

## 論文内容の要旨

Japanese cedar (*Cryptomeria japonica*; CJ) pollinosis is one of the most common IgE-mediated type I allergies in Japan, causing allergic rhinitis, conjunctivitis and asthma as clinical symptoms; it has been considered as a national affliction. Approximately 27% of Japanese population is afflicted with this disease from February to April each year. Two major allergenic proteins of CJ pollen, Cry j 1 and Cry j 2, have been isolated, characterized and basic immunologic studies have exhibited their potency as immunotherapeutic agents. IgE specific to Cry j 1 was detected in up to 95% of patients suffering from CJ pollinosis while Cr j 2 was about 70%. The study suggested that both Cry j 1 and Cry j 2 are believed to be important in the pathogenesis of CJ pollinosis.

One of promising approaches to the administration of allergy is desensitization by vaccination with peptides which are derived from allergens. To avoid allergic reactions due to the presence of allergen-specific IgE binding sites in the whole antigen, the novel antigen lacking the epitopes reactive to IgE should be utilized. Peptides only containing T-cell epitopes that function to induce T-cell tolerance is a safe treatment strategy for controlling allergies. Whereas, a major difficulty with this approach is the diversity of MHC class II molecules in individuals, resulting in patients with different MHC class II molecules responding to different allergen-derived peptides. Therefore, as many T cell epitopes of allergens as possible should be included to achieve sufficient efficacy in a large population of allergic patients.

So far recombinant peptides, in which multiple T-cell epitopes from Japanese cedar allergenic proteins were linked, have been developed, and basic immunologic studies have exhibited their potency as immunotherapeutic agents. Recently, several studies have reported the generation of recombinant allergens including T-cell epitopes from Japanese cedar pollen such as oral administration of transgenic rice seed containing destructed Cry j 1 and Cry j 2 and production of a major Japanese cedar pollen allergen Cry j 1 in *Lactobacillus plantarum*, and their prophylactic effect *in vivo*. However, not many T-cell epitopes of Cry j 1 and Cry j 2 were included in the developed immunotherapeutic peptides. In addition, GM (genetically modified) crops have not been approved in Japan; the production of recombinant allergens in transgenic rice seed must be grown separately from regular rice to prevent accidental release of the transgene into the environment and possible contamination of the food chain. Moreover, plant production is dependent on seasonal variation and unable continuous supply of product. In this study, much more epitopes, i.e. five major T-cell epitopes from Cry j 1 and seven from Cry j 2 were selected. The selection bases on considerations that epitopes is able to stimulate strong proliferative response of T-cell line.

Cholera toxin B subunit (CTB) is a highly efficient carrier molecular for the generation of mucosal immune responses and/or induction of systemic T-cell tolerance to linked antigens. CTB binds with high affinity to the ganglioside GM1 which is found in membrane microdomains on the plasma membrane of host cells, and to be able to cluster five GM1 molecules at once. Due to this property of CTB, it can be used as an adjuvant and transporter for the effective delivery of antigens as oral vaccine with reduced toxicity and high efficacy (Lebens *et al.*, 1994). In this study, genes encoding major T-cell epitopes from Cr j 1 and/or Cry j 2 were fused to *ctb* gene in tandem by overlap extension PCR. Then the fusion genes were introduced into pET-28a(+) vector for the expression purpose in *E. coli* system with many advantages such as potentially

very high expression levels, low cost, simple culture conditions, rapid growth and scaleable.

## MATERIAL AND METHODS

### *Bacterial strains, plasmid and media*

Plasmid pET28a(+) and *E. coli* BL21(DE3) strain used for expression of antigen peptides were purchased from Merk Japan (Tokyo, Japan). *E. coli* JM109 strain (Takara Bio, Ohtsu, Japan) was used for DNA manipulation. The *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C with agitation. Ampicillin and kanamycin were used at the final concentration of 100 µg/ml and 50 µg/ml, respectively.

### *Construction of cry j 1 epi and cry j 2 epi using ctb as a scaffold*

The *ctb* gene was amplified from the genomic DNA of *Vibrio cholera* 569B strain (supplied by Osaka University). Fusion genes of *ctb-cry j 1 epi* and *ctb-cry j 2 epi* were acquired by stepwise PCR method: *ctb* gene was used as the template of the first PCR and the resultant PCR product was used as the template of the next reaction.

### *Construction of the fusion gene of BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII*

In order to provide structural flexibility as well as to improve fusion protein stability, a linker with 15 bp in length (a.a. sequence, GGGSG) (Amet *et al.*, 2009; Arai *et al.*, 2001) was inserted between the C-terminal end of *ctb* and the N-terminal end of *cry j 1 epi-cry j 2 epi*. In addition, a FLAG sequence with 24 bp in length (a.a. sequence, DYKDDDDK) (Hopp *et al.*, 1988) was used at the C-terminal end of *cry j 1 epi-cry j 2 epi* as an epitope tag for the detection of target fusion protein. The fusion gene of *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* was constructed by

linking five sequences, including *BamHI-ctb*, *linker*, *cryj1 epi*, *cryj2 epi* and *flag-HindIII* using stepwise PCR and overlap extension PCR method .

#### *Construction of recombinant plasmids for E. coli*

pET28a(+) plasmid was used as expression vector, which provides two 6×His-tag coding sequence at both termini for one-step purification of expressed proteins. The expression vectors were constructed as follows. The *BamHI/HindIII*-digested fragments of the fusion genes *ctb-cry j1 epi*; *ctb-cry j2 epi* and *BamHI-ctb-linker-cry j 1 epi- cry j 2 epi -flag-HindIII* were cloned into corresponding sites of pET28a(+) vector to produce three recombinant plasmids separately pET28a- *ctb-cry j1 epi*; pET28a-*ctb-cry j2 epi* and pET28a-*BamHI-ctb-linker-cry j 1 epi- cry j 2 epi -flag-HindIII*. The expression vectors were constructed in *E. coli* JM109 strain and then transformed into *E. coli* BL21(DE3) strain for expression purpose.

Transformation of *E. coli* was performed according to the manufacturer's instruction (TaKaRa Code 9052, Novagen Code TB009). Nucleotide sequences and orientation of the fusion genes in the recombinant cloning plasmids were analyzed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

#### *SDS-PAGE and Western blot*

Protein samples were analyzed by SDS-PAGE on a commercial 12.5% acrylamide gel (ePAGEL) (Atto, Tokyo, Japan). Proteins were visualized by the staining solution of Coomassie brilliant blue R-250.

Western blot analysis was performed to test antigenicities of expressed antigen peptides. Proteins insolated on SDS-PAGE were electro-blotted onto Hybond-P PVDF membrane (GE healthcare Japan, Tokyo, Japan) by using semi-dry method. After blocked with 5% skim milk in TBS containing 0.5% Tween-20 for 2h at room

temperature, the membrane was incubated with the primary antibody for overnight at 4°C. Rabbit anti-cholera toxin B subunit antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as the primary antibody for both CTB-Cry j1 epi and CTB-Cry j 2 epi, and rabbit anti-Cry j 1 antibody (Hayashibara) and rabbit anti-Cry j2 antibody (LSL) were used as primary antibodies for CTB-Cry j 1 epi and CTB-Cry j 2 epi, respectively. Then the incubation with the secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit IgG, Amersham) was performed for 1h at room temperature. For the detection of CTB-Linker-Cry j 1 epi-Cry j 2-Flag, Rabbit anti-Flag antibody (Sigma) and Goat anti-cholera toxin B subunit antibody (Sigma) were used as primary antibodies. And, horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) and horseradish peroxidase-conjugated anti-goat IgG (Funakoshi) were used as secondary antibodies, respectively. Signal was detected by ECL detection reagents (Amersham). Commercial CTB (Sigma), commercial Cry j 1 antigen (Hayashibara) or commercial Cry j 2 antigen (Hayashibara) were used as the positive controls.

#### *Expression and purification of antigen peptides*

For optimal production of the fusion proteins, the conditions such as IPTG (Takara) concentration and time course were conducted. A single positive colony of *E. coli* BL21(DE3) transformed by pET28a-*ctb-cry j1 epi*; pET28a-*ctb-cry j2 epi* or pET28a-*BamHI-ctb-linker-cry j 1 epi- cry j 2 epi -flag-HindIII* was grown in LB medium supplemented with kanamycin (50 µg/ml) at 37°C until the OD<sub>600</sub> reached 0.1~1.5, and protein expression was induced at 27°C for 3h by the addition of various concentration of IPTG ranging from 0.1 to 3 mM. After the optimum IPTG concentration was determined, the time course study of 0-20h was carried out to find the best condition for protein induction. Cells were harvested by centrifugation and bacterial pellets were lysed with HisTALON xTractor buffer (Clontech Code 635656) supplemented with

DNaseI and lysozyme. The suspension was mixed with 2-fold SDS-PAGE sample buffer and heated at 95°C for 5 min. Aliquots of the total cellular proteins were analyzed by SDS-PAGE, followed by western blot assay.

The purification of the recombinant proteins (his-tagged proteins) using TALON Spin Columns (Clontech Code 635601) was performed according to the manufacture's instruction. Protein yield was measured with BCA Protein Assay Reagent Kit (Pierce) using bovine serum albumin (BSA) as the standard.

## **RESULTS**

### *Selection of major T-cell epitopes from Cry j 1 and Cry j 2*

On the basis of references (Hirahara *et al.*, 2001; Sone *et al.*, 1998), major T cell epitopes from Cry j 1 and Cry j 2 were selected. Selection based on considerations that epitopes should be able to stimulate strong proliferative response of T-cell line which is Cry j 1 or Cry j 2-specific, and various MHC class II types, the molecules of which are capable of presenting T-cell epitopes, should be included to obtain reliable applicability in a large population of patients.

Major T cell epitopes from Cry j 1 and Cry j 2 were selected based on the features of epitopes, which contains available epitopes from Cry j 1 and Cry j 2 for the design of immunotherapeutic, MHC class II types of human (HLA) presenting the epitopes, the reactivity of Cry j 1 or Cry j 2-specific T-cell line against the epitopes as well as the finally selected epitopes in the study based on the literature (Sone *et al.*, 1998).

All the epitopes with reactivity higher than 50% as well as the strong reactivity higher than 15% were selected. Meanwhile, epitopes with the reactive ratio of 50% or less and undetermined HLA type were eliminated. In addition, Cry j 2-96-107 possesses

very strong reactivity based on another literature (Hirahara *et al.*, 2001); it was chosen instead of Cry j 2-90-104. Because Cry j 2-245-259 is presented by DR15 that was the sole type among the selected HLA class II molecules, it was used as an epitope in order to achieve sufficient efficacy in a large population of allergic patients in spite of the reactivity of 50%. Cry j 2-345-374 is an important region for the design of the antigen peptide; therefore it was also selected in the study.

#### *Expression and antigenicities of CTB-Cry j 1 epi and CTB-Cry j 2 epi*

To assess antigenicities of antigen peptides, western blot was performed. The purified recombinant antigen peptides were proved antigenic against corresponding antibodies, i.e. CTB-Cry j 1 epi was recognized by anti-cholera toxin and anti-Cry j 1 antibodies; and CTB-Cry j 2 epi was recognized by anti-cholera toxin and anti-Cry j 2 antibodies. The result suggested that the recombinant antigen peptides of CTB-Cry j 1 epi and CTB-Cry j 2 epi were successfully expressed in *E.coli* BL21(DE3).

#### *Construction of the fusion gene of BamHI-ctb-linker-cry j 1 epi- cry j 2 epi -flag-HindIII*

Since the antigenicity of peptide antigens expressed from *cry j 1 epi* and *cry j 2 epi* genes were confirmed, we intended to construct the recombinant gene containing both the *cry j 1* and *cry j 2*. Fusion gene, *BamHI-ctb-linker-cry j 1 epi- cry j 2 epi -flag-HindIII*, was constructed by overlap extension PCR as described above. Sizes of resultant PCR products visualized on the agarose gel are in good agreement with the calculated ones, suggesting that the fusion gene were successfully constructed. The final product was cloned into pET28a(+) plasmid for protein expression. Results of sequencing analysis of the inserted DNA showed that the sequence was identical to the designed one and had correct orientation in the expression plasmid.

#### *Expression and immunoblot analysis of CTB-linker-Cry j 1 epi-Cry j 2 epi-Flag*

### *Purification of the fusion antigen peptide*

SDS-PAGE analysis was performed to analyze the expressed fusion protein in *E. coli* BL21(DE3). A single band with high homogeneity was obtained from eluted fractions on His-tag affinity column. Molecular mass of the fusion protein visualized on SDS-PAGE was in good agreement with the theoretical one (34.65 kDa).

To achieve the optimal expression of the fusion protein, the conditions such as the induction time and different IPTG concentration were tested. The selected clone was grown in LB broth with kanamycin at 37°C, and protein expression was induced at 27°C with various concentration of IPTG. Changes in growth and the fusion protein expression were monitored during incubation. The expression level of the fusion protein was not dependent on IPTG concentration ranging from 0.1-3 mM. The time-course of the fusion protein production was also examined. As the result show that, during the initial 3h, the fusion protein increased markedly and the expression level reached maximum followed by a gradual decrease. The estimated expression level of more than 120 mg/L was achieved with the addition of 1 mM IPTG to the culture that had been incubated for 4h at 37°C (at OD<sub>600</sub> ~ 1).

### *Immunoblot analysis of the fusion antigen peptide*

Western blot was performed to confirm expression of CTB-linker-Cry j 1 epi-Cry j 2 epi-Flag. The purified recombinant antigen peptide was recognized by anti-Flag and anti-CTB antibodies. The result suggested that the recombinant antigen peptide of CTB-Linker-Cry j 1 epi -Cry j 2 epi-Flag was successfully expressed in *E.coli* BL21(DE3).



## DISCUSSION

The production of edible vaccines by plants is a significant innovation since plant-derived vaccines offer the advantages of low cost and easy control of production scale. However, the long growth period and the possibility of gene diffusion to surrounding plants by pollination limit the application of antigen-producing transgenic plants. Therefore, expression of vaccines using *E. coli* system is one of the most attractive alternatives because its genetics and physiology are well understood and quantities up to 50% of the total cell protein can be produced.

Japanese cedar pollinosis is one of the major allergic diseases in Japan (Kaneko *et al.*, 2005). Allergen-specific immunotherapy is safer and more effective than conventional immunotherapy for the treatment of IgE-mediated allergic diseases. Peptide vaccines using T-cell epitopes would be an effective and safe immunotherapy for allergic diseases, since recombinant antigen peptides can be designed by selecting T-cell epitopes which lack IgE-binding activity.

In this study, up to five major T cell epitopes from Cry j 1 and seven from Cry j 2 were selected on the basis of references in the study. To utilize the ability of CTB as an adjuvant and carrier, genetically engineered fusion peptide, CTB-Linker-Cry j 1 epi-Cry j 2 epi-Flag was designed. Genes encoding epitopes from Cry j 1 and Cry j 2 were fused to CTB gene in tandem by PCR. Results of sequencing analysis showed that the sequence was identical to the designed one and had correct orientation in expression plasmid. The fusion gene was expressed in *E. coli* system. The expressed CTB-Linker-Cry j 1 epi-Cry j 2 epi-Flag was purified to a high homogeneity with Hig-tag affinity column, giving approximately 120 mg/L of the recombinant protein in the culture. Reactivities to anti-CTB and anti-FLAG antibodies were confirmed by western blot analysis. The present study indicated that the production of sufficient

amount of recombinant proteins for immunotherapy may become possible by the recombinant techniques using *E. coli* and other bacterial strains for protein expression.