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Structural and immunological characterization of recombinant ovomucoid expressed in *Escherichia coli*

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Abstract

The expression of recombinant allergens is becoming new insights of an important diagnosis and the therapy of allergies as well as molecular approaches to immunological and structural studies of allergens. Ovomucoid is a major food allergens in the hen's egg white which causes immediate food-hypersensitivity reactions mainly in children. A gene coding for the cDNA representing an entire ovomucoid molecule has been cloned in *Escherichia coli* under the control of T5 promoter fused with six-Histidine tag at the amino terminal end. Upon induction, the *E. coli* cells, harbouring this construct, expressed the recombinant protein as a soluble fraction and the recombinant ovomucoid protein was purified to electrophoeretic homogeneity using Ni^{2+} nitrilotriacetic acid agarose affinity chromatography. Immunoblot analysis showed that human IgE and IgG binding activities of the recombinant ovomucoid was identical to that of native analogue. The antigenicity and allergenicity of recombinant ovomucoid were almost same as that of native form when tested with an ELISA using six individual patient's serum. CD spectra indicated that that the recombinant ovomucoid has more α-helix and less β-structure than native form. These results show that the recombinant ovomucoid constructed in this study could be used for further studies on the immunological and structural studies of ovomucoid.

Introduction

Food allergy is an immune-mediated state of hypersensitivity resulting from exposure to food allergens and is most often mediated by immunoglobulin, IgE. Egg white is the most common source of allergic reactions mainly in children (Bleumink & Young 1971, Anet *et al*. 1985, Sampson & Ho 1997). Ovomucoid Gal d1 is the dominant allergen in hen's egg (Bernhisel-Broadbent *et al.* 1994, Urisu *et al*. 1997, Mine & Zhang 2002a). It is a highly glycosylated protein comprising of 186 amino acids arranged in three tandem domains. The carbohydrate molecules represent about 25% of the molecule (Kato *et al*. 1987). IgE antibodies have an important role in egg hypersensitivity. Several immunogenic studies of peptides derived by chemical or enzymatic fragmentation of ovomucoid have been carried out by different groups using mouse IgE or rabbit anti-ovomucoid antibody (Konishi *et al*. 1985, Matsuda *et al*. 1985). Antibody specific to ovomucoid is detected frequently in the sera from egg allergic patients (Crespo *et al*. 1995, Cook & Sampson 1997, Mine & Zhang 2002a). Studies have been done on IgG as well as IgE antibodies from allergic patients to each domain fragment of ovomucoid (Matsuda *et al*. 1986, Cook & Sampson 1997, Zhang & Mine 1998, 1999).

Recombinant DNA technology offers an insight for the study structure-function relationships of different food allergens. It provides an molecular approach to study immunological and structural functions of allergenic proteins (Baldo 1990). Studies on structure-function relationships of the third domain of ovomucoid have been carried out by comparing natural and semi-synthetic variants (Scheurer *et al*. 1999, Son *et al*. 1999). A number of recombinant food allergens have been cloned and expressed (Lorenz *et al*. 2001). The involvement of the third domain of ovomucoid on

its protease inhibitory activity using site specific mutagenesis has been studied (Kojima *et al*. 1999). The expression of an entire ovomucoid gene and the characterization of recombinant ovomucoid as an antigen, however, has not been successfully investigated due to its unique structure. Our group had earlier reported IgE binding properties of the recombinant ovomucoid third domain expressed in *E. coli* (Sasaki & Mine 2001). Also, our previous work has shown that there is significantly more human IgG and IgE binding activity to the third domain than to the first and second domain against patients' sera (Zhang & Mine 1998). Recently, fine mapping of IgG and IgE epitopes of ovomucoid has been done by several groups (Cook & Sampson 1997, Mine & Zhang 2002b). This provides molecular approaches to the study of ovomucoid allergen for altering allergenic epitope by site-directed mutagenesis to reduce its allergenicity. Therefore, construction of recombinant whole ovomucoid expression system is an important process for the purpose.

The present study was undertaken to express an entire ovomucoid gene, consisting of all the three domains, in *E. coli* and its secretory expression as a soluble form. The recombinant ovomucoid was purified to electrophoretic homogeneity and was investigated for its structural and immunological characterization in comparison to its native analogue.

Materials and methods

Cloning of recombinant ovomucoid

Poly(A^+) RNA was obtained from chicken oviduct. The poly(A^+) RNA was isolated by polyAT tract mRNA isolation kit (Promega, Madison, WI). Using random primers and AMV reverse transcriptase, the first strand was synthesized. PCR was performed using the first strand cDNA as template. The oligonucleotide primers used were 5′-AGACGCAGACCATTACCTTG-3′ and 5′-CTCAG CCAGCATCAGCAGTT-3′. The PCR conditions used for amplification were 40 cycles of denaturing, each of 1 min at 94 °C, 1 min annealing at 57 °C and 1 min elongation at 72 °C. The amplified product was subcloned into a pGEM-T-easy vector (Amersham Pharmacia Biotech, Uppsala, Sweden) and sequenced. The cDNA coding for the chicken ovomucoid gene was gel purified using gene clean spin kit (BIO 101, LaJolla, CA) and further cloned into the pQE-UA cloning expression vector (QIA expression kit, Qiagen, Hilden, Germany). This vector allows direct insertion of the amplified fragment into the prelinearized ends which has a U overhang on each 3′-end. The gene was cloned under the control of T5 promoter with an amino-terminal His-tag.

Expression of the fusion protein

The recombinant plasmid fused to six His-tag was expressed by transforming *E. coli* [M15pREP4] cells. The cells harbouring the construct were grown in Luria broth (LB) media (Difco Laboratories, Sparks, MD) at 37 °C under agitation overnight with ampicillin (100 μg ml^{-1}) and kanamycin (25 μg ml^{-1}) (Sigma). The overnight grown culture was further subcultured and grown to a turbidity of 0.3–0.6 at 600 nm and the expression was induced by adding 1 mM IPTG (Amersham Pharmacia Biotech. Uppsala, Sweden). The culture was harvested at 7000 g for 15 min after growing for 4 h. The soluble and insoluble fractions were separated and analysed on a 12.5% (v/v) SDS-PAGE gel (Laemmli 1970).

Purification of the recombinant fusion protein

The soluble fraction containing the expressed recombinant protein was loaded onto Ni^{2+}-nitrilotriacetic acid (Ni-NTA) resin and purified according to the manufacturer's instructions (Qiagen). In brief, the six His tagged recombinant ovomucoid fraction was loaded to the Ni-NTA resin equilibrated with the lysis buffer (50 mM sodium phosphate, 300 mM NaCl and 10 mM imidazole, pH 8). The resin was washed with the washing buffer (50 mM sodium phosphate, 300 mM NaCl and 20 mM imidazole, pH 8) and eluted with the elution buffer (50 mM sodium phosphate, 300 mM NaCl and 250 mM imidazole, pH 6.3). The eluate was dialyzed against 20 mM Tris/HCl buffer, pH 8 and loaded onto Mono Q column (Amersham Pharmacia Biotech) on HPLC (Waters, Ventura, CA) and eluted against 20 mM Tris/HCl buffer containing 1 M NaCl, pH 8. The fractions were analysed on a 12.5% (v/v) SDS-PAGE gel.

Immunoblot analysis for the recombinant protein

Electrophoretic transfer of the protein onto a nitrocellulose membrane (Micron Separations Inc., Westborough, MA) was done using slight modifications of the earlier published protocol (Towbin *et al.*, 1979). The membrane was blocked with 2% (w/v) BSA in 20 mM Tris/HCl buffered saline (TBS, pH 7.5) and probed with six pooled human egg allergic patient's

serum (diluted 1:100 for the detection of IgG and 1:20 for IgE) for 2 h at room temperature. The membrane was washed three times with TBST (TBS containing 0.05% Tween) and probed with alkaline phosphatase-conjugated goat anti human IgG (1:2000 dilution) and IgE (1:1000 dilution) antibodies (Sigma) and was further washed with TBST and developed with Nitro-Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Life Technologies, Gaithersburg, MD). Colour development was terminated by washing the membranes with deionized water.

Circular dichroism (CD) analysis

The secondary structure of the chicken recombinant ovomucoid antigen and chicken egg white ovomucoid (native) were estimated using a Jasco J 600 spectropolarimeter (Easton, MD). The samples were dissolved in 10 mm phosphate buffer, pH 7 and each sample was scanned four times from $\lambda = 190$–250 nm wavelength in quartz cuvette (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with a 0.1 cm path length. A buffer baseline (containing 10 mM phosphate buffer, pH 7) was subtracted from the spectra of each sample. The percentages of the different secondary structures (α-helix, β-structure and random coil) were calculated using a Jasco protein structure estimation program, based on earlier published method (Yang & Wu 1986).

Enzyme-linked immunosorbent assay (ELISA)

Antigenic and allergenic properties of the ovomucoid antigen were determined by an indirect ELISA. The recombinant and the native ovomucoid antigens were coated to a 96-well microtiter plate in 0.1 M sodium carbonate buffer (pH 9.6) at 0.5 μg well^{-1} and incubated overnight at 4 °C. The plate was washed four times with TBS containing 0.05% of Tween 20 (TBST). The plate was blocked with 150 μl of 2% (w/v) BSA in TBS for 2 h at 37 °C. The plate was washed with TBST three times and incubated with 100 μl of egg allergenic patients sera (1/20 dilution for IgE detection and 1/100 dilution for IgG detection in TBS containing 1% (w/v) BSA) at room temperature for 2 h. The plate was further washed with TBST three times and incubated with 100 μl of monoclonal anti human IgE conjugated alkaline phosphatase (1/1000) (Sigma) for IgE detection and goat anti-human IgG conjugated alkaline phosphatase (1/2000) (Sigma) for IgG detection at room temperature for 2 h. The plate was washed six times with TBST and developed with 100 μl of *p*-nitrophenol phosphate (1 mg ml^{-1}) (Sigma) in 0.1 m diethanolamine buffer (pH 9.8) for 60 min at room temperature. The reaction was terminated by adding 25 μl of 3 N NaOH. Absorbance at 405 nm was read by the microplate reader (Model 550, Bio-Rad laboratories, Hercules, CA).

Human serum

Human serum was collected from six patients exhibiting class 2–4 radioallergosorbent test towards eggs. All of the patients were allergic to egg white. All serum samples were kindly provided by Dr A. Urisu (Department of Pediatrics, Fujita Health University, Japan) and Dr Morikawa (Department of Pediatrics, Gunma University, Japan) and stored at -80 °C until use. A normal serum from a non-allergic donor was used as a control serum.

Statistical analysis

Data were analysed by using analysis of variance (ANNOVA-SPSS version 7.5 for windows: SPSS Chicago, IL) and the level of significance between antibody activity was defined at $p < 0.05$.

Results and discussion

Expression of chicken ovomucoid gene in E. coli

The gene coding for the protein sequence of the whole chicken ovomucoid, consisting of all the three tandem repeats was successfully expressed as a recombinant fusion construct, with a six-His tag, under the control of T5 promoter in *E. coli* M15 [pREP4]. The amplified PCR product was directly cloned into the U overhang of the pQE-UA cloning vector and upon transformation and induction of the *E. coli* cells harbouring the plasmid, the recombinant protein was expressed in soluble form which were analysed on SDS-PAGE and uninduced fraction were maintained as controls (Figure 1A, lanes 1 and 2).

Purification of the fusion protein

The expression and purification of recombinant proteins facilitates production and detailed characterization of virtually any proteins. Although a large number of recombinant proteins have been expressed earlier, purification has always been complicated and cumbersome. Attaching affinity tags simplifies the procedure for purification. His tag has been preferred by many

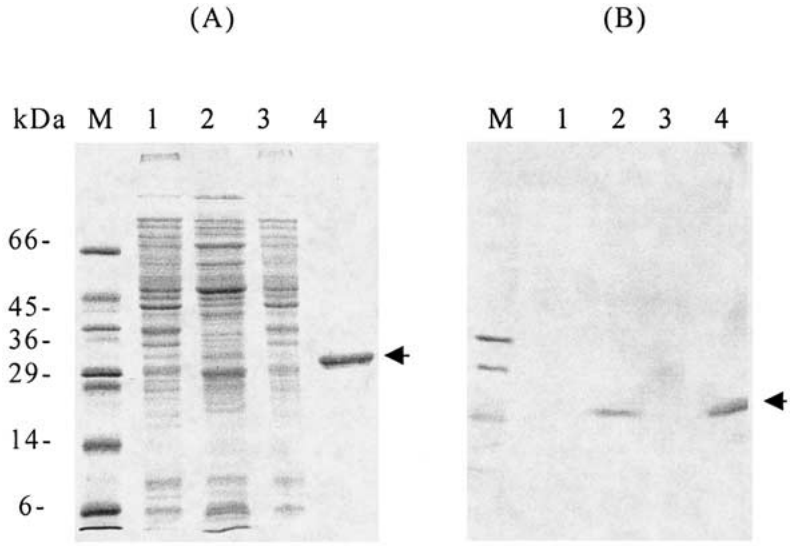

Fig. 1. Expression and purification of the recombinant ovomucoid. (A) SDS-PAGE analysis. Lane 1 uninduced fraction (no IPTG); lane 2 induced fraction (with IPTG); lane 3 unbound flowthrough fractions; lane 4 purified recombinant ovomucoid. M, molecular mass markers and the arrow points to the purified protein. (B) Immunoblot analysis. The western blotting order of samples is similar to that of the SDS-PAGE gel. The rabbit anti-ovomucoid IgG antibody was used to prove the ovomucoid.

researchers because of its non-interference with the structure and function of the protein (Sulkowski 1985). We used the Ni-NTA resin for affinity chromatography and the recombinant ovomucoid was purified to almost 90% homogeneity and was further purified by ion exchange chromatography which yielded a molecular mass of 28 kDa (Figure 1A, lane 4). Approximately 4–5 mg of purified recombinant ovomucoid was obtained from 1 l of media.

Western immunoblotting of the recombinant protein

Authenticity of the recombinant ovomucoid was confirmed by immunoblot analysis using rabbit anti-ovomucoid IgG antibodies raised in our laboratory previously (Sasaki & Mine 2001). Figure 1B shows the purified recombinant ovomucoid fractions (lanes 2 and 4) were identical in molecular mass to its native analogue purified from egg white. Furthermore, the both native and recombinant ovomucoid were subjected to western immunoblot analysis using six pooled human sera from egg allergic patients. Both the antigens showed the same corresponding band with immunblot analysis (Figures 2A and B) suggesting that the recombinant antigen had sequential epitopes similar to that of the native protein and the antigenicity and allergenicty of both the antigens were recognized on the immunblot.

Circular dichroism spectral analysis

The recombinant ovomucoid had a different CD profile to that of native analogue, resulting that π-π^* II transition was shifted to lower energy, a red shifted and was centred at 222 nm (Figure 3). The CD spectrum of the α-helix is comprised of the π-π^* and n-π^* transition bands. The perpendicularly polarized (π-π^*) $\perp$ transition at 192 nm is positive in sign, whereas the lower energy transition is the paralleled polarized (π-π^*) II transition at 209 nm and the n-π^* transition at 222 nm, both are negative in sign (Mulkerrin 1995). These results showed that the structure of the α-helix side chain residues of recombinant ovomucoid has different feature from that of native form. Table 1 summerized the percentage of secondary structure of both native and recombinant ovomucoid. The secondary structure based on CD analysis showed that the α-helix of the native ovomucoid was less when compared to the recombinant ovomucoid, while the β-structure seems to be higher in the native when compared to the recombinant antigen. This indicates that the recombinant ovomucoid obtained by the present method has a slightly rigid structure when compared to the native protein, but as pointed out earlier (Lorenz *et al*. 2001) the recombinant food allergens are in general not identical copies of their respective native analogue and the heterologous proteins may have slight modification in their structures without any loss of biological activ-

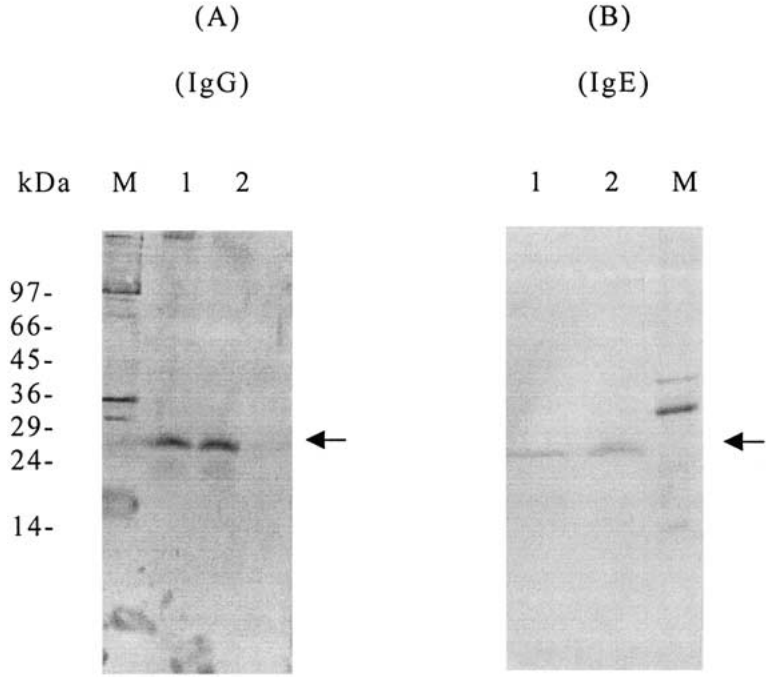

Fig. 2. Immunoblotting analysis of IgG and IgE against pooled patient's sera. (A) IgG Immunoblot. Lane 1 corresponds to the native protein and lane 2 represents the recombinant ovomucoid fraction. M denotes the molecular mass markers. (B) IgE Immunoblot. The order of the samples is the same as the IgG immunoblot. Six pooled human sera from egg allergic patients were used.

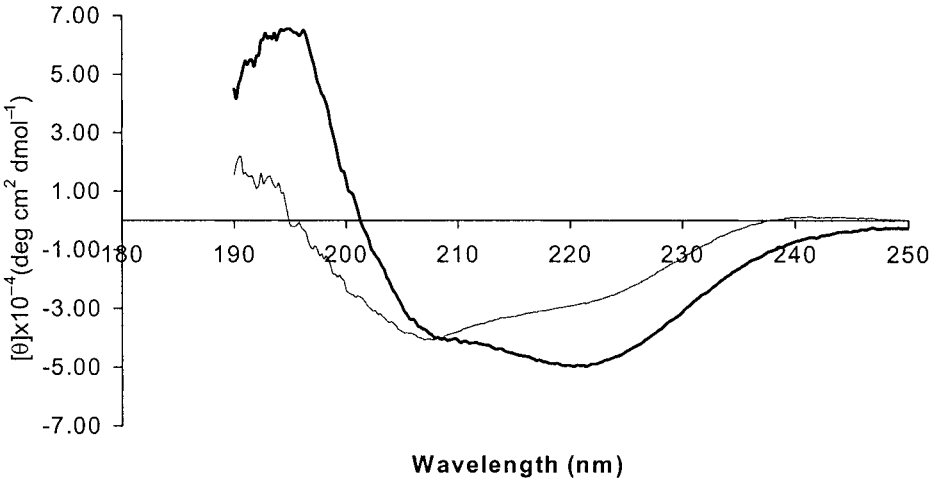

Fig. 3. Circular dichroism spectra of recombinant and native ovomucoid. The far-UV spectra was measured by scanning four times from 190 nm to 250 nm. The proteins (0.2 mg ml^{-1}) were dissolved in 10 mM phosphate buffer, pH 7 and blank containing phosphate buffer was measured and subtracted from the spectra. The bold line denotes the recombinant ovomucoid spectrum, the fine line denotes the native ovomucoid spectrum.

Table 1. Secondary structure fractions of native and recombinant oromucooid.

Forms	Secondary structure (%)[a]			
	α-Helix	β-Sheet	β-Turn	Random coil
Native ovomucoid	16.5	26.4	18.8	38.4
Recombinant ovomucoid	25.6	23.9	18.9	31.5

[a]The percentage of each of the secondary structures were estimated using the Jasco protein secondary structure estimation program, based on the method of Yang *et al.* (1986).

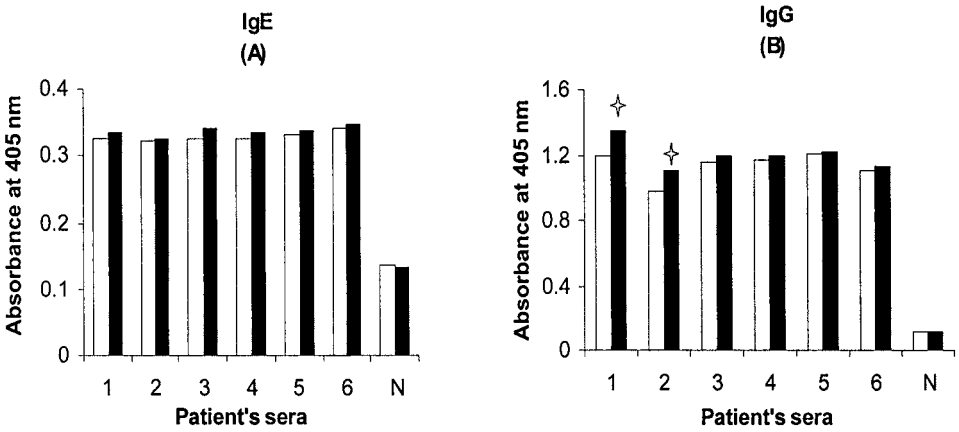

Fig. 4. Comparison of the binding activity of human specific IgE and IgG antibodies with the native and recombinant ovomucoid. (A) IgE binding activity. The open bars denote the native ovomucoid and the closed bars denote the recombinant ovomucoid. (B) IgG binding activity. Numbers 1–6 represent from different serum and N denotes the control. ✧, Recombinant ovomucoid where the binding activity is higher than its native analogue.

ity. So the IgG and IgE binding properties were to be considered in detail.

Enzyme linked immunosorbent assay

The importance of linear and conformational epitopes was obtained by determining ovomucoid-specific IgG and IgE antibody binding activities to the native and the recombinant ovomucoid on ELISA. The ovomucoid specific IgE and IgG antibodies from different patients showed similar ability to recognize the native and recombinant ovomucoid antigens (Figure 4) when tested individual serum. The IgG binding activity of the recombinant ovomucoid with some sera (numbers 1 and 2) was significantly higher ($p < 0.05$) than that of native form. This may be due to the differences of recognition of tertiary structure of native and recombinant ovomucoid under ELISA since both types of specific antibodies (against sequential and tertiary epitopes) can be recognized by ELISA. Our group has recently identified the major IgG and IgE epitopes (sequential) in the entire ovomucoid protein backbone, and investigated the essential amino acid residues and structurally analysed their allergenic properties, however, the role of tertiary structure of ovomucoid on allergic responses has not been investigated. The information obtained from the critical amino acids in the previous work (Mine & Zhang 2002b) could be used as a useful tool in construction of synthetic mutants by site directed mutagenisis and to the studies of structure-function relationships of allergenic epitopes in ovomucoid. This molecular approach would further give us a better understanding to reduce the risk involved with food-hypersensitivity in humans.

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