Effective reduction of antigenicity of hen egg lysozyme by site-specific glycosylation

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Abstract Various mutant lysozymes were constructed by genetic modification and secreted in yeast expression system to evaluate the changes in the antigenicity of hen egg lysozyme (HEL). Although Arg68, the most critical residue to antigenicity of HEL, was substituted with Gln, the binding of monoclonal antibodies (mAbs) with the mutant lysozyme did not critically reduce, remaining 60% of the binding with mAb. In contrast, glycosylated mutant lysozyme G49N whose glycine was substituted with asparagine dramatically reduced the binding with mAb. The oligomannosyl type of G49N lysozyme reduced binding with mAb to one-fifth, while the polymannosyl type of G49N lysozyme completely diminished the binding with mAb. This suggests that the site-specific glycosylation of lysozyme in the interfacial region of lysozyme–antibody complex is more effective to reduce the antigenicity than the mutation of single amino acid substitution in the interfacial region.

Key words: Reduction of antigenicity; Glycosylated lysozyme; Site-specific glycosylation

1. Introduction

Human allergies to food and pollen proteins are becoming of a concern as the number of patients is greatly increasing. We have reported that the antigenicity of proteins in soybean [1] and Japanese cedar pollen [2] can be masked by polysaccharide attachment. The immunoblotting analysis with patient's sera revealed that Maillard-type soy protein–polysaccharide conjugate was very effective to mask the allergen structure of soy 34-kDa protein [1]. A similar observation was obtained for the reduction of allergen structure of allergen CJ1 in Japanese cedar pollen [2]. The Maillard-type polysaccharide conjugate was formed between the ε-amino group in the protein surface and the reducing end carbonyl group in polysaccharide [3]. Although a limited number (one or two) of polysaccharides is bound to protein, the binding is non-site-specific. This arises the difficulties to elucidate the molecular mechanism of the masking of the antigenic structure of allergen proteins by polysaccharide attachment. In order to elucidate the molecular mechanism of the masking of allergenic structure of allergen proteins with polysaccharide, hen egg lysozyme (HEL) was used as a model protein. HEL is one of the first proteins of which the antigenic structure has been defined by X-ray analysis of the HEL–antibody complex [4]. Major epitopes consist of the 14–17 amino acids spanning two or more non-contiguous segments that contact with antibody in the X-ray structure of the complex [4,5]. Therefore, it seems likely that the dramatic reduction of antigenic structure does not result from only the single amino acid substitution of antigen HEL, although a partial lowering of affinity with antibody may be observed by increasing dissociation rates. We have reported that the binding of HEL with monoclonal antibody (mAb) was dependent on the conformation and that the mutation of the surface region showed no dramatic reduction of the binding [6]. The site-specific glycosylation of antigen in the interfacial region with antibody may be one of the most promising methods to dramatically reduce the allergen structure of HEL, because the broad steric hindrance occurs in the interfacial region. We have developed the genetic engineering using a yeast expression system to attach an oligosaccharide and a polysaccharide to HEL by the introduction of the N-linked glycosylation signal sequence Asn-X-Thr/Ser, and succeeded to construct oligomannosyl and polymannosyl lysozymes [7].

This paper describes the effects of genetic glycosylation to HEL on the antigenic structure. This study may contribute to the development of non-allergenic food and pollen proteins.

2. Materials and methods

2.1. Preparation of mAbs

7-week-old male BALB/c mice were immunized intraperitoneally with 50 μg lysozyme in complete Freund's adjuvants (500 μl/mouse). After 3 weeks, they were boosted with antigen in 500 μl of phosphate-buffered saline (PBS(-)). The preparation of hybridoma was carried out as previously described [6]. The cloned antibody-producing hybridoma were grown by mass culture in NS-1 medium. To purify γ-globulin fraction from the culture medium, the medium was adjusted to 40–50% saturation with ammonium sulfate and allowed to stand overnight at 4°C. After centrifugation, the precipitate was dissolved in water, dialyzed against distilled water and freeze-dried.

2.2. Binding of mAbs 4G5 and TL G1100 with various mutant lysozymes

The binding of mAbs with mutant lysozymes was determined by competitive enzyme-linked immunosorbent assay (ELISA) [8]. 100 μl of antigen solution (100 μg/ml) was mixed with an equal volume of mAb solution (100 μg/ml). The free antibodies were detected with solid phase ELISA as follows. The wells of microtiter plates were preliminary seeded with 100 μl of the antigens (100 μg/ml) and then coated with 1% bovine serum albumin in PBS(-). The plates were rinsed with PBS(-) containing 0.05% Tween 20 (PBS(-) Tween), and then 100 μl of mAbs (100 μg/ml) in PBS(-) were added to each well.

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After the plates were washed with PBS(-) Tween, 100 μl of diluted goat anti-mouse antibody conjugated with peroxidase was added to each well. After washing with PBS(-) Tween, 100 μl of substrate (0.04% *O*-phenylenediamine and 0.02% H_2O_2 in 0.05 M citrate – 0.1 M phosphate buffer at pH 5.0) was added. The reaction was stopped by the addition of 50 μl 2.5 M H_2SO_4, and the absorbance of each well was measured at 490 nm on a microplate reader (Bio-Rad Model 450).

2.3. Preparation of mutant HEL

For construction of the yeast expression plasmids, the wild-type and mutant HEL (R68Q, K13D, C94/76A) cDNAs were inserted into the *Sal*I site of pYG-100, as described previously [7,9]. The expression vectors were introduced into *Saccharomyces cerevisiae* AH22. Leu^+ transformants were screened by subculturing in the modified Burkholder minimum medium plates supplemented with histidine (20 μg/ml) at 30°C. After cultivation, well growing colonies were replica cultivated in the yeast medium on a small scale (5 ml) and the overexpression subclones with the highest level of lysozyme activity were screened and propagated from single colonies. The overexpression colonies were directly subcultured in a large scale in the yeast medium at 30°C for 5 days. 6 l of the growth medium of the host cell was centrifuged at 6000×*g* for 15 min to remove cells at 4°C and diluted with deionized water at least twice. The solution was directly applied to a CM Toypearl 650M column equilibrated with 50 mM Tris–HCl buffer (pH 7.5). After rechromatography, the mutant HEL was collected and used for the experiment.

2.4. Preparation of glycosylated mutant HEL

The genetic modification was attempted to construct oligomannosyl and polymannosyl lysozymes as reported by Nakamura et al. [7]. To introduce the potential N-linked glycosylation site (Asn-X-Ser/Thr), the conversion of the Gly49 codon to Asn was carried out by site-directed mutagenesis. The mutant HEL cDNA was inserted into the *Sal*I site of pYG-100 and secreted in the yeast medium as described above. The oligomannosyl and polymannosyl lysozymes were separated by gel filtration on a column of Sephadex G-50.

2.5. *Endo*-b-N-*acetyl glucosaminidase (Endo-H) treatment*

The method of Tarentino and Maley [10] was slightly modified for the enzymatic deletion of saccharide chains of glycosylated HEL with Endo-H. The glycosylated lysozyme was incubated with an equal volume of 50 mM citrate buffer (pH 5.5) with 0.02 units of Endo-H at 37°C for 24 h.

2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out according to the method of Laemmli [11] using 15% acrylamide separating gel and 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 2-β-mercaptoethanol. Electrophoresis was carried out at a constant current of 10 mA for 5 h using an electrophoresis buffer of Tris–glycine containing 0.1% SDS. After electrophoresis, the gel sheet was stained for protein with 0.025% Coomassie brilliant blue R-250 solution.

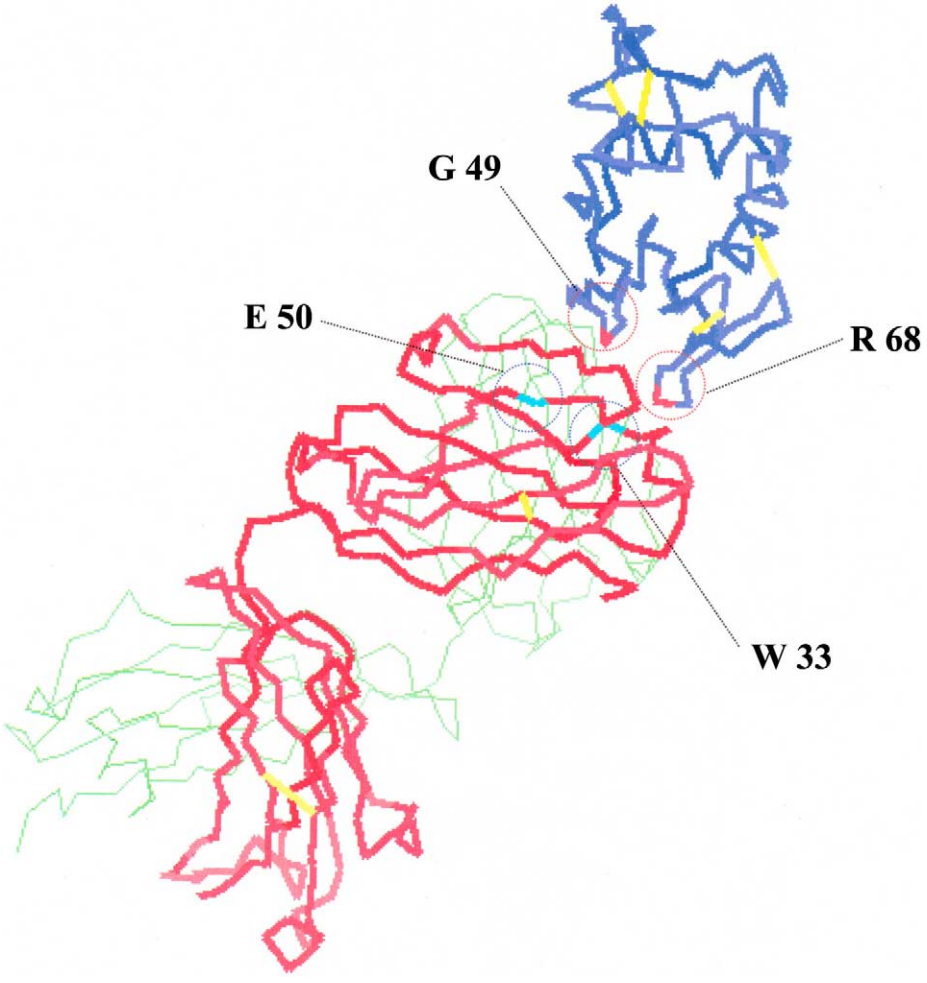

Fig. 1. Three-dimensional (3D) line image for the interfacial region of lysozyme–Fab (HyHEL-5) complex. W33 and E50 (cyan in blue circle) in VH chain of HyHEL-5 (red) bind to one surface of lysozyme (blue). Critical residues of antigenicity of lysozyme (R68) and glycosylation point (G49) are indicated in red circle. Yellow lines indicate disulfide bonds, and the VL chain of HyHEL-5 is in green. The 3D line image was drawn with reference to Molecules RUS form (NIH) – WEBMOL Java Viewer (File name: IgG1 Fab Fragment(HyHEL-5) Complexed with Lysozyme Mutant R68).

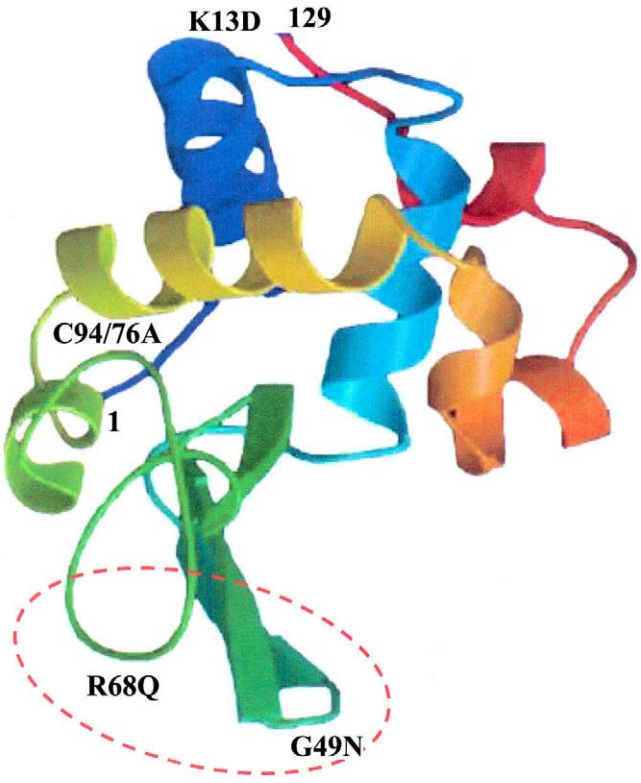

Fig. 2. Structure of HEL and the position of mutation. 1 and 129 indicate N- and C-terminal residues, respectively. Four positions of mutation were shown in the figure. The interfacial region with antibody was indicated by circle dotted line.

2.7. Preparation of Maillard-type lysozyme–polysaccharide conjugates

The attachment of galactomannan or dextran (average molecular weight: 10∼20 kDa) to lysozyme was carried out using Maillard reaction in dry state according to the method of Kato et al. [6]. Lysozyme–polysaccharide powder mixtures in the molar ratio of 1:4 were dissolved in water at 10% (w/v) and lyophilized. Lysozyme–polysaccharide mixtures were dry-heated at 60°C under 65% relative humidity (RH) in a desiccator containing saturated KI solution in the bottom for 2 weeks. Maillard reaction between the ε-amino groups in protein and the reducing end carbonyl groups in polysaccharide is accelerated in the low water activity described above. The conjugate thus obtained was purified as described below. To remove polysaccharide-free lysozyme–polysaccharide, the conjugates were purified by cation-exchange chromatography using a CM Toypearl 650 column. Elution was done with a linear gradient with NaCl (0–0.5 M) in 0.01 M phosphate buffer (pH 7.0). The peak of lysozyme–polysaccharide conjugate was collected and dialyzed against distilled water and then lyophilized.

3. Results and discussion

3.1. Construction and secretion of mutant HEL to reduce the antigenicity

Various mutants were designed for the reduction of the antigenic structure of HEL. Major epitopes contain the 14–17 amino acid residues that contact with antibody in the X-ray structure of the lysozyme–antibody complex [4]. Among these residues, the importance of Arg68 in the antigenicity of HEL was confirmed by many investigations. As shown in Fig. 1, the antigenic determinants of HEL were identified as Arg45 and Arg68 that were spatially adjacent on the surface of the groove of Fab (HyHEL-5) in the antibody [4]. Thus, R68Q was constructed to reduce the antigenicity of HEL. In addition, the glycosylation mutant (G49N) close to the interfacial region of HEL with antibody may be one of the most promising methods to reduce the antigenicity. The HEL (K13D and C94/76A) mutated at residues far from the interfacial region with antibody were also constructed to evaluate the effect of conformational stability on the reduction of antigenic structure. The mutated positions were shown in Fig. 2.

The SDS–PAGE patterns of these mutants were shown in Fig. 3. The mutants R68Q, K13D, C94/76A showed a single band having the same size as wild-type lysozyme, while mutant G49N showed non-glycosylated and glycosylated bands having higher molecular size of oligomannosyl and polymannosyl lysozymes. The carbohydrate chain of oligomannosyl lysozyme was $Man_{18}GlcNAc_2$ and that of polymannosyl lysozyme was $Man_{310}GlcNAc_2$ [7]. These mutant HELs were used in the experiments.

3.2. Reduction of antigenic structure of lysozyme by genetic modifications

We reported that the mAb of HEL could sensitively detect the conformational changes [6]. The binding of mAb with HEL was decreased both by denaturation with heat and guanidine–HCl, corresponding to the denaturation curves of lysozyme. This suggests that the epitope region of HEL is specific to the tertiary structure around two loops containing Arg45 and Arg68. Therefore, mAbs used here seem to be HyHEL-5 [5]. As shown in Fig. 4, R68Q mutant HEL reduced the binding with mAbs 4G5 and TL G1100 in a similar manner as the unstable mutant lysozymes, such as helix-destabilized mutant (K13D) and disulfide bond deletion mutant (C94/76A). However, the reduction was in part and still remained the reactivity with mAbs. On the other hand, the oligomannosyl and polymannosyl lysozymes (G49N) greatly decreased the binding capacity with mAbs, suggesting that the

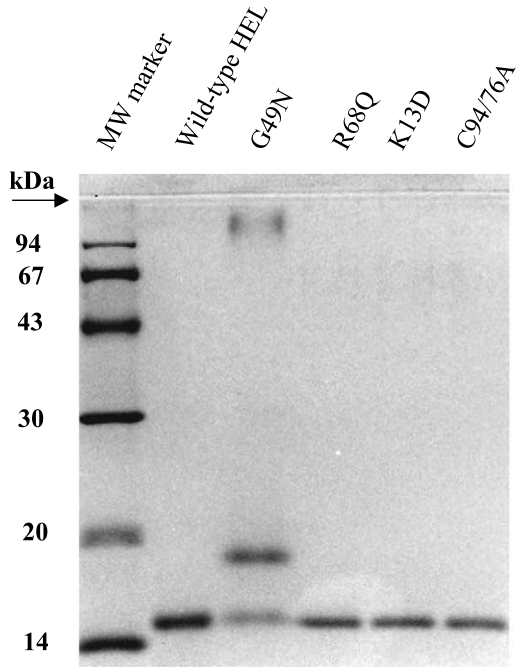

Fig. 3. SDS–PAGE patterns of wild-type and mutant lysozymes secreted in the cultivation medium of *S. cerevisiae*. Molecular markers used are as follows: 94 000 Da, phosphorylase b; 67 000 Da, bovine serum albumin; 43 000 Da, ovalbumin; 30 000 Da, carbonic anhydrase; 20 100 Da, trypsin inhibitor; 14 300 Da, α-lactalbumin.

extended carbohydrate chain may result in the steric hindrance around the epitope region near Arg45, as shown in Fig. 5. The binding capacity with mAbs of glycosylation mutant was almost recovered by Endo-H treatment, although the binding capacity with mAbs was not recovered completely, because lysozyme was slightly denatured or one molecule of sugar (GlcNAc) on Arg49 still remained to attach after Endo-H treatment. The site-specific glycosylation of HEL in the

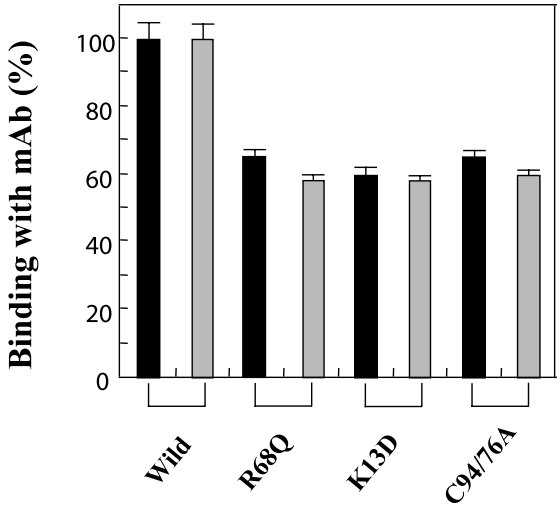

Fig. 4. Binding of various mutant lysozymes with mAbs. Binding was represented as the percentage of the absorbance of mutant lysozymes to that of wild-type lysozyme in solid phase ELISA. The dark bars indicate the binding with mAb 4G5, and the light bars indicate the binding with mAb TL G1100. The data are shown as mean ± S.D. of five replicates.

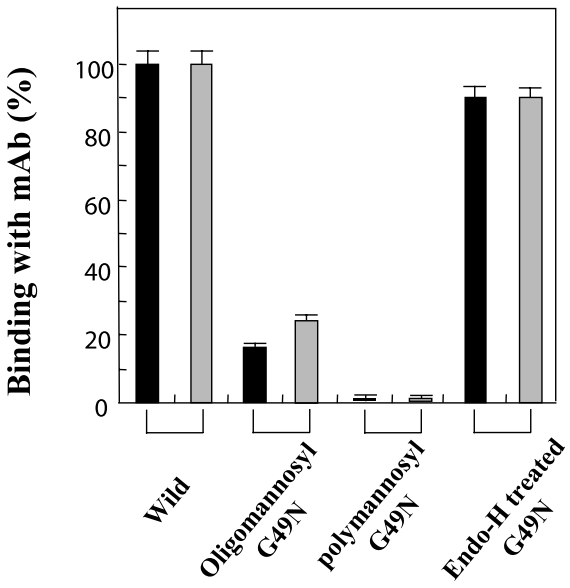

Fig. 5. Binding of glycosylated mutant lysozymes with mAbs. Binding was represented as the percentage of the absorbance of mutant lysozymes to that of wild-type lysozyme in solid phase ELISA. The dark bars indicate the binding with mAb 4G5, and the light bars indicate the binding with mAb TL G1100. The data are shown as mean ± S.D. of five replicates.

binding interfacial region with antibody seems to be very effective to reduce the reduction of antigenicity.

3.3. *Effect of Maillard-type polysaccharide attachment on the antigenicity of HEL*

Effect of non-site-specific glycosylation on the reduction of antigenicity was investigated. Maillard-type polysaccharide–

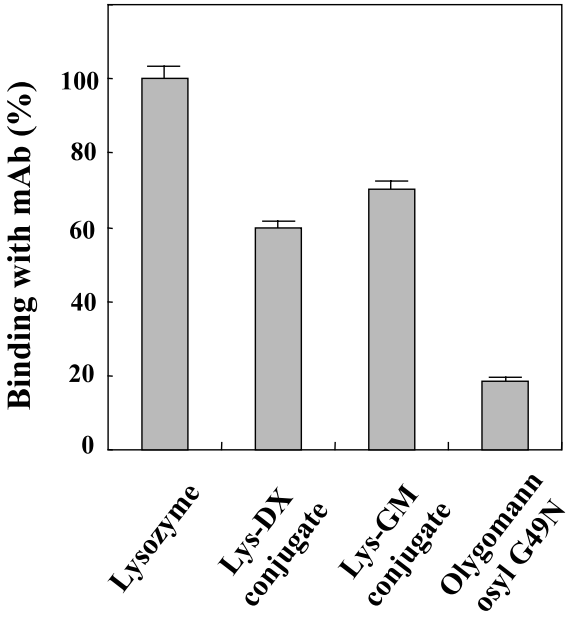

Fig. 6. Binding of Maillard-type lysozyme–polysaccharide conjugates with mAb 4G5. Binding was represented as the percentage of the absorbance of lysozyme–polysaccharide conjugates to that of native lysozyme in solid phase ELISA. The data are shown as mean ± S.D. of five replicates.

lysozyme conjugate can be formed by dry-heating at 60°C between the lysyl residue at position 97 and reducing end carbonyl group in polysaccharide [12]. Therefore, it seems likely that polysaccharides attach to the residue far from the interfacial region with antibody. Thus, the binding of Maillard-type lysozyme–dextran and lysozyme–galactomannan conjugates with mAbs was shown in Fig. 6. The binding with mAb significantly reduced, but dramatic reduction was not observed. This observation suggests that non-specific glycosylation is not necessary for reducing the antigenicity, but site-specific glycosylation is essential for the reduction of antigenicity of HEL.

In conclusion, a single amino acid mutation in the interfacial region of the HEL–antibody complex did not critically reduce the antigenicity, because of the broad contact area between HEL and antibody. In contrast, the site-specific glycosylation of HEL at the interfacial region dramatically reduced the antigenicity.

We previously reported that the production of antibodies (IgG and IgE) against lysozyme was greatly suppressed by the intraperitoneal injection of site-specific polymannosyl lysozymes (G49N), while the production of IgG was enhanced by the intraperitoneal injection of galactomannan–lysozyme conjugate, although the production of IgE was suppressed [13]. These observations suggest that masking the epitope of antigen proteins by site-specific polysaccharide attachment may be effective to reduce antigenicity and can be used as an immune tolerogen that induces the immune tolerance.

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