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Site-specific Glycosylation at Asn-292 in Ovalbumin is Essential to Efficient Secretion in Yeast

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Chicken ovalbumin (OVA) exists as mono-*N*-glycosylated form with a carbohydrate chain on Asn-292 in egg white, despite the possession of two potential *N*-glycosylation sites. To investigate the roles of *N*-glycosylation of OVA, we constructed a series of *N*-glycosylation mutants deleted *N*-glycosylation site and compared the secretion level of the mutants in *Pichia pastoris*. N292Q and N292/311Q mutants resulted in greater lowering of the secretion level as compared with wild-type, whereas N311Q mutant was secreted in approximately equal amounts to wild-type. However, secretion of wild-type and N311Q mutant was inhibited completely by tunicamycin treatment. All the *N*-glycosylation mutants have been expressed in the cells, as well as wild-type. Circular dichroism and fluorescence spectra of secreted N311Q mutant were almost identical to those of wild-type, while those of N292Q and N292/311Q mutants were different from wild-type; and, N292Q and N292/311Q mutants showed considerably lower denaturation temperature than wild-type. The results indicate that *N*-glycosylation at Asn-292 of OVA is required for the folding and secretion.

Key words: folding, *N*-glycosylation, ovalbumin, *Pichia pastoris*, secretion.

Abbreviations: OVA, ovalbumin; Endo H, endoglycosidase H; PNGase F, peptide:*N*-glycanase F; CD, circular dichroism.

N-glycosylation of proteins is known to be a biologically important post-translational modification. Recent studies have shown that the *N*-glycosylation plays pivotal roles in the folding, subcellular trafficking and apical sorting of glycoproteins (*1–4*). Chicken ovalbumin (OVA) is a major protein component of the egg white and is well characterized structurally. This protein is known to contain two potential *N*-glycosylation sites at Asn-292 and Asn-311, whereas it exists as mono-glycosylated form with a carbohydrate chain on only Asn-292 in egg white (*5*). On the other hand, it has been reported that di-glycosylated form, which has two carbohydrate chains on Asn-292 and Asn-311, was expressed only transiently in hen oviduct (*6, 7*). However, the di-glycosylated form has not been found in egg white. Hence, the *N*-glycosylation of OVA it can be expected to function for its synthesis and secretion. Recently, we have reported that two glycosylation forms of OVA, which are mono-glycosylated form with a carbohydrate chain on Asn-292 and di-glycosylated form with two carbohydrate chains on Asn-292 and Asn-311, were secreted into culture medium on methylotrophic yeast *Pichia pastoris* expression system of OVA (*8*). Thus, we thought that using *P. pastoris* expression system of OVA it is able to reveal the functions of *N*-glycosylation of OVA.

In this study, we constructed a series of glycosylation mutants (N292Q, N311Q and N292/311Q) of OVA deleted *N*-glycosylation site, examined the levels of secretion and synthesis of the *N*-glycosylation mutants, and then discuss the significance of *N*-glycosylation for the folding and secretion of OVA.

MATERIALS AND METHODS

Materials—Restriction enzymes, ligase and polymerase were purchased from Takara Shuzo (Kyoto, Japan). Oligonucleotide primers were provided by Sigma-Aldrich Japan K.K. Genosys Division. The DNA sequence kit was obtained from PE Biosystems (Foster City, CA, USA). Mouse IgG monoclonal antibody to hen egg OVA and goat antimouse IgG conjugated peroxidase were from Cosmo-Bio Chemicals (Tokyo, Japan) and Sigma-Aldrich Co. (St. Louis, MO, USA), respectively. Tunicamycin, the *N*-glycosylation inhibitor, was purchased from Sigma-Aldrich Co. An enhanced chemiluminescence (ECL + Plus) western blotting detection kit was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA). Endoglycosidase H (Endo H), peptide:*N*-glycanase F (PNGase F) and *Dpn* I were purchased from New England Biolabs (Beverly, MA, USA). All other chemicals used in the experiments were of analytical grade.

Strains and Plasmids—Escherichia coli XL1-blue and TOP10F′ were used as host cells for all cloning experiments. *P. pastoris* X-33 as a yeast expression strain of OVA was purchased from Invitrogen (Carlsbad, CA, USA). pT7 Blue T vector obtained from Novagen Merck (Darmstadt, Germany) was used for the

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subcloning of PCR products. Yeast expression plasmid pGAPZαA was purchased from Invitrogen. This plasmid contains a glucose-inducible GAP promoter, α-factor secretion signal derived from *Saccharomyces cerevisiae* and a Zeocin™ resistance gene.

Construction of Yeast Expression Vector for N-glycosylation Mutants—OVA cDNA is mutated in *N*-glycosylation consensus sequence (Asn-Xaa-Ser/Thr) by point mutation using synthetic oligonucleotide primers, 5′-AAATACCAACTCACATCTGTC-3′, 5′-TGTG AGTTGGTATTTTTCCTC-3′ (N292Q sense and antisense) and 5′-GCCCAGCTGTCTGGCATCTCC-3′, 5′-CAGCTGGGCTGAAGAGCTAAACA-3′ (N311Q sense and antisense) (underlines indicate mutation site). The point mutation was carried out with Ex *Taq* polymerase using pT7 Blue containing OVA cDNA as a template. After the template was digested with *Dpn* I, the pT7 Blue carrying mutated OVA cDNA was transformed into *E. coli* XL1-blue competent cells according to the method of Hanahan (*9*). Transformants were selected on LB agar plate with carbenicillin. Subsequently, the OVA cDNA was excised from pT7 Blue by a double digestion with *Xho* I and *Xba* I, and then ligated to the same restriction enzymes-digested pGAPZαA with T4 DNA ligase. The vector carrying OVA cDNA was transformed into *E. coli* TOP10F′ competent cells. Transformants were selected on a low-salt LB agar plate with Zeocin™.

Yeast Transformation and Screening of Transformant Colonies—The pGAPZαA containing mutated cDNA was linearized with *Bln* I (*Avr* II) and then transformed into *P. pastoris* competent cells by electroporation using a Gene Pulser™ (Bio Rad Model No. 1652098, 1652075). The transformed cells well-grown on YPDS plate with Zeocin™ were cultured for confirmation of OVA expression.

Comparison of Secretion Level—After cells were pre-cultured for 48 h, the cell number was adjusted to about OD = 15 at optical density (OD) of 600 nm. The aliquots (100 μl) of the cell solution were inoculated into 2 ml of fresh yeast extract-peptone-dextrose (YPD) medium. After further culture for 48 h, the aliquots (20 μl) of the supernatant of culture medium were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and used for western blotting.

SDS-gel Electrophoresis—SDS–PAGE was performed using 5% acrylamide stacking gel and 10% acrylamide separating gel containing 1% SDS, according to the method of Laemmli (*10*). The gel sheet was stained with 0.025% Coomassie brilliant blue R-250 solution.

Western Blotting Analysis—After SDS–PAGE, proteins were blotted on polyvinylidene difluoride (PVDF) membrane using an electroblot apparatus (ATTO, Model AE-6675). The membrane was exposed to the monoclonal antibody against OVA in Tween phosphate buffer solution (PBS) after blocking with 1% bovine serum albumin (BSA) solution, and then further exposed to the second antibody in Tween PBS. Immunodetection was performed with an ECL + Plus western blotting kit.

Treatment with Tunicamycin—The cells pre-cultured for 48 h were transferred into fresh YPD medium with tunicamycin (100 μg/ml) and further cultured for 48 h. After that, cells were transferred into the same medium without tunicamycin and cultured for additional 48 h. The supernatant (20 μl) of each culture medium was subjected to SDS–PAGE and used for western blotting.

Purification of N-glycosylation Mutants—The selected yeast strain was grown in YPD medium with Zeocin™. This pre-culture was transferred into the same fresh medium at large scale and cultured for 48 h, and then the culture medium was centrifuged for the removal of cells. The obtained supernatant was concentrated by ultrafiltration, fractionated using ammonium sulphate at about 75% saturation, and then dialysed against a 10 mM potassium phosphate buffer (pH 7.0). The dialysed protein solution was purified on a column of DEAE Sepharose™ FF (GE Healthcare Bio-Sciences Corp.), according to the method described in our previous work (*8*). The purified OVA proteins were lyophilized and used for characterization.

Digestion with Endo H and PNGase F—Protein samples (0.025% protein) were boiled for 10 min in denaturing buffer containing 1% 2-mercaptoethanol and 0.5% SDS to expose fully all glycosylation sites, and then deglycosylation was done by treatment with Endo H or PNGase F at 37°C for 3 h. The buffers used in these enzyme reactions were 50 mM sodium citrate (pH 5.5) for Endo H and 50 mM sodium phosphate (pH 7.5) containing 1% Nonidet P-40 for PNGase F.

Comparison of Intracellular Expression Level—The culture medium (2 ml) pre-cultured for 48 h was transferred into 2 ml of fresh YPD medium after adjusting cell number, and then cultured over 30 h. The yeast cells were collected every 6 h during culture, lysed with Yeast Buster™ (Novagen Merck), and then the aliquots (5 μl) of cell lysates were subjected to SDS–PAGE and used for western blotting to monitor intracellular OVA protein.

Circular Dichroism (CD) Spectra and Fluorescence Spectra—CD spectra were taken on a Jasco spectropolarimer (J-600), which was continuously purged with nitrogen. Measurement was performed at 25°C for a final concentration of 1 mg/ml in 10 mM potassium phosphate buffer (pH 7.0) using the cell with 1.0 mm pathlength for far-ultraviolet CD spectra (200–260 nm). An average of three to five consecutive scans were taken for each sample. Each spectrum was normalized for protein concentration and represented as the mean residue ellipticity (degree cm^2/dmol). The protein concentration was estimated from the absorption at 280 nm using $E^{1\%}_{1cm} = 7.12$ (*11*).

Tryptophan fluorescence spectra of proteins were monitored with a Jasco fluorescence spectrometer (FP-6300) for excitation at 295 nm. A 0.2 mg/ml protein solution in 10 mM potassium phosphate buffer (pH 7.0) was followed by recording the spectra in the range of 300–400 nm at 25°C.

Thermal Unfolding—Thermal unfolding curves were obtained by monitoring the CD value at 222 nm and the temperature indicated. The water-jacket cell containing the sample was heated at a linear rate of 1°C/min from 45°C to 85°C with a thermostatically regulated circulating water bath. The fraction of native protein was calculated from CD values by linearly extrapolating the pre- and post-transition base lines, based on the

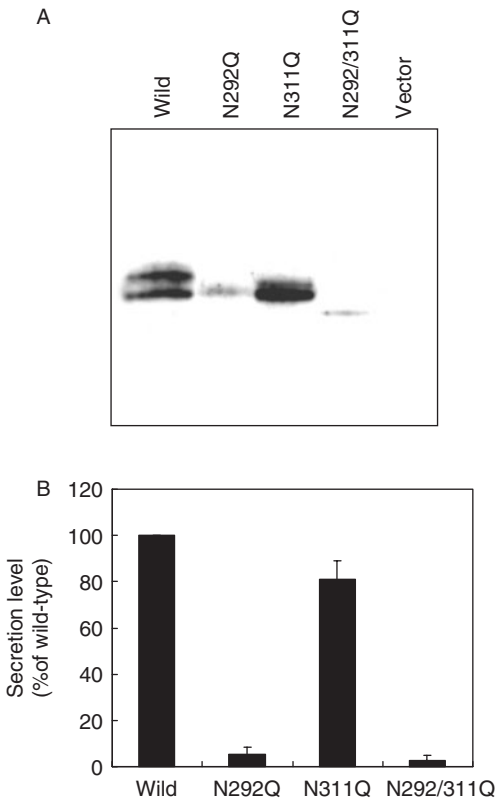

Fig. 1. **Comparison of the secretion level of wild-type and *N*-glycosylation mutants. (A)** Western blotting of culture medium after 48 h of cuture. **(B)** the ratio of secretion level of *N*-glycosylation mutants to wild-type, which was determined from the band intensity using densitometer. The values presented in the figure are obtained from eight independent experiments, and error bar shows SD.

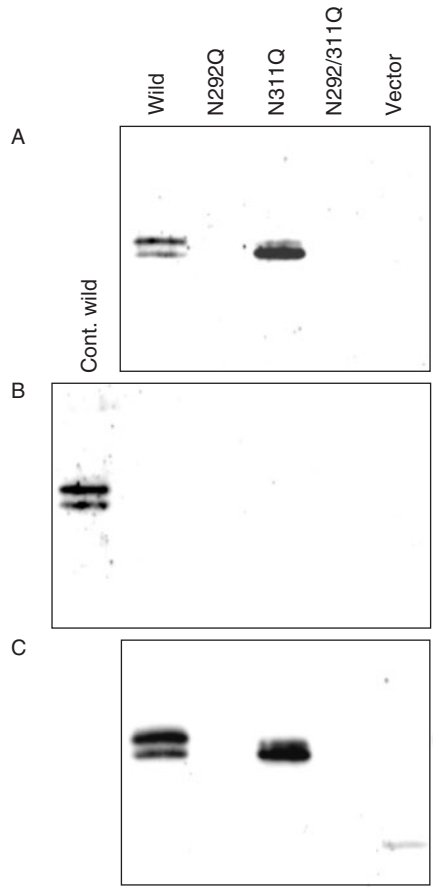

Fig. 2. **Effect of tunicamycin treatment on secretion of wild-type and *N*-glycosylation mutants.** Each strain was cultured in the absence of tunicamycin for 48 h **(A)**, and then in the presence of tunicamycin for 48 h **(B)**, and the tunicamycin-treated cells were cultured again in fresh medium without the inhibitor for 48 h **(C)**. The 'Cont. wild' shows the sample from culture medium of wild-type cultured for 48 h without tunicamycin.

assumption that the CD values of the pre- and post-transition reflect those of the folded and unfolded proteins, respectively. Assuming that the unfolding equilibrium follows a two-state mechanism, the denaturation temperature (T_d) was determined from the unfolding curves obtained.

RESULTS AND DISCUSSION

Secretion Level of Wild-type and N-glycosylation Mutants—To investigate the roles of carbohydrate chain attached to OVA, we first compared the secretion level of wild-type and *N*-glycosylation mutants in *P. pastoris*. The growth and copy number of each *N*-glycosylation mutant were almost equal to that of wild-type (data not shown). After culture for 48 h, each culture medium was subjected to SDS–PAGE, and western blotting was performed for detection of *N*-glycosylation mutants (Fig. 1A). The amounts of the immunoreactive product were determined from the band intensity using densitometer (Fig. 1B). As observed in Fig. 1, the secretion level of N311Q mutant was slightly lower than that of wild-type, while that of N292Q mutant greatly decreased to less 10%. Moreover, N292/311Q double mutant was rarely secreted into culture medium.

Effect of treatment of tunicamycin, *N*-glycosylation inhibitor, on the secretion levels of *N*-glycosylation mutants was investigated. Yeast strains harbouring each *N*-glycosylation mutant were cultured for 48 h in the presence of tunicamycin, and then the strains were cultured for 48 h in the fresh culture medium without the inhibitor. At the each step, culture medium was subjected to SDS–PAGE, and western blotting was performed for detection of *N*-glycosylation mutants (Fig. 2). As shown in Fig. 2B, during treatment with tunicamycin, secretion of N292Q and N292/311Q mutants was completely inhibited. Secretion of wild-type and N311Q mutant was also suppressed greatly by tunicamycin treatment (Fig. 2B); however, their secretion levels were restored when the tunicamycin-treated yeast cells were cultured again in fresh culture medium without the inhibitor (Fig. 2C). This result, together with Fig. 1, suggests that the deletion of *N*-glycosylation site at Asn-292 strongly inhibits secretion of OVA in *P. pasotris*.

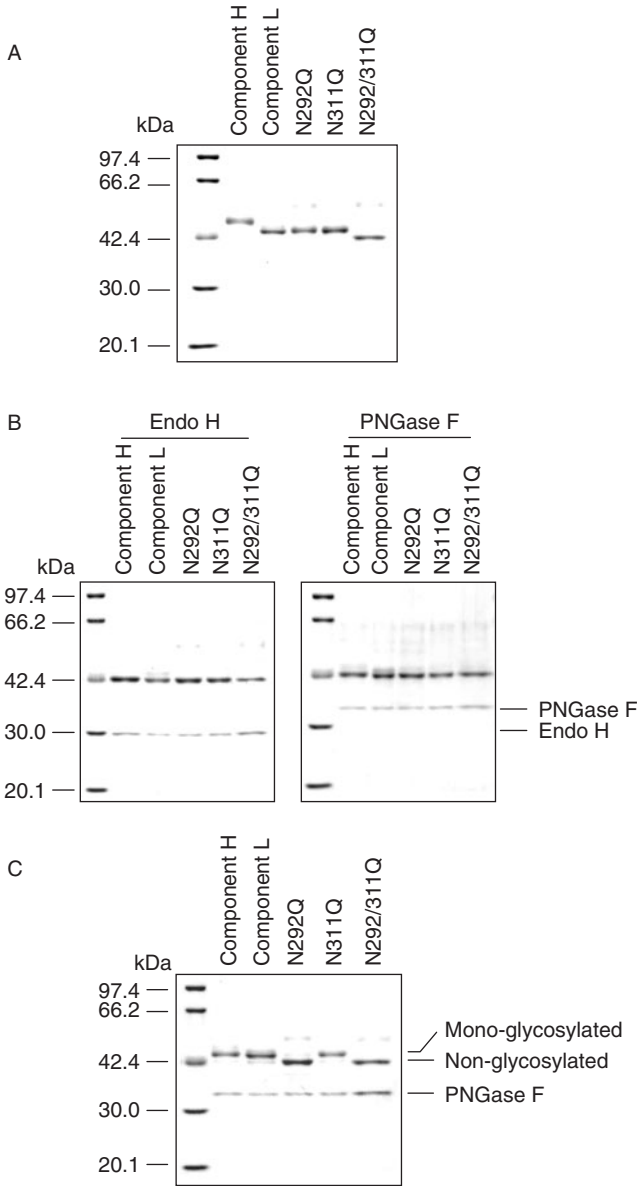

Fig. 3. **SDS–PAGE patterns of wild-type and *N*-glycosylation mutants expressed in *P. pastoris*.** **(A)** SDS–PAGE pattern of purified *N*-glycosylation mutants. **(B)** effects of digestion by Endo H and PNGase F. The digestion was performed after heating for 10 min with 2-mercaptoethanol and SDS. **(C)** effects of digestion by PNGase F without protein denaturation due to 2-mercaptoethanol and SDS. Components H and L of wild-type are di-glycosylated form and mono-glycosylated form, respectively.

Characteristics of N-glycosylation Mutants—Secreted wild-type and *N*-glycosylation mutants were isolated from culture medium, and then characterized with regard to *N*-glycosylation (Fig. 3). Figure 3A shows SDS–PAGE pattern of wild-type and *N*-glycosylation mutants after purification. Components H and L of wild-type show di-glycosylated form and mono-glycosylated form, respectively, as reported in our previous work (*8*). The mobility of N292Q and N311Q mutants on SDS–PAGE was identical to that of component L, corresponding to mono-glycosylated form. N292/311Q mutant deleted two glycosylation sites was estimated as a non-glycosylated form from the mobility. Wild-type and *N*-glycosylation mutants were subjected to the digestion with Endo H or PNGase F (Fig. 3B). By both Endo H and PNGase F digestions, wild-type, N292Q and N311Q mutants migrated to the same position as N292/311Q mutant corresponding to non-glycosylated form. From the result, we confirmed that N292Q and N311Q mutants are secreted as mono-*N*-glycosylated form, and that N292/311Q mutant is non-glycosylated form. Next, in order to examine the position of *N*-glycosylation site, another PNGase F digestion was performed in the absence of SDS and reducing reagent (Fig. 3C). We have reported previously that the carbohydrate chain on Asn-311 of component H of wild-type is eliminated specifically with the glycosidase PNGase F in the non-denaturing condition, but not Asn-292 (*8*). After the digestion with PNGase F, component H and N292Q mutant migrated with a slightly faster mobility than their untreated proteins, suggesting the loss of a carbohydrate unit on Asn-311 (compare A and C in Fig. 3). On the other hand, component L and N311Q mutant, which are probably glycosylated on Asn-292, did not change, as expected, in the mobility on SDS–PAGE before and after the glycosidase treatment. It was confirmed by these results that *N*-glycosylation site of N292Q and N311Q mutants is positions Asn-311 and Asn-292, respectively.

Expression of N-glycosylation Mutants in Yeast Cells—N292Q and N292/311Q mutants, in which glycosylation site at Asn-292 was deleted, were rarely secreted into culture medium, as shown in Fig. 1. Therefore, we tried to examine whether N292Q and N292/311Q mutants are expressed in yeast cells or not. Figure 4A shows the secretion level of wild-type and *N*-glycosylation mutants over 48 h of culture. Wild-type and N311Q mutant were found to be secreted into culture medium at incubation of 4–6 h. On the other hand, secretion of N292Q and N292/311Q mutants was detected at incubation of 12–18 h, and their secretion levels were very low as compared with those of wild-type and N311Q mutant. Thus, the cells harbouring each *N*-glycosylation mutants were harvested every 6 h up to 30 h during culture and the cell lysates were subjected to SDS–PAGE, and then western blotting was performed for detection of expression of each *N*-glycosylation mutants (Fig. 4B). The expression level in the cells for all *N*-glycosylation mutants was almost the same as that of wild-type, despite such differences in levels of their secretion (Figs 1 and 4A). However, when the culture time was extended up to 24 h, the amount of expression in the cells clearly decreased and was not detectable after the culture for 30 h. This decrease means that wild-type and N311Q mutant were almost secreted into culture medium, and that most of N292Q and N292/311Q mutants were not secreted but were degraded in the cell. The result of the expression level in the cell revealed that N292Q and N292/311Q mutants are normally synthesized in the cell but are degraded prior to the secretion. On the other hand, N311Q mutant appears to be secreted in a similar extent as wild-type.

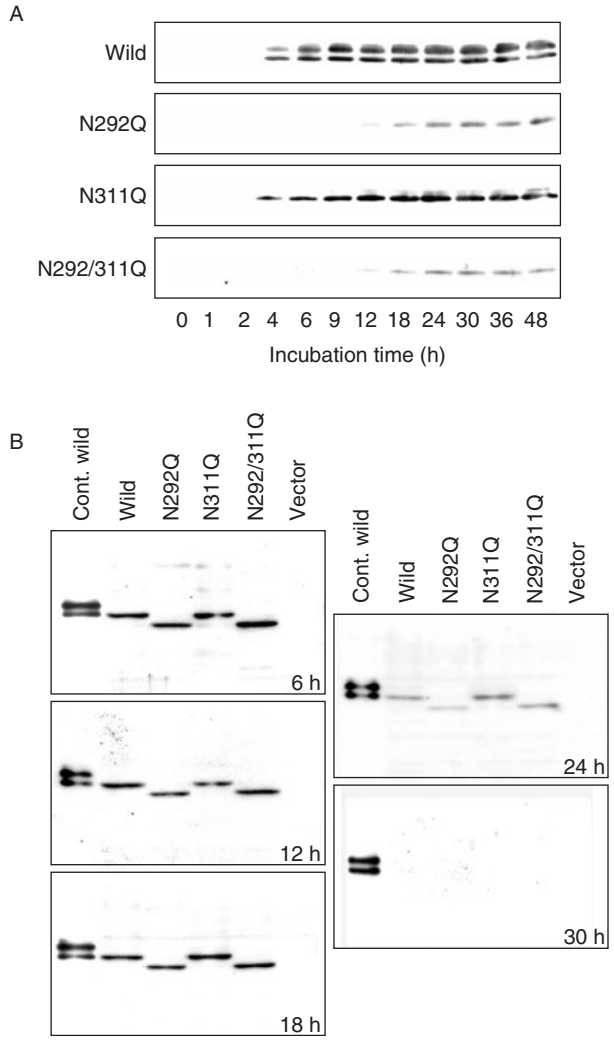

Fig. 4. **Intracellular expression level of wild-type and *N*-glycosylation mutants. (A)** western blotting of the sample from culture medium of wild-type and *N*-glycosylation mutants at time intervals during culture for 48 h. **(B)** the cells were harvested every 6 h and lysed. The 'Cont. wild' shows the sample from culture medium of wild-type cultured for 48 h.

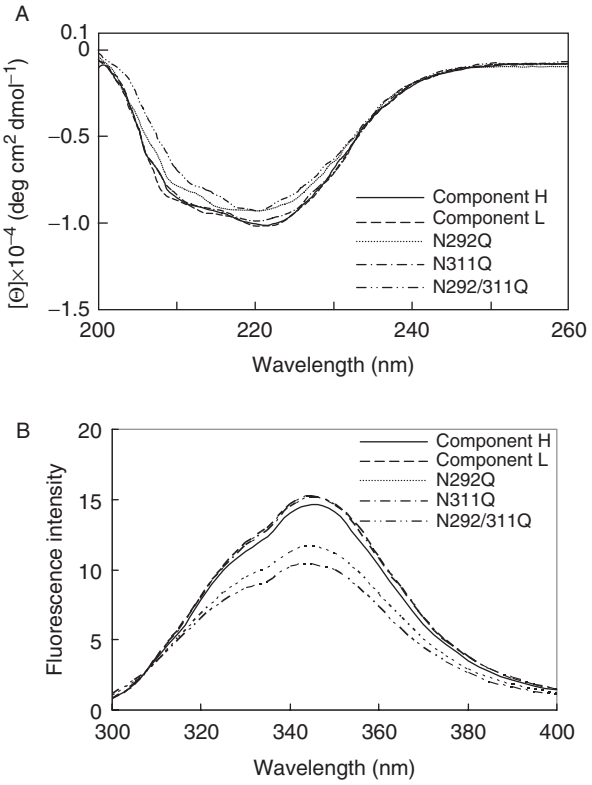

Fig. 5. **Conformational analysis of secreted wild-type and *N*-glycosylation mutants. (A)** Far-UV CD spectra. **(B)** Fluorescence spectra. The presented spectra are average of five independent measurements.

Strikingly, it was presumed from the mobilities on SDS–PAGE that in cell lysate, wild-type exists as only mono-glycosylated form corresponding to component L and that N292Q mutant as non-glycosylated form such as N292/311Q mutant. It is likely that the conversion of component H into mono-glycosylated form and of N292Q mutant into non-glycosylated form may be caused by the digestion with endogenous glycosidase in *P. pastoris* during cell lysis for the detection of the intracellular recombinant OVAs. As shown in Fig. 3C, we demonstrated that the digestion with glycosidase PNGase F in the absence of SDS and reducing reagent *in vitro* resulted in deglycosylation of component H and N292Q mutant, but not of component L and N311Q mutant. This result suggests a possibility that the specific deglycosylation at Asn-311 of OVA may occur in the cells. In addition, carbohydrate chain attached to Asn-311 may have been a recognition signal for degradation of OVA in the cells, because N292Q mutant was rarely secreted into culture medium. This might explain the reason that OVA exists as a singly glycosylated form with a carbohydrate chain on Asn-292 in egg white. On the other hand, as di-glycosylated form of wild-type with the carbohydrate chain on Asn-292 and Asn-311 was secreted effectively into culture medium, *N*-glycosylation of Asn-292 on the di-glycosylated form may predominate for a secretion, even if the carbohydrate chain was attached to Asn-311. In conclusion, N292Q and N292/311Q mutants seem to be degraded in the cells prior to the secretion, and the *N*-glycosylation at Asn-292 is required for efficient secretion.

Structural Characteristics of Secreted N-glycosylation Mutants—The structure of secreted *N*-glycosylation mutants was characterized by CD spectra in the far-UV region (Fig. 5A) and intrinsic fluorescence spectra analyses (Fig. 5B). CD spectrum of N311Q mutant was almost identical to those of wild-type (components H and L), indicating the similarity of the secondary structure. But the spectra of N292Q and N292/311Q mutants, in which *N*-glycosylation site at Asn-292 is deleted, gave a blue-shift in wavelength range of 200–230 nm, that is specific for α-helix and β-sheet structures. The change may be attributed to the conformational changes of N292Q and N292/311Q mutants, compared with wild-type. Furthermore, fluorescence emission intensity (F_{max})

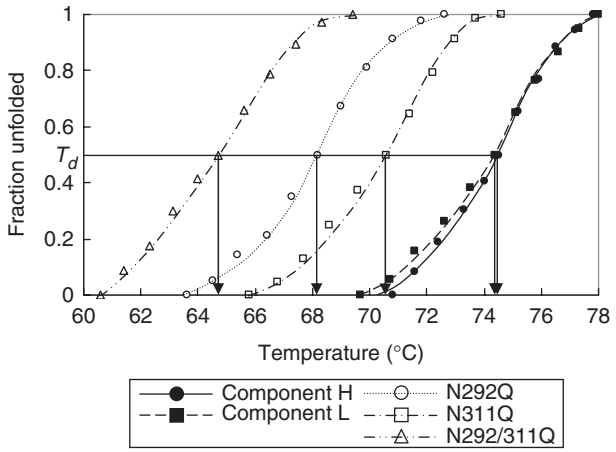

Fig. 6. **Thermal stability of secreted wild-type and *N*-glycosylation mutants.** Thermal unfolding curves were obtained by monitoring the CD value at 222 nm at pH 7.0. The transition temperature of denaturation was determined as T_d, midpoint temperature of the unfolding curve. These curves were determined from data of five independent measurements.

of N292Q and N292/311Q mutants was low significantly. Mills reported that quenching of F_{max} is attributed to the conformational changes leading to the exposure of tryptophan groups to the polar environment (*12*). Therefore, the decrease in F_{max} means that N292Q and N292/311Q mutants had undergone significantly conformational changes.

Thermal stability of secreted *N*-glycosylation mutants was measured by monitoring the magnitude of the CD band at 222 nm observed at different temperatures (Fig. 6). The transition temperature of denaturation (T_d) was determined from the each unfolding curve. Components H and L of wild-type displayed T_d of 74.5 and 74.4°C, respectively. The almost identical T_d values for components H and L indicate that an additional *N*-glycosylation at Asn-311 may have no effect on the thermostabilization. T_d of N311Q mutant (70.6°C) decreased by about 4°C as compared with that of component L, although the difference in structure of these proteins is only one amino acid residue at position 311, that is Gln for N311Q mutant and Asn for component L. Although the reason is not clear, since there are some α-helices near side chain of Asn-311 (*13*), the decrease in T_d may be caused by some negative interactions between Gln-311 and the adjacent α-helices. T_d values for N292Q and N292/311Q mutants were 68.2°C and 64.7°C, respectively, displaying considerably lower temperature than wild-type. The results suggest that the attachment of carbohydrate chain to Asn-292 may be required for a correct protein folding. It is likely that the lacking of *N*-glycosylation at Asn-292 results in incorrect and loose conformation, thus facilitating degradation of the protein. As a result, N292Q and N292/311Q mutants appear to be rarely secreted in the cell surface.

It is well-known that lectin-like chaperones, calnexin or calreticulin interact with *N*-linked carbohydrate chain of glycoproteins to promote the correct protein folding (*14–16*). Thus, it was presumed that the carbohydrate chain on Asn-292 of OVA functions to facilitate the folding and secretion through interactions with calnexin in *P. pastoris*. In contrast, N292Q and N292/311Q mutants, in which the glycosylation site of Asn-292 is deleted, was not correctly folded (Figs 5 and 6), thus resulting in protein degradation through ERAD (ER associated degradation) (Fig. 4). However, further studies focusing on quality control of glycoprotein is needed to reveal whether the site-specific carbohydrate chain on Asn-292 of OVA contributes to the protein folding.

Recent studies have reported that *N*-glycosylation is important for secretion of glycoproteins (*17–21*). On the other hand, it has been reported in an earlier study that *N*-glycosylation of OVA was not related to the secretion in vertebrate cells (*22, 23*). However, in their reports, not only a strict comparison of the secretion level was not performed, but also structure of non-glycosylated OVA was not investigated in detail. Therefore, we tried to elucidate functional role of the carbohydrate chain of OVA, especially to reveal why OVA in egg white exists as mono-glycosylated form at Asn-292, despite the existence of two potential *N*-glycosylation sites (Asn-292 and Asn-311). But the recombinant OVA attached carbohydrate chain to Asn-311 was not so far identified in the expression system of vertebrate cells including chicken. Therefore, using the OVA expression system of yeast *P. pastoris* it is possible to gain a better understanding of the functional role of glycosylation of OVA. In *P. pastoris*, we demonstrated in this study that site-specific glycosylation at Asn-292 of OVA is required for correct folding and efficient secretion of the protein in yeast. In addition, we described the reason that the carbohydrate chain of OVA has been attached predominantly to Asn-292.

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