



Immunological comparison of native and recombinant egg allergen, ovalbumin, expressed in *Escherichia coli*

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Abstract

Chicken ovalbumin is one of the major egg white allergens which causes IgE-mediated food hypersensitivity. A gene encoding for chicken ovalbumin (*Gad d1*) was isolated from chicken oviduct by PCR amplification and was cloned under the control of T5 promoter fused with a six-histidine tag at the *N*-terminal end. *Escherichia coli* harbouring this construct expressed high quantities of the recombinant protein in the form of soluble fraction. The protein was purified using affinity chromatography on a Ni²⁺-nitrilotriacetic acid agarose column and was further purified to homogeneity by ion exchange chromatography. Homogeneity was confirmed through SDS-PAGE, Western blot and secondary conformation analysis. The reactivity of the recombinant and native protein was tested against six egg allergic human patient's sera and the IgE and IgG binding activity was tested using both Western blot and ELISA. When compared to native ovalbumin, the recombinant protein had similar binding activity in immunoblotting, but slightly increased activity by ELISA. Circular dichroism revealed that the recombinant protein had a slightly less compact structure than the native form. Both antigens exhibited a similar immunogenicity in mice.

Introduction

Egg allergy is more frequent in young children than in adults (Anet *et al.* 1985). All major allergenic proteins in hen's egg white are well characterized (Holen & Elsayed 1990). Out of 40 different egg white proteins, only four major allergens, ovalbumin, ovomucoid, ovotransferrin and lysozyme, are associated with egg allergy (Hoffman 1983, Hefle 1996, Mine & Zhang 2002). The presence of hen's egg-specific IgE against ovomucoid seemed to be a predictive marker at early infancy for allergic sensitization to common allergens that cause asthma, allergic rhinitis and atopic dermatitis (Aabin *et al.* 1996, Urisu *et al.* 1997, Cooke & Sampson 1997). Ovalbumin is the most abundant globular protein of egg white. It is a disulfide protein and a member of the serpin super family (Huber & Carrel 1989). The molecular weight of ovalbumin is 45 kDa and it has 385 amino acids (Nisbet *et al.* 1981).

Most studies in the last few years have focused on allergenic structures of ovalbumin (Kahlert *et al.* 1992, Honma *et al.* 1996). A number of studies have been done on its structural (Stein *et al.* 1990, Takahashi *et al.* 1991), functional (Doi & Kitabatake 1997) and immunological properties (Kim *et al.* 2002).

Many recombinant food allergens have been previously expressed and characterized (Lorenz *et al.* 2001). However, recombinant proteins are in general not identical copies of their respective native analogue and they may have slight modification in their structures. Therefore, it is a critical step to investigate the immunological comparison of native and recombinant allergens before subjecting to further studies (Baldo 1990, Petersen *et al.* 1997). Ovalbumin has been cloned and expressed earlier to study its structural attributes (McReynolds *et al.* 1978, Takahashi *et al.* 1995) but, somewhat surprisingly, studies on the allergenic properties of recombinant ovalbumin have not

been documented. The IgE-mediated immunological reaction is the most frequent and the best known form of food allergy. Comparative investigations of recombinant food allergens and their natural counterparts may offer a better understanding into the structural basis of allergenicity. Here, the chicken ovalbumin gene was cloned and expressed as a soluble fraction and comparative studies with native ovalbumin were carried out using ELISA and Western blot analysis, using human sera from egg allergic patients. The antigenicity and allergenicity recognized by the human sera in response to the recombinant antigen was determined. IgG and IgE binding properties of both the recombinant and the native ovalbumin are discussed.

Materials and methods

Reagents

All restriction enzymes and DNA size markers were purchased from New England Biolabs (Beverly, MA). Antibiotics, IPTG, Freund's complete and incomplete adjuvants were obtained from Sigma. The kits for PCR and DNA purification were obtained from Promega (Madison, WI). The gel elution gene clean spin kit was purchased from Bio 101 (Bio/Can Scientific, Vista, CA). The QIA express UA Cloning kit was purchased from Qiagen (Qiagen GmbH, Hilden, Germany).

PCR amplification and cloning

Poly(A⁺) RNA was isolated from chicken oviducts using PolyATtract mRNA isolation kit (Promega), following the manufacturer's protocol. The first-strand cDNA was synthesized from 1 µg poly(A⁺) RNA using random primers and AMV reverse transcriptase. Following reverse transcription, the ovalbumin gene cDNA was amplified by PCR using the first-strand cDNA as template and oligonucleotide primers 5'-AGCTCGAAAGACAACCTCAGA-3' and 5'-GCAGCAGAGAACATACTTTT-3'. The PCR parameters were 40 cycles of denaturing, each for 1 min at 94 °C, 1.5 min annealing at 57 °C and 1 min elongation at 72 °C. Chicken ovalbumin encoding gene obtained from PCR amplification was gel purified using Gene Clean Spin Kit (BIO 101, La Jolla, CA) and directly cloned into pGemT-easy vector (Amersham Pharmacia Biotech, Uppsala, Sweden) and sequenced. pQE-30 UA cloning vector (QIA express kit, Qiagen, Hilden, Germany) for direct cloning of the PCR product was used to clone the amplified

cDNA. This vector allows the 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 a N-terminal 6X Histag.

Expression of the recombinant construct

The gene encoding the protein sequence to be expressed was ligated using T4 DNA ligase under standard conditions (Sambrook *et al.* 1989). The construct harbouring the ovalbumin gene was expressed by transforming the plasmid into competent *E. coli* M15 [pREP4] cells. The cells containing the plasmid were grown in Luria broth (LB) at 37 °C overnight with ampicillin (100 µg ml⁻¹) and kanamycin (25 µg ml⁻¹). The culture was inoculated into fresh LB media and grown at 37 °C until the optical density reached 0.3–0.6 at 600 nm when the expression was induced by adding 1 mM IPTG. After allowing the culture to grow for 4 h, the soluble and insoluble fractions were separated by centrifugation at 7000 g for 5 min. The expression levels were checked on 12.5% SDS-PAGE gel (Laemmli 1970). Un-induced culture fractions were run as controls. The specificity of the recombinant protein was further confirmed by immunoblotting the sample with antibody raised in rabbit against native ovalbumin.

Purification of the recombinant protein

The recombinant ovalbumin protein, which contained a 6×-His affinity tag, was purified on Ni²⁺-nitrilotriacetic (Ni-NTA) resin according to manufacturer's instructions (Qiagen GmbH). In brief, the 6×-His tagged ovalbumin soluble protein was loaded on the Ni-NTA resin equilibrated with the lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole, pH 8). The resin was washed with the wash buffer (50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole, pH 8) and elution was done using the elution buffer (50 mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole, pH 6.3). The eluate was dialysed against 20 mM Tris/HCl buffer, pH 8 and the protein was loaded onto a Mono Q column (Amersham Pharmacia Biotech) on HPLC (Waters, Ventura, CA) and eluted with 20 mM Tris/HCl buffer containing 1 M NaCl, pH 8. The fractions were analysed on 12.5% SDS-PAGE gel.

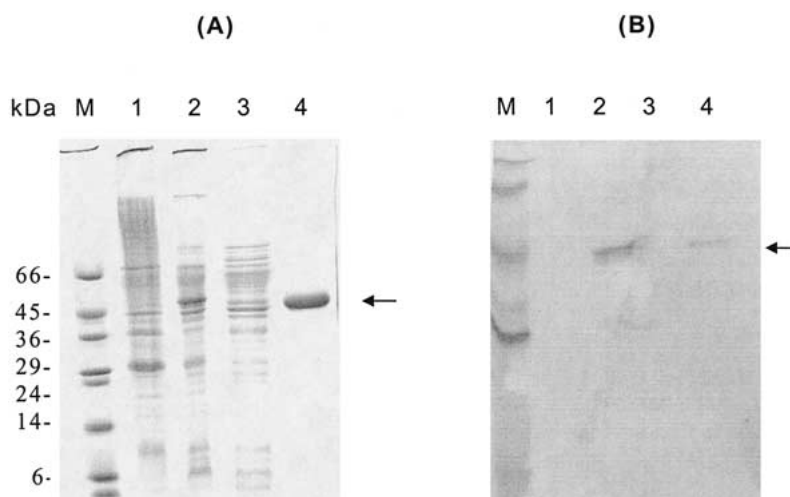


Fig. 1. Expression and purification of recombinant ovalbumin. (A) SDS-PAGE. Lane 1 represents the uninduced control fraction (no IPTG) and lane 2 represents the lysate of the IPTG induced fraction. Lane 3 represents the flow through after column binding and lane 4 represents the purified protein. (B) Immunoblotting for expression and purification of the recombinant ovalbumin. Lane 1 represents the uninduced fraction and lane 2 represents the IPTG induced fraction. Lane 3 represents the flow through fraction after column binding and lane 4 represents the purified protein. M denotes the molecular weight markers. Arrow points to the recombinant protein.

Western immunoblotting

SDS-PAGE was performed on a 12.5% (v/v) polyacrylamide gel, after heating the samples at 95 °C in 1% SDS and 5 mM 2-mercaptoethanol. The proteins were immediately electrophoretically transferred to nitrocellulose membrane (Micron Separations Inc., Westborough, MA) using slight modifications of the earlier published method (Towbin *et al.* 1979). The membrane was blocked with 2% bovine serum albumin (BSA) in 20 mM Tris/buffered saline (TBS, pH 7.5) and probed with pooled human egg allergic patients serum. The serum was diluted 1:100 in TBS containing 0.05% Tween (TBST) for IgG blot and 1:20 for IgE blot. The membrane was washed twice with TBST 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 stained with nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Life Technologies). Colour development was terminated by washing the membranes with deionized water.

Immunization

Immunization with the recombinant and the native ovalbumin antigens were done using Balb/c mice. Four mice for each group of antigen were used at 6–8 weeks of age. 100 µg of the antigen was injected sub-

cutaneously with complete Freund's adjuvant (Sigma). Control mice were injected with normal saline (0.9% NaCl). Fourteen days after the primary immunization the mice were administered a booster consisting of 100 µg of protein in incomplete Freund's adjuvant. The animals were bled 7 d after the primary and secondary immunization. Blood from mice was stored at 4 °C for 24 h to form a clot. Antisera were collected from each blood sample after clot formation. The total and specific IgG and IgE levels were checked using an enzyme-linked immunosorbent assay (ELISA) as described below.

Determination of IgG and IgE production

To determine the production of total IgG and IgE levels in the antisera raised in mice against the native and recombinant ovalbumin, a sandwich ELISA was performed. One hundred micro litres of goat anti-mouse IgG (Cedarlane, Hornby, ON, Canada) and anti-mouse IgE monoclonal antibodies (BD Bio Science, San Diego, CA) (1 µg well⁻¹) in 50 mM sodium carbonate buffer, pH 8.5 was added to each well of a 96-well ELISA microtiter plate (Corning, Cambridge, MA). The plate was kept at 4 °C overnight. The plate was washed three times with TBST, followed by incubation with 150 µl of 2% BSA in TBS for 2 h at 37 °C. The plate was washed with TBST three times and incubated for 2 h at 37 °C with 100 µl of the serum (× 500 diluted each) obtained from mice. Standard

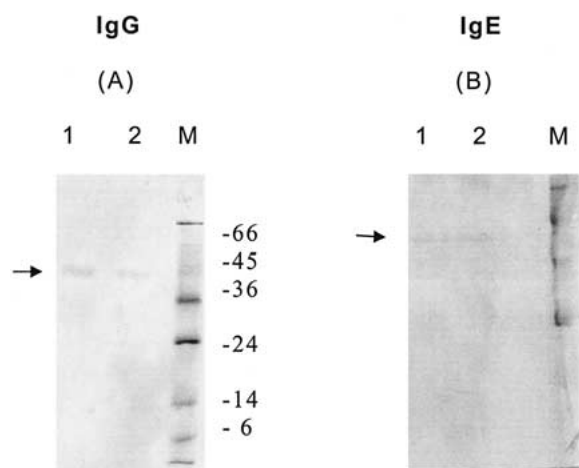


Fig. 2. Western immunoblot for reactivity of recombinant and native ovalbumin against egg allergic patients' sera. (A) Immunoblot for IgG reactivity. Lane 1 represents the native ovalbumin and lane 2 represents the recombinant ovalbumin. M represents the molecular weight markers and the arrow points to the protein. (B) Immunoblot for IgE reactivity. Lane 1 represents the native protein and lane 2 represents the recombinant ovalbumin. M denotes the molecular weight markers and arrow points to the protein.

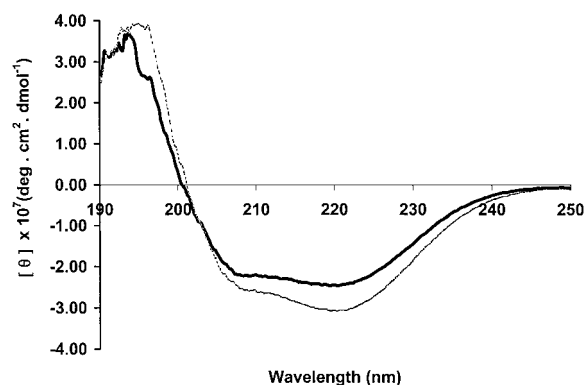


Fig. 3. Circular dichroism spectra of recombinant and native ovalbumin. The far-UV spectra was measured by scanning four times from 190 nm to 250 nm. The proteins (0.2 mg ml^{-1}) were dissolved in 10 mM phosphate buffer, pH 7 and blank containing phosphate buffer was measured and subtracted from the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

pure mouse IgG and IgE antibodies (BD Bio Science) were used as controls. After the plate was washed with TBST four times, $100 \mu\text{l}$ of alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma) (1:2000 dilution) for measuring IgG level and monoclonal anti-mouse IgE conjugated to biotin (1:1000 dilution) (Sigma) for determining IgE level were added to each well and incubated 2 h at 37°C . For IgE assay, the plate was further incubated with avidin conjugated al-

kaline phosphatase (1:3000 dilution) (Sigma) for 1 h at 37°C . The plate was washed six times and developed with $100 \mu\text{l}$ of *p*-nitrophenol phosphate (1 mg ml^{-1}) (Sigma) in 0.1 M diethanolamine buffer (pH 9.8) for 60 min at 37°C . The reaction was terminated by adding $25 \mu\text{l}$ of 3 M NaOH. Absorbance at 405 nm was read by microplate reader (Model 550, Bio-Rad Laboratories). For specific IgG and IgE determination, recombinant and native ovalbumin was coated ($1 \mu\text{g well}^{-1}$) instead of anti-mouse IgG and IgE antibodies and the same procedures followed.

Circular dichroism

Circular dichroism (CD) spectra of the recombinant ovalbumin and native ovalbumin were determined in 20 mM phosphate buffer, pH 7.5 using a Jasco J 600 spectropolarimeter (Easton, MD). Each sample was scanned four times from 190 to 250 nm in a quartz cuvette with a 0.1 cm path length. A buffer baseline 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 secondary structure estimation program, based on earlier published method (Yang et al. 1986).

Enzyme-linked immunosorbent assay to measure allergenicity and antigenicity

Indirect ELISA was performed to determine the allergenicity and antigenicity of the native and recombinant protein, according to a modification of the earlier published method (Engvall & Pernern 1972). In brief, medical grade high binding polystyrene 96-well microplates (Corning, Cambridge, MA) were pre-coated with $100 \mu\text{l}$ of the native and recombinant ovalbumin ($1 \mu\text{g well}^{-1}$ in 100 mM carbonate buffer, pH 9.6) and incubated overnight at 4°C . The plates were washed three times with TBST. The plates were blocked with 2% BSA in TBS for 2 h at 37°C and washed with TBST three times. The plates were then incubated with $100 \mu\text{l}$ of pooled sera from egg allergic patients (diluted 1:20 for IgE and 1:100 dilution for IgG in TBS containing 1% BSA). The plates were further washed three times with TBST and incubated with $100 \mu\text{l}$ of alkaline phosphatase-conjugated rabbit anti-human IgG (Sigma) diluted to 1:2000 and monoclonal anti-human IgE conjugated alkaline phosphatase (Sigma) diluted to 1:1000 for 2 h at room temperature. The plates were washed six times and developed with *p*-nitrophenol phosphate (Sigma) in 0.1 M diethanolamine buffer (pH 9.8) for 60 min at room temperature.

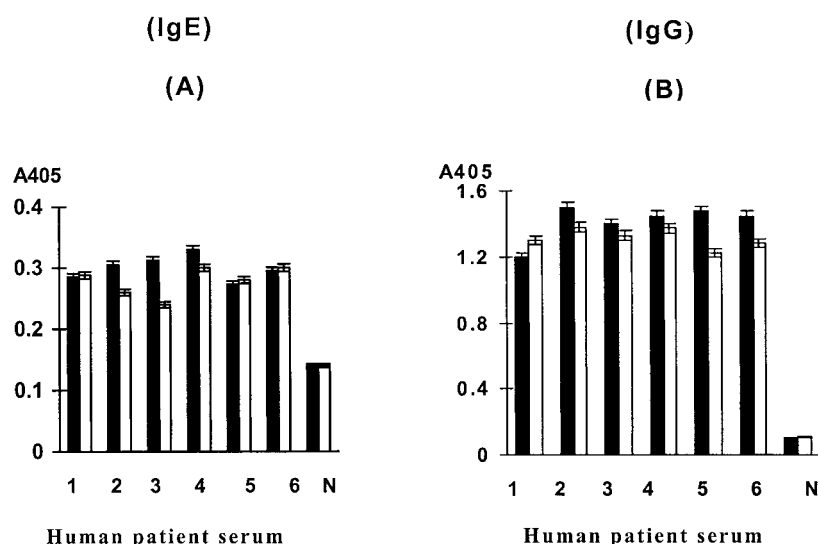


Fig. 4. Comparison of the binding activity of human specific IgE (A) and IgG (B) with native and recombinant ovalbumin. Open box represents recombinant antigen reactivity and closed box represents the native antigen reactivity. N denotes the normal serum and numbers 1–6 denote different patient's sera. Data are average of triplicate measurements $\pm$ SD.

The reactions were terminated by adding 3 M NaOH ($25 \mu\text{l well}^{-1}$) and the plate was read at 405 nm using a Bio-Rad Microplate Reader.

Human patient's sera

Human serum was collected from six patients exhibiting class 2–4 radioallergosorbent test towards egg. All the patients showed high degree of allergenicity to egg white. All serum samples were kindly provided by Dr A. Urisu (Department of Paediatrics, Fujita Health University, Japan) and stored at -80°C until use.

Production of anti-ovalbumin polyclonal antibodies

Two rabbits (Central Animal Facility, University of Guelph) were injected intramuscularly with 0.2 mg native ovalbumin that had been re-suspended in 0.2 ml of 0.9% sterile saline and emulsified with an equal amount of Complete Freund's Adjuvant (CFA; Sigma). Two weeks later the rabbits were given a second intramuscular injection, again with the same proteins in CFA. Two more injections were administered in the subsequent two weeks. Antibody titers were monitored by ELISA. The collected antisera were precipitated with saturated ammonium sulfate to a final saturation of 35%. The precipitates were dissolved in PBS, dialyzed against PBS for 48 h, and then stored at -80°C until use.

Statistical analysis

The significant difference in binding of the human sera to the antigens were estimated by analysis of variance test (ANNOVA-SPSS version 7.5 Software for Windows: SPSS, Chicago, IL) and the level of significance was defined at $p < 0.05$.

Results and discussion

Expression of a protein fused to a short polypeptide tag can provide an affinity handle by which purification of the fusion protein can be facilitated, or it can provide a function by which the fusion protein can be detected (Bujard *et al.* 1987). The ovalbumin gene was cloned from the chicken oviduct with the histidine tag at the *N*-terminal end under the control of the T5 promoter. *E. coli* cells harbouring the plasmid upon expression with 1 mM IPTG, showed a predominant band of 45 kDa corresponding to the expected size of ovalbumin in the total cell extract (Figure 1A, lane 2). These data suggest that the expression of the recombinant protein was efficiently directed by the T5 promoter. Fractionation of the induced culture revealed that the protein was secreted in soluble form and the histidine tagged fusion protein was purified to homogeneity by affinity chromatography and ion exchange chromatography (Figure 1A, lane 4). Heterogeneous proteins are highly prone to proteolysis which may result in

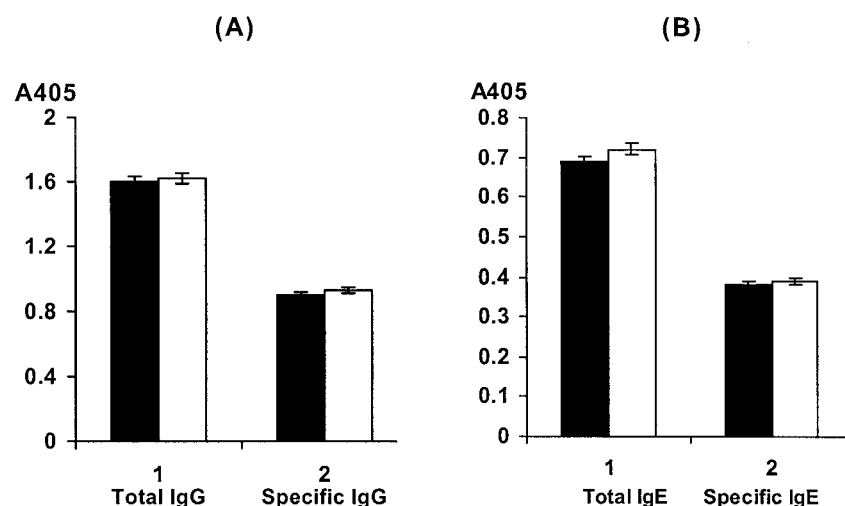


Fig. 5. Total and specific levels of IgG and IgE in mice antisera (A) Represents the IgE levels and (B) represents the IgG levels. The closed box represents the levels of native ovalbumin and the open box represents the recombinant ovalbumin. Data are average of duplicate measurements $\pm$ SD of four mice each.

Table 1. Secondary structure of native and recombinant ovalbumin analysed by circular dichroism.

Forms	Secondary structure (%)			
	α -Helix	β -Sheet	β -Turn	Random coil
Native ovalbumin	40.2	39.3	10.2	10.3
Recombinant ovalbumin	38.2	37.2	10.5	14.1

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).

complete hydrolysis of the product, and different approaches to prevent this have been tried (Enfors 1992). However, in our study no such degradation of the recombinant protein was detected.

The possible transfer of a gene coding for an allergen was confirmed for its authenticity by immunoblot analysis using polyclonal anti-ovalbumin antibody raised in rabbit (Figure 1B, lanes 2 and 4). We showed that recombinant ovalbumin has structural and functional properties similar to those of the natural protein, including the conservation of its antigenic and allergenic properties. Specific protein recognition by the human immune system was shown by immunoblotting the native and the recombinant protein with egg white patient's sera against human IgG and IgE antibodies (Figure 2). Binding of the native and the recombinant antigens to human patients' sera revealed that the immunoreactivity of both antigens was the same, suggesting that the recombinant ovalbumin has sequential epitopes similar to that of the native form.

Changes have been revealed earlier in the antigenic and allergenic properties of ovalbumin after heat and gamma irradiation treatment (Kim *et al.* 2002). But the recombinant ovalbumin did not show any change in its reactivity.

The secondary structure of ovalbumin, using denatured protein in comparison to the native protein, has been discussed earlier (Takahashi *et al.* 1991, Onda *et al.* 1997). However, the comparison of native and recombinant ovalbumin using CD technique has not been conducted. Figure 3 shows CD spectrum of native and recombinant ovalbumin. The $n-\pi^*$ transition at 222 nm of the recombinant ovalbumin was decreased compared to the native analogue. Based on CD analysis, the CD ellipticity in our study showed that the α -helix and the β -structure content of the recombinant ovalbumin was slightly lower than the native ovalbumin (Table 1), which indicates that the recombinant protein may have a slightly less compact structure than the native form. It has been previously suggested

(Lorenz *et al.* 2001) that recombinant food allergens are, in general, not identical copies of the natural analogue, since changes to the IgE binding properties of the recombinant protein may occur. In this study, the 6×-Histag at the *N*-terminus of recombinant ovalbumin may decrease the α -helix of the molecule and reduced its similarity to the native form.

The IgG and IgE class antibodies have been shown to exist in sera from patients with allergy (Fallstrom *et al.* 1986). To define the allergenic epitopes of ovalbumin against patient's sera the reactivity of IgG and IgE were determined using ELISA. The binding activities of six patients' sera against the native and the recombinant ovalbumin antigens were compared. The IgG and IgE-binding activity of the recombinant ovalbumin was found to be increased ($p < 0.05$) against some of the human patients sera samples (serum numbers 2–4 for IgE and numbers 2–6 for IgG-binding activity) in comparison with that of the native protein (Figure 3). The increased binding activity of the sera against the recombinant antigen could be due to a structural change in the recombinant protein. During sensitization, antigen-presenting cells in the human body are presented with denatured form of allergens to be processed (Kaminogawa 1996). It is also expected that food allergens could be partially hydrolyzed or denatured before being absorbed from the intestine and following sensitization by the gut immune system. In fact, we previously reported that human IgG antibody from egg allergic patients sera recognized partially denatured ovalbumin more strongly than its native form (Mine & Zhang 2002).

We also compared immunogenicity of native and recombinant ovalbumin in mice. The total and specific IgG and IgE levels in the antisera raised in mice is shown in Figure 4. All mice studied showed a similar total IgG and IgE response against both the native and recombinant ovalbumin and this was further confirmed by the reactivity of both the antigens towards the detection levels of specific IgG and IgE. This indicated that both the recombinant and native ovalbumin could have similar immunogenicity.

In conclusion the recombinant ovalbumin produced was similar to its native analogue and could be used as a tool to characterize sequential and conformational epitopes and to discuss food allergy with regards to the IgE binding specificity. More recently, our group has completely mapped immunodominant IgE allergenic sequential epitopes in ovalbumin (personal communication). The results of this study are important to develop molecular approaches to deter-

mine structural variants using site directed mutagenesis for the study of structure-function relationships of allergenic epitopes in ovalbumin.

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References

- Aabin B, Poulsen LK, Ebbehøj K, Norgaard A, Frøkiær H, Bindslev-Jensen C, Barkholt V (1996) Identification of IgE binding egg white proteins; comparison of results obtained by different methods. *Int. Arch. Allerg. Immunol.* **109**: 50–57.
- Anet J, Back JF, Baker RS, Barnetter D, Burley RW, Howden MEH (1985) Allergens in the white and yolk of hen's eggs. A study of IgE binding by egg proteins. *Int. Arch. Allerg. Immunol.* **77**: 364–371.
- Baldo BA (1990) *Molecular Approaches to the Study of Allergens*. Basel, Switzerland: Karger.
- Bujard H, Gentz R, Lanzer M, Stuber D, Müller M, Ibrahimi I, Hauptle MT, Dobberstein B (1987) A T75 promoter based transcription-translation system for the analysis of protein *in vitro* and *in vivo*. *Meth. Enzymol.* **155**: 416–433.
- Cooke SK, Sampson HA (1997) Allergenic properties of ovomucoid in man. *J. Immunol.* **159**: 2026–2032.
- Doi E, Kitabatake N (1997) Structure and functionality of egg proteins. In: Damodaran S, Paraf A, eds. *Food Proteins and their Applications, Structure and Functionality of Egg Proteins*. New York: Marcel Dekker, pp. 325–340.
- Enfors SO (1992) Control of *in vivo* proteolysis in the production of recombinant proteins. *Trends Biotechnol.* **10**: 310–315.
- Engvall E, Pernmann P (1972) Enzyme-linked immunosorbent assay, ELISA. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen coated tubes. *J. Immunol.* **109**: 129–135.
- Fallstrom SP, Ahlstedt S, Carlsson B, Lönnerdal B, Hanson IA (1986) Serum antibodies against native, processed and digested cow's milk proteins in children with cow's milk protein intolerance. *Clin. Allerg.* **16**: 417–423.
- Hefle SL (1996) The chemistry and biology of food allergens. *Food Technol.* **50**: 86–92.
- Hoffman DR (1983) Immunochemical identification of the allergens in egg white. *J. Allerg. Clin. Immunol.* **71**: 481–486.
- Holen S, Elsayed S (1990) Characterization of four major allergens in hen-egg white by IEP/SDS-PAGE combined with electrophoretic transfer and IgE-immunoblotting. *Int. Arch. Allerg. Appl. Immunol.* **9**: 136–141.
- Honma K., Kohno Y, Saito K, Shimojo N, Horiuchi T, Hayashi H, Suzuki N, Hosoya T, Tsunoo H, Niimi H (1996) Allergenic epitopes of ovalbumin in patients with hen's egg allergy: inhibition of basophil histamine release by haptenic ovalbumin peptide. *Clin. Exp. Immunol.* **103**: 446–453.
- Huber R, Carrel RW (1989) Implications of the 3-dimensional structure of α 1-antitrypsin for the structure and function of serpins. *Biochemistry* **28**: 8951–8966.

- Kahlert H, Petersen A, Becker W-M, Schlaah M (1992) Epitope analysis of the allergen ovalbumin (Gal d II) with monoclonal antibodies and patients' IgE. *Mol. Immunol.* **29**: 1191–1201.
- Kaminogawa S (1996) Food allergy, oral tolerance and immuno modulation-their molecular and cellular mechanisms. *Biosci. Biotechnol. Biochem.* **61**: 1749–1756.
- Kim MJ, Lee JW, Yook HS, Lee SY, Kim MC, Byun MW (2002) Changes in the antigenic and immunoglobulin E binding properties of hen's egg albumin with the combination of heat and gamma irradiation treatment. *J. Food Prot.* **65**: 1192–1195.
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–695.
- Lorenz AR, Haustein D, Vieths S (2001) IgE binding properties of recombinant food allergens. *Int. Symp. Food Allerg.* **3**: 1–36.
- McReynolds L, O'Malley BW, Nisbet AD, Fothergill JE, Givol D, Fields S, Robertson M, Brownlee GG (1978) Sequence of chicken ovalbumin mRNA. *Nature* **273**: 723–728.
- Mine Y, Zhang JW (2002) Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins. *J. Agric. Fd. Chem.* **50**: 2679–2683.
- Nisbet AD, Sandry RH, Moir A JG, Fothergill LA, Fothergill JE (1981) The complete amino acid sequence of hen ovalbumin. *Eur. J. Biochem.* **115**: 335–345.
- Onda M, Tatsumi E, Takahashi N, Hirose M (1997) Refolding process of ovalbumin from urea-denatured state. Evidence for the involvement of monoproductive side chain interactions in an early intermediate. *J. Biol. Chem.* **272**: 3973–3979.
- Petersen A, Grobe K, Lindner B, Schlaak M, Becker WM (1997) Comparison of natural and recombinant isoforms of grass pollen allergens. *Electrophoresis* **18**: 819–825.
- Sambrook J, Fritsch EF, Maniatis F (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.
- Stein PE, Leslie AG, Finch JT, Carrell RW (1991) Crystal structure of uncleaved ovalbumin at 1.95 Å resolution. *J. Mol. Biol.* **221**: 941–959.
- Stein PE, Leslie AG, Finch JT, Turnell WG, McLaughlin PJ, Carrell RW (1990) Crystal structure of ovalbumin as a model for the reactive centre of serpins. *Nature* **347**: 99–102.
- Takahashi N, Koseki T, Doi E, Hirose M (1991) Role of an intrachain disulfide bond in the conformation and stability of ovalbumin. *J. Biochem.* **109**: 846–851.
- Takahashi N, Orita T, Hirose M (1995) Production of chicken ovalbumin in *Escherichia coli*. *Gene* **161**: 211–216.
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**: 4350–4354.
- Urisu A, Ando H, Morita Y, Wada E, Yasaki T, Yamada K, Komada K, Torii S, Goto M, Wakamatsu T (1997) Allergenic activity of heated and ovomucoid-depleted egg white. *J. Allerg. Clin. Immunol.* **100**: 171–176.
- Yang JT, Wu CSC, Martinez HM (1986) Calculation of protein conformation from circular dichroism. *Meth. Enzymol.* **130**: 208–268.