Structural Characteristics of Hen Egg Ovalbumin Expressed in Yeast *Pichia pastoris*

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The recombinant ovalbumin (OVA) produced in yeast *Pichia pastoris* was purified from the culture medium by anion exchange chromatography, and its structural characteristics were compared with those of hen egg OVA, mainly from the point of view of posttranslational modification. The expressed OVA consisted of two molecular species immunoreactive with antibody for hen egg OVA. The two molecular species, 45 and 47 kDa in molecular size, were thought to correspond to mono-glycosylated form and di-glycosylated form respectively. The non-glycosylated form was not produced in the system. The other posttranslational modifications (N-terminal acetylation and phosphorylation) observed in hen egg OVA were not detected in either of the molecular species. The two recombinant proteins displayed almost exactly the same circular dichroism and intrinsic tryptophan fluorescence spectra as hen egg OVA. The melting temperature, $T_m$, which was determined from the thermal unfolding curve, was almost identical in the two recombinant proteins, despite the difference in glycosylation levels, while it decreased by about 2.5°C as compared with that of hen egg OVA (77.3°C). These data indicate that the additional glycosylation to Asn-311 in the recombinant protein does not affect protein conformation or thermostability.

**Key words:** ovalbumin; *Pichia pastoris* expression; N-linked glycosylation; phosphorylation

Ovalbumin (OVA) is the most abundant protein of hen egg white (comprising 58%), and is well characterized structurally. It is a globular, acidic protein which comprises a single polypeptide chain of 385 amino acid residues with a molecular weight of 45 kDa and has a single carbohydrate chain linked to Asn-292 and an acetyl group at the N-terminus. It also exists in three forms, $A_1$, $A_2$, and $A_3$-OVA with two, one, and no phosphate groups per molecule respectively. Most of hen egg OVA is of the $A_1$-form. This protein is widely used as an food ingredient by the food industry to enhance and improve the functionality of various food products.

In order to investigate the relationship of the structure and the functional properties of hen egg OVA, we attempted to obtain the protein using genetic engineering, because it is possible to get numerous mutants of hen egg OVA. An expression system using *Escherichia coli* has been attempted to produce a large amount of recombinant OVA, but the posttranslational modifications in the expression system did not occur. There are no reports that recombinant OVA was expressed in an eukaryote system. Recently, a methylotrophic yeast, *Pichia pastoris*, known to possess a high-level production system, has been developed as a host for the correct folding and the posttranslational modifications of recombinant protein. *P. pastoris* is a desirable expression system because it has a highly efficient and inducible GAP (glyceraldehyde-3-phosphate dehydrogenase) promoter for high-level expression of proteins. Hence *P. pastoris* was used for the high-level production of recombinant OVA.

In the present study, we attempted to construct a secretory production system of recombinant OVA in *P. pastoris*, and described characteristics of the expressed OVA in terms of posttranslational modification. The result should provide a better understanding of the structure–function relationship of hen egg OVA.

**Materials and Methods**

**Materials.** Restriction enzymes, ligase, and polymerase were purchased from Takara Shuzo (Kyoto). Oligonucleotide primers were synthesized by Hitachi Keisoku (Hiroshima). The DNA sequence kit was obtained from PE Biosystems (Tokyo). Rabbit antiserum to hen egg OVA and goat anti-rabbit IgG were from Cosmo-Bio Chemicals (Tokyo). An enhanced chemiluminescence (ECL+Plus) Western blotting detection kit was purchased from Amersham Pharmacia Biotech (UK). Endoglycosidase-H (Endo-H) and Peptide N-Glycosidase F (PNGase F) was purchased from New...
England Biolabs (Beverly, MA). Acid phosphatase (from wheat germ, type I, EC 3.1.3.2) was obtained from Sigma Chemical (St. Louis, MO). Hen egg OVA was prepared from fresh hen egg white by crystallization five times in sodium sulfate according to the method of Kekwick and Cannan. All other chemicals used in the experiments were of analytical grade.

Strains and plasmids. *E. coli* XL1-blue and TOP10F’ were used as host cells for all cloning experiments. *P. pastoris* X-33 as a yeast expression strain of hen egg OVA was purchased from Invitrogen (Carlsbad, CA). pT7 Blue T-vector obtained from Novagen Merck (Darmstadt, Germany) was used for the 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 plasmid. Synthetic oligonucleotide primers, 5'-AAACTCGAGAAAAAGGCTCCATCAGGCAAG-3' (*Xho* I site primer) and 5'-GGGTCTAGATCTCGCTTGAGGAG-3' (*Xba* I site primer), were introduced to the upstream and downstream of hen egg OVA cDNA to construct cDNA with the new restriction enzyme sites underlined, respectively. The PCR was carried out with Ex *Taq* polymerase according to the manufacturer’s manual. The PCR products obtained were ligated to pT7 Blue T-vector with T4 DNA ligase at 4°C overnight. The plasmid carrying hen egg OVA cDNA was transformed into *E. coli* XL1-blue competent cells according to the method of Hanahan. Transformsants were selected on LB agar plate with carbenicillin (50μg/ml). Subsequently, the OVA cDNA was excised from pT7 Blue/ova by a double digestion with *Xho* I and *Xba* I, and then ligated to the same restriction enzyme-digested pGAPZαA with T4 DNA ligase at 4°C overnight. The plasmid carrying hen egg OVA cDNA was transformed into *E. coli* TOP10F’ competent cells. Transformants were selected on a low-salt LB agar plate with Zeocin™ (25μg/ml). The construction map is shown in Fig. 1.

Yeast transformation and screening of transformant colonies. The plasmid containing pGAPZαA/ova 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). The transformed cells well-grown on YPDS plate with Zeocin™ (100μg/ml) were cultivated for 2d at 30°C for confirmation of recombinant OVA expression.

Purification of expressed protein. The selected yeast cell was cultivated in 3ml of YPD medium with Zeocin™ (100μg/ml) at 30°C. This preculture was transferred into 400ml of the same fresh medium and incubated at 30°C for 2d, and then the yeast culture medium was centrifuged for the removal of cells. The supernatant obtained was fractionated using ammonium sulfate at 40 to 75% saturation, and dialyzed against a 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM PMSF as protease inhibitors. The dialyzed protein solution was purified on a column of DEAE Sepharose™ FF (Amersham Biosciences). The two components thus obtained were used for characterization of the recombinant OVA from *P. pastoris*.

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. In order to assess the degree of phosphorylation, native-PAGE without SDS and reducing agent was performed using 5% acrylamide stacking gel and 7% acrylamide separating gel, as described by Davis. The gel sheet was stained with 0.025% Coomassie brilliant blue R-250 solution and then destained.

Immunodetection. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using an electroblot apparatus (Bio Rad, Model AE-6675). The transferred membrane was exposed to the primary antibody (rabbit antiserum to hen egg OVA) in Tween PBS after blocking with 1% BSA solution. The membrane was further exposed to the second antibody in Tween PBS. Immunodetection was performed with an ECL+Plus Western blotting kit.

N-terminal amino acid sequencing. The protein samples were transferred to a PVDF membrane after SDS–PAGE, and the amino-terminal amino acids were
sequenced with a Shimadzu protein sequencer (PPSQ-21A, Kyoto).

Deglycosylation treatment. After protein samples (0.025% protein) were boiled for 10 min in denaturing buffer containing 1% 2-mercaptoethanol and 0.5% SDS, 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.

Measurement of molecular weight. For TOF-MS, the protein samples (0.025% protein) were mixed with a matrix, 3,5-dimethoxyl-4-hydroxyximamic acid (sinapinic acid) dissolved in a solution containing equal volumes of acetonitrile and 0.1% trifluoroacetic acid. Spectra were obtained using a Voyager™ RP mass spectrometer (PerSeptive Biosystems, Framingham, MA).

Dephosphorylation treatment. Dephosphorylation of protein samples (0.1% protein) was carried out using acid phosphatase (from wheat), as described by Kitabatake et al.6

Circular dichroism (CD) spectra. CD spectra were taken on a Jasco spectropolarimeter (J-600, Tokyo), 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 was taken for each sample. Each spectrum was normalized for protein concentration and represented as the mean residue ellipticity (degree cm²/dmol). The protein concentration was estimated from the absorption at 280 nm using ε¹⁰₀ = 7.12.16

Fluorescence spectra. Tryptophan fluorescence spectra of proteins were monitored with a fluorescence spectrometer (650-10S, Hitachi, Japan) 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 60 to 95 °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 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 midpoint temperature, Tm, was determined from the unfolding curves obtained.

Results and Discussion

Purification of recombinant OVA

As shown in Fig. 2A, two proteins were found to be secreted in the supernatant of the culture medium after induction for 2 d. Then the protein fraction prepared from the culture medium by ammonium fractionation was purified by DEAE-Sepharose™ FF column chromatography. As shown in Fig. 2B, the protein fraction was separated into two protein components by chromatography. Moreover, since each protein component reacted against hen egg OVA antibody (Fig. 2C), it was found that the expression system of recombinant OVA in P. pastoris produced the two varieties of OVA. On the basis of molecular size on SDS–PAGE, the high molecular species and the low molecular species were designated components H and L respectively. The molecular weight of component L was almost identical with that of hen egg OVA. The total secretion amount was approximately 10 mg/l culture medium, and the ratio of the expressed components H and L was almost identical.

Analysis of N-terminal amino acid sequences

Determination of the N-terminal sequences of recombinant OVA was performed. The N-terminal sequences of each component (Gly-Ser-Ile-Gly-Ala-Ala-Ser-) were identical with those of hen egg OVA predicted from the cDNA sequences, except for the lack of N-terminal acetylation. The N-terminal sequences of hen egg OVA were not possible to determine because of the acetylation of N-terminal Gly residue. Base on the results, the possibility of modification in the N-terminal Gly residue of the expressed components H and L was excluded.

Deglycosylation and glycosylation profiling

As shown in Fig. 2, the difference in molecular mass between component H and component L (or hen egg OVA) on SDS–PAGE suggested that component H underwent a modification that causes an increase in molecular weight. Hen egg OVA is known to contain two potential N-glycosylation sites, at Asn-292 and Asn-
311, whereas it exists in a singly N-glycosylated form with a carbohydrate chain on Asn-292 in hen egg white.\(^1,3\) In addition, Kato et al.\(^{17,18}\) isolated and characterized biosynthetic intermediates of hen egg OVA, one of which was shown to have been di-N-glycosylated only transiently at both of Asn-292 and Asn-311 in the hen oviduct. Hence the difference in molecular size between components H and L might be due to the difference in the number of the attached carbohydrate chain. We attempted to evaluate the glycosylation levels of the two components. The deglycosylation of each component together with hen egg OVA was performed by treatments with two glycosidases (Fig. 3A, B, and C). It has been reported that Endo-H cleaves between two \(\text{N}\)-acetylglucosamines of \(\text{N}\)-linked glycoproteins, and that PNGase F cleaves between the \(\text{N}\)-acetylglucosamine and the asparagine residue of \(\text{N}\)-linked glycoproteins.\(^{19}\) Because hen egg OVA has one carbohydrate chain, it gave a band having slightly high mobility on SDS–PAGE after digestion with two glycosidases (lane 1 in Fig. 3A, B, and C). It has been reported that Endo-H cleaves between two \(\text{N}\)-acetylglucosamines of \(\text{N}\)-linked glycoproteins.\(^{19}\) The composition of the attached \(\text{N}\)-linked carbohydrate chain unit on OVA is distinct from that in mammalian cells, and that the basic mode of the carbohydrate chain is a high mannose type (\(\text{Man}_{8,14}\text{GlcNAc}_{2}\)).\(^{25}\) While PNGase F can fully cleave a \(\text{N}\)-linked carbohydrate chain added to Asn residue in a protein, in the case of Endo-H digestion, one \(\text{N}\)-acetylglucosamine residue remains on the protein. Hence the molecular mass of the actual carbohydrate chain bounded to the protein must be increased by 442 and 221 (the molecular mass of \(\text{N}\)-acetylglucosamine) to 3686.5 and 1867.9 (the difference in molecular mass before and after Endo-H digestion) respectively. By combining our data with the reference data, the \(\text{N}\)-linked carbohydrate chain unit on both components H and L was estimated to be \(\text{Man}_{0}\text{GlcNAc}_{2}\).
Dephosphorylation profiling

Hen egg OVA is a protein phosphorylated at Ser-68 and Ser-344. The protein exists in three forms, A1-, A2-, and A3-OVA, with two, one, and no phosphate group per molecule respectively; the ratio is A1:A2:A3 = 85:12:3. To evaluate the phosphorylation levels, dephosphorylation of both the recombinant OVA species together with hen egg OVA was performed under identical conditions with acid phosphatase, and then analyzed by native-PAGE (Fig. 5). After digestion, hen egg OVA gave a main band corresponding to A3-OVA (the dephosphorylated form), while components H and L did not show any change in electrophoretic mobilities on native-PAGE. This result indicates that the two recombinant OVA species did not contain a phosphate group on Ser and/or Thr residues in their molecules.

CD and fluorescence spectra analysis

The structural characteristics of the recombinant OVA species were evaluated by comparing their CD spectra in the far ultraviolet region (200–260 nm) with those of hen egg OVA (Fig. 6). The CD spectra of components H

### Table 1. TOF-MS Analysis and Possible Carbohydrate Chain Composition for Recombinant OVA

<table>
<thead>
<tr>
<th>Component</th>
<th>Component H</th>
<th>Component L</th>
</tr>
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<tbody>
<tr>
<td>1. Intact</td>
<td>46926.7</td>
<td>44882</td>
</tr>
<tr>
<td>2. Endo-H treatment&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43240.2</td>
<td>43014.1</td>
</tr>
<tr>
<td>3. PNGase F treatment&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>44812.7</td>
<td>44920.4</td>
</tr>
</tbody>
</table>

Difference between 1 and 2 (actual carbohydrate) 3686.5 1867.9
Difference between 1 and 3 2114 -38.4
Possible carbohydrate chain (Man<sub>9</sub>GlcNAc<sub>2</sub>)<sub>2</sub> Man<sub>9</sub>GlcNAc<sub>2</sub> (molecular mass) (4124) (2062)

<sup>a</sup>One GlcNAc of N-linked oligosaccharide core remains the glycoprotein.
<sup>b</sup>Carbohydrate chains are completely cleaved from the glycoprotein.
<sup>c</sup>Under the non-denaturing condition (without 2-ME and SDS).

Fig. 4. TOF-MS Analysis of Hen Egg OVA and the Recombinant OVA Proteins before and after Glycosidase Treatment.
(A) before glycosidase treatment; (B) after Endo-H treatment; (C) after PNGase F treatment under non-denaturing. The top, middle, and bottom in each pattern show hen egg OVA, component H, and component L respectively.

Fig. 5. Native-PAGE Pattern of Hen Egg OVA and the Recombinant OVA Proteins Dephosphorylated with Phosphatase.
(A) without phosphatase; (B) with phosphatase. A1, A2, and A3 show OVA molecular species with two, one, and no phosphate groups per molecule respectively. Lane 1, hen egg OVA; lane 2, component H; lane 3, component L.
and L were almost the same as that of hen egg OVA. This result indicates that the secondary structure of these recombinant proteins is virtually the same as that of hen egg OVA, despite the lack of N-terminal acetyl group and phosphate groups. In addition, the attachment of an extra carbohydrate chain detected in component H also hardly affects the protein conformation. Trp fluorescence is also a sensitive index of alteration in protein conformation. Hence the fluorescence spectra of the recombinant proteins after excitation at 295 nm were compared with those of hen egg OVA (Fig. 7). The fluorescence maxima for the recombinant proteins were identical with that of hen egg OVA, whereas the fluorescence intensities of the recombinant proteins decreased considerably. These results suggest that the molecular structure of both the recombinant OVA species might cause a slight structural perturbation in the tertiary structure without alteration in gross structure.

**Thermal unfolding**

Thermal unfolding was analyzed by following the magnitude of the CD band at 222 nm observed at different temperatures. The thermal unfolding curves of hen egg OVA and the recombinant proteins as a function of temperature at pH 7.0 are shown in Fig. 8. The melting temperature, $T_m$, was determined from the each unfolding curve. Hen egg OVA displayed a $T_m$ of 77.3°C under the experimental conditions. The $T_m$ of components H and L was 74.9 and 74.8°C and decreased by 2.4 and 2.5°C respectively, as compared with that of hen egg OVA. The almost identical $T_m$ of components H and L suggests that additional glycosylation has no effect on thermostability. On the other hand, the $T_m$ of completely dephosphorylated hen egg OVA was also measured with phosphatase. The dephosphorylated sample showed a $T_m$ of 75.1°C, decreasing by 2.2°C as compared with hen egg OVA. This result suggests that phosphate groups play an important role in the thermostability of OVA, although there is a slight difference between dephosphorylated hen egg OVA and components H and L. This view is supported by a previous paper.5)

**Conclusion**

This expression system for hen egg OVA in *P. pastoris* secreted two molecular species immunoreactive with hen egg OVA antibody into the culture medium. To our knowledge, this is the first study of the production of recombinant OVA protein in *P. pastoris*. The two recombinant OVA proteins, components H and L, were in the di-glycosylated form and the mono-glycosylated form respectively. The di- and mono-glycosylated forms were found to be secreted at the almost same ratio, while the non-glycosylated form was not detected. The
acetylation and phosphorylation observed in hen egg OVA did not occur in the recombinant OVA proteins, but the conformation of both recombinant proteins was almost the same as that of hen egg OVA. The thermostability of the recombinant proteins was almost identical, although it was lower by about 2.5°C than hen egg OVA. These results suggest that an additional glycosylation to the Asn-311 dose not affect conformation or thermostability. The reason of which a singly N-glycosylated form and a di-glycosylated form were secreted at the same ratio from *P. pastoris* remains to be elucidated. Further studies are being conducted to clarify why the non-glycosylated form cannot be secreted normally, by focusing on the quality control system for newly synthesized glycoproteins.

**References**