Relationship between the stability of lysozymes mutated at the inside hydrophobic core and secretion in *Saccharomyces cerevisiae*

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The relationship between the stability of lysozymes mutated at the inside hydrophobic core and secretion was investigated to understand the optimal secretion of mutant lysozymes in the *Saccharomyces cerevisiae*. S91T mutant lysozyme increased in the methyl residue inside the core greatly increased the conformational stability. The secretion amount of S91T in *S. cerevisiae* increased greatly compared with wild-type lysozyme. On the other hand, I55V and T40S/I55V mutant lysozymes decreased in methyl residue inside the core brought about their unstable conformation. The secretion amounts of these unstable mutant lysozymes significantly decreased. In addition, the effect of glycosylation on the secretion of these mutants was investigated. The secretion amounts of glycosylated lysozyme S91T/G49N with stable hydrophobic core greatly increased compared with that of glycosylated lysozyme G49N, while those of mutant I55V/G49N and T40S/I55V/G49N with unstable hydrophobic core greatly decreased. These results indicate that the secretion amounts of mutant lysozymes increase in proportion to the hydrophobic core stabilities and that a similar good correlation was obtained with glycosylated lysozymes.

1 Introduction

The yeast *Saccharomyces cerevisiae*, a eukaryotic organism that is easy to manipulate, is valuable and useful as a model system to study the expression and function of foreign proteins in mammalian cells. Furthermore, *S. cerevisiae* is widely used to express foreign proteins which are rare or difficult to be extracted from original tissue. The advantages of the expression system in yeast are to get the correctly folded proteins through endoplasmic reticulum (ER) and to process accurately the signal peptide to have the same *N*-terminal amino acid as mammalian original proteins. When the foreign proteins are secreted in *S. cerevisiae*, the secretion amounts of foreign proteins are very variable depending on the protein species. This is probably a result that the incompletely folded or unfolded nascent proteins are degraded by the quality control system in the ER. Recent studies have been elucidated that the folding and quality control of proteins carried out though the molecular chaperones including binding protein (BiP), protein disulfide isomerase (PDI), calnexin, calreticulin, ERp57 (a homologue of PDI which exhibits thiol-disulfide oxidoreductase activity on glycoproteins) in the ER in mammalian cells [1–3]. Foreign proteins may be subjected to the quality control of ER in yeast depending on the stability of protein conformation. It has been reported that the hydrophobic core stability is very important for the stability of protein conformation [4, 5]. In order to elucidate the relationship between the secretion amount and the stability of protein hydrophobic core in yeast, various mutant hen egg white lysozymes (HEWLs) with different stabilities of hydrophobic cores were used as model proteins.

HGWL has been well studied with respect to structural and functional properties. The wild and mutant lysozymes are correctly processed in the yeast expression system as well as in a hen and secreted in their soluble forms in the yeast medium. The secreted forms of lysozymes are easily collected and purified by the CM-Toyopearl adsorption method with good reproducibility and little loss. In addition, we have reported that HEWL is glycosylated in a yeast expression system when the *N*-glycosylation signal sequence, Asn-X-Thr/Ser, is introduced by site-directed mutagenesis of lysozyme cDNA and that the secretion of glycosylated lysozymes greatly increased [6, 7]. The glycosylated mutant G49N, whose glycine at position 49 was substituted with asparagine, was used as a typical glycosylated lysozyme. Thus, we attempt to construct various mutant nonglycosylated and glycosylated lysozymes having different hydrophobic core stabilities. Malcom *et al.* [4, 5] reported that the inside core of lysozyme molecule consisting of hydrophobic residues at positions The-40, Ile-55 and Ser-91 are replaced to have different stabilities. These residues lie together and form a structural unit within the core of the enzyme, just beneath the active site cleft, at the vertex of the putative hinge between the two globular domans [8, 9]. S91T mutant lysozyme increasing in hydrophobic residue inside the core showed conformational stability [4]. On the other hand, I55V and T40S/I55V mutant lysozymes decreasing in hydrophobic residue inside the core brought about their unstable conformation [4]. Study on the secretion of these nonglycosylated and glycosylated mutant lysozymes with different hydrophobic core stabilities may give useful information on yeast secretion mechanism of foreign proteins.

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Abbreviations: ER, endoplasmic reticulum; **HEWL**, hen egg white lysozyme

Keywords: *Saccharomyces cerevisiae* / Mutant lysozyme / Wild-type lysozyme / Secretion

2 Materials and methods

2.1 Materials

Restriction endonuclease, T4 DNA ligase, alkaline phosphatase, the DNA sequencing kit and DNA blunting kit were purchased from Takara Shuzo (Kyoto, Japan). Quick change™ site-directed mutagenesis kit was from Stratagene (La Jolla, CA, USA). Synthetic oligonucleotides were purchased from Kurabo (Osaka, Japan). CM-Toyopearl resin was a product of Toso (Tokyo, Japan). The Centricon centrifugal concentrator was purchased from Amicon (Beverly, MA, USA). All other chemicals were of analytical grade for biochemical use.

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2.2 Bacterial strains and plasmids

E. coli XL-1 blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac (F′ proAB, lacI^{q}Z ΔM15, Tn10 (tet^r)^c), which was used as host cells in all cloning experiments, was supplied by Amersham Japan. *S. cerevisiae* AH22 (Mat α, Leu, His4, Cir$^{+/-}$), used as another yeast expression strain of mutant lysozymes, was provided by Dr. I. Kumagai, Tohoku University. The recombinant plasmid pKK-1, which contains a full-length HEWL cDNA, was also provided from Dr. I. Kumagai, Tohoku University. pYG100, an *E. coli*-yeast shuttle vector, was provided by Dr. K. Matsubara of Osaka University.

2.3 PCR site-directed mutagenesis of hen lysozyme cDNA

The site-directed mutagenesis of hen lysozyme cDNA was carried out using the Quick change™ site-directed mutagnesis kit. The primers of site-directed mutagenesis for constructing various mutants are as follows. 5′-ATAACAGCGACCGTGAACTGC-3′ (sense) and 5′-GCAGTTCACGGTCGCTGTTAT-3′ (antisense); 5′-GACTACGGAGTCCTACAGATC-3′ (sense) and 5′-GATCTGTAGGACTCCGTAGTC-3′ (antisense); 5′-AACTTCAACAGCCAGGCTACA-3′ (sense) and 5′-TGTAGCCTGGCTGTTGAAGTT-3′ (antisense), 5′-AACACCGATAACAGTACCGAC-3′ (sense) and 5′-GTCGGTACTGTTATCGGTGTT-3′ (antisense) were for S91T, I55V and T40S, G49N, respectively. These colonies are selected by LB agar plate with 60 μg/mL ampicillin. The presence of the mutation was confirmed by dideoxynucleotide chain termination method [10] using Thermo Sequence Core Sequencing kit from Amersham.

2.4 Construction of *S. cerevisiae* AH22 expression plasmids of the mutant hen lysozymes

After the PCR site-directed mutagenesis, the plasmid pKK-1 was digested with *Eco*RI/*Hin*dIII. The fragment of mutant lysozyme cDNA was purified by low-melting agarose gel. By using blunt end approach, the cDNA of mutant lysozymes were inserted into the *Sal*I site in the pYG100 vector between the GPD promoter and terminator which is the expression plasmid of *S. cerevisiae* AH22.

2.5 Expression of mutant hen lysozymes in *S. ceevisiae* AH22

The expression vector was introduced into *S. cerevisiae* AH22 according to the lithium acetate procedure. His$^+$ transformants were screened by subculturing n modified Burkholder minimum medium (BMM) supplemented with 60 μg/mL of leucine at 30 °C. The overexpressing subclones with the highest levels of lysozyme activity were screened and propagated from single colonies were inoculated into 3 mL of BMM and incubated for 2 days at 30 °C with shaking. This preculture was subcultured to 100 mL of the same medium in a flask (500 mL) and incubated another 2 days at 30 °C under shaking, and then 10 mL of the second preculture was transferred to fresh 1 L of BMM in a 3 L flask and cultured under the same conditions.

2.6 Purification of the mutant hen lysozymes

The growth medium of the host cell was centrifuged at 7000 rpm for 10 min at 4 °C. The supernatant was applied to a CM-Toyopearl 650M column (1.5 × 5.0 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5), and then adsorbed proteins were step-eluted using the same buffer containing 0.5 M sodium chloride [11]. Fraction containing the protein was collected, and then the samples which were desalted and concentrated by Centricon centrifugal concentrators (Amicon) were used in the experiments.

2.7 Lytic activity of mutant lysozymes

The lytic action of mutant lysozymes derivatives against *M. lysodeikticus* cells was determined with turbidimetric methods [12] based on the decrease in turbidity of a cell suspension following the addition of lysozyme derivatives (0.01%). *M. lysodeikticus* cell suspension optical density (OD = 0.8) was prepared as a substrate in 0.5 M acetic acid –0.5 M sodium phosphate buffer at pH 4.0–9.0. 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 was 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 A450 per min.

2.8 Determination of Δ*G*

The Gibbs free energy change (Δ*G*) was determined from the denaturation curves which were drawn by following the changes in the ellipticity at 222 nm during heating according to [13]. The temperature was controlled during all the measurements by circulating water in the cell holder from a thermostated bath with a heating rate of 1 °C/min from 25–80 °C. Using the data for the thermal denaturation curves, the transition temperature (*Td*) and the apparent fraction (fapp) of unfolding were represented as a function of temperature to show clearly the denaturation curves. The thermal denaturation of the lysozymes at pH 3.0 was completely reversible, therefore, we can calculate the equilibrium constant between the native and the denaturated forms by determining the fraction of unfolding, fd, from the denaturation curves by Eq. (1).

$$K = \text{fapp}/(1 - \text{fapp}) \tag{1}$$

The unfolding enthalpy change (Δ*H*) was calculated by the van't Hoff Eq. (2) as a function of temperature near *Td*, the transition point of denaturation.

$$\ln K1/K2 = -\Delta H^*/R(1/T1 - 1/T2) \tag{2}$$

where Δ*H** is the enthalpy change at *Td*, *T*1 or *T*2 is the temperature near *Td*, *K*1 or *K*2 is the equilibrium constant at *T*1 or *T*2, respectively, and *R* is the gas constant. Entropy (Δ*S*) can be calculated from

$$\Delta G = \Delta H - T\Delta S \tag{3}$$

Because Δ*G* is 0 at *Td*, Δ*S** = Δ*H**/*Td* where Δ*S** is the entropy change at *Td*. The Gibbs energy change (Δ*G*) of unfolding can be calculated by

$$\Delta G = \Delta H^* - T\Delta S^* + \Delta Cp[T - Td - T\ln(T/Td)] \tag{4}$$

where Δ*Cp* is the denaturational increment of the heat capacity; the value for the lysozyme is 1.594 kcal/mol.

3 Results and discussion

3.1 Conformation stability of mutant lysozymes constructed by genetic modification

The conformational stability of the mutant lysozymes was estimated from the denaturation curves by monitoring the change in ellipticity at 222 nm of the CD spectrum during heating at 35–80 °C, because a negative CD value at 222 nm is characteristic of an ordered secondary structure. As shown in Fig. 1 a, the unfolding transition midpoints for nonglycosylated lysozymes S91T, I55V, T40S/I55V and wild-type are 76.6, 71.3, 71.0, and 74.0 °C, respectively. This indicates that S91T is more stable than wild-type lysozyme, while I55V and T40S/I55V are less stable than wild-type lysozyme. The effect of glycosylation on the stability of mutant and wild-type lysozymes was investigated. As shown in Fig. 1 b, the unfolding transition midpoints for glycosylated lysozymes G49N, S91T/G49N, I55V/G49N and T40S/I55V/G49N were 72.1, 74.9, 65.1 and 60.4 °C, respectively. In a similar manner as the nonglycosylated lysozymes, S91T/G49N was heat-stable, while I55V/G49N and T40S/I55V/G49N are unstable compared with the control glycosylated mutant G49N. Although the overall stabilities of glycosylated lysozymes were lower than nonglycosylated lysozymes, the unstable mutants (I55V/G49N and T40S/I55V/G49N) become further more unstable than the stable mutants (G49N, S91T/G49N).

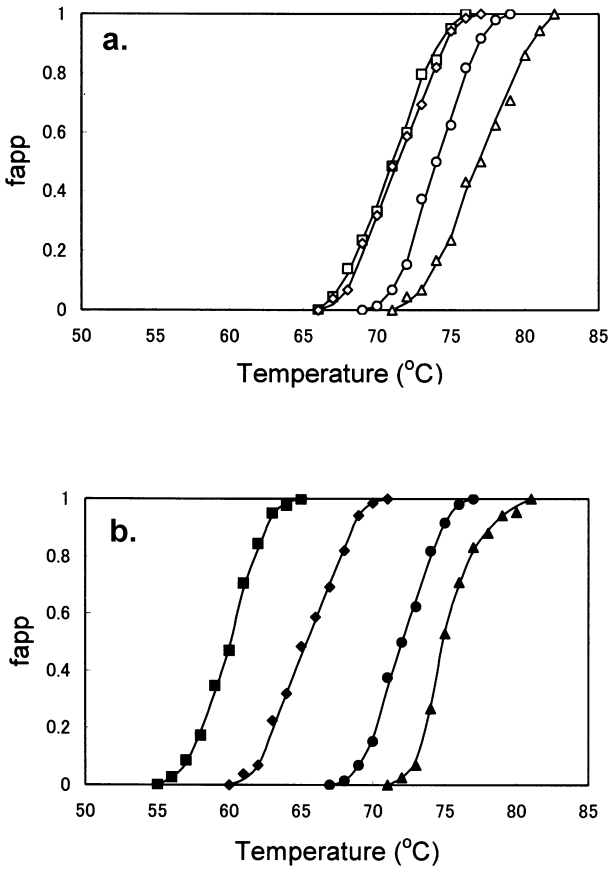

Figure 1. Thermal denaturation curves of wild-type and varies mutant lysozymes. (a) Thermal denaturation curves of wild-type (open cycles) and mutant S91T (open triangles), I55V (open diamonds) and T40S/I55V (open squares) lysozymes; (b) thermal denaturation curves of glycosylated mutant G49N (solid cycles), S91T/G49N (solid triangles), I55V/G49N (solid diamonds) and I55V/T40S/G49N (solid squares) lysozymes. The protein solution was prepared at the concentration of 0.05 mg/mL in 50 mM glycine-HCl buffer (pH 3.5). The thermal denaturation was measured by monitoring the change in ellipticity of CD spectrum at 222 nm from 35 to 80 °C. Fapp indicates the apparent fraction of denaturation.

The Gibbs energy change (ΔG) of denaturation was estimated from the thermal denaturation curve as a quantitative measure of the conformation stability [13]. The thermodynamic parameters of mutant and wild-type lysozymes were calculated from the equation described in Section 2.8. As shown in Table 1, the ΔG for the methyl group inserted mutant (S91T) increased and that for the methyl group removed mutants (I55V, T40S/I55V) decreased. It is reasonable that the addition of hydrophobic (methyl) residue inside the core causes stable conformation and the removal of that causes unstable conformation. The difference in ΔG of glycosylated lysozymes (G49N, S91T/G49N, I55V/G49N and T40S/I55V/G49N) indicated almost the same tendency as that of the nonglycosylated, although the values of ΔG of glycoproteins were commonly lower than that of the nonglycosylated lysozymes.

Since the position of mutation is the surrounding of the active site of lysozyme, the effects of mutant lysozymes on structure were further investigated by following the pH-activity profile against *M. lysodeikticus*. As shown in Fig. 2, the max-

Table 1. Thermodynamic parameters of wild and mutant lysozymes

	Td (°C)	ΔH (kcal · mol^{-1})	$T\Delta S$ (kcal · mol^{-1})	$\Delta G^{a)}$ (kcal · mol^{-1})
Wild	74.0	134.46	387.5	13.85
S91T	76.6	161.60	462.2	18.50
I55V	71.3	107.32	311.7	9.57
T40S/I55V	71.0	105.70	307.2	9.34
G49N	72.1	131.55	381.2	13.31
S91T/G49N	74.9	135.06	391.0	14.19
I55V/G49N	65.1	100.26	296.6	8.35
T40S/I55V/G49N	60.4	94.03	282.2	7.33

Td (transition point of denaturation) was determined by the denaturation curves drawn by following changes in the ellipticity at 222 nm during heating at 35–80 °C in glycine-HCl buffer (pH 3.5). The thermodynamic parameters were determined from the equation in Section 2.8.

a) Values are extrapolated to 20.0 °C

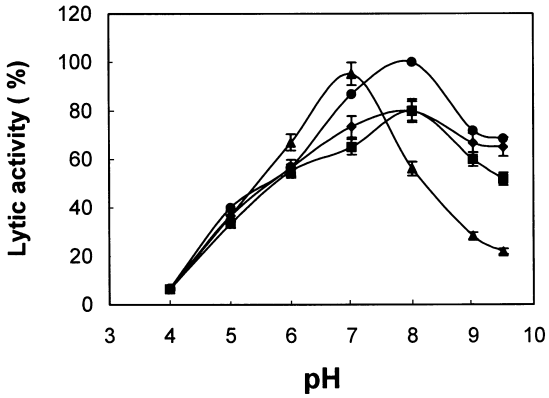

Figure 2. Lytic activity against *M. lysodeikticus* cells of wild-type (solid cycles) and mutant S91T (solid triangles), I55V (solid diamonds) and T40S/I55V (solid squares) lysozymes on pH. The vertical bars indicate the standard deviations (n = 3) of lytic activity.

imal lytic activities of S91T, I55V and T40S/I55V exhibited 94.7, 81.6, 80.2% of that of wild-type lysozyme, respectively. The I55V and T40S/I55V mutants had lower activity than that of wild-type lysozyme, suggesting slight changes in tertiary structure. On the other hand, the lytic activity of S91T mutant lysozyme was almost the same as that of wild-type, although the optimal pH shifted to lower pH.

3.2 Secretion amounts of mutant lysozymes in *S. cerevisiae*

The wild-type and mutant lysozymes were secreted in the culture medium of *S. cerevisiae* AH22 carrying the lysozyme expression vectors. The secreted proteins in the medium were collected and purified by the cation-exchange chromatography on CM-Toyopearl. The SDS-PAGE analysis shows that mutant lysozymes S91T, I55V and T40S/I55V are the same mobility as wild-type lysozyme (Fig. 3a). Taking into account the mobility of SDS-PAGE, they had the same molecular size as wild-type lysozyme (14.3 kDa). Figure 4a shows the secretion amounts of these mutant lysozymes in *S. cerevisiae* AH22. The secretion amouns of S91T greatly increased compared with wild-type lysozymes, while the secretion amounts of mutant lysozymes I55V and T40S/I55V significantly decreased. The SDS-PAGE analysis of glycosylated lysozymes (G49N) showed that the large molecular size of *N*-glycosylated lysozymes with a polymannose chain was secreted in the yeast medium [6]. Figure 4b shows the secretion amounts of glycosylated lysozymes in *S. cerevisiae* AH22. In a similar manner as nonglycosylated lysozymes, the secretion amount of more stable hydrophobic core mutant S91T/G49N was higher than that of mutant G49N, while the secretion amounts of less stable hydrophobic core mutants I55V/G49N

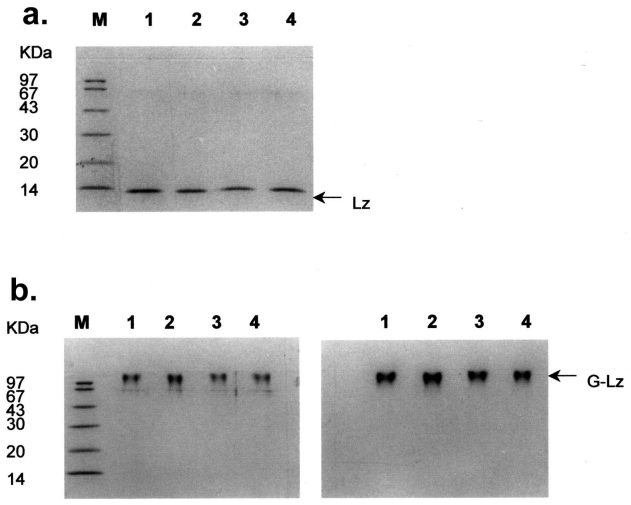

Figure 3. SDS-PAGE patterns of wild-type and mutant lysozymes secreted in *S. cerevisiae* AH22. The peaks of CM-Toyopearl column chromatography were applied to SDS-PAGE. (a) The eluates of unstable mutants (I55V, I55V/G49N, T40S/I55V, T40S/I55V/G49N) were 5-fold concentrated; those of stable lysozymes (wild-type, S91T, G49N, S91T/G49N) were not concentrated before applying to lysozyme; lane 4, T40S/I55V mutant lysozyme. M, molecular marker. Lz, lysozymes. (b) The gel sheets were stained for proteins and carbohydrates with Coomassie Brilliant Blue (left panel) and periodic acid-fuchsin (right panel). Lane 1, S91T/G49N mutant lysozyme; 2, G49N lysozyme; 3, I55V/G49N mutant lysozyme; 4, T40S/I55V/G49N mutant lysozyme. M, molecular marker; G-Lz, glycosylated lysozymes.

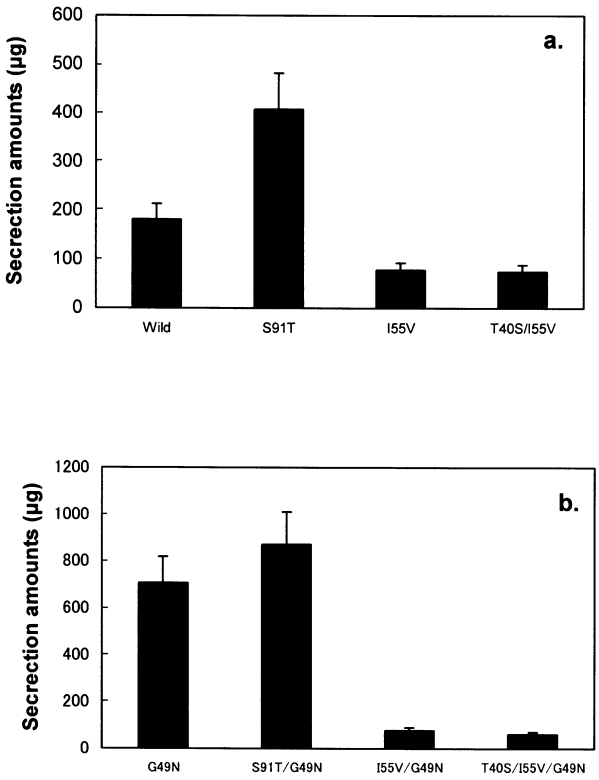

Figure 4. Secretion amounts of (a) nonglycosylated and (b) glycosylated lysozymes expressed in *S. cerevisiae* AH22. Wild-type and mutant lysozymes secreted to the supernatants of growth medium were adsorbed on the CM-Toyopearl 650M column equilibrated with 50 mM Tris-HCl buffer (pH 7.5) and then eluted with the same buffer containing 0.5 M NaCl. The vertical axis indicates the secretion amounts per 1 L of growth medium. The vertical bars indicate the standard deviations ($n = 3$) of secretion amounts.

and T40S/I55V/G49N were much lower than that of mutant G49N. These data strongly suggest the close relationship between the conformational stabilities and secretion amounts of both nonglycosylated and glycosylated lysozymes.

3.3 Relationship between hydrophobic core stabilities and secretion amounts of lysozymes

Interestingly, as shown in Fig. 5, the linear relationship between conformational stabilities (ΔG) and secretion amounts of these mutant lysozymes was observed in these nonglycosylated and glycosylated lysozymes. This result suggests that the secretion amounts closely depend on the hydrophobic core stability of proteins in *S. cerevisiae*. It is probable that the stable lysozyme (S91T) can decrease the energy for folding, thus making the lysozyme folded form easily in the ER. Therefore, the more stable conformation may be possible to pass through the quality control easily in yeast cell. Although the glycosylated lysozymes have undergone the folding and quality control in a different pathway from nonglycosylated lysozymes, the similar good correlation between ΔG and secretion was obtained, suggesting that the secretion amount is closely related with hydrophobic core stability. We have previously reported that *N*-glycosylation could enhance the secretion of wild-type, amyloidogenic and hydrophobic peptide-fused lysozymes in *S. cerevisiae* [6, 7, 14]. The similar result was obtained in these mutant lysozymes (S91T/G49N, I55V/G49N

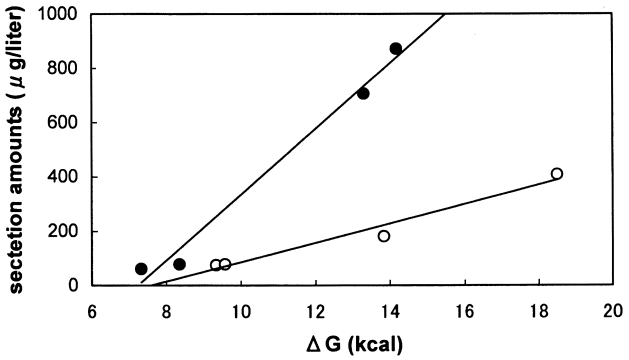

Figure 5. Relationship of the conformational stability (ΔG) with the secretion amounts of mutant lysozymes.

and T40S/I55V/G49N). This suggests that *N*-glycosylation enhancing secretion amounts of lysozymes can be widely applied to mutant lysozymes in *S. cerevisiae*.

Many other mutant lysozymes were well studied in our previous study [7, 15, 16], including mutant K13D (which destabizes α-helix 5–15), C76A (which lacks a disulfide bridge between cysteine 76 and 94), I55T and D66H (which are amyloidogenic variants), hydrophobic peptide-fused lysozyme (in which a hydrophobic pentapeptide Phe-Phe-Val-Ala-Pro was genetically inserted into the *C*-terminus), and so on. These mutations resulted in unstable conformation to lysozymes and then led to the decrease of the secretion amount of the mutant lysozymes, although a linear relationship between the conformational stability (ΔG) and secretion amounts for these mutants was not obtaind. It seems likely that the linear relationship can be obtained only in the mutations at the steric sites around the hydrophobic core. Interestingly, the mutant lysozyme S91T which increases the stability of hydrophobic core can enhance the secretion amount of lysozyme no matter it is glycosylated compared with wild-type lysozyme. This finding directs to the development of the stable lysozyme mutants. It is general that useful foreign proteins suffered from the low secretion when they were expressed in *S. cerevisiae*. The molecular design of enhancing the stabilities of proteins hydrophobic core can be widely available for increasing the products of foreign proteins expressed in *S. cerevisiae*.

4 References

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