmids—The pT7 Blue T-vector was used for the cloning of PCR products. Vector pPICZαA is an *E. coli–P. pastoris* shuttle vector with sequences required for selection in each host. Vector pPICZαA contains an α-factor secretion signal that allows the efficient secretion of most proteins from *P. pastoris*. Vector pPICZαA contains 5′-AOX, a 942 bp fragment containing the AOX 1 promoter that allows methanol-inducible and high-level expression in *P. pastoris*, and the Zeocin™ resistance gene for positive selection in *E. coli* and *P. pastoris*.

Growth Media—*E. coli* cells were grown at 37°C in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) and low salt LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5). These media were supplemented with 50 μg/ml carbenicillin and 25 μg/ml Zeocin™, respectively, for the selection of transformants. The yeast cells were grown at 30°C in YPD medium (2% tryptone, 1% yeast extract, 2% glucose) and on YPDS plates (2% tryptone, 1% yeast extract, 2% glucose, 1 M sorbitol, 2% agar). The media and plates were supplemented with 100 μg/ml Zeocin™ for selection of *Pichia* transformants. Yeast cells were induced at 30°C in BMM (Buffered Minimal Methanol) medium (100 mM potassium phosphate, pH 7.5, 1.34% YNB, 4 × 10^{-5}% biotin, 0.5% methanol) for small scale expression, and SM (Synthetic Minimal) medium (trace element, salt stock, vitamin, methanol) for large scale expression (*6*).

Quick Change Site-Directed Mutagenesis of α-Lactalbumin cDNAs—Quick Change site-directed mutagenesis of α-lactalbumin cDNAs was carried out using PCR methods, as follows. Template DNA (50 μg), Ex Taq DNA polymerase (1 μl:2.5 U/μl), Ex Taq reaction buffer (5 μl), and a dNTP mixture (1 μl:2.5 mM) were used for PCR. *Dpn*I restriction enzyme (1 μl:10 U/μl) was used for the digestion of template DNA. PCR was performed by three-temperature cycling (95°C for 30 s, 50°C for 1 min, and 68°C for 9 min) for 16 cycles. *E. coli* XL-1 blue competent cells were used for the ligation of PCR products. The synthetic oligonucleotide primers were 5′-AGTACAAAACGATGACAGCAC-3′ (sense) and 5′-GTGCTGTCATCGTTTTGTACT-3′ (antisense) for mutant N45D, and 5′-GTACAAAACAATAACAG-CACAG-3′ (sense) and 5′-CTGTGCTGTTATTGTTTTGT-AC-3′ (antisense) for mutant D46N. After PCR the methylated parental DNA template was digested with *Dpn*I for 2 h at 37°C, and then the reaction mixture containing DNA with the designed mutation was transformed into *E. coli* XL-1 blue. After transformation, the *E. coli* XL-1 blue cells repair the nicked DNA. These colonies were selected on LB agar plates with 50 μg/ml carbenicillin. The mutations were confirmed by DNA sequencing.

Construction of an Expression Vector (pPICZαA / α-LA)—Synthetic oligonucleotide primers 5′GGGCTCGAGAAAAG-AGAACAATTAACAAAATGTGAGG-3′ (A) and 5′-GGGTC-TAGATTTTATTCAGGACAGAAGTGGA-3′ (B) were used for PCR to construct an α-lactalbumin cDNA with the new restriction enzyme sites underlined, *Xho*I and *Xba*I, respectively. PCR was carried out as follows. α-Lactalbumin derived pT7 blue plasmids were linearized with *Eco*RI. Mixtures contain linearized template dsDNA of α-lactalbumin (100 ng), primer A (10 pmol), primer B (10 pmol), Ex Taq DNA polymerase (1 μl:2.5 U/μl), Ex Taq reaction buffer (5 μl), and dNTP (5 μl) were subjected to PCR. PCR was performed by three-temperature cycling (94°C for 30 s, 58°C for 30 s, and 72°C for 1 min) for 25 cycles. The PCR products were confirmed by 1.0% agarose gel electrophoresis, and the gel pattern of α-lactalbumin cDNAs was confirmed, with a band at around 600 base pairs. Then the cDNA bands were extracted with glass milk and ligated into the pT7 blue T-vector. These plasmids were transformed into *E. coli* XL-1 blue. After transformation, these colonies were selected on LB agar plates with carbenicillin (50 μg/ml). Subsequently, these pT7 blue plasmids including α-lactalbumin cDNAs were purified from *E. coli* XL-1 blue on a large scale, digested with restriction enzymes *Xho*I and *Xba*I for 1 h at 37°C, and then confirmed by 1.0% agarose gel electrophoresis. The gel pattern showed two bands at around 2.9 k and 600 base pairs, which correspond to the band of α-lactalbumin cDNA.

For construction of *P. pastoris* X-33 expression plasmids, pPICZαA/α-LA, an α-lactalbumin cDNA derived pT7 blue plasmid, and pPICZαA vector were digested with *Xho*I and *Xba*I, respectively. When pPICZαA was digested with *Xho*I and *Xba*I, a band corresponding to 3.6 k base pairs appeared, and this band was extracted from the 1.0% agarose gel with glass milk. Subsequently, the extracted cDNA was inserted into the *Xho*I and *Xba*I sites of the pPICZαA vector. The constructed plasmid containing α-lactalbumin was transformed into *E. coli* Top10F′. After transformation, colonies were selected on low salt LB agar plates (pH 7.5) with Zeocin™ (25 μg/ml). Subsequently, to confirm transformation, the selected colonies were cultured on a small scale, and then expression plasmid pPICZαA was purified from *E. coli* Top10F′.

Transformation of the Wild-Type and Mutant α-Lactalbumin cDNA into P. pastoris X-33—Finally, these plasmids were isolated on a large scale in low salt LB medium including Zeocin™, and then linearized with *Bst*XI for 2 h at 45°C for efficient transformation in *P. pastoris* cells. The constructed plasmid linearized with *Bst*XI was introduced into *P. pastoris* X-33 by electroporation using Gene Pulser™ (Bio Rad). The linearization was confirmed by 1.0% agarose electrophoresis.

α-Lactalbumin cDNA is coded from the kex 2 signal cleavage site to the *Xba*I site region. The α-factor prepro-sequence is cleaved at kex 2 signal cleavage site during secretion of α-lactalbumin. Zeocin™-resistant transformants were screened by subculturing on YPDS agar plates with Zeocin™ (100 μg/ml) for 2–3 days at 30°C. After cultivation, well-growing colonies were then replica-cultivated in YPD medium with Zeocin™ (100 μg/ml) on a small scale (4 ml) at 30°C with shaking.

Secretion and Purification of the Wild-Type and Mutant α-Lactalbumins—*P. pastoris* transformed with α-lactalbumin cDNA was cultured in YPD medium with Zeocin™ (100 μg/ml) on a small scale (4 ml) at 30°C with shaking. One milliliter of this culture was transferred to 200 ml of fresh YPD medium in a one liter triangular flask and cultured under the same conditions with shaking for 16–18 h until the turbidity (OD_{600}) of the culture reached 2–6. Subsequently, the cells were harvested by centrifugation (1,500 ×*g*, 5 min) and the cell pellets were resuspended to 200 ml of SM medium in a 3 liter Sakaguchi flask. To induce expression of the wild-type and mutant α-lactalbumin, the

cultures in 200 ml of SM medium were incubated for 2–5 days at 30°C with shaking, methanol being added to a final concentration of 0.5% every 24 h. All growth medium of the host cells was centrifuged at 5,000 rpm for 5 min to remove the cells at 4°C. The supernatant was adjusted at pH 7.5 with 1 N NaOH and diluted 10 times, and then applied to a DEAE-Sepharose FF column (2 cm × 20 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was washed with the same buffer until the washing solution was free from protein. The absorbed α-lactalbumin was eluted with a gradient of 0.5 M sodium chloride in 20 mM Tris-HCl buffer, pH 7.5. The protein content of each fraction was determined by measuring the absorbance at 280 nm. The fraction containing the protein was collected. The purified protein was used for TOF-MS analysis.

SDS–Polyacrylamide Gel Electrophoresis (SDS-PAGE)—SDS-PAGE was conducted according to the method of Laemmli (7) using a 15% acrylamide separating gel and a 5% stacking gel containing 1% SDS. Samples were heated at 100°C for 5 min in Tris-glycine buffer (pH 8.8) containing 1% SDS and 1% 2-mercaptoethanol. Electrophoresis was carried out at a constant current of 10 mA for 5 h using an electrophoretic Tris-glycine buffer containing 0.1% SDS.

Endo-H Treatment—The method of Tarentino and Maley (8) was slightly modified for the digestion of glycosylated α-lactalbumin with endo-β-*N*-acetylglucosaminidase (Endo-H). The glycosylated α-lactalbumin (0.2 mg/ml) was boiled in 50 mM citrate buffer (pH 5.5) containing 1% SDS and 200 μg/ml of phenylmethylsulfonyl fluoride (PMSF) for 5 min. After cooling, samples were supplemented with an equal volume of 50 mM citrate buffer (pH 5.5) with 0.02 units of Endo-H or without the enzyme, and subsequently incubated at 37°C for 20 h. After incubation, the samples were analyzed by SDS-PAGE.

Western Blotting—After 15% SDS polyacrylamide gel electrophoresis, protein bands were transferred to a polyvinylidene difluoride (PVDF) membrane for 1 h at 100 mA in blotting buffer (20 mM glycine, 25 mM Tris, 20% methanol). Subsequently, non-specific binding sites were blocked by immersing the PVDF membrane in 0.05% Tween 20 in phosphate buffer (PBS) containing 1% BSA for 1 h at room temperature on an orbital shaker and then exposed to rabbit anti–bovine α-lactalbumin antiserum (1×10^{-4} in 0.05% Tween 20) in Tween-PBS for 1 h at room temperature. The membrane was further exposed to goat anti-mouse peroxidase conjugate (1×10^{-4} in Tween-PBS) for 1 h at room temperature. Immunodetection of α-lactalbumin was carried out with an ECL+Plus kit [Western blotting detection reagents (Amersham Pharmacia Biotech)]. The chemiluminescence intensities associated with the various protein bands were determined by exposing the filter to X-ray film with Hypercassette™.

TOF-MS—TOF-MS analysis was performed using Voyager™ DE/PROJ (PerSeptive Biosystems, USA). The matrix, 3,5-dimethoxyl-4-hydroxyciammic acid (sinapinic acid), was dissolved in a reaction solution containing equal volumes of acetonitrile and 0.1% TFA for the wild-type and N45D mutant. The sample concentration was 1.5 mg/ml for the wild-type and N45D mutant.

RESULTS

Expression of Wild-Type α-Lactalbumin—The transformed yeast, *P. pastoris*, carrying α-lactalbumin cDNA was grown at 30°C for 5 days. The secretion of α-lactalbumin was followed by Western blotting analysis of culture medium at intervals of 24 h (Fig. 1A). The bands on Western blotting of SDS-PAGE patterns were measured with a densitometer to determine the secreted amounts (Fig. 1B). As shown in the SDS-PAGE pattern time course, the band of wild-type α-lactalbumin (14 kDa) increased with culture time, while the higher molecular weight (16 kDa) band further increased with culture time (lanes 3–7). In addition to the 16 kDa band, a faint band near 20 kDa appeared on prolonged culture. The 16 and 20 kDa bands became a 14 kDa band on treatment with Endo-H, which cleaved the *N*-linked carbohydrate chain (lane 8), suggesting that the band is glycosylated α-lactalbumin. Since the wild type α-lactalbumin has two glycosylation sites at positions 45 and 74, both single and double glycosylated α-lactalbumins seem to appear on long term cultivation. The secreted amount of glycosylated α-lactalbumin was higher than that of nonglycosylated protein. Thus, it was confirmed that α-lactalbumin was predominantly secreted in the glycosy-

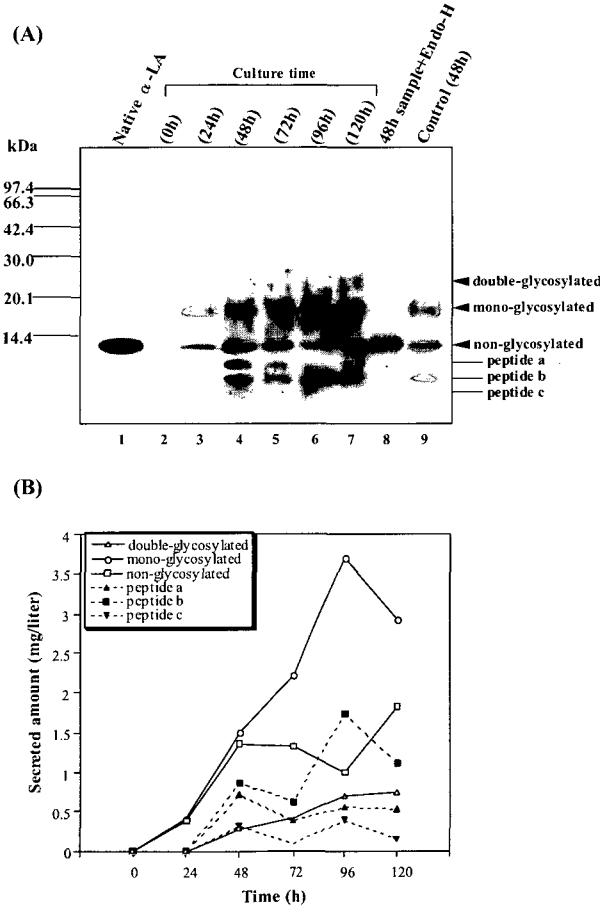

Fig. 1. **Time-course of SDS-PAGE patterns (A) and secreted amount (B) in the culture medium of wild-type α-lactalbumin secreted in *Pichia pastoris*.** The SDS-PAGE (A) bands were transferred to a PVDF membrane and then immuno-reacted with α-lactalbumin antibodies for the culture medium at intervals of 24 h (lanes 2–7). Endo-H treatment was carried out with the culture medium for 48 h (lane 8). The secreted amounts of the corresponding proteins (B) were determined by densitometric measurement of the bands in the SDS-PAGE patterns.

lated form in *P. pastoris*. The lower molecular weight bands seem to be peptide fragments due to digestion by serine proteases in *P. pastoris* (*6*). Therefore, to obtain a sufficient amount of sample, the optimal culture time for nonglycosylated α-lactalbumin is 48 h and that for the glycosylated protein is 96 h.

Expression of Mutant α-Lactalbumin (N45D)—In order to determine the position of *N*-linked glycosylation, an α-lactalbumin mutant, N45D, with the *N*-linked glycosylation site deleted was constructed and secreted in *P. pastoris*. As shown in Fig. 2, the secretion of glycosylated α-lactalbumin was greatly decreased, while the nonglycosylated protein was predominantly secreted. The mutant secreted a small amount of the glycosylated form in which the carbohydrate chain was removed by Endo-H (lane 8) within 48 h, while a considerable amount of the glycosylated form was secreted on prolonged cultivation (lane 7). The carbohydrate chain seems to be attached to the remaining glycosylation site at position 74. This suggests that the Asn at position 45 is the predominant *N*-linked glycosylation site and is a more sensitive glycosylation site than the Asn at position 74. As shown in Fig. 2B, the secretion of the nonglycosylated N45D mutant was remarkably high. This suggests that deletion of the glycosylation site at position 45 is effective for high secretion of α-lactalbumin. Despite the high secretion, the degradation of α-lactalbumin into low molecular peptides was also observed in SDS-PAGE patterns.

Expression of Glycosylated Mutant α-Lactalbumin (D46N)—In contrast to the deletion of an *N*-linked glycosylation site, an attempt to increase the number of *N*-linked glycosylation sites was carried out to further investigate the effect of the attachment of the carbohydrate chain on the secretion of α-lactalbumin. The substitution of Asp at position 46 with Asn resulted in an additional glycosylation signal sequence, Asn[46]-Ser[47]-Thr[48]. Figure 3 shows the secretion pattern of the additional glycosylated α-lactalbumin mutant (D46N). This SDS-PAGE pattern differs from that of the wild-type. The band of nonglycosylated α-lactalbumin completely disappeared and the broad band of glycosylated α-lactalbumins increased with culture time. The broad band was overlapped by two other bands, 16 and 20 kDa.

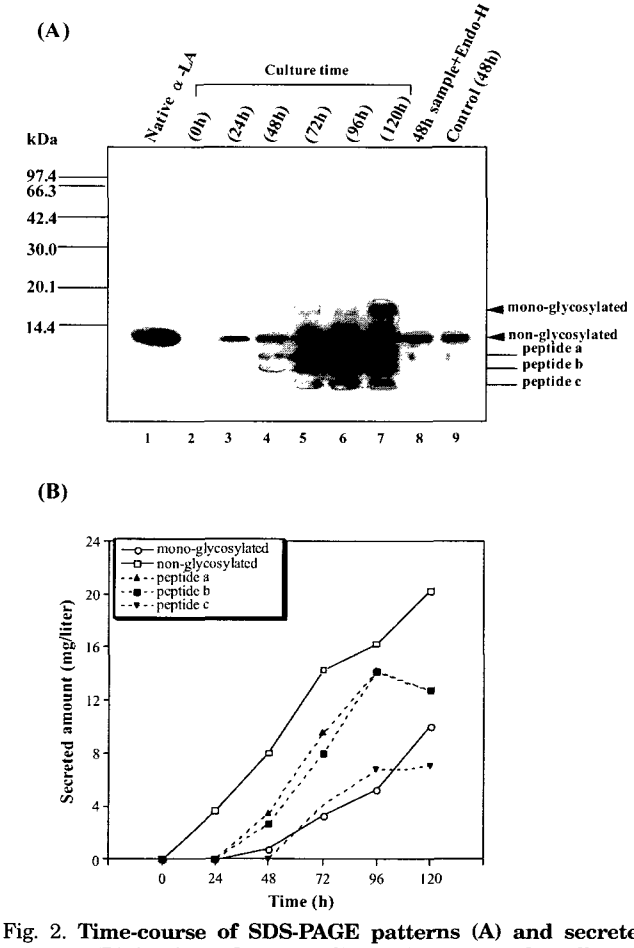

Fig. 2. **Time-course of SDS-PAGE patterns (A) and secreted amount (B) in the culture medium of mutant α-lactalbumin (N45D) secreted in *Pichia pastoris*.** The SDS-PAGE (A) bands were transferred to a PVDF membrane and then immuno-reacted with α-lactalbumin antibodies for the culture medium at intervals of 24 h (lanes 2–7). Endo-H treatment was carried out with the culture medium for 48 h (lane 8). The secreted amounts of the corresponding proteins (B) were determined by densitometric measurement of the bands in the SDS-PAGE patterns.

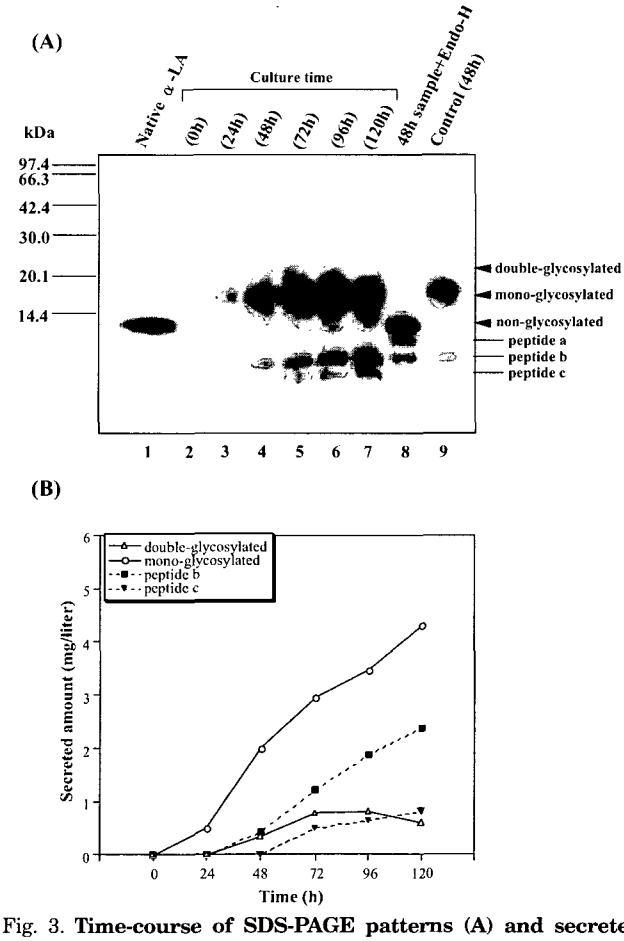

Fig. 3. **Time-course of SDS-PAGE patterns (A) and secreted amount (B) in the culture medium of glycosylated mutant α-lactalbumins (D46N) secreted in *Pichia pastoris*.** The SDS-PAGE (A) bands were transferred to a PVDF membrane and then immuno-reacted with α-lactalbumin antibodies for the culture medium at intervals of 24 h (lanes 2–7). Endo-H treatment was carried out with the culture medium for 48 h (lane 8). The secreted amounts of the corresponding proteins (B) were determined by densitometric measurement of the bands in the SDS-PAGE patterns.

TABLE I. **Mass data and possible monosaccharide compositions for glycosylated forms of α-lactalbumin.**

Peak	Measured mass	Calculated mass	Carbohydrate composition based on measured mass
(Wild type)			
1	14,176	14,177	
2	16,523	16,524	$Man_{12}NAG_2$
3	17,904	17,900	$(Man_9NAG_2)_2$
(N45D)			
2	16,041	16,040	Man_9NAG_2
3	16,227	16,202	$Man_{10}NAG_2$
4	16,369	16,364	$Man_{11}NAG_2$

One band is the single glycosylated α-lactalbumin with oligosaccharide attached at Asn 45, Asn 46 or Asn 74, and another is one double glycosylated at either Asn 45 and Asn 46 or Asn 74. The degradation of α-lactalbumin into peptides was observed in a similar manner to as for the wild type α-lactalbumin.

Determination the Molecular Weights of the Wild Type and Mutant α-Lactalbumins—To determine the molecular weights of various α-lactalbumins, TOF-MS was carried out. The numbers of attached oligosaccharides were estimated from the molecular masses (Table I). The basic structure of *N*-linked carbohydrate chains in *P. pastoris* is $Man_{8-14}NAG_2$. The molecular mass spectrum of the wild type α-lactalbumin showed the presence of single ($Man_{12}NAG_2$: 16,523 Da) and double [$(Man_9NAG_2)_2$: 17,904 Da] oligosaccharide chains. The molecular mass of mutant N45D showed only the presence of a single oligosaccharide ($Man_{9-11}NAG_2$) chain. The molecular mass of α-lactalbumin is consistent with the SDS-PAGE patterns, although the band of double glycosylated α-lactalbumin appeared near 20 kDa due to the molecular sieving effect, *i.e.* the carbohydrate chains slightly suppress movement unlike in the case of the nonglycosylated protein.

DISCUSSION

Goat α-lactalbumin has two *N*-linked glycosylation signal sequences, Asn^{45}-Asp^{46}-Ser^{47} and Asn^{74}-Ile^{75}-Ser^{76}. Although α-lactalbumins in cow and goat commonly have the *N*-linked glycosylation signal sequence at positions 45 and 74, there is little information about glycosylated α-lactalbumin. To elucidate the functional significance of glycosylated α-lactalbumin in mammalian milk, the secretion of nonglycosylated and glycosylated α-lactalbumins was attempted in the expression system of yeast *P. pastoris* carrying goat α-lactalbumin cDNA. As expected, wild type α-lactalbumin was secreted in both single and double glycosylated forms in the *P. pastoris* expression system. The results of mass spectrometry suggested that the mode of glycosylation of α-lactalbumin is $Man_{9-12}NAG_2$. The glycosylation of proteins in *P. pastoris* has been reported to occur in a different manner from in mammalian cells (*9*). The basic mode of glycosylation has been reported to be $Man_{8-14}NAG_2$ (*10*). Therefore, the glycosylation of α-lactalbumin seems to be consistent with that of other proteins.

Although the *N*-linked glycosylation sites of α-lactalbumin exist at both positions 45 and 74, the former site seems to the predominant glycosylation site judging from the data in Fig. 2. The glycosylation of mutant N45D was greatly decreased and occurred only at a late cultivation time. This suggests that the glycosylation site at position 45 is more easily recognized as the signal sequence than that at position 74.

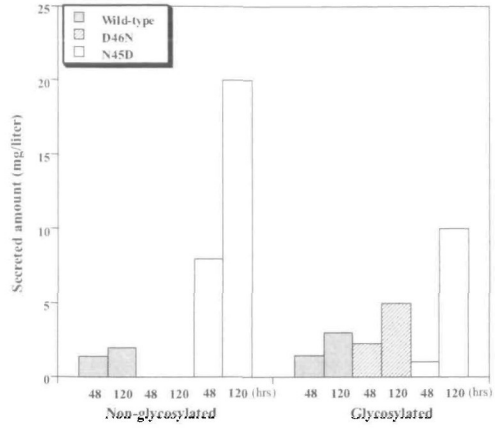

Fig. 4. **Changes in the secreted amounts of the wild type, and mutant D46N and N45D α-lactalbumins in *Pichia pastoris* during cultivation for 48 and 120 h.** The secreted amounts of the nonglycosylated and glycosylated forms of various α-lactalbumin are cited from Figs. 1–3, B.

Mutant N45D with the *N*-linked glycosylation signal sequence at position 45 deleted showed greatly suppressed secretion of glycosylated α-lactalbumin, while the mutant α-lactalbumin predominantly secreted a large amount of nonglycosylated protein. On the other hand, mutant D46N with a further *N*-glycosylation signal site at position 46 secreted only *N*-linked glycosylated α-lactalbumin and the secretion was greatly decreased. It is very interesting that the secretion of α-lactalbumin is suppressed with an increase in the number of *N*-linked glycosylation sites. In order to determine the exact relation between the secreted amounts and the *N*-linked glycosylation site of various α-lactalbumins, determination of the secreted amount was carried out by Western blotting to avoid the contaminant proteins, as shown in Figs. 1–3. The results are summarized in Fig. 4. The secreted amounts of mutant N45D with the *N*-linked glycosylation site at position 45 deleted were much higher than those of the wild type and mutant D46N with the increased number of *N*-linked glycosylation sites. It is noteworthy that the secretion of α-lactalbumins (wild-type and D46N) having the glycosylation signal sequence at position 45 was very suppressed, while that of α-lactalbumins lacking the sequence at position 45 was greatly enhanced. These results suggest the glycosylation at position 45 in α-lactalbumin is involved in the suppression of the secretion. It has been reported that *N*-linked glycoproteins are subjected to quality control in ER, in which unfolded proteins are degraded by proteasome through a retrotranslocation system (*11, 12*). We found that calnexin, the molecular chaperone specific for glycoproteins, is involved in the quality control in yeast *Saccharomyces cerevisiae*, using a calnexin-deleted strain (*13*). It seems likely that glycosylated α-lactalbumins are unstable or have misfolded conformations in ER, thus being degraded by the quality control system in yeast even if it is not as severe as in mammalian cells. The effect of the expression level on the secretion of α-lactalbumin was evaluated as the accumula-

tion of mutant proteins in the yeast cells. The glycosylated α-lactalbumins were not accumulated in the cells, although a low level of accumulation was observed in a similar manner as in the case of the non-glycosylated protein. Although most α-lactalbumins are secreted in a nonglycosylated form in mammalian milk (*5*), α-lactalbumin was greatly secreted in the glycosylated form in yeast *P. pastoris*. This observation suggests that glycosylated α-lactalbumin in mammalian cells may be subjected to quality control, thus resulting in low levels of secretion, while the quality control may be tolerant in yeast.

It has been suggested (*5*) that in the Golgi region of the mammary gland, α-lactalbumin is wholly secreted as a glycoprotein for export from the cell and subsequently degraded by milk glycoside hydrolases. This hypothesis is in conflict with our results, because deletion of the *N*-linked glycosylation site at position 45 enhanced the secretion of nonglycosylated α-lactalbumin, while an increase in the number of glycosylation sites greatly decreased the secretion of both nonglycosylated proteins. Although the biological function of glycosylated α-lactalbumin remains unclear, our studies may indicate its functional significance.

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