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Polymannosylation to asparagine-19 in hen egg white lysozyme in yeast

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Abstract Complementary DNA encoding hen egg white lysozyme (HEWL) was subjected to site-directed mutagenesis to have the N-glycosylation signal sequence (Asn^{19}-Tyr^{20}-Thr^{21}) by substituting Arg with Thr at position 21. The mutant lysozyme (R21T) was expressed in *Saccharomyces cerevisiae* carrying the yeast expression plasmid inserting the mutant HEWL cDNA. The mutant lysozyme was expressed in the glycosylated forms which are mainly a polymannosyl form with a small amount of oligomannosyl form. The polymannosyl lysozyme was susceptible to Endo H cleavage of the carbohydrate chain. The length of the polymannose chain was predicted to be approximately 340 residues/mol of lysozyme from carbohydrate analysis. According to the estimation with low-angle laser light scattering combined with HPLC, the average molecular weight of polymannosyl lysozyme was 75 kDa, which is consistent with the value obtained from the carbohydrate analysis. The size of polymannosyl lysozyme R21T is similar or somewhat larger than that of G49N reported previously. Thus, it was confirmed that the unusually large polymannose chain was attached to heterologous mutant lysozyme, regardless of the N-linked position, in yeast.

Key words: Lysozyme; Polymannosylation; Glycosylation; Genetic engineering; Hen egg white lysozyme

1. Introduction

The yeast *Saccharomyces cerevisae* has been used as a model eukaryotic organism to produce useful heterologous proteins. The glycosylation of proteins using the yeast expression system is one of the most promising approaches to strenghten the stability to heating and protease attack. We reported that the polymannosyl and oligomannosyl lysozymes were expressed in the yeast carrying the mutant lysozyme expression plasmid in which the signal sequence (Asn-X-Thr/Ser) for N-glycosylation was inserted in hen egg white lysozyme (HEWL) cDNA by site-directed mutagenesis [1]. When the signal for N-linked glycosylation was created at position 49, 67, 70 and 103, only the mutant lysozyme (G49N) in which glycine-49 was substituted with asparagine was expressed in the two types of glycosylated forms, a small oligomannose chain ($Man_{14}GlcNAc_2$)-linked form and a large polymannose chain ($Man_{310}GlcNAc_2$)-linked form, whereas other mutants were not glycosylated. As expected, the polymannosyl lysozyme revealed a striking heat stability in that no coagulation was observed under conditions in which the wild-type lysozyme coagulated. Thus, it was proved that the polyglycosylation of proteins was a very useful approach to enhance the structural stability.

In order to elucidate the polymannosylation mechanism of heterologous proteins in yeast, some questions to this finding still remain to be solved: e.g. the reason why only the residue at position 49 was glycosylated should be elucidated, although other mutants were also designed to have a signal sequence for N-linked glycosylation at the molecular surface. In addition, it should be elucidated whether the unusual polymannosylation occurred at the other N-glycosylation site of mutant lysozyme. In *S. cerevisiae*, the core oligosaccharides are elongated in the Golgi cisternae through the stepwise addition of mannose residues catalyzed by several mannosyltransferases, leading to highly branched outer chains consisting of 50–150 mannose residues in the intrinsic proteins of yeast [2,3]. The degree of polymannosylation of mutant lysozyme (G49N) is more than twice that of the intrinsic mannoproteins in yeast. The mechanism of unusual polymannosylation may be further elucidated by the construction of mutants glycosylated at a site other than position 49. Our previous result, that mutant lysozyme was glycosylated at a position in the most N-terminal site of the mutant proteins attempted in the experiment, supports the proposal that the efficiency of the glycosylation decreases with the distance of the N-terminus in proteins [4–6]. Therefore, we attempted to construct a mutant lysozyme having an N-glycosylation signal sequence at a more N-terminal site and on the molecular surface. As a result, we were successful in constructing another polymannosyl lysozyme (R21T) the arginine-21 of which was substituted with threonine and asparagine-19 was glycosylated.

This paper describes the characteristics of the R21T mutant lysozyme to elucidate the mechanism of polymannosylation of mutant lysozymes.

2. Materials and methods

2.1. Materials

The yeast expression plasmid pYG-100 was supplied by Dr. K. Matsubara, Osaka University. The recombinant plasmid pKK-1, which contains a full-length hen egg-white lysozyme cDNA [7] was from Dr. I. Kumagai, University of Tokyo. T4 DNA ligase, alkaline phosphatase and restriction enzymes were purchased from Takara Shuzo (Kyoto). The DNA sequencing kit was also purchased from Takara Shuzo (Japan). The oligonucleotide-directed in vitro mutagenesis system (version 2) for site-directed mutagenesis was purchased from Amersham Japan. CM-Toyopearl 650M resin was from Tosoh (Tokyo). Concanavalin A-Sepharose and α-methylmannoside were supplied by Pharmacia and Wako (Tokyo), respectively. Sephadex G-50 was from Pharmacia. *Micrococcus lysodeikticus* dried cells and ethylene glycol chitin for the lysozyme assay were from Sigma and Nakarai Co. (Japan), respectively. Endo-β-N-acetylglucosaminidase (endo-H) was supplied by Genzyme. All other chemicals were of analytical grade for biochemical use.

2.2. Site-directed mutagenesis of HEWL cDNA

The conversion of the Arg-21 codon to Thr to introduce the asparagine-linked (N-linked) glycosylation sites (Asn-X-Ser/Thr) at the lysozyme molecular surface (positions 19–21) was carried out by a

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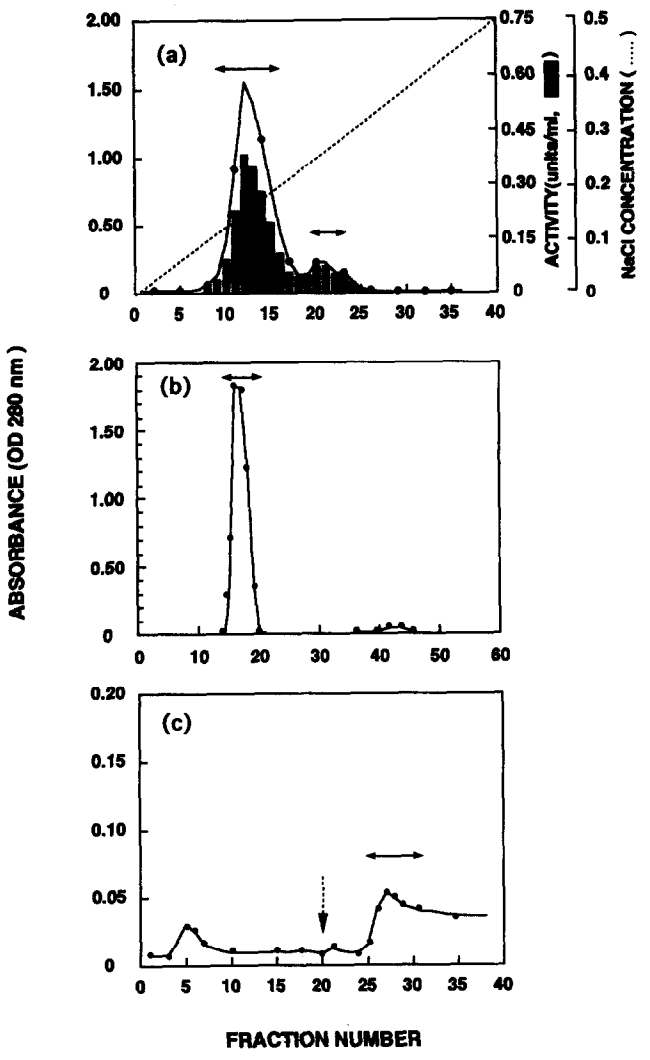

Fig. 1. Isolation of mutant lysozymes secreted from *S. cerevisiae* carrying R21T lysozyme cDNA with CM-Toyopearl column (panel A), Sephadex G-50 column (panel B), and concanavalin A-Sepharose column (panel C). Panel A, The lysozyme secreted in the yeast medium was adsorbed to CM-Toyopearl column. The adsorbed lysozyme was eluted in a gradient elution with 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl. The fractions indicated by horizontal arrows were pooled and used for further purification. Panel B, The first peak in panel A was applied to Sephadex G-50 column and then eluted with 50 mM Tris-HCl buffer (pH 7.5). Panel C, The second and third peaks in panel A were together applied to concanavalin A-Sepharose column prewashed with 10 bed volume of 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl and were eluted with the same buffer. A vertical arrow indicates the start of elution with 100 mM α-methylmannoside. The fractions indicated by horizontal arrow were pooled and used for the experiments.

site-specific mutagenesis using the bacteriophage vector M13mp19. The *Eco*RI/*Hin*dIII fragment of the pKK-1 plasmid was subcloned into the *Eco*RI/*Hin*dIII site of the bacteriophage vector M13mp19. The mutant HEWL cDNA (R21T) was constructed in M13mp19 vector by the Amersham oligonucleotide-directed mutagenesis system. The mutagenic oligonucleotide primer, 5'-GGACTTGATAACTATCGGGGATACAGCCTG-3', was synthesized by the phosphoamidate method using a Pharmacia DNA synthesizer to convert the Arg-21 (CGG) codon to Thr (ACG). The presence of the mutation was confirmed by the dideoxy-DNA sequencing analysis [8].

2.3. Expression of the mutant HEWLs

For construction of the yeast expression plasmids, the mutant HEWL cDNAs were inserted into the *Sal*I site of pYG-100, as previously described [7,9]. The expression vector was introduced into *S. cerevisiae* AH22 (*MAT*a, *Leu2*, *His4*, *Cir*$^+$) according to the lithium acetate procedure [10]. Leu$^+$ transformants were screened by subculturing in modified Burkholder minimum medium [11] supplemented with histidine (20 μg/ml) at 30°C. After cultivation, well-growing colonies were then replica-cultivated in the yeast medium on a small scale (5 ml), and the over-expressing subclones with the highest levels of lysozyme activity were screened and propagated from single colonies. The over-expressing colonies were directly subcultured on a large scale in yeast minimum medium at 30°C for 5 days.

2.4. Purification of the mutant HEWLs

Six liters of the growth medium of the host cell was centrifuged at 6000 × *g* for 15 min at 4°C to remove the cells, and diluted with deionized water at least twice. The solution was directly applied to a CM-Toyopearl 650M opened column (1.8 × 5 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5), and the column was washed with the same buffer until the washing solution was free from proteins. The adsorbed lysozyme was eluted with 0.5 M NaCl in 50 mM Tris-HCl buffer (pH 7.5). The protein solution that was diluted with deionized water at least 5 times was again applied to the regenerated CM-Toyopearl 650M opened column (1.8 × 2 cm) and then gradient elution was carried out with 0.5 M NaCl. The main peak of lysozyme was then applied to a Sephadex G-50 gel filtration column (1.1 × 45 cm). The small peak of lysozyme was applied to a concanavalin A-Sepharose column prewashed with 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl and was subsequently eluated with 100 mM α-methylmannoside. The protein content in each of the fractions was detected by measuring the absorbance at 280 nm, and the carbohydrate content was determined by measuring the absorbance at 490 nm after color development with the phenol-sulfuric acid reaction. All of the fractions containing glycoprotein were collected together and concentrated using a regenerated cation-exchange column.

2.5. Endo-H treatment

The method of Tarentino and Maley [12] was slightly modified for the digestion of the glycosylated lysozymes with endo-β-*N*-acetylglucosaminidase (endo-H). The glycosylated lysozymes (0.2 mg/ml) were boiled in a 50 mM sodium citrate buffer (pH 5.5) containing 1% SDS and 200 μg/ml of phenylmethylsulfonyl fluoride for 5 min. After cooling, samples were supplemented with an equal volume of either 0.02 U of endo-H in 50 mM sodium citrate buffer (pH 5.5) or the same buffer without the enzyme and were sequentially incubated 37°C for 20 h. After incubation, the samples were analyzed by SDS-PAGE.

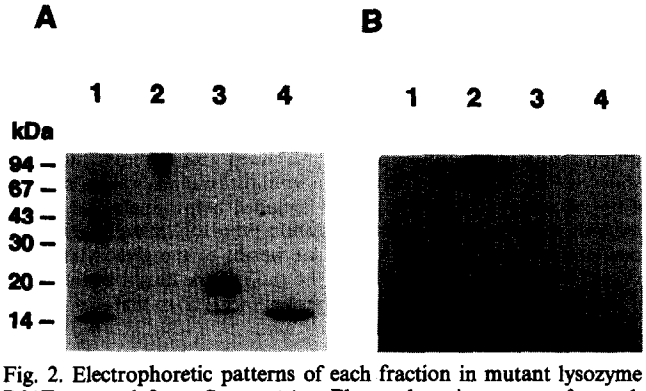

Fig. 2. Electrophoretic patterns of each fraction in mutant lysozyme R21T secreted from *S. cerevisiae*. Electrophoretic patterns for each peak in Fig. 1a were represented. The gel sheets were stained for proteins and carbohydrates with Coomassie Brilliant Blue (panel A) and periodic acid-Fuchsin (panel B), respectively. Lane 1, molecular weight markers (94,000, phosphorylase b; 67,000, bovine serum albumin; 43,000, ovalbumin; 30,000, carbonic anhydrase; 20,100, trypsin inhibitor; 14,300, α-lactalbumin); Lane 2, first peak (fraction 10 to 15 in Fig. 1a); Lane 3, second peak (fraction 19 to 21 in Fig. 1a); Lane 4, third peak (fraction 24 to 25 in Fig. 1a).

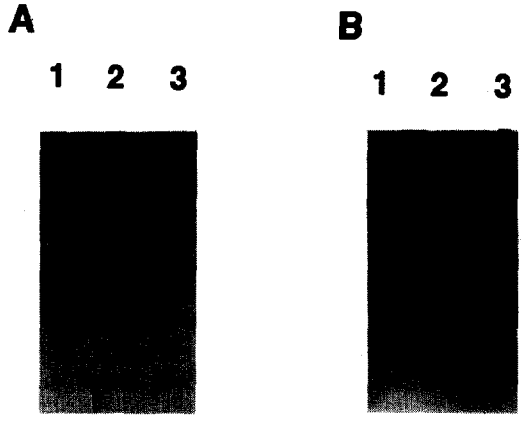

Fig. 3. Endo-H treatment of polymannosyl lysozyme R21T secreted from *S. cerevisiae*. Endo-H digest was subjected to SDS-PAGE. The gel sheets were stained for proteins and carbohydrates with Comassie Brilliant Blue (panel A) and periodic acid-Fuchsin (panel B), respectively. Lane 1, wild lysozyme; Lane 2, polymannosyl lysozyme treated with endo-H; Lane 3, polymannosyl lysozyme.

2.6. SDS-PAGE

SDS-PAGE was conducted according to the method of Laemmli [13] using a 15% acrylamide separating gel and a 5% stacking gel containing 1% SDS. Samples were heated at 100°C for 5 min in a 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 buffer of Tris-glycine containing 0.1% SDS. After electrophoresis, the gel sheets were then stained for protein and carbohydrate with 0.025% Coomassie brilliant blue-R250 solution and 0.5% periodic acid-Fuchsin solution [14], respectively.

2.7. Chemical analysis

The total sugar content of the glycosylated lysozymes was estimated by using the phenol-sulfuric acid reaction employing mannose as a standard. HPLC analysis serves as a method of identification of the hexose liberated from glycosylated lysozymes by hydrolysis with 2 N HCl at 100°C for 3 h in a sealed glass ampule. The hydrolysates were then dried, dissolved in water, and chromatographed on an Asahipak NH2P-50 column (Asahi Chemical, Tokyo) in 75% acetonitrile using the Hitachi HPLC system equipped with an RI detector. For the analysis of hexosamine, the hydrolysates with 3 N HCl at 100°C for 4 h were dried and then analyzed using an amino acid analyzer.

2.8. Determination of the molecular weight

The molecular weight of the polyglycosyl lysozyme was determined according to the low-angle laser scattering method [15]. The glycosylated lysozyme solution (0.5%) in 200 mM sodium phosphate buffer (pH 6.9) was applied to a high performance gel-chromatography column, connected with a TSK gel G3000SW column (Tosoh; 0.75 × 60 cm) equilibrated with the above buffer, and eluted with the same buffer at a flow rate of 0.3 ml/min. The elution from the columns was monitored using an array of detectors which included a low-angle laser light scattering photometer (Tosoh; LS-8) and a precision differential refractometer (Tosoh; RI-8). The molecular weight of the glycosylated lysozyme (MW) was estimated from the ratio of the height of the peak of a low-angle laser light scattering photometer (LS) to that of a refractometer (RI), according to [15].

2.9. Enzymatic assay

Lysozyme activity was measured by a lysis and glycolysis assay using *M. lysodeikticus* cells and ethylene glycol chitin as substrates, respectively. Bacteriolytic activity of lysozyme was assayed by the method of Parry et al. [16] with slight modification. The suspensions of *M. lysodeikticus* cells (OD_{450} = 0.7) were prepared in 100 mM acetic acid-sodium acetate buffer at pH 6.0. After first adjusting its absorbance at 280 nm to 0.050, the assay lysozyme solution (0.1 ml) was added to 2.4 ml of the cell suspension in each buffer. The initial decrease in the absorbance at 450 nm of the mixture caused by lysis of *M. lysodeikticus* cells was measured at 20°C for 1 min with a Hitachi U-2000 spectrophotometer. A decrease of 0.001 was defined as one unit. Hydrolytic activity with glycolysis was measured by following the reducing procedure [17]. To 0.5 ml of lysozyme solution in 10 mM acetic acid-sodium acetate buffer (pH 4.5) was added 1.0 ml of 0.05% solution of ethylene glycol chitin. The mixture was incubated at 40°C for 30 min. After the reaction, 2 ml of the color reagent (made by dissolving 0.5 g potassium ferricyanide in 1 l of 0.5 M sodium carbonate) was added, and the mixture was immediately boiled for 15 min to estimate the reducing power resulting from hydrolysis of ethylene glycol chitin. The amounts (μmol/min) of reducing groups produced by lysozyme action was defined as one unit.

3. Results and discussion

Mutant lysozymes were constructed so as to have the signal sequence (Asn-X-Thr/Ser) for N-glycosylation at position 16–24 in the region of the N-terminal site. This is located on the molecular surface and forms a β-turn structure, because this region is the most promising site for N-glycosylation. Among the mutants constructed, R21T, the arginine-21 of which was substituted with threonine was successfully secreted in the glycosylated forms. This mutant has a signal sequence for N-glycosylation sequence of Asn-Tyr-Thr at position 19–21, thereby the glycosylation occurs at the position of asparagine-19. The mutant lysozyme (R21T) secreted in the yeast medium was collected and purified by a cation-exchange chromatography. The elution pattern of R21T on a CM-Toyopearl column is shown in Fig. 1a. The first main peak was applied to a Sephadex G-50 column (Fig. 1b), and the second and third peaks were together applied to a concanavalin A-Sepharose column (Fig. 1c). The main peak was eluted in the void volume, suggesting the presence of polysaccharide chain-linked lysozymes (Fig. 1b). On the other hand, the second and third peaks were separated into the adsorbed and non-adsorbed fractions on a concanavalin A-Sepharose column, indicating the presence of oligosaccharide chain-linked lysozyme. Fig. 2 shows the SDS-PAGE patterns of the first (lane 2), second (lane 3) and third (lane 4) peaks. The SDS-PAGE analysis (panel A) reveals that the first and second peaks are a much larger and slightly larger size of lysozyme, respectively, and the third peak corresponds to the normal size of lysozyme. The carbohydrate-stained patterns (panel B) shows that the first peak is strongly stained, whereas the second peak is slightly stained. The results suggest that the mutant R21T secretes the polymannosyl form, with a small amount of the oligomannosyl

Table 1
Enzymatic activity of glycosylated lysozymes

Lysozyme	Enzymatic activity	
	Glycolysis[1] (U/mg)	Lysis[2] (U/mg)
Wild	0.90	17,518 ± 43
Oligomannosyl	0.90 ± 0.02	13,839 ± 438
Polymannosyl	0.76 ± 0.01	394 ± 228

The enzymatic activities were determined by using the polymannosyl lysozyme purified by gel filtration on a Sephadex G-50 column (Fig. 1b) and the oligomanosyl lysozyme purified by a concanavalin A-Sepharose column (Fig. 1c).

[1] Micromoles of reducing groups in glycol chitin per min.

[2] Decreases in the turbidity (OD450) of *M. lysodeikticus* per min, when decrease of 0.001 was defined as are unit.

form, of lysozyme into the medium. Approximately 80% of lysozyme was secreted outside, while 20% was retained within the yeast cells.

To prove the type of N-linked glycosylation, polymannosyl lysozyme was treated with endo H, which is a specific enzyme for the cleavage of the N-linked glycosylated chain in glycoprotein. As shown in Fig. 3, the polysaccharide chain of R21T was completely digested by endo-H. The same result was obtained in the case of oligomannosyl lysozyme (data not shown). Thus, it was confirmed that R21T was glycosylated with an N-linked polysaccharide chain.

Enzymatic activity of mutant lysozyme was measured by both the lysis of *M. lysodeikticus* and the glycolysis of glycol chitin. Table 1 shows the lysozyme activities of polymannosyl lysozyme purified by gel-filtration on a Sephadex G-50 column (Fig. 1b) and oligomannosyl lysozyme purified by a concanavalin A-Sepharose column (Fig. 1c). Although the lytic activity for *M. lysodeikticus* was diminished in the polymannosyl lysozyme, probably due to the steric hindrance of the polysaccharide chain, the glycolysis activity of polymannosyl lysozyme was kept to about 70% of that of the wild-type. On the other hand, both lytic and glycolysis activity were completely conserved in the oligomannosyl form.

The carbohydrate analysis of the first peak showed that R21T had 338 mannose residues per one mole lysozyme (Table 2). About 10% longer mannose chains were additionally polymerized in R21T than G49N [1]. This was further confirmed by estimating the molecular weight of the polymannosyl lysozyme R21T. The molecular weight distribution of polymannosyl lysozyme R21T was estimated by the low angle laser light scatting technique combined with HPLC, which is a promising procedure for the estimation of molecular mass of heterogeneous biopolymers such as protein–polysaccharide conjugates [15]. Fig. 4 shows the elution patterns of the polymannosyl lysozymes obtained with a low-angle laser light scatting photometer (LS) and a differential refractometer (RI). The molecular weight at each elution position was calculated from the ratio of LS to RI in Fig. 4, as described previously [15]. The molecular weights of polymannosyl lysozyme R21T were distributed in the range of 40–90 kDa, indicating that the degree of polymerization of mannose is about 150–420. Thus, it was confirmed that the same degree or slightly higher degree of polymannosylation occurred in R21T, compared to G49N [1].

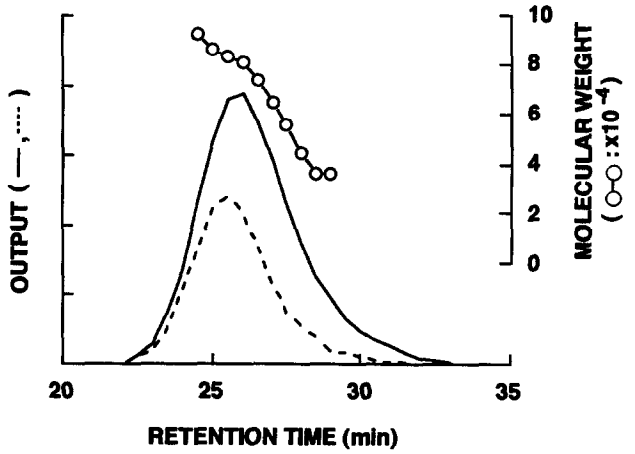

Fig. 4. Molecular weight distribution of polymannosyl lysozymes R21T using low angle laser light scattering technique combined with HPLC. The polymannosyl lysozymes (0.5%) in 200 mM sodium phosphate buffer (pH 6.9) was applied to TSK-gel G3000SW column (0.75 × 60 cm, Tosoh) at a flow rate of 0.3 ml/min. The elution was monitored with a low angle laser light scattering detector (----) and a precision differential refractometer (—). The molecular weight distribution (o–o) for polymannosyl lysozyme was determined from the ratio of the height of the LS peak to the RI peakby the equation described in a previous paper [14].

Table 2
Carbohydrate analysis of polymannosyl and oligomannosyl lysozymes

Glycosylated lysozyme	Contents (moles/mol lysozyme)	
	N-Acetylglucosamine	Mannose
Polymannosyl	2	338 ± 20
Oligomannosyl	2	14 ± 1

The carbohydrate analysis was carried out with the polymannosyl lysozyme purified by gel filtration on a Sephadex G-50 column (Fig. 1b) and the oligomannosyl lysozyme purified by a concanavalin A-Sepharose column (Fig. 1c).

The highly polymannosylated lysozyme was expressed in *S. cerevisiae* carrying the yeast expression plasmid inserting the HEWL cDNA mutated to have the N-glycosylation signal sequence Asn^{19}-Tyr^{20}-Thr^{21} by substituting Arg-21 with Thr. Therefore, it was confirmed that the extremely large polymannose chain was commonly attached to heterologous lysozyme in yeast as well as other mutant lysozyme G49N [1]. The length of polymannose residues of R21T and G49N lysozymes was more than twice that of the general mannoproteins in yeast. Therefore, it seems likely that hen egg white lysozyme may have the capability of being highly polymannosylated. Since the elongation of the polymannose chains occurs in the Golgi apparatus, it is most probable that the time interval that mutant lysozyme is present in Golgi cisternae is longer than that of the general mannoproteins, because of such structural properties as a highly basic isoelectric point and a rigid conformation.

The cDNA of lysozyme encoding the signal sequence for N-glycosylation, can be used as a reporter gene in studies of the processing or trafficking of N-linked glycoproteins in yeast, which is still unclear. We are now studying some of the interesting changes in the glycosylation patterns with various stresses such as pH and heat for yeast, to further elucidate the mechanism of polymannosylation of heterologous lysozyme.

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