Enhanced secretion of hydrophobic peptide fused lysozyme by the introduction of *N*-glycosylation signal and the disruption of calnexin gene in *Saccharomyces cerevisiae*

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Abstract The insertion of a hydrophobic pentapeptide (Phe-Phe-Val-Ala-Pro) into the C-terminus in hen egg white lysozyme by genetic modification resulted in an unstable structure which caused little secretion in a yeast expression system, although this modification is useful to enhance bactericidal action to Gram-negative bacteria [Ibrahim et al. (1994) J. Biol. Chem. 269, 5059–5063]. To enhance the secretion of the unstable hydrophobic pentapeptide fused lysozymes (H5-Lz), we attempted to introduce the signal sequence (Asn-X-Ser/Thr) of *N*-linked glycosylation into lysozyme and to suppress the quality control of the unstable mutant in the yeast expression system. The polymannosyl hydrophobic fused lysozyme (H5/G49N-Lz) having the *N*-glycosylation signal sequence was expressed in the medium at 3.4 times that of unglycosylated lysozyme. Further, the secretion of the unstable mutant lysozyme was done in the *Saccharomyces cerevisiae* disrupted calnexin gene to avoid the degradation of the unstable mutant by the quality control. Although disruption of the calnexin gene did not lead to gross effects on the levels of growth of *S. cerevisiae* (W303-1b), the secretion amount of H5/G49N-Lz in calnexin disrupted *S. cerevisiae* was 2.5 times larger than that in wild type *S. cerevisiae*. These results suggest that the secretion of unstable glycosylated lysozyme (H5/G49N) was suppressed by the quality control function of calnexin and that the disruption of calnexin is effective to increase the secretion of unstable glycosylated protein.

Key words: Hydrophobic peptide; *N*-Glycosylation; Calnexin; *Saccharomyces cerevisiae*

1. Introduction

The antimicrobial action of hen egg white lysozyme is limited to Gram-positive bacteria. If the antimicrobial action were broadened to Gram-negative bacteria, it would bring about a breakthrough development for the food and pharmaceutical industries. For this purpose, the molecular design of lysozyme was attempted using genetic engineering. The lysozyme having a hydrophobic pentapeptide (Phe-Phe-Val-Ala-Pro) at its C-terminus was constructed by genetic modification and it was secreted in the medium of *Saccharomyces cerevisiae*. The hydrophobic peptide fused lysozyme (H5-Lz) inhibits the growth of Gram-negative bacteria (*Escherichia coli*) [1,2]. But the problem is that the secretion of H5-Lz is very low in the yeast expression system. This problem should be solved by the development of a new strategy. The stable polymannosyl mutant lysozyme (G49N-Lz) having an *N*-glycosylation signal sequence was highly expressed in the medium [3]. Therefore, the introduction of an *N*-glycosyl carbohydrate chain was attempted in order to increase the secretion of H5-Lz. However, it is general that according to the quality control in the endoplasmic reticulum (ER) in yeast, secreted amounts of unstable proteins are very low. Recent studies have demonstrated that calnexin is a major molecular chaperone associating transiently with numerous newly synthesized glycoproteins during their maturation in the ER [4–6]. Calnexin is a component of the ER quality control system for glycoproteins and retains misfolding intermediates through their oligosaccharide moieties until these substrates fold properly or until misfolded protein are degraded [7–10]. Therefore, we devised a method to obtain a considerable amount of mutant lysozymes using *S. cerevisiae* disrupted calnexin. It has been reported that *S. cerevisiae* disrupted calnexin normally grows without any morphological change [11]. Therefore, it is expected that the secretion of unstable glycosylated fusion lysozymes can be enhanced in the yeast disrupted calnexin without quality control. Thus, we attempted to secrete glycosylated hydrophobic peptide fused lysozyme (H5/G49N) in *S. cerevisiae* disrupted calnexin. This attempt must shed light on the solution of the high secretion of useful proteins with unstable conformations.

2. Materials and methods

2.1. Materials

Authentic lysozyme was purified from fresh hen egg white and recrystallized five times as previously reported [12]. Microbial substrate of lysozyme, *Micrococcus lysodeikticus* cells, was purchased from Sigma Chemical (St. Louis, MO). Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co. Ltd. (Kyoto). Centricon centrifugal concentrators was purchased from Amicon (USA). CM Toyopearl resin was a production of Tosoh (Tokyo).

2.2. Bacterial strains and plasmids

E. coli TG1 (K12, ΔCNE (*lac-pro*) *supE thi hsd D5/F′ tra D36 proA*$^+$*B*$^+$ *lacIq lacZ′′M15*), which was used as the host cells for propagation of plasmid (pRS426), was supplied by Amersham Japan. *S. cerevisiae* diploid strain W303-1b (*Mat ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15*) and W303-1b (Δ*cne 1*::*Leu2*) were provided by Dr. Parlati, McGill University, Canada. The recombinant plasmid pKK-1, which contains a full length hen egg white lysozyme cDNA, was provided by Dr. I. Kumagai, Tohoku University. This plasmid contains 16 bp of the 5′ non-coding region, 440 bp of the coding region, and about 120 bp of the 3′ non-coding region of hen prelysozyme cDNA in the same orientation as lacZ′ in pUC18. pYG-100, an *E. coli*-yeast shuttle vector, was provided by Dr. K. Matsubara of Osaka University. The yeast episomal vector pRS426 was

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provided by Dr. R. Akada, Department of Applied Chemistry and Chemical Engineering, Yamaguchi University.

2.3. Growth media

The *E. coli* cells were grown in LB broth (1% Bactotryptone, 0.5% yeast extract, 1% NaCl). The medium was supplemented with 60 μg/ml of ampicillin for selection of transformation. For growth of yeast, YPD (1% yeast extract, 2% polypeptone, 2% glucose) was routinely used. The *S. cerevisiae* W303-1b cells bearing the expression vector pRSKK-1 were grown on yeast minimum medium (JMM) supplemented with 40 μg/ml of adenine, 60 μg/ml of leucine, 40 μg/ml of tryptophan and 20 μg/ml of histidine.

2.4. Construction of expression plasmids of the mutant HEWLs

The mutants were inserted into the *Sal*I site in the pYG-100 vector between the GPD promoter and terminator. The pYG-100 carrying mutant lysozyme genes was treated with *Hin*dIII, thus obtained from the promoter to terminator region containing the mutant HEWL cDNA. This fragment was inserted into the *Hin*dIII site of pRS426 which is the expression plasmid of yeast *S. cerevisiae* W303-1b.

2.5. Expression of glycosylated fusion lysozyme in yeast S. cerevisiae W303-1b

The expression vector was introduced into *S. cerevisiae* W303-1b according to the lithium acetate procedure [13]. *Ura*$^+$ transformants were screened by subculturing in yeast minimal medium (JMM) supplemented with 40 μg/ml of adenine, 60 μg/ml of leucine, 40 μg/ml of tryptophan and 20 μg/ml of histidine. The over-expressing colonies were inoculated into 3 ml of JMM and incubated for 2 days at 30°C with shaking. This preculture was subcultured to 100 ml of the same medium in a Sakaguchi flask (500 ml) and incubated for another 2 days at 30°C with shaking. To the main culture medium in a jar fermenter, 100 ml of the seed culture was inoculated by aseptic transfer.

2.6. Analysis

The growth of *S. cerevisiae* W303-1b was measured as the absorbance at 660 nm using a Hitachi U-2000 spectrophotometer. Glucose and ethanol in the culture medium were measured with a high pressure liquid chromatograph (HPLC, Hitachi, L-6000) using a Shodex sugar column SH 1011 (8×300 mm) with 0.01 N H_2SO_4 as the mobile phase at a flow rate of 1 ml/min.

2.7. Purification of glycosylated fusion lysozyme

The growth medium of *S. cerevisiae* W303-1b described above was centrifuged at 8000 rpm for 5 min at 4°C. The supernatant was applied to a CM-Toyopearl 650 column (1.5×5.0 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The polymannosyl hydrophobic peptide fused lysozyme was then eluted with the same buffer containing 0.5 M NaCl. Fractions containing the lysozyme were pooled, diluted 10 times with the same buffer and rechromatographed on a CM-Toyopearl 650 column (1.5×10 cm). Lysozyme was eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. The fractions containing the polymannosyl hydrophobic peptide fused lysozyme were collected, and then concentrated with Centricon centrifugal concentrators.

2.8. Conformational stability of H5-Lz

The thermal denaturation was monitored by circular dichroism (CD) spectroscopy in a Jasco Model J-500 spectropolarimeter. The denaturation curves of 65 μg/ml protein in 50 mM glycine-HCl buffer (pH 2.85) were recorded at 222 nm in a 1.0 cm cuvette with a temperature increase of 1°C/min from 30°C to 90°C, to obtain the thermodynamic parameters of wild type and fusion lysozymes. The values of entropy, enthalpy and Gibbs free energy were determined from the thermal transition curves as described previously [14]. The Gibbs free energy function was drawn as reported [15].

2.9. Endo-H treatment of glycosylated fusion lysozyme

The method of Tarentino and Maley was slightly modified for the digestion of glycosylated fusion lysozyme with endo-α-*N*-acetyl glucosaminidase H (Endo-H). The glycosylated lysozyme (0.2 mg/ml) was dissolved in 50 mM sodium citrate buffer (pH 5.5) containing 200 μg/ml of phenylmethylsulfonyl fluoride. Then samples were mixed with an equal volume of 0.02 unit of Endo-H in 50 mM sodium citrate buffer (pH 5.5), and subsequently incubated at 37°C for 24 h.

2.10. Lytic activity of fusion lysozyme

The lytic action of fusion lysozyme derivatives against *M. lysodeikticus* cells was determined according to turbidimetric methods [16] based on the decrease in turbidity of a cell suspension following the addition of lysozyme derivatives (0.01%). *M. lysodeikticus* cell suspension was prepared as a substrate in 50 mM sodium phosphate buffer (pH 5.5). A 100 μl portion of the lysozyme derivative solution was added into 2.4 ml of the substrate and the decrease in absorbance at 450 nm monitored for 1 min using a Hitachi U-2000 spectrophotometer. One unit of lysozyme is defined as the amount of enzyme that causes a decrease of 0.001 in A_{450} per min.

3. Results and discussion

3.1. Conformational stability of hydrophobic fusion lysozyme (H5-Lz)

Since insertion of a hydrophobic domain into proteins may cause perturbation of the interior hydrophobic interaction, it is predicted that the conformational stability of lysozyme (H5-Lz) may decrease by hydrophobic peptide fusion. Therefore, the conformational stability of the protein was estimated from the denaturation curves by monitoring the change in ellipticity at 222 nm of the CD spectrum, because a negative CD value at 222 nm is characteristic of an ordered structure. The thermodynamic parameters were calculated from the denaturation curves. Table 1 shows the thermodynamic parameters of wild type and fusion lysozymes (H5-Lz) calculated from the denaturation curves. Although the hydrophobic extension had a slight effect on the melting point, a substantial decrease in the Gibbs free energy of lysozyme was observed. It appears that the hydrophobic extension led to a marked decrease in the conformational stability of lysozyme, likely by interfering with the intramolecular hydrophobic bonding potential. Since a slight decrease in T_m value with considerable reduction in the enthalpy of denaturation has been accompanied by the fusion of such a hydrophobic pentapeptide, it is probable that the fusion lysozyme may exist as a compact intermediate form. This form has a significant secondary structure and a relatively unstable form, a translocation competent form [17]. Becktel and Schellman refer to plots of ΔG vs. temperature as protein stability curves [15]. This interesting temperature dependence is described by a form of the Gibbs-Helmholtz equation:

$$\Delta G(T) = \Delta H_m(1-T/T_m)-\Delta C_p[(T_m-T) + T\ \ln(T/T_m)]$$

where $\Delta G(T)$ is ΔG at temperature T, T_m is the midpoint of the thermal unfolding curve, ΔH_m is the enthalpy change for unfolding measured at T_m, and ΔC_p is the difference in heat capacity between the unfolding and folded conformations (for lysozyme $\Delta C_p = 51.7$ J/K per mol of amino acid residue). As shown in Fig. 1, wild type lysozyme exhibited maximum

Table 1
Thermodynamic parameters of wild type and fusion lysozymes[a]

Protein	T_m[b] (°C)	ΔG (25°C)[c] (kcal/mol)	$\Delta(\Delta G)$[d] (kcal/mol)
WT-Lz	69.80	7.76	–
H5-Lz	68.80	5.70	2.06
G49N-Lz	68.02	5.61	2.15
H5/G49N-Lz	65.01	5.08	2.68

[a]Methods used to measure conformational stability are described in Section 2. Determination was performed for 2 ml (65 μg/ml protein in 50 mM glycine-HCl buffer, pH 2.85).
[b]Midpoint of the CD thermal denaturation curve.
[c]Change in Gibbs energy at the given temperature.
[d]Difference of ΔG between the wild type lysozyme and the mutant.

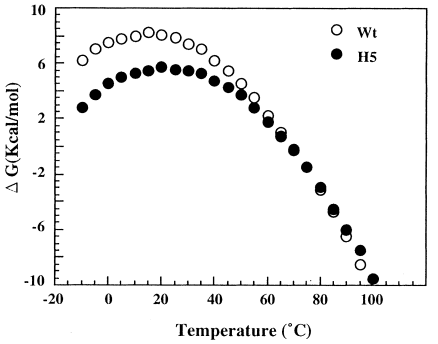

Fig. 1. Temperature dependence of Gibbs free energy change (ΔG) for unfolding of wild type and fusion lysozyme. Proteins were prepared in 50 mM glycine-HCl buffer, pH 2.85 (65 μg/ml). The stability curves of wild type (○) and fusion lysozyme (●) were computed according to the methods described by Becktel and Schellman [15].

stability (T_{max}) at 14°C, whereas ΔG is 8.07 kcal/mol, whereas the fusion protein showed a maximum stability (T_{max}) at the much higher temperature of 22°C, and ΔG is 5.73 kcal/mol. The data suggest that the hydrophobic extension resulted in a shift of the maximum stabilization of lysozyme to a higher temperature. This result is in full agreement with the effect of water ordering in the vicinity of exposed non-polar groups. These results indicate that the fusion lysozyme constructed in this study is a conformationally unstable protein having a hydrophobic sequence, which assumes a β-strand configuration as deduced from secondary structure prediction. Thus, the hydrophobic peptide fused lysozyme (H5) was confirmed to be unstable mutant.

3.2. *Effect of glycosylation on the secreted amounts of hydrophobic peptide fused lysozymes*

We reported that the polymannosyl mutant lysozyme (G49N-Lz) having an *N*-glycosylation signal sequence (Asn-X-Thr/Ser) was highly expressed in the yeast medium [3]. To enhance the secretion of the hydrophobic pentapeptide fused lysozyme (H5-Lz), we introduced the signal sequence of *N*-glycosylation into the hydrophobic pentapeptide fused lysozyme (H5/G49N-Lz). The secreted protein was purified by two steps of cation exchange chromatography with CM-Toyopearl (Fig. 2). The secretion of H5/G49N-Lz (Fig. 2B) was increased compared to that of H5-Lz (Fig. 2A). Despite the introduction of the *N*-glycosylation signal, the secretion is not enough to improve. Since the mutant lysozyme was not found in the cytosol fraction, the expressed mutant lysozyme may be subjected to degradation by the quality control in ER.

3.3. *Conformational stability of glycosylated hydrophobic fusion lysozyme (H5/G49N-Lz)*

It is considered that the quality control works for unstable mutant proteins in yeast ER. Therefore, the loss of secretion amount of glycosylated hydrophobic fusion lysozyme may be due to the unstable structure. Thus, the stability of the polymannosyl lysozyme (G49N-Lz) and the fusion lysozyme (H5/G49N-Lz) was measured from the denaturation curves by monitoring the ellipticity at 222 nm during heating. Table 1 shows the melting point (T_m) and changes in Gibbs energy (ΔG) reflecting the conformational stability. As shown in Table 1, the T_m and ΔG were decreased by the glycosylation and synergically lowered in the H5/G49N mutant. It seems likely that the instability of the H5/G49N mutant may bring about the lowering of the secretion.

3.4. *Secretion of unstable mutant in wild type and calnexin disrupted S. cerevisiae*

Calnexin plays a major role in the quality control as a retention mechanism for incompletely folded or misfolded glycoproteins in ER. To enhance the secretion of H5/G49N-Lz, we investigated the secretion of H5/G49N-Lz in *S. cerevisiae* strain disrupted calnexin gene (W303-1b). As shown in Fig. 2, the secretion amounts of the H5/G49N-Lz in the calnexin disrupted *S. cerevisiae* were larger than those in the wild type *S. cerevisiae* W303-1b with calnexin (Fig. 2C). The secretion amounts of H5/G49N-Lz in *S. cerevisiae* W303-1b with and without calnexin were 17.2 and 44.3 μg/l, respectively. These results suggest that the unstable glycosylated protein was degraded by the quality control function of calnexin and that a considerable amount of secretion of the unstable glycosylated protein may be enhanced using the yeast strain disrupted calnexin.

3.5. *Effect of calnexin gene deletion on the growth of S. cerevisiae W303-1b*

The effect of calnexin gene deletion on the growth of *S. cerevisiae* W303-1b was investigated. As shown in Fig. 3, growth was normal. Moreover, glucose consumption and ethanol production were also almost the same in both wild type and calnexin disrupted strains. This suggests that disrup-

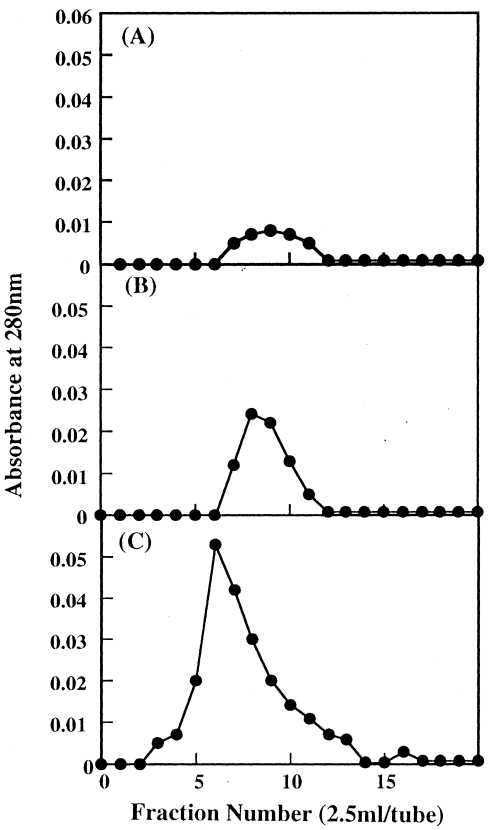

Fig. 2. Elution pattern of unstable mutant HEWL secreted with and without calnexin on CM-Toyopearl with 0.5 M NaCl in 50 mM Tris-HCl buffer (pH 7.5). A: H5-Lz (wild yeast strain W303-1b). B: H5/G49N-Lz (wild yeast strain W303-1b). C: H5/G49N-Lz (calnexin deleted yeast strain W303-1b).

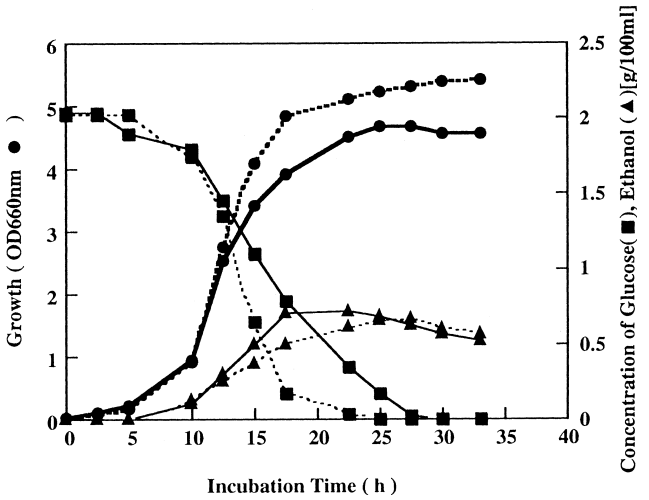

Fig. 3. Effect of calnexin deletion on growth (●), glucose concentration (■), and ethanol concentration (▲) of *S. cerevisiae* W303-1b. Wild strain W303-1b (solid line) and the calnexin disrupted strain (dotted line)were incubated in a jar fermenter at 30°C for the period indicated.

tion of the calnexin gene did not lead to gross effects at the level of growth and metabolism. This indicates that the increase in the secretion of mutant unstable lysozyme comes from the disappearance of the quality control of calnexin.

3.6. *Characteristic of glycosylated hydrophobic fusion lysozyme (H5/G49N)*

The secreted H5/G49N-Lz was the glycosylated form, as judged from the SDS-PAGE pattern (data not shown). As shown in a previous paper [3], the large molecular size of *N*-glycosylated lysozyme with a polymannose chain was predominantly secreted in yeast medium. Similarly, the high molecular bands were shown in H5/G49N-Lz for protein and carbohydrate stains. The glycosylated form was digested by Endo-H. The lytic activity was recovered by the treatment with Endo-H. The lytic activity of H5 lysozyme was 80% of that of wild type protein, suggesting a conformational change due to hydrophobic peptide fusion. In conclusion, the secretion of unstable mutant protein can be improved by glycosylation and the use of calnexin deleted yeast.

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