Expression of recombinant T-cell epitopes of major Japanese cedar pollen allergens fused with cholera toxin B subunit in bacterial strains

HOANG VAN VINH

A dissertation submitted to

Kochi University of Technology

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

The Graduate School of Engineering

Kochi University of Technology

Kochi, Japan

September, 2014

Expression of recombinant T-cell epitopes of major Japanese cedar pollen allergens fused with cholera toxin B subunit in bacterial strains HOANG VAN VINH

TABLE OF CONTENTS

ABSTRACT

Peptides containing T-cell epitopes from allergens, which are not reactive to allergen-specific IgE, are appropriate candidates as antigens for specific immunotherapy against allergies. To develop a vaccine that can be used in practical application to prevent and treat Japanese cedar pollen allergy, four major T-cell epitopes from the Cry j 1 antigen and six from the Cry j 2 antigen were selected to design *cry j 1 epi* and *cry j 2 epi*, DNA constructs encoding artificial polypeptides of the selected epitopes. To apply cholera toxin B subunit (CTB) as an adjuvant and carrier for the effective delivery of antigen peptide, *cry j 1 epi* and *cry j 2 epi* were linked and then fused to the CTB gene in tandem by overlap extension PCR. Then, the fusion gene was expressed in two expression systems using *Escherichia coli* strain BL21(DE3) and *Brevibacillus choshinensis*, respectively.

PART-I

Expression of recombinant T-cell epitopes of major Japanese cedar pollen allergens fused with cholera toxin B subunit in *Escherichia coli*

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*, *cry j 1 epi*, *cry j 2 epi*, and *flag-HindIII*, using stepwise and overlap extension PCR methods. Then, the fusion gene was introduced into a pET-28a(+) vector for expression in an *Escherichia coli* system that exhibits various advantages such as potentially high expression levels, low cost, simple culture conditions, rapid growth, and scalability. In addition, it is known that *E. coli* expression system exhibited almost no proteolytic activity. Thus, the structural stability of expressed recombinant protein is guaranteed. The aim of this study using *E. coli* as host was to investigate expression of the recombinant antigen peptide in bacterial expression system as well as to examine property and antigenicity of the recombinant

antigen peptide.

Expression of the recombinant fusion antigen peptide induced with 1 mM IPTG continued for 3-5 h, and then the concentration of the expressed protein decreased gradually. Recovery of the recombinant protein was approximately 120 mg/L of culture. The expressed recombinant protein was purified by a His-tag affinity column and confirmed by western blot analysis using anti-CTB and anti-FLAG antibodies. The purified recombinant antigen peptide also proved antigenic against anti-Cry j 1 and anti-Cry j 2 antibodies. Thus it was shown that the whole recombinant protein was expressed and existed stably in *E. coli* expression system.

The present study indicates that production of sufficient amounts of recombinant protein for immunotherapy may be possible by recombinant techniques using *E. coli* or other bacterial strains for protein expression.

PART-II

Expression of recombinant T-cell epitopes of major Japanese cedar pollen allergens fused with cholera toxin B subunit in *Brevibacillus choshinensis*

E. coli expression system is the most commonly used organism for heterologous protein production. However, there is a disadvantage for therapeutic use of expressed recombinant protein in *E. coli* due to the presence of endotoxin, lipopolysaccharide (LPS) which locates in the outer membrane of Gram-negative bacteria. Endotoxin that is well-known as a pyrogen causes fever, shock and other various symptoms in humans and animals. Therefore, recombinant proteins produced in *E. coli* must be purified until they become endotoxin-free.

In contrast to *E. coli*, the expression of recombinant antigen peptide in *Brevibacillus choshinensis (Bacillus brevis)* seems to be an interesting alternative system. Gram-positive bacterium *B. choshinensis* is a well-established host-vector system for the production of foreign proteins, especially secretory proteins. Thus, the expressed recombinant protein can be easy

purified from culture supernatant. Moreover, *B. choshinensis* produces little or no detectable extracellular proteases. Therefore, the secreted and produced recombinant protein is not degraded by the protease activity and the yield of the recombinant protein can be maintained stably. These unique characteristics of *B. choshinensis* make it one of the most promising hosts for the production of recombinant proteins.

In this study, *B. choshinensis* and pNC-HisT were used as a host for overproduction and as an expression-secretion vector, respectively. Fusion antigen gene of *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* was constructed by stepwise PCR and overlap extension PCR methods to investigate the expression of the fusion antigen peptide CTB-Linker-Cry j 1 epi-Cry j 2 epi-Flag. The recombinant *B. choshinensis* was grown under various culture conditions. However, expression of the recombinant fusion antigen peptide was not detected by SDS-PAGE analysis.

Therefore, to utilize the ability of secretion of *Bacillus licheniformis* α-amylase (BLA) in *B. choshinensis*, the fusion antigen was linked to BLA to design a genetically engineered fusion antigen gene *BamHI-BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII.* Then, the fusion antigen genes were introduced into pNC-HisT vectors and expressed in *B. choshinensis.* Unexpectedly, only BLA was secreted to the culture medium, suggesting that BLA was cleaved from the recombinant fusion protein by proteolytic activity of unidentified protease(s). When the recombinant *B. choshinensis* was cultured in the presence of protease inhibitors, whole recombinant protein and degradated antigen peptide were detected in the bacterial cells by western blot with anti-CTB and anti-FLAG antibodies.

These results indicate that the recombinant protein could not exist stably in intracellular space of *B. choshinensis* due to cellular proteolytic activity. In addition, it is suggested that unique proteolytic cleavage between BLA and CTB might occur during the secretion process of the recombinant protein.

CHAPTER I

General Introduction

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. Approximately 27% of the Japanese population is afflicted by this disease from February to April each year [1,2]. Two major allergenic proteins have been isolated and characterized from CJ pollen, Cry j 1 [3,4] and Cry j 2 [5-7]. In a clinical study, IgE antibodies specific to Cry j 1 and Cry j 2 in sera were detected in 134 of 145 (92%) patients suffering from CJ pollinosis, while the remainder contained IgE reactive to one of the two major antigens [8]. This study suggested that both Cry j 1 and Cry j 2 play important roles in the pathogenesis of CJ pollinosis.

A promising approach to prevent and treat allergies is desensitization by vaccination with peptides derived from allergens. To avoid allergic reactions due to the presence of allergen-specific IgE-binding sites in the whole antigen, novel antigens have been developed that lack epitopes reactive to IgE. The application of peptides only containing T-cell epitopes that induce T-cell tolerance is a safe treatment strategy to control allergies [9,10]. However, a major obstacle with this approach is the diversity of MHC class II molecules among individuals, leading to patients with different MHC class II molecules responding to unique allergen-derived peptides [11]. Therefore, as many T-cell epitopes of allergens as possible should be included to achieve sufficient efficacy in a larger population of sensitized patients [12]. T-cell epitopes in Cry j 1 and Cry j 2 were determined by epitope mapping using synthetic peptides covering their amino acid sequences, followed by proliferation assays using these synthetic peptides and peripheral blood mononuclear cells (PBMCs) from CJ pollinosis sufferers [13-15].

Thus far, recombinant peptides consisting of multiple linked T-cell epitopes from Japanese cedar allergenic proteins have been developed, and basic immunological studies have revealed

their potential as immunotherapeutic agents [12,15,16]. Several recent studies have described transgenic organisms that express recombinant allergens including T-cell epitopes from Japanese cedar pollen. Takaiwa and colleagues reported transgenic rice seeds containing T-cell epitopes in Cry j 1 and Cry j 2, which were designed as an oral (edible) vaccine [17-19]. Using a different approach, egg white containing T-cell epitopes has been produced by transgenic chickens [20]. Another report has described *Lactobacillus plantarum* producing Cry j 1 and its prophylactic effect *in vivo* [21]. However, few T-cell epitopes of Cry j 1 and Cry j 2 have been included in previously developed immunotherapeutic peptides. In this study, we selected four major T-cell epitopes from Cry j 1 and six from Cry j 2 based on the ability of the epitopes to stimulate a strong proliferative response in T-cell lines [12,15].

Cholera toxin (CT) is secreted by the gram-negative bacterium *Vibrio cholera.* CT is an oligomeric protein with molecular weight of 84 kDa containing a single A subunit (CTA), and five B subunits (CTBs). CTA with molecular weight of 27 kDa is composed of the toxic domain CTA_1 and a short sequence CTA_2 which are generated by proteolytic cleavage of CTA between residues 192 and 195. These two peptide chains are still connected by a disulfide bond between residues 187 and 199. Before CTA_1 enter the cytosol of host cells, the disulfide bond must be cleaved. CTB is a peptide of 11.5 kDa and forms a stable pentamer through non-covalent interaction. The CTB pentamer binds ganglioside GM_1 on the plasma membranes [22]. Then the CTB-GM_1 complex carries the CTA_1 into the endoplasmic reticulum [23]. The three-dimensional structure of CT is shown in Fig. 1.1

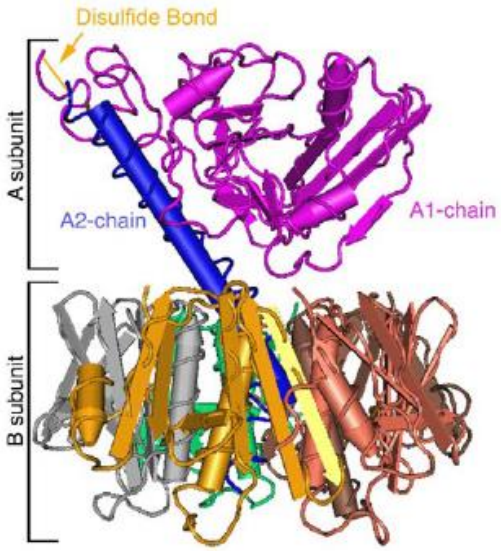

Figure 1.1. Three-dimensional structure of CT [24]

CTB has been used as an efficient carrier molecule to generate mucosal immune responses and induce T-cell tolerance to antigens linked to CTB [25-27]. CTB binds with high affinity to the ganglioside GM_1 that is found in membrane microdomains on the plasma membrane of host cells, and is able to cluster five GM_1 molecules at once [28]. Owing to this property of CTB, it can be employed as an adjuvant and transporter for effective delivery of antigens as a mucosal vaccine with reduced toxicity and high efficacy [27].

CHAPTER II

Expression of recombinant T-cell epitopes of major Japanese cedar pollen allergens fused with cholera toxin B subunit in *Escherichia coli*

2.1. Introduction

Production of edible vaccines by plants is a significant innovation because plant-derived vaccines offer advantages such as low cost and easy control of production scale [17,29,30]. However, a 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* is one of the most attractive alternatives because its genetics and physiology are well understood and recombinant proteins can be produced at up to 50% of the total cellular protein [31].

CJ pollinosis is one of the major allergic diseases in Japan [1,2]. Allergen-specific immunotherapy is safer and more effective than conventional immunotherapy for the treatment of IgE-mediated allergic diseases [32,33]. Peptide vaccines using T-cell epitopes would be an effective and safe immunotherapy for allergic diseases, because recombinant antigen peptides can be designed by selecting T-cell epitopes that lack IgE-binding activity [9,10].

In the previous study, genes encoding T-cell epitopes from major Japanese cedar pollen allergens, Cry j 1 and Cry j 2, were assembled to generate fusion genes, *cry j 1 epi* and *cry j 2 epi*, respectively. These fusion genes of T-cell epitopes were fused to CTB gene by stepwise PCR to construct two fusion genes in separate, *ctb-cry j 1 epi* and *ctb-cry j 2 epi*. Antigenicities of the recombinant T-cell epitope fusion peptides, CTB-Cry j 1 epi and CTB-Cry j 2 epi, expressed in *E. coli* system were confirmed by western blotting analysis [34].

In this study, in order to achieve sufficient efficacy in a larger population of sensitized patients, *cry j 1 epi* encoding four major T-cell epitopes from Cry j 1 and *cry j 2 epi* encoding six epitopes from Cry j 2 were fused to *ctb* in tandem by PCR. Then, the fusion gene was expressed in *Escherichia coli* strain BL21(DE3) to produce the recombinant protein of CTB-Linker-Cry j 1 epi-Cry j 2 epi-FLAG.

2.2. Materials and Methods

2.2.1. Bacterial strains, plasmid, and reagents

The pET28a(+) plasmid and *E. coli* strain BL21(DE3) used for expression of antigen peptides were purchased from Merck Japan (Tokyo, Japan). *E. coli* strain JM109 (Takara Bio, Otsu, Japan) was used for DNA manipulation. Restriction enzymes, a DNA ligation kit (Mighty mix), and IPTG were purchased from Takara Bio. HisTALON xTractor buffer and TALON columns were purchased from Clontech Laboratories (Mountain View, CA, USA). PCR primers were supplied by Life Technologies Japan (Tokyo, Japan).

2.2.2. Construction of cry j 1 epi and cry j 2 epi using ctb as a scaffold

The *ctb* gene amplified from the genomic DNA of *Vibrio cholera* strain 569B was supplied by Professor Takeshi Honda (Osaka University). Fusion genes of *ctb* and four T-cell epitopes from Cry j 1 (*ctb-cry j 1 epi*) and six T-cell epitopes from Cry j 2 (*ctb-cry j 2 epi*) were acquired by stepwise PCR, in which *ctb* was used as a template for the first PCR and the resultant PCR product was used as a template for the next reaction. The reactions were carried out with the forward primer ctb-F (5'-ACACCTCAAAATATTACTGATTTGT-3') and a reverse primer from P1-P5 to construct *ctb-cry j 1* or P'1-P'7 to construct *ctb-cry j 2*. Each reverse primer possessed an overlapping sequence homologous to the template and an overhang of the epitope gene, which was based on the codon usage in *E. coli* (http://www.kazusa.or.jp/codon, July 28 2014). The sequences and reaction order of the reverse primers are shown in Table 2.1. DNA constructs *cry j 1 epi* and *cry j 2 epi* amplified by PCR were employed to construct the fusion gene. All PCR were performed with fidelity KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). PCR products were purified using a PCR purification kit or gel extraction kit (Qiagen K.K., Tokyo, Japan).

Table 2.1. Reverse primers used to construct the fusion gene *ctb-cry j 1 epi* and *ctb-cry j 2 epi*

Name	Position in Cry j antigen	Sequence* (5'-3')
P1	Cry j 1-16-30	CGATCCAAAGCCCACCGCGCAATCCGCCAGTTTCATACGG TTCTG*ATTTGCCATACTAATTGCGG*
P2	Cry j 1-81-95	ACGGCCGTCGAACGTTTTATAGCCCGCAATATACATCGGCA TTTT*CGATCCAAAGCCCAC*
P3	Cry j 1-106-125-1	GTTGCTCACACGTTTAATGAACACGCACGG*ACGGCCGTCG AACGTTTTAT*
P4	Cry j 1-106-125-2	GCCATACAGATGCAGGCCATGAATAATCAC*GTTGCTCACAC GTTTAATGA*
P5	Cry j 1-212-224	CGGGCCGAACTGGTTGAACGCCACCGTCACTTTCATGCT*G CCATACAGATGCAGGCCAT*
P'1	Cry j 2-68-80	TTTCCAGCTCGCCGGGTTCTGATACGCCGCAATAATGCC*AT TTGCCATACTAATTGCGG*
P'2	Cry j2-87-98	GCCCATCAGCGTGAAGCCCGTCAGTTTCGCGAACTG*TTTCC AGCTCGCCGGGTTCT*
P'3	Cry j 2-182-200-1	ATGGAAGTTTTTGCTCGCGAAAATATCAAT*GCCCATCAGCG TGAAGCCCG*
P'4	Cry j 2-182-200-2	GCCCGTGCCAATCGTGTTTTTCTGCAG*ATGGAAGTTTTTGCT CGCGA*
P'5	Cry j 2-236-250	GAATTTCGCGCCGTTCACATGCACATAGCTCACTTCCGCAC GGCT*GCCCGTGCCAATCGT*
P'6	Cry j 2-336-365-1	GCTCGTCAGTTTCAGGCTAATATCGCTCAGTTTAATATCTTT GCA*GAATTTCGCGCCGTT*
P'7	Cry j 2-336-365-2	GAAATAGCCGTTCGCGTTATCGTTCAGGCAGCTCGCAATTT TGCC*GCTCGTCAGTTTCAG*

*Sequences in red and italics are complementary to the corresponding template.

2.2.3. Construction of fusion gene BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII

To provide structural flexibility and improve fusion protein stability, a linker of 15 bp in length (aa sequence: GGGSG) [35,36] 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 of 24 bp in length (aa sequence: DYKDDDDK) [37] was inserted at the C-terminal end of *cry j 1 epi-cry j 2 epi* as an epitope tag for detection of the target fusion protein. The fusion gene *BamHI-ctb-linker-cry j 1*

epi-cry j 2 epi-flag-HindIII (Fig. 2.1) was constructed by linking five sequences, including *BamHI-ctb*, *linker*, *cry j 1 epi*, *cry j 2 epi*, and *flag-HindIII*, using stepwise and overlap extension PCR methods [38] as follows:

Step #1. Construction of BamHI-ctb-linker-(overlap cry j 1 epi)

The *ctb* gene was used as a template for the first PCR. The sequence of the *linker* was linked to *ctb* by PCR using the forward primer *Bam*HI-ctb-F (5'-GCG**GGATCC***ACACCTCAAAATATTACTGAT*-3', *Bam*HI site is indicated in bold and the sequence in italics corresponds to the beginning of the *ctb* gene) and reverse primer linker-ctb-R (5'-**TCCGCTACCTCCGCC***ATTTGCCATACTAATTGCGG*-3', the sequence indicated in bold corresponds to the linker and the sequence in italics is complementary to the *ctb* gene) (Fig. 2.2A). Then, using the *BamHI-ctb-linke*r fusion gene as the template, PCR was performed to link the 5' end sequence of *cry j 1 epi* (*overlap cry j 1 epi*) to the 3' end of the *BamHI-ctb-linker* fusion gene using the forward primer *Bam*HI-ctb-F and reverse primer (overlap cry j 1 epi)-linker-ctb-R (5'-**GCCAGTTTCATACGGTTCTG***TCCGCTACCTCCGCCATTTG*-3', the sequence indicated in bold overlaps with the first 20 bp of the 5' end sequence of the *cry j 1 epi* gene, and the sequence in italics is complementary to the linker and *ctb* gene) (Fig. 2.2A).

Step #2. Construction of cry j 1 epi-(overlap cry j 2 epi)

The *cry j 1 epi* gene was used as a template for the first PCR. The 5' end sequence of *cry j 2 epi* (*overlap cry j 2 epi*) was linked to the 3' end of the *cry j 1* gene by PCR using the forward primer cry j1 epi-F (5'-CAGAACCGTATGAAACTGGCGGATT-3') and reverse primer (overlap cry j 2 epi)-cry j 1 epi-R (5'-**CAATAATGCC***CGGGCCGAACTGGTTGAACG*-3', the sequence indicated in bold overlaps with the first 10 bp of the 5' end sequence of the *cry j 2 epi* gene, and the sequence in italics is complementary to the *cry j 1 epi* gene) (Fig. 2.2B).

Step #3. Construction of (overlap cry j 1 epi)-cry j 2 epi-flag-HindIII

The *cry j 2 epi* gene was used as a template for the first PCR. The sequence of (*overlap cry j*

1 epi) was linked to the 5’ end of the *cry j 2 epi* gene, and the sequence of *flag-HindIII* was linked to the 3’ end of the *cry j 2* gene by stepwise PCR using the forward primer (overlap cry j 1 epi)-cry j 2 epi-F (5’-**GTTCGGCCCG***GGCATTATTGCGGCGTATCA*-3’, the sequence indicated in bold overlaps with the last 10 bp of the 3’ end sequence of the *cry j 1 epi* gene, and the sequence in italics corresponds to the *cry j 2 epi* gene), and reverse primer flag 1-cry j 2 epi-R (5’-**CGTCGTCTTTGTAGTC***GAAATAGCCGTTCGCGTTA*-3’, the sequence indicated in bold corresponds to the first 16 bp of the 5’ end sequence of the *flag*, and the sequence in italics is complementary to the *cry j 2 epi* gene) (Fig. 2C). Then, the first PCR product was used as the template to construct (*overlap cry j 1 epi*)-*cry j 2 epi-flag-HindIII* using the forward primer (overlap cry j 1 eip)-cry j 2 eip-F and reverse primer *Hind*III-flag 2-flag 1-R (5'-GCG**AAGCTT**CTTGTCAT*CGTCGTCTTTGTAGTC*-3', *Hind*III site is indicated in bold, the underlined sequence corresponds to the last 8 bp of the 3’ end of the *flag*, and the sequence in italics is complementary to the template) (Fig. 2.2C).

Step #4. Linking of the three constructs by overlap extension PCR

The fusion gene *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* was constructed by overlap extension PCR using the purified PCR products of steps #1, #2, and #3 as DNA templates. First, the two PCR products of steps #1 (Fig. 2A) and #2 (Fig. 2B) were mixed and subjected to an overlapping extension reaction for 10 cycles without the addition of flanking primers to generate *BamHI-ctb-linker-cry j 1 epi*-(*overlap cry j 2 epi*). Then, the forward primer *Bam*HI-ctb-F and reverse primer (overlap cry j 2 epi)-cry j 1 epi-R were added to amplify the PCR product for 25 cycles. Second, the purified PCR product of *BamHI-ctb-linker-cry j 1 epi*-(*overlap cry j 2 epi*) and PCR product of step #3 (Fig. 2C) were mixed and an overlapping extension reaction was carried out in the same manner to generate *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII*. The final fusion gene was amplified by adding the forward primer *Bam*HI-ctb-F and reverse primer *Hind*III-flag 2-flag 1-R. The nucleotide sequence of the final construct DNA has been deposited in the DDBJ (accession no. AB978369).

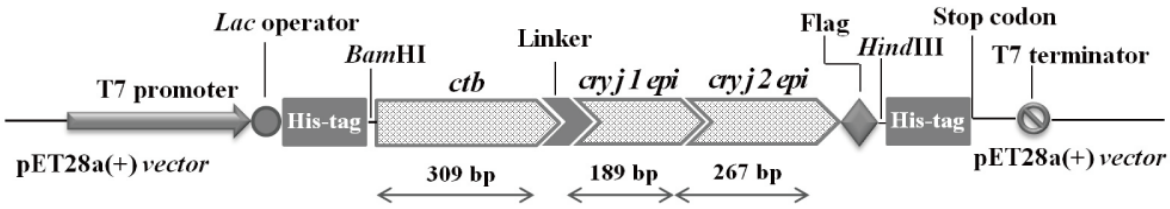

Fig. 2.1. Schematic diagram of the fusion gene in the pET28a(+) vector

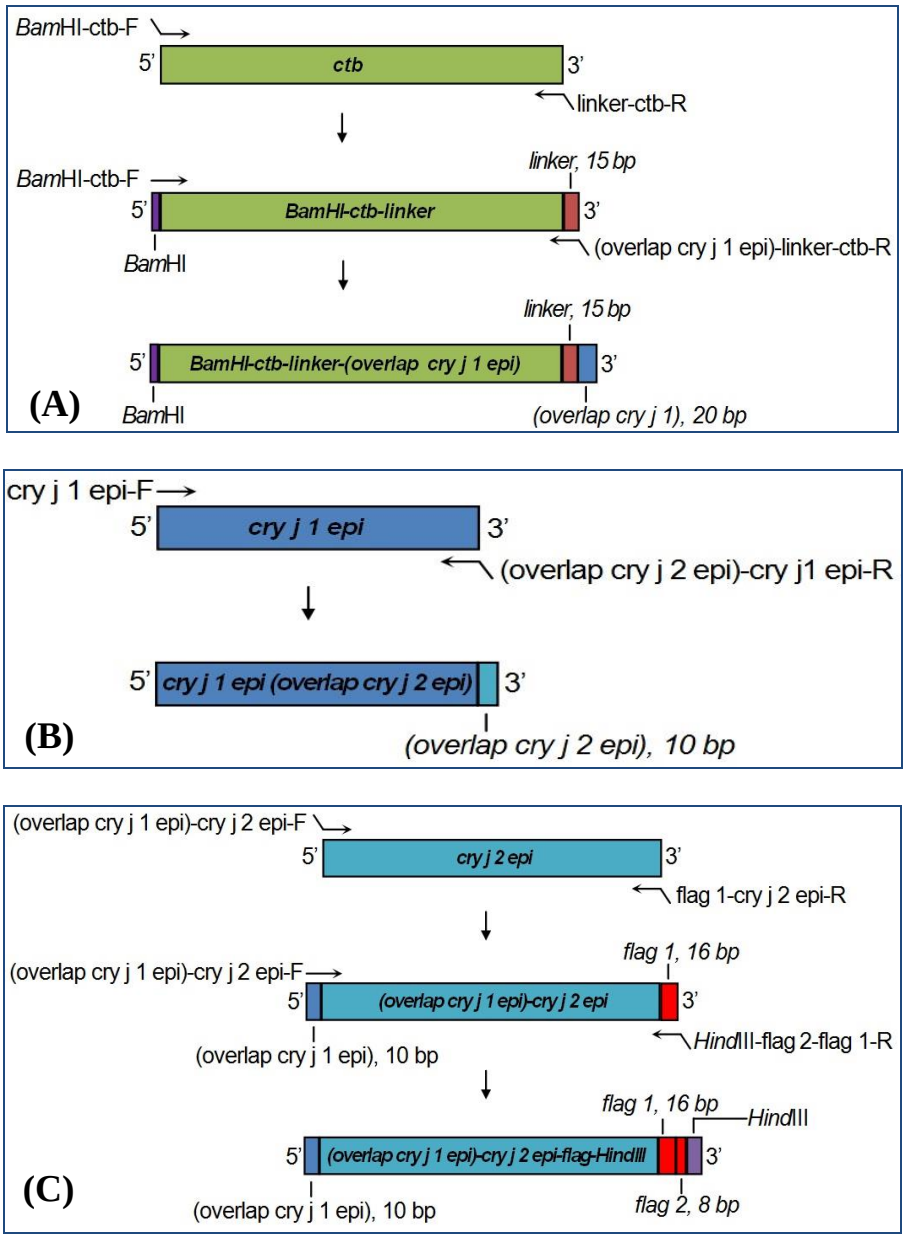

Fig. 2.2. Construction of DNA templates for overlap extension PCR

Three DNA constructs for overlap extension PCR were prepared as described in the Materials and Methods. **(A)** Construction of *BamHI-ctb-linker-(overlap cry j 1 epi)*. **(B)** Construction of *cry j 1 epi-(overlap cry j 2 epi)*. **(C)** Construction of *(overlap cry j 1 epi)-cry j 2 epi-flag-HindIII.*

2.2.4. Construction of the recombinant plasmid for E. coli

The pET28a(+) plasmid was used as an expression vector, which provides two 6×His-tag coding sequences at both N- and C-termini for one-step purification of expressed proteins. The expression vector was constructed as follows. *Bam*HI/*Hind*III-digested fragments of the fusion gene *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* were cloned into the corresponding sites of the pET28a(+) vector to produce a recombinant plasmid, pET28a-*BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* (Fig. 2.1). The expression vector was constructed in *E. coli* strain JM109 and then transformed into the *E. coli* strain BL21(DE3) for expression.

Transformation of *E. coli* was carried out according to the manufacturers' instructions using *E. coli* competent cells of strains JM109 (Takara Bio) and BL21(DE3) (Merck Japan). DNA sequencing was performed using the dye-terminator method by Bio Matrix Research (Nagareyama, Japan).

2.2.5. SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting

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

Western blot analysis was carried out to test the antigenicities of the expressed peptide. Proteins resolved by SDS-PAGE were electro-blotted onto a Hybond-P PVDF membrane (GE Healthcare Japan, Hino, Japan) using the semi-dry method. After blocking with 5% skim milk powder in Tris-buffered saline (TBS) containing 0.5% Tween-20 for 2 h at room temperature, the membrane was incubated with primary antibodies at 4 °C overnight. Then, the membrane was

incubated with secondary antibodies for 1 h at room temperature. A rabbit anti-Flag antibody (1:5000 dilution) (Sigma-Aldrich Japan, Tokyo, Japan), goat anti-CTB antibody (1:5000 dilution) (List Biological Laboratories, Campbell, CA, USA), rabbit anti-Cry j 1 antibody (1:500 dilution) (Hayashibara, Okayama, Japan) and rabbit anti-Cry j2 antibody (1:5000 dilution) (Hayashibara) were used as primary antibodies. A horseradish peroxidase-conjugated anti-rabbit IgG (1:5000 dilution) (Cell Signaling Technology Japan, Tokyo, Japan) and horseradish peroxidase-conjugated anti-goat IgG (1:5000 dilution) (R & D Systems, Minneapolis, MN, USA) were used as secondary antibodies. Signals were detected by ECL detection reagents (GE Healthcare Japan). Commercial Cry j 1 and Cry j 2 antigens (Hayashibara) were used as the positive controls.

2.2.6. Expression and purification of antigen peptides

For optimal production of the recombinant fusion protein, we examined conditions such as the IPTG concentration and time course of protein production. A single positive colony of *E. coli* BL21(DE3) transformed with pET28a-*BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* was grown in Luria-Bertani (LB) medium containing kanamycin (50 μg/ml) at 37°C until the OD_{600} reached 0.1-1.5. Protein expression was then induced at 27°C for 3 h by addition of various IPTG concentrations ranging from 0.1 to 3 mM. After determination of the optimal IPTG concentration, a time course study for 20 h was performed to find the optimal conditions for protein expression. Cells were harvested by centrifugation and bacterial pellets were lysed with HisTALON xTractor buffer containing DNaseI and lysozyme. The suspension was combined with 2× 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 blotting.

Purification of the recombinant His-tagged protein using TALON Spin Columns was performed according to the manufacturer's instructions. Protein yield was measured by a Pierce BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as a standard.

2.3. Results

2.3.1. Selection of major T-cell epitopes from Cry j 1 and Cry j 2

Based on previous studies [12,15], we selected major T-cell epitopes from Cry j 1 and Cry j 2. Selection based on parameters that epitopes should be able to stimulate strong proliferative responses in T-cell lines, which are Cry j 1 or Cry j 2 specific, and capable of being presented by various MHC class II (HLA class II) types, should be considered to obtain reliable applicability in large populations of patients.

Major T-cell epitopes from Cry j 1 and Cry j 2 were selected based on the features summarized in Table 2.2, including available epitopes from Cry j 1 and Cry j 2, HLA types that present the epitopes, and the reactivity of epitope peptides in PBMC proliferation assays [15].

Epitopes were selected with reactivity higher than 50% and strong reactivity higher than 15%, whereas epitopes were eliminated with a reactive ratio of ≤50% and an undetermined HLA type. In addition, Cry j 2-87-98 possesses very strong reactivity according to a previous study [12], and was therefore chosen instead of Cry j 2-81-95. Because Cry j 2-236-250 is presented by DR15, which was the only HLA type among the selected HLA class II molecules, it was used as an epitope to achieve sufficient efficacy in a large population of allergic patients despite reactivity of 50%. Cry j 2-336-365 is an important region that contains two epitopes with high reactivity, and was therefore selected for the study.

Table 2.2. Selection of T-cell epitopes in Cry j 1 and Cry j 2 based on the literature [15]

Allergen	Epitope position	HLA type	Positive response (%) (Strong positive response)	Selected peptide	Epitopes included**
Cry j 1	16-30	DQ6	61 (28)	Cry j 1-16-30	1
	66-80	ND*	50 (11)		
	81-95	ND*	61 (17)	Cry j 1-81-95	1
	91-105	DQ6	50 (11)		
	106-120	DR51	72 (28)	Cry j 1-106-125	1
	111-125	ND*	56 (28)		
	211-225	DP5	72 (33)	Cry j 1-212-224	1
	301-315	ND*	56 (11)		
	316-330	ND*	50 (22)		
Cry j 2	66-80	DR51	61 (39)	Cry j 2-68-80	1
	81-95	DP5	50 (28)	Cry j 2-87-98	1
	141-155	ND*	56 (0)		
	186-200	DR53	67 (28)	Cry j 2-182-200	1
	236-250	DR15	50 (17)	Cry j 2-236-250	1
	336-350	DP5	50 (22)		
	346-360	DQ6	67 (28)	Cry j 2-336-365	2
	351-365	ND*	61 (22)		

*Not determined.

**Two overlapping epitope peptides were counted as one epitope.

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

The fusion gene *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* (Fig. 2.1) was constructed by overlap extension PCR as described in the Materials and Methods. The sizes of resultant PCR products visualized on the agarose gel were in good agreement with the calculated sizes (Fig. 2.3), suggesting that the fusion gene was constructed successfully. The final product was cloned into pET28a(+) for protein expression. The results of sequencing analysis of the inserted DNA showed that the nucleotide and amino acid sequences were identical to the designed sequences and had the correct orientation in the expression plasmid (Fig. 2.4). The nucleotide sequence has been deposited in the DDBJ under accession no. AB978369.

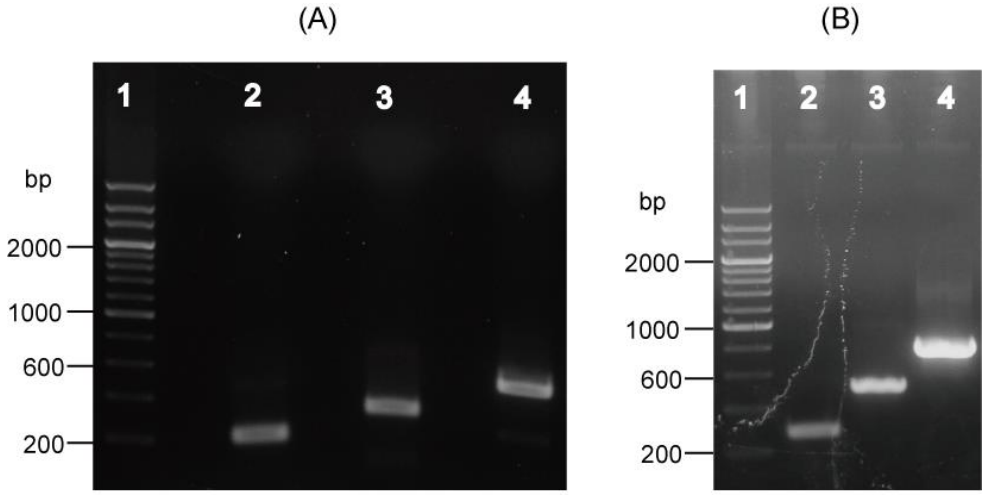

Fig. 2.3. Electrophoretic analysis of products acquired from overlap extension PCR

(A) PCR products in the construction of *BamHI-ctb-linker-cry j 1 epi-(overlap cry j 2 epi)*: Lane 1, 200 bp marker; Lane 2, cry j 1 epi-(overlap cry j 2 epi) fusion gene (199 bp); Lane 3, *BamHI-ctb-linker-(overlap cry j 1 epi)* fusion gene (353 bp); Lane 4, *BamHI-ctb-linker-cry j1 epi-(overlap cry j 2 epi)* fusion gene (532 bp). **(B)** PCR products in the construction of *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII*: Lane 1, 200 bp marker; Lane 2, *(overlap cry j 1 epi)-cry j 2 epi-flag-HindIII* (310 bp); Lane 3, *BamHI-ctb-linker-cry j1 epi-(overlap cry j 2 epi)* fusion gene (532 bp); Lane 4, The final product of *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* fusion gene (822 bp).

TPQNITDLCAEYHNTQIHTLNDKIFSYTESLAGKREMAIITFKNGATFQVEVPGSQHIDSQ
KKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAISMAN**GGGSG***QNRMKLADCAV*
GFGS---KMPMYIAGYKTFDGR---PCVFIKRVSNVIIHGLHLYG---SMKVTVAFNQFGP---GIIAA
YQNPASWK---QFAKLTGFTLMG---IDIFASKNFHLQKNTIGTG---SRAEVSYVHVNGAKF-
--CKDIKLSDISLKLTSGKIASCLNDNANGYF**DYKDDDDK**

Fig. 2.4. Amino acid sequence of CTB-Linker-Cry j 1 epi-Cry j 2 epi-Flag

The amino acid sequences are indicated as follows: CTB (103 aa, underlined); linker (5 aa, bold) Cry j 1 epi (63 aa, italics); Cry j 2 epi (69 aa, no emphasis); Flag (8 aa, bold and underlined). The order of linkage is CTB, linker, Cry j 1-16-30, Cry j 1-81-95, Cry j 1-106-125, Cry j 1-212-224, Cry j 2-68-80, Cry j 2-87-98, Cry j 2-182-200, Cryj 2-236-250, Cry j 2-336-365, and Flag.

2.3.3. Expression and purification of the fusion antigen peptide

To achieve optimal expression of the fusion protein, we tested conditions such as induction time and various IPTG concentrations. The selected clone was cultured in LB medium with kanamycin at 37°C until the OD_{600} reached ~1. Protein expression was then induced at 27°C with various concentrations of IPTG. Bacterial growth and expression of the fusion protein were monitored during incubation.

SDS-PAGE analysis was carried out to analyze the expressed fusion protein in *E. coli* BL21(DE3). A single band with high homogeneity was obtained from eluted fractions on the His-tag affinity column. The molecular mass of the fusion protein visualized on SDS-PAGE was in good agreement with the theoretical mass (34.65 kDa) (Fig. 2.5A).

Figure 2.5B shows that the expression level of the fusion protein did not depend on IPTG concentrations ranging from 0.1-3 mM. The time course of fusion protein production was examined further. As shown in Fig. 2.6, during the initial 3 h, expression of the fusion protein increased markedly and reached a maximum after 3-5 h followed by a gradual decrease. An estimated expression level of approximately 120 mg/L was achieved by addition of 1 mM IPTG to the culture that had been incubated for 4 h at 37°C (at OD_{600} ~1).

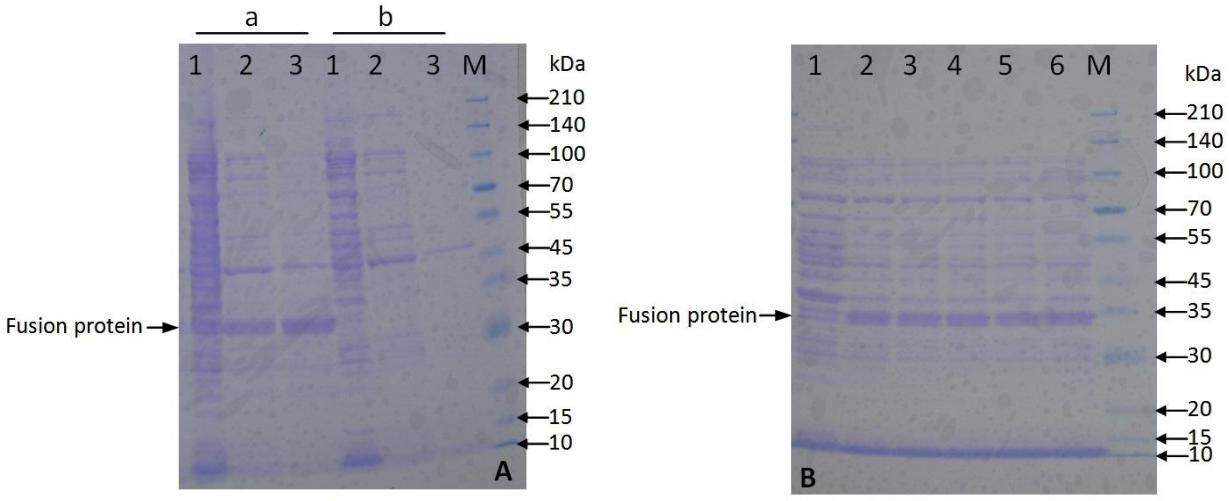

Fig. 2.5. SDS-PAGE analysis and purification of the fusion antigen peptide

(A) Expression and purification of the recombinant antigen peptide: (a) E. coli BL21(DE3) harboring pET28a-BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIIII; (b) Negative control harboring pET28a(+) without the DNA insert. Lane 1, total intracellular protein; Lane 2, eluted fraction I; Lane 3, eluted fraction II; Lane M, molecular weight marker. (B) Effect of IPTG concentrations on induction: Lanes 1–6, total cellular protein at IPTG concentrations of 0, 0.1, 0.5, 1, 2, and 3 mM, respectively; Lane M, molecular weight marker. After IPTG addition, culture continued for 3 h at 27°C. Arrows show the position of the recombinant peptide.

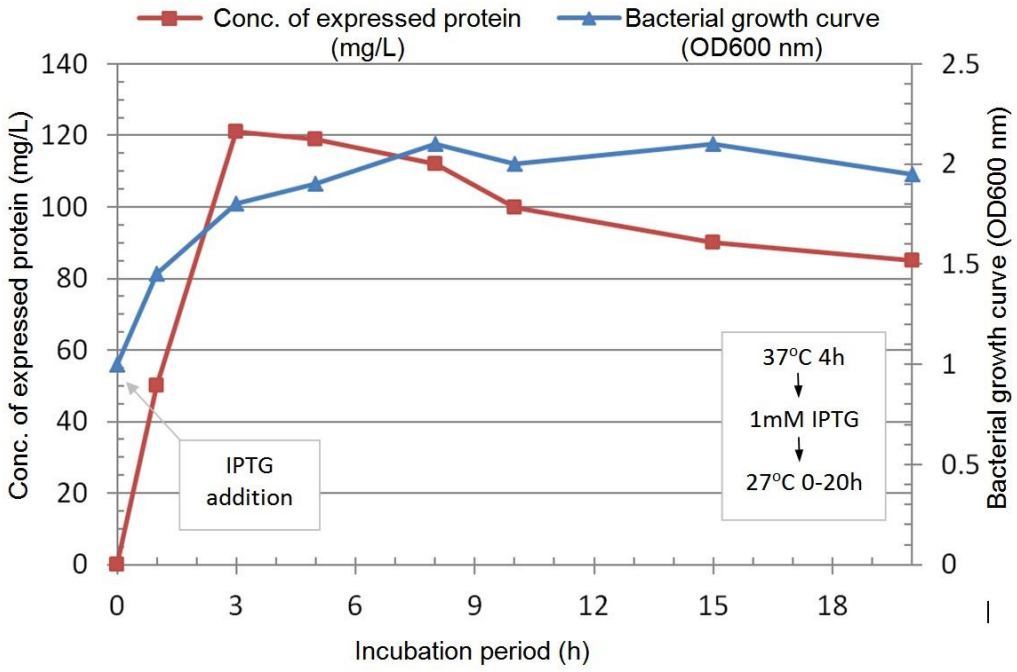

Fig. 2.6. The time-course of the fusion protein production

E. coli BL21(DE3) harboring pET28a-*BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIIII* was inoculated at a ratio of 1:100 (vol:vol) into 60 ml LB medium and incubated at 37°C for about 4 h up to an OD_{600} value of 1. Then, protein expression was induced by addition of 1 mM IPTG followed by culture at 27°C for 20 h. The fusion protein was purified by a His-tag column from samples obtained at the indicated times. The expression level of the fusion protein was monitored by a protein assay.

2.3.4. Western blot analysis of the fusion antigen peptide

Western blotting 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 (Fig. 2.7A) and anti-CTB antibodies (Fig. 2.7B). This result demonstrated that the recombinant antigen peptide Cry j 1 epi-Cry j 2 epi flanked by CTB and Flag was successfully expressed in *E.coli* BL21(DE3).

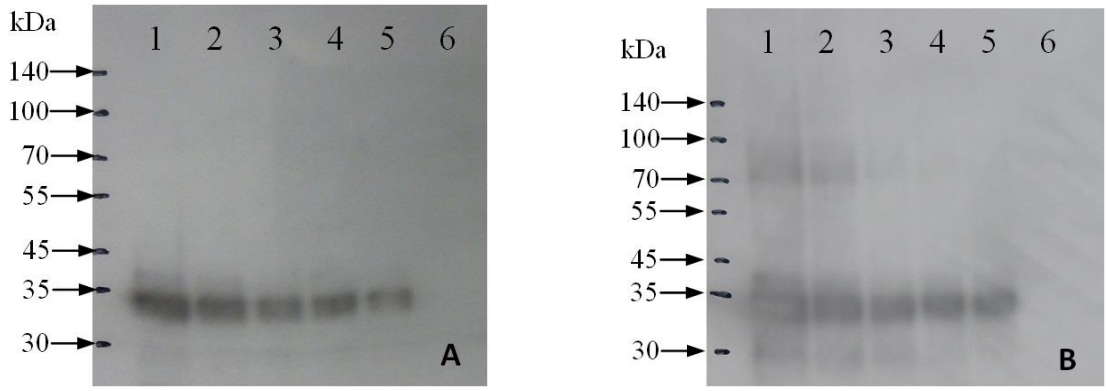

Fig. 2.7. Western blot analysis of the purified fusion antigen peptide

(A) Western blot with an anti-Flag antibody. (B) Western blot with an anti-CTB antibody. Lanes 1-5, purified CTB-Linker-Cry j 1 epi- Cry j 2 epi-Flag (34.65 kDa). Lanes contained 2.5, 1.25, 0.85, 0.63, and 0.5 μg of the purified protein, respectively. Lane 6, purified fraction from *E. coli* harboring pET28a(+) without the DNA insert (negative control).

To examine antigenicity of the antigen peptide, western blot experiments using anti-Cry j 1 and anti-Cry j 2 as primary antibodies were also performed (Fig. 2.8). The result showed that the purified recombinant antigen peptide with molecular weight of 34.65 kDa was reactive against anti-Cry j 1 (Fig. 2.8A) and anti-Cry j 2 (Fig. 2.8B) antibodies. In addition, a false positive band with a molecular weight of ~45 kDa observed in Fig. 2.8A is considered to be a protein from *E. coli* host that could not be removed by the His-tag affinity column purification. As shown in Fig. 2.5A, both the affinity-purified protein fractions from the recombinant *E. coli* and the negative control (*E coli* containing pET28a(+) without insert) contained the ~45 kDa band.

It should be also noted that a false band corresponding to the size of the commercial Cry j 1 antigen (Lane 1, Fig. 2.8B) is a result of cross-reactivity between Cry j 1 antigen and anti-Cry j 2 antibody.

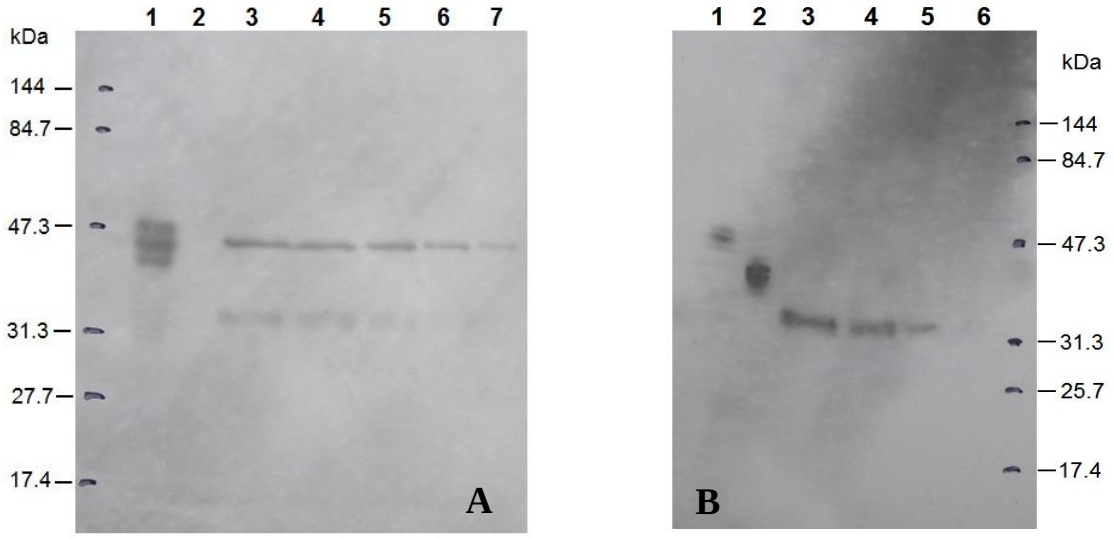

Fig. 2.8. Antigenicities of the purified fusion antigen peptide

(A) Western blot with an anti-Cry j 1 antibody (1:500 dilution). Lanes 1, commercial Cry j 1 antigen (0.075 μg, 50 kDa). Lanes 2, commercial Cry j 2 antigen (0.075 μg, 37 kDa); Lanes 3-7, purified CTB-Linker-Cry j 1 epi- Cry j 2 epi-Flag (34.65 kDa). Lanes contained 2.40, 1.80, 1.20, 0.60 and 0.12 μg of the purified protein, respectively.

(B) Western blot with an anti-Cry j 2 antibody (1:5000 dilution). Lanes 1, commercial Cry j 1 antigen (0.075 μg, 50 kDa). Lanes 2, commercial Cry j 2 antigen (0.075 μg, 37 kDa); Lanes 3-6, purified CTB-Linker-Cry j 1 epi- Cry j 2 epi-Flag (34.65 kDa). Lanes contained 1.44, 0.72, 0.36 and 0.12 μg of the purified protein, respectively.

2.4. Discussion

The current treatments of CJ pollinosis are mostly medication using H_1-antihistamines corticosteroids and other drugs to suppress allergic and inflammatory reactions. However, these symptomatic therapies have no curative effect and the pollinosis returns in the next season. Therefore, allergen vaccination is an alternative to control this pollinosis effectively by decreasing allergy through immunotolerance. However, administration of whole antigens may cause anaphylaxis because the antigens contain epitopes which react with allergen-specific IgE on the cell surface of mast cells. Thus, peptide vaccines using T-cell epitopes which lack IgE-binding activity would be an effective and safe immunotherapy for allergic diseases.

The fusion gene, *ctb-linker-cry j 1 epi- cry j 2 epi-flag* expressed in *E. coli* was purified to high homogeneity by a His-tag affinity column, providing approximately 120 mg/L of the recombinant protein in the culture. Reactivity of the antigen protein to anti-CTB and anti-FLAG antibodies as well as antigenicity against anti-Cry j 1 and anti-Cry j 2 antibodies were confirmed by western blot analysis. Thus it was shown that the whole recombinant protein was expressed and existed stably in *E. coli* expression system.

The result also indicated that anti-Cry j 1 antibody recognized the purified recombinant antigen peptide much more weakly than anti-Cry j 2 antibody. This seems to be low amount of paratopes in the polyclonal anti-Cry j 1 antibody which can recognize epitopes of the recombinant antigen peptide. Actually, the antibody diluted to 1:5000 did not give a positive band while the same antibody diluted to 1:500 revealed a positive band corresponding to the recombinant antigen peptide (34.65 kDa) (Fig. 2.8A). It should be noted that a false band of ~45 kDa which appeared in Fig. 2.8A is considered to be a protein from *E. coli* host. In Fig. 2.5A, both the affinity-purified protein fractions from the recombinant *E. coli* and the negative control (*E coli* containing pET28a(+) without insert) contained the ~45 kDa band.

In addition, we found that the commercial Cry j 1 antigen and anti-Cry j 2 antibody crossreacted, resulting in a false positive band corresponding to the size of the Cry j 1 antigen

(Lane 1, Fig. 2.8B). The result also indicated that the apparent molecular weight of the commercial Cry j 1 antigen visualized on SDS-PAGE was much lower than 50 kDa (Code No. HBL-C-1, Hayashibara, Japan).

The present study indicates that production of sufficient amounts of recombinant proteins for immunotherapy may be possible by recombinant techniques using *E. coli* or other bacterial strains for protein expression. This efficient production system will help us to obtain purified recombinant peptides containing T-cell epitopes, which may be used in oral or sublingual administration for immunotherapy of pollinosis [39,40]. The DNA construct of the T-cell epitope peptide prepared in this study may be employed to generate GRAS (generally recognized as safe) bacterial strains expressing T-cell epitopes for edible vaccines.

CHAPTER III

Expression of recombinant T-cell epitopes of major Japanese cedar pollen allergens fused with cholera toxin B subunit in *Brevibacillus choshinensis*

3.1. Introduction

One of the most popular organisms used for recombinant protein production is the Gram-negative bacterium *E. coli*. Despite its advantages, there are some well-known drawbacks of using *E.coli* for recombinant protein expression, including contamination by pyrogen. *E. coli* contains lipopolysaccharide (LPS) referred to as endotoxin which locates in the outer membrane of *E. coli* and causes fever in humans and animals. In addition, another disadvantage for production of recombinant protein in *E. coli* is an inefficient system for translocation and secretion of expressed proteins, which make it difficult to purify a large amount of expressed proteins by simple purification procedures.

In contrast to *E. coli*, the expression of recombinant antigen peptide in *Brevibacillus choshinensis (Bacillus brevis)* seems to be an interesting alternative system. The Gram-positive bacterium *B. choshinensis* is well-known for its contributions to produce industrially relevant recombinant proteins, enzymes, antigens and cytokines [41,42]. It has been developed as an attractive host because it is non-pathogenic to humans and is considered as a safe microorganism, which is a considerable advantage when the protein is purified for medical purposes. Furthermore, it exhibits a well-established host-vector system for the production of foreign proteins, especially secretory proteins. Thus, the expressed recombinant protein can be easy purified from culture supernatant. Moreover, *B. choshinensis* produces little or no detectable extracellular proteases. Therefore, the secreted and produced recombinant protein is not degraded by the protease activity and the yield of the recombinant protein can be maintained stably. These unique characteristics of *B. choshinensis* make it one of the most promising hosts for the production of recombinant proteins [43,44].

Recently, a great number of research papers have been published on the cloning and expression of α-amylase from *B. licheniformis* (BLA) [45-48]. It was shown that a high-level production of approximately 0.2 mg/ml recombinant BLA was obtained in the culture supernatant when *B. choshinensis* was used as a host bacterium [49-52]. To utilize the effective secretion of BLA, the fusion antigen was linked to BLA to design a genetically engineered fusion antigen peptide, BLA-Linker-CTB-Linker-Cry j 1 epi-Cry j 2 epi-FLAG.

In this study, *B. choshinensis* and pNC-HisT were used as a host for overproduction and as an expression-secretion vector, respectively. Two fusion antigen genes, *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* and *BamHI-BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* were constructed by stepwise PCR and overlap extension PCR methods. Then, the fusion antigen genes were introduced into pNC-HisT vectors and expressed in *B. choshinensis.*

3.2. Materials and Methods

3.2.1. Bacterial strains, plasmid, and reagents

Plasmids and bacterial strains used in the study are listed in Table 3.1.

The pNC-HisT plasmid and *Brevibacillus choshinensis* used for expression of antigen peptides and α-amylase (BLA) were purchased from Takara Bio (Ohtsu, Japan). *E. coli* JM109 (Takara Bio) was used for DNA manipulation. The *E. coli* strain was grown in LB medium at 37 ^{o}C. The *B. choshinensis* was grown in various media including MT, TM and 2SY (Appendix) at 30 ^{o}C. Ampicillin (Sigma-Aldrich Japan, Tokyo, Japan), kanamycin (Wako Pure Chemical Industries, Osaka, Japan) and Neomycin (Wako Pure Chemical Industries) were used at the final concentration of 100 µg/ml, 50 µg/ml and 10 µg/ml, respectively. Restriction enzymes and a DNA ligation kit (Mighty mix) were purchased from Takara Bio. HisTALON xTractor buffer and TALON columns were purchased from Clontech Laboratories (Mountain View, CA, USA). All the PCR primers were supplied by Life Technologies Japan (Tokyo, Japan).

Table 3.1. Plasmids and bacterial strains used in the study

Plasmid	Description	Bacterial strain	Antibiotic resistance*	Purpose
pET28a(+)-*ctb-linker-cry j 1 epi-cry j 2 epi-flag*	Containing *ctb-linker-cry j 1 epi-cry j 2 epi-flag*	*E. coli* JM109	Kan^R	Cloning
pNC-HisT	-	*B. choshinensis*	Nm^R	Negative control for expression
pNC-HisT-*BLA*	Containing *α-amylase (BLA)*	*E. coli* JM109	Amp^R	Cloning
		B. choshinensis	Nm^R	Positive control for expression
pNC-HisT-*ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop -terminator*	Containing *ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop -terminator*	*E. coli* JM109	Amp^R	Cloning
		B. choshinensis	Nm^R	Expression
pNC-HisT-*BLA-linker-ctb -linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator*	Containing *BLA-linker-ctb -linker-cry j 1 epi-cry j 2 epi-flag-stop -terminator*	*E. coli* JM109	Amp^R	Cloning
		B. choshinensis	Nm^R	Expression

*Antibiotics indicated are: Kan - Kanamycin; Nm - Neomycin; Amp - Ampicillin

3.2.2. Construction of fusion genes by PCR amplification

3.2.2.1. Construction of BamHI-BLA-stop-terminator-HindIII

The fusion gene of *BamHI-BLA-stop-terminator-HindIII* (Fig. 3.2) was acquired by PCR amplification using pNY326-BLA plasmid (Code No. HB114, Takara Bio) as the template which has the gene encoding *Bacillus licheniformis α-amylase* (BLA). The forward primer *Bam*HI-BLA-F (5'- GAT**GGATCC***GCAGCAGCGGCGGCAAATCTT*-3', *Bam*HI site is indicated in bold and the sequence in italics corresponds to the beginning of the *BLA* gene) and reverse primer *Hind*III-terminator-R (5'-CCG**AAGCTTC***AAAGAAATTTATAAGACGGG*-3', *Hind*III site

is indicated in bold, and the sequence in italics is complementary to the 3' end of the *terminator*). PCR was accomplished with fidelity KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). PCR profiles included an initial step at 94 ^{o}C for 2 min, followed by 25 cycles of denaturing at 94 ^{o}C for 15 sec, annealing at 60 ^{o}C for 30 sec and elongation at 68 ^{o}C for 1.5 min. The PCR product was purified by using PCR purification kit (Qiagen K. K., Tokyo, Japan).

3.2.2.2. Construction of fusion gene BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII

The fusion gene *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* (Fig. 3.3) was constructed by linking two sequences, including *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag* and *stop-terminator-HindIII* by overlap extension PCR method as follows:

Step #1. Construction of BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-(overlap stop-terminator)

Recombinant plasmid of pET28a(+)-*ctb-linker-cry j 1 epi-cry j 2 epi-flag* from the recombinant *E. coli* JM109 (chapter II) was used as the template to amplify the fusion gene *ctb-linker-cry j 1 epi-cry j 2 epi-flag*. Then, the sequence of *(overlap stop-terminator)* was linked to the 3' end of the *ctb-linker-cry j 1 epi-cry j 2 epi-flag* using the forward primer *Bam*HI-ctb-F (as described in 2.2.3 of chapter II) and reverse primer (overlap stop-terminator)-flag-R (5'-**TGCTCTTCTA***CTTGTCATCGTCGTCTTTGT*-3', the sequence indicated in bold overlaps with the first 10 bp of the 5' end sequence of the *stop-terminator-HindIII* gene, and the sequence in italics is complementary to the *flag* gene).

Step #2. Construction of (overlap flag)-stop-terminator-HindIII

The *stop-terminator-HindIII* fusion gene was amplified from the template *BamHI-BLA-stop-terminator-HindIII* which was constructed as described in 3.2.2.1. Then, the sequence of *(overlap flag)* was linked to the 5' end of the *stop-terminator-HindIII* using the forward primer (overlap flag)-stop-terminator-F

(5’-**CGATGACAAG***TAGAAGAGCAGAGAGGACGG*-3’, the sequence indicated in bold overlaps with the last 10 bp of the 3’ end sequence of the *flag*, and the sequence in italics corresponds to the to the *stop-terminator*) and the reverse primer *Hind*III-terminator-R.

Step #3. Linking of the two constructs by overlap extension PCR

The fusion gene *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* was constructed by overlap extension PCR using the purified PCR products of steps #1 and #2 as DNA templates. The final fusion gene was amplified by adding the forward primer *Bam*HI-ctb-F and the reverse primer *Hind*III-terminator-R.

3.2.2.3. Construction of fusion gene BamHI-BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII

The BLA-linked fusion antigen gene of *BamHI-BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* (Fig. 3.4) was constructed by linking two sequences which are *BamHI-BLA-linker* and *ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* by overlap extension PCR method as follows:

Step #1. Construction of BamHI-BLA-linker-(overlap ctb)

The *BamHI-BLA-stop-terminator-HindIII* fusion gene was used as a template for the first PCR to construct *BamHI-BLA-linker* fusion gene. The sequence of the *linker* was linked to *BLA* by PCR using the forward primer *Bam*HI-BLA-F and reverse primer linker-BLA-R (5’-**TCCGCTACCTCCGCC***TCTTTGAACATAAATTGAAA*-3’, the sequence indicated in bold corresponds to the *linker* and the sequence in italics is complementary to the *BLA* gene). Then, using the *BamHI-BLA-linker* fusion gene as the template, PCR was performed to link the sequence of *(overlap ctb)* to the 3’ end of the *BamHI-BLA-linker* fusion gene using the forward primer *Bam*HI-BLA-F and reverse primer (overlap ctb)-linker-BLA-R (5’-**TTTGAGGTGT***TCCGCTACCTCCGCCTCTTT*-3’, the sequence indicated in bold overlaps

with the first 10 bp of the 5' end sequence of the *ctb* gene, and the sequence in italics is complementary to the linker and *linker-BLA* gene).

Step #2. Construction of (overlap linker)-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII

The *ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* fusion gene was amplified from the template *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* which was constructed as described in 3.2.2.2. Then, the sequence of *(overlap linker)* was linked to the 5' end of the *ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* fusion gene using the forward primer (overlap linker)-ctb-F (5'-**AGGTAGCGGA***ACACCTCAAAATATTACTGA*-3', the sequence indicated in bold overlaps with the last 10 bp of the 3' end sequence of the *linker*, and the sequence in italics corresponds to the to the *ctb* gene) and the reversed primer *Hind*III-terminator-R.

Step #3. Linking of the two constructs by overlap extension PCR

The fusion gene *BamHI-BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-Terminator-HindIII* was constructed by overlap extension PCR using the purified PCR products of steps #1 and #2 as DNA templates. The final fusion gene was amplified by adding the forward primer *Bam*HI-BLA-F and the reverse primer *Hind*III-terminator-R.

3.2.3. Construction of recombinant plasmids and transformation

B. choshinensis and pNC-HisT (Fig. 3.1) were used as a host for overproduction and as an expression-secretion vector, respectively. pNC-HisT is a shuttle vector between *B. choshinensis* and *E. coli* derived from partially altered pNCMO2, a secretory expression vector. The pNC-HisT contains P2 promoter, which is one of five promoters driving transcription of cell wall protein (HWP) gene and does not work in *E. coli*. In *B. choshinensis* cells, P2 promoter works as very strong promoter, enabling efficient protein production. This vector is constructed with insertion of a His-Tag sequence (6x His) located at the downstream of the secretion signal peptide and Thrombin cleavage site is inserted so that the His-Tag can be removed from the

fusion expression protein.

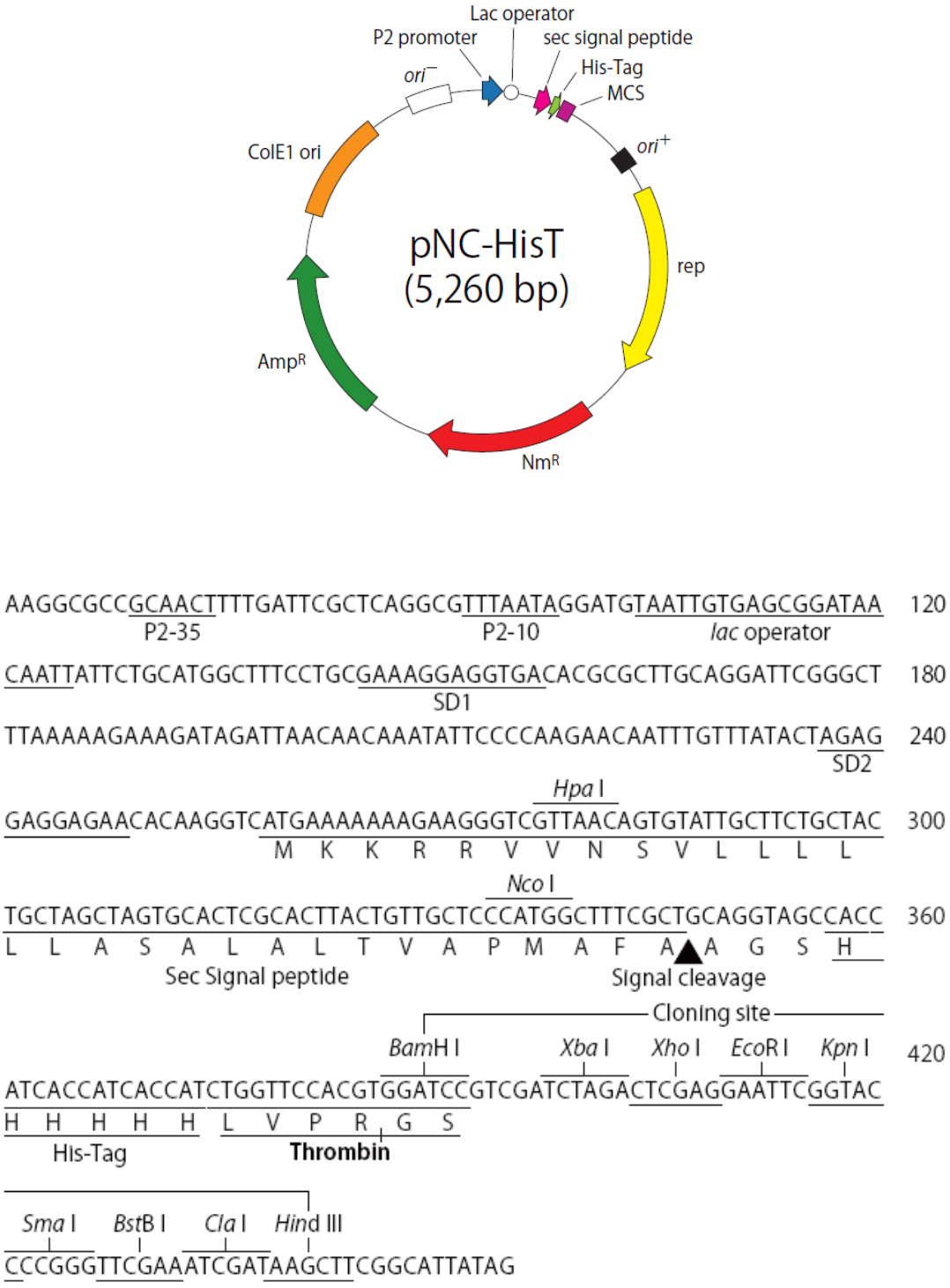

Fig. 3.1. Schematic structure of pNC-HisT vector

(Code No. HB121, Takara Bio)

E. coli JM109 was used for DNA manipulation as well as cloning. The fusion antigen peptide expression vectors were constructed as follows. The *Bam*HI/*Hind*III-digested fragments of *BamHI-BLA-stop-terminator-HindIII*; *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* and *BamHI-BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* were cloned into *Bam*HI/*Hind*III sites of pNC-HisT vectors using a DNA ligation kit (Takara Bio) to construct pNC-HisT-*BLA-stop-terminator* (Fig. 3.2), pNC-HisT-*ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator* (Fig. 3.3) and pNC-HisT-*BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator* (Fig. 3.4), respectively. All the restriction enzyme digestion products were purified from agarose gel using Gel Extraction Kit (Qiagen).

E. coli JM109 competent cells were transformed with the recombinant cloning plasmids. Positive colonies were selected on LB/ampicillin plates and confirmed by colony PCR using the forward primer pNC-F (5'-CGCTTGCAGGATTCGG-3') and the reverse primer pNC-R (5'-CAATGTAATTGTTCCCTACCTGC-3'). The recombinant plasmids were extracted using a Spin Miniprep Kit (Qiagen) and DNA sequencing was performed using the dye-terminator method by Bio Matrix Research (Nagareyama, Japan) to analyze the nucleotide sequences and the orientations of the inserts in the recombinant plasmids.

Then, *B. choshinensis* competent cells were transformed with the expression plasmids, according to the manufacturer's instructions using *B. choshinensis* competent cells (Cat. #HB116, Takara Bio) as below:

B. choshinensis competent cells were thawed quickly in a water bath at 37 ^{o}C for 30 sec, followed by centrifugation at 12,000 rpm for 1 min to form a cell pellet. The recombinant plasmids (50 ng) extracted from *E. coli* JM109 in 50 µl of Solution A (Appendix) were incubated with the bacterial cell pellets at room temperature for 5 min. Then, 150 µl of Solution B (PEG solution, Appendix) was added to the mixtures and mixed by vortex until the solution was uniform. After centrifugation of the cells at 5,000 rpm for 5 min 1 ml of MT medium (Appendix)

was added to the cell pellet and suspended completely with a micropipette. The competent cells were incubated at 37 ^{o}C with shaking (120 rpm) for 2 h and then applied on a MT medium plate supplemented with neomycin and cultured overnight at 37 ^{o}C.

Positive colonies were selected on LB/neomycin plates and confirmed by colony PCR.

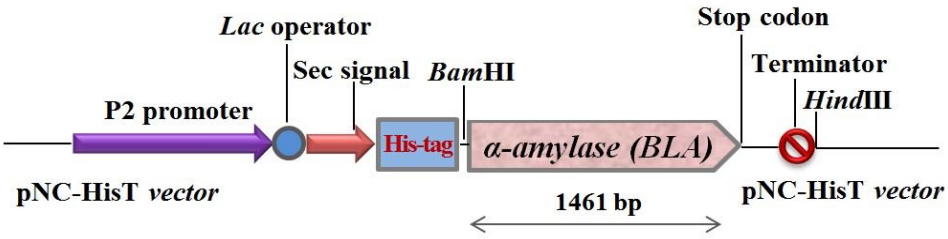

Fig. 3.2. Schematic diagram of the BLA gene in the pNC-HisT vector

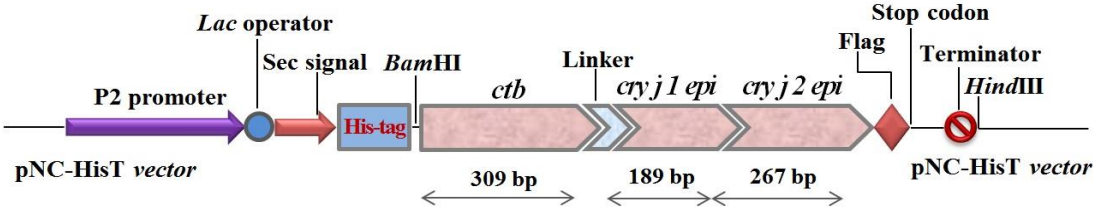

Fig. 3.3. Schematic diagram of the fusion gene in the pNC-HisT vector

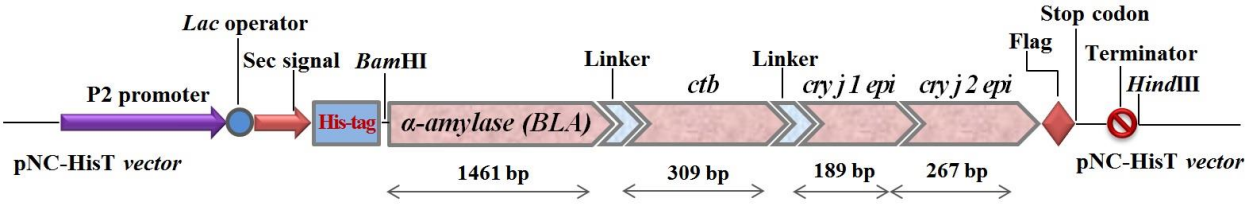

Fig. 3.4. Schematic diagram of the BLA-linked fusion gene in the pNC-HisT vector

3.2.4. Expression of target proteins by Brevibacillus recombinants

The production of the target proteins may vary across transformants depending on the nature of the target proteins. The size of colonies may also vary. Thus, six to ten colonies were selected at random (including both large and small colonies) for incubating in culture tubes. A single positive colony of *B. choshinensis* transformed by pNC-HisT-*BLA-stop-terminator*, pNC-HisT-*ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator* or pNC-HisT-*BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator* was inoculated into both 10 ml of 2SY and TM liquid media (Appendix) containing neomycin (10 μg/ml) in culture tubes (φ16 mm). Incubation was conducted at 30 ℃ with shaking at 120 rpm for 24-72 h. During the incubation, aliquots of the culture were taken every 24 h to confirm target protein production. At the end of incubation, the supernatant fractions were recovered by centrifugation at 5,000 ×g for 5 min. The cell pellets were suspended and lysed with an equal volume of HisTALON xTractor buffer containing DNaseI and lysozyme.

A negative control including pNC-HisT vector without insert DNA was used to confirm protein production by comparative analysis. SDS-PAGE analysis was performed on both the supernatant and pellet fractions, followed by western blot analysis to detect the expression of the target proteins.

Purification of the recombinant His-tagged protein using TALON Spin Columns was performed according to the manufacturer's instructions. Protein yield was measured by a Pierce BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as a standard.

3.2.5. SDS-PAGE

The protein samples mixed with an equal volume of 2× SDS-PAGE sample buffer were heated at 95°C for 5 min and loaded on on a commercial 12.5 % polyacrylamide gel (ePAGEL)

(Atto, Tokyo, Japan). Gel electrophoresis was carried out at 180V, 40A for 1h. Proteins were visualized by staining with Coomassie brilliant blue R-250.

3.2.6. Western blotting

Western blot analysis was carried out to test the antigenicities of the expressed peptide as described in Materials and methods 2.2.5 of chapter II.

3.3. Results

3.3.1. Construction of fusion genes

The fusion genes were constructed by stepwise PCR and overlap extension PCR as described in the Materials and Methods. The sizes of resultant PCR products visualized on the agarose gel were in good agreement with the calculated sizes (Fig. 3.5), suggesting that the fusion genes were constructed successfully by these methods.

The final products were cloned into pNC-HisT vector for protein expression. The results of sequencing analysis of the inserted DNA showed that the nucleotide sequences and amino acid sequences were identical to the designed sequences and had the correct orientation in the expression plasmids (Fig. 3.6).

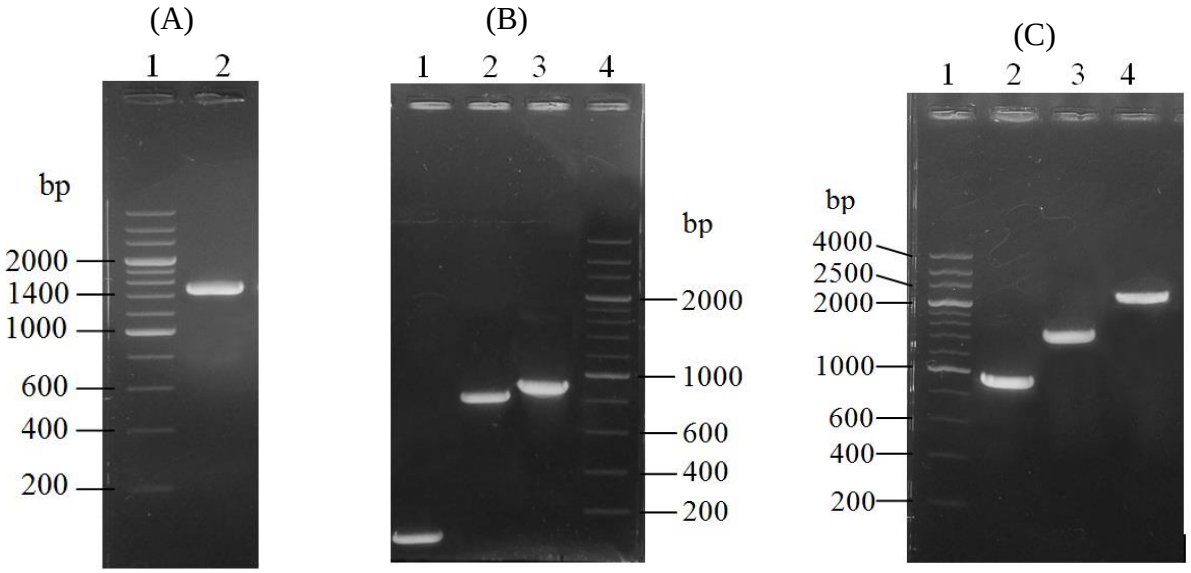

Fig. 3.5. Electrophoretic analysis of products acquired from overlap extension PCR

(A) PCR product in the construction of *BamHI-BLA-stop-terminator-HindIII*: Lane 1, 200 bp marker; Lane 2, The final product of *BamHI-BLA-stop-terminator-HindIII* (1552 bp). **(B)** PCR products in the construction of *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII*: Lane 1, *(overlap flag)-stop-terminator-HindIII* (92 bp); Lane 2, *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-(overlap stop-terminator)* (823 bp); Lane 3, The final product of *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* (895 bp); Lane 4, 200 bp marker. **(C)** PCR products in the construction of *BamHI-BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII*: Lane 1, 200 bp marker; Lane 2, *(overlap linker)-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* (896 bp); Lane 3, *BamHI-BLA-linker-(overlap ctb)* (1495 bp); Lane 4, The final product of *BamHI-BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII* (2371 bp).

(A)

Nucleotide sequence of *BamHI-BLA-stop-terminator-HindIII*

GAT***GGATCC***---GCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTA
CATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATATTTGGCTGAACA
CGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTA

CGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTA

CGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTA

CGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTTG

AAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCGAATTAAAGCCTGGACA

CATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTGGTACCATTTTGA

CGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAAGGAAAGGCTT

GGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTATGCCGACATCGATTAT

GACCATCCTGATGTCGCAGCAGAAATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAA

TTGGACGGTTTCCGTCTTGATGCTGTCAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAA

TCATGTCAGGGAAAAAACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTT

GGGCGCGCTGGAAAACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCT

TCATTATCAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAAC

AGTACGGTCGTTTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGC

CGGGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCT

CACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTC

CCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGT

ATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAG

GCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCA

AAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCG

TTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGG

TTTCAATTTATGTTCAAAGA---TAG---*AAGAGCAGAGAGGACGGATTTCCTGAAGGAAATCCGTTTT*

*TTTATTTTGCCCGTCTTATAAATTTCTTTG---**AAGCTT**CGG*

Amino acid sequence of BLA

AAAAANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGA

YDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPA

DRNRVISGEHRIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGKAWDWEV

SNENGNYDYLMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSFLRDWVNHVR

EKTGKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNST

VVSKHPLKAVTFVDNHDTQPGQSLESTVQTWFKPLAYAFILTRESGYPQVFYGDMYGTKGDSQRE

IPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWTREGDSSVANSGLAALITDGPGGAKRMYVG

RQNAGETWHDITGNRSEPVVINSEGWGEFHVNGGSVSIYVQR

(B)

Nucleotide sequence of *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII*

GCG---***GGATCC***---ACACCTCAAAATATTACTGATTTGTGTGCAGAATACCACAACACACAAATAC

ATACGCTAAATGATAAGATATTTTCGTATACAGAATCTCTAGCTGGAAAAAGAGAGATGGCTATC

ATTACTTTTAAGAATGGTGCAACTTTTCAAGTAGAAGTACCAGGTAGTCAACATATAGATTCAC

AAAAAAAAGCGATTGAAAGGATGAAGGATACCCTGAGGATTGCATATCTTACTGAAGCTAAAG

TCGAAAAGTTATGTGTATGGAATAATAAAACGCCTCATGCGATTGCCGCAATTAGTATGGCAAAT

---GGCGGAGGTAGCGGA---CAGAACCGTATGAAACTGGCGGATTGCGCGGTGGGCTTTGGATC

GAAAATGCCGATGTATATTGCGGGCTATAAAACGTTCGATGGCCGTCCGTGCGTGTTCATTAAA

CGTGTGAGCAACGTGATTATTCATGGCCTGCATCTGTATGGCAGCATGAAAGTGACGGTGGCG

TTCAACCAGTTCGGCCCG---GGCATTATTGCGGCGTATCAGAACCCGGCGAGCTGGAAACAGT

TCGCGAAACTGACGGGCTTCACGCTGATGGGCATTGATATTTTCGCGAGCAAAAACTTCCATCT

GCAGAAAAACACGATTGGCACGGGCAGCCGTGCGGAAGTGAGCTATGTGCATGTGAACGGCG

CGAAATTCTGCAAAGATATTAAACTGAGCGATATTAGCCTGAAACTGACGAGCGGCAAAATTG

CGAGCTGCCTGAACGATAACGCGAACGGCTATTTC---GACTACAAAGACGACGATGACAAG---T

AG---AAGAGCAGAGAGGACGGATTTCCTGAAGGAAATCCGTTTTTTTATTTTGCCCGTCTTATA

AATTTCTTTG***AAGCTT***CGG

Amino acid sequence of CTB-Linker-Cry j 1 epi-Cry j 2 epi-FLAG

TPQNITDLCAEYHNTQIHTLNDKIFSYTESLAGKREMAIITFKNGATFQVEVPGSQHIDSQKKAIER

MKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAISMAN*GGGSG*QNRMKLADCAVGFGSKMPMYIA

GYKTFDGRPCVFIKRVSNVIIHGLHLYGSMKVTVAFNQFGPGIIAAYQNPASWKQFAKLTGFTLMG

IDIFASKNFHLQKNTIGTGSRAEVSYVHVNGAKFCKDIKLSDISLKLTSGKIASCLNDNANGYFDY

KDDDDK

(C)

Nucleotide sequence of *BamHI-BLA-linker-ctb-linker-cry j 1 epi-cry j 2 epi-flag-stop-terminator-HindIII*

GAT***GGATCC***---GCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTA

CATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATATTTGGCTGAACA

CGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTA

CGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTA

CGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTA

CGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTTG

AAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCGAATTAAAGCCTGGACA

CATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTGGTACCATTTTGA

CGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAAGGAAAGGCTT

GGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTATGCCGACATCGATTAT

GACCATCCTGATGTCGCAGCAGAAATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAA

TTGGACGGTTTCCGTCTTGATGCTGTCAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAA

TCATGTCAGGGAAAAAACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTT

GGGCGCGCTGGAAAACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCT

TCATTATCAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAAC

AGTACGGTCGTTTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGC

CGGGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCT

CACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTC

CCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGT

ATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAG

GCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCA

AAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCG

TTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGG

TTTCAATTTATGTTCAAAGA---GGCGGAGGTAGCGGA---ACACCTCAAAATATTACTGATTTGTG

TGCAGAATACCACAACACACAAATACATACGCTAAATGATAAGATATTTTCGTATACAGAATCTC

TAGCTGGAAAAAGAGAGATGGCTATCATTACTTTTAAGAATGGTGCAACTTTTCAAGTAGAAGT

ACCAGGTAGTCAACATATAGATTCACAAAAAAAAGCGATTGAAAGGATGAAGGATACCCTGAG

GATTGCATATCTTACTGAAGCTAAAGTCGAAAAGTTATGTGTATGGAATAATAAAACGCCTCAT

GCGATTGCCGCAATTAGTATGGCAAAT---GGCGGAGGTAGCGGA---CAGAACCGTATGAAACTG

GCGGATTGCGCGGTGGGCTTTGGATCGAAAATGCCGATGTATATTGCGGGCTATAAAACGTTCG

ATGGCCGTCCGTGCGTGTTCATTAAACGTGTGAGCAACGTGATTATTCATGGCCTGCATCTGTAT

GGCAGCATGAAAGTGACGGTGGCGTTCAACCAGTTCGGCCCGGGCATTATTGCGGCGTATCAG

AACCCGGCGAGCTGGAAACAGTTCGCGAAACTGACGGGCTTCACGCTGATGGGCATTGATATT

TTCGCGAGCAAAAACTTCCATCTGCAGAAAAACACGATTGGCACGGGCAGCCGTGCGGAAGT

GAGCTATGTGCATGTGAACGGCGCGAAATTCTGCAAAGATATTAAACTGAGCGATATTAGCCTG

AAACTGACGAGCGGCAAAATTGCGAGCTGCCTGAACGATAACGCGAACGGCTATTTC---GACT

ACAAAGACGACGATGACAAG---TAG---AAGAGCAGAGAGGACGGATTTCCTGAAGGAAATCC

GTTTTTTTATTTTGCCCGTCTTATAAATTTCTTTG---***AAGCTT***CGG

Amino acid sequence of BLA-Linker-CTB-Linker-Cry j 1 epi-Cry j 2 epi-FLAG

AAAAANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGA

YDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPA

DRNRVISGEHRIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGKAWDWEV

SNENGNYDYLMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSFLRDWVNHVR

EKTGKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNST

VVSKHPLKAVTFVDNHDTQPGQSLESTVQTWFKPLAYAFILTRESGYPQVFYGDMYGTKGDSQRE
IPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWTREGDSSVANSGLAALITDGPGGAKRMYVG
RQNAGETWHDITGNRSEPVVINSEGWGEFHVNGGSVSIYVQR*GGGSG*TPQNITDLCAEYHNTQIH
TLNDKIFSYTESLAGKREMAIITFKNGATFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKL
CVWNNKTPHAIAAISMAN*GGGSG*QNRMKLADCAVGFGSKMPMYIAGYKTFDGRPCVFIKRVSN
VIIHGLHLYGSMKVTVAFNQFGPGIIAAYQNPASWKQFAKLTGFTLMGIDIFASKNFHLQKNTIGTG
SRAEVSYVHVNGAKFCKDIKLSDISLKLTSGKIASCLNDNANGYFDYKDDDDK

Fig. 3.6. Nucleotide and amino acid sequences of fusion proteins

BamHI and HindIII sites are shown in bold italic and the stop codon in red. In amino acid sequences, the linker and FLAG are indicated in blue and red, respectively.

(A) DNA sequence (1552 bp) and amino acid sequence of BLA (487 aa). **(B)** DNA sequence (895 bp) and amino acid sequence of CTB-Linker-Cry j 1 epi-Cry j 2 epi-FLAG (268 aa). **(C)** DNA sequence (2371 bp) and amino acid sequence of BLA-Linker-CTB-Linker-Cry j 1 epi-Cry j 2 epi-FLAG (760 aa).

3.3.2. Expression and purification of fusion antigen peptides

3.3.2.1. Expression of CTB-Linker-Cry j 1 epi-Cry j 2 epi-Flag

To examine the expression of the fusion antigen peptide, *B. choshinensis* harboring pNC-HisT-*BamHI-ctb-linker-cry j1 epi-cry j2 epi-flag-stop-terminator-HindIII* was grown in TM and 2SY liquid media under varying culture conditions. SDS-PAGE was carried out on samples from the culture supernatants and the pellets for expressed protein analysis. However, the expression of the recombinant fusion protein with a molecular weight of 31.13 kDa was not detected by SDS-PAGE analysis (Fig. 3.7). The experiments might have resulted in low-level expression of the recombinant protein that can be detected only by western blotting. However, to be applied to the practical purposes such as oral vaccines and purification of the antigen peptide from the supernatant, expressionlevel was too low to meet this requirement.

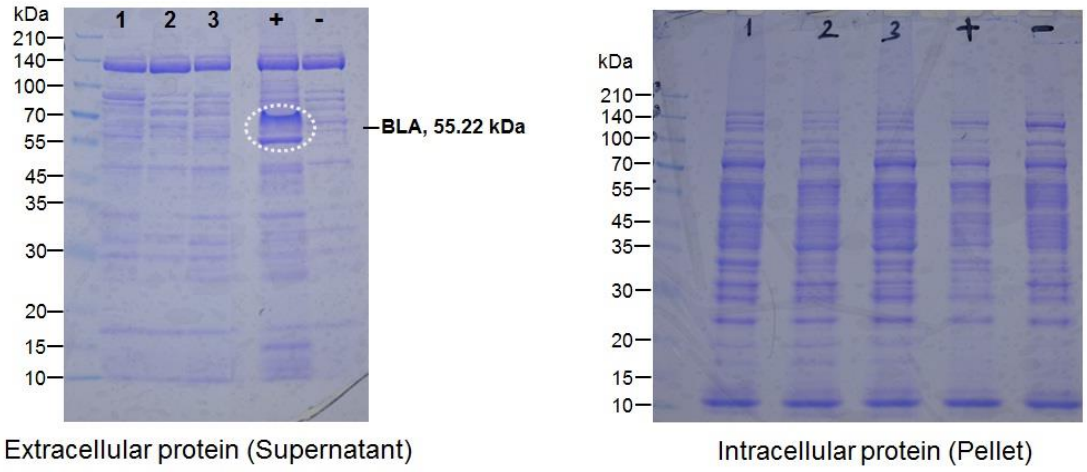

Fig. 3.7. Expression and purification of CTB-Linker-Cry j 1 epi-Cry j 2 epi-Flag

(1~3), recombinant *B. choshinensis* harboring pNC-HisT-*ctb-linker-cry j 1 epi- cry j 2 epi*; (+), Positive control harboring pNC-*HisT-BLA-stop-terminator*; (-), Negative control harboring pNC-HisT vector without insert DNA.

3.3.2.2. Expression and purification of BLA

To increase the expression level of the recombinant antigen protein, fusion of the antigen protein to one of secretary proteins was attempted.

As shown in Figure 3.8, BLA (α-amylase) can be efficiently expressed into the supernatant in the *B. choshinensis* expression system. Therefore, BLA can be a candidate of the fusion partner of the recombinant antigen protein.

The recombinant *B. choshinensis* harboring *pNC-HisT-BLA-stop-terminator* was inoculated in TM/neomycin and incubated at 30 ^{o}C for 48 h. SDS-PAGE analysis was carried out on the culture supernatant to analyze the expression of BLA. A single band with high homogeneity was obtained from eluted fractions on the His-tag affinity column. The molecular mass of the fusion protein visualized on the gel was in good agreement with the theoretical mass (55.22 kDa) (Fig. 3.8). The result indicated that the efficient production and secretion of BLA into the culture supernatant was achieved using this expression system.

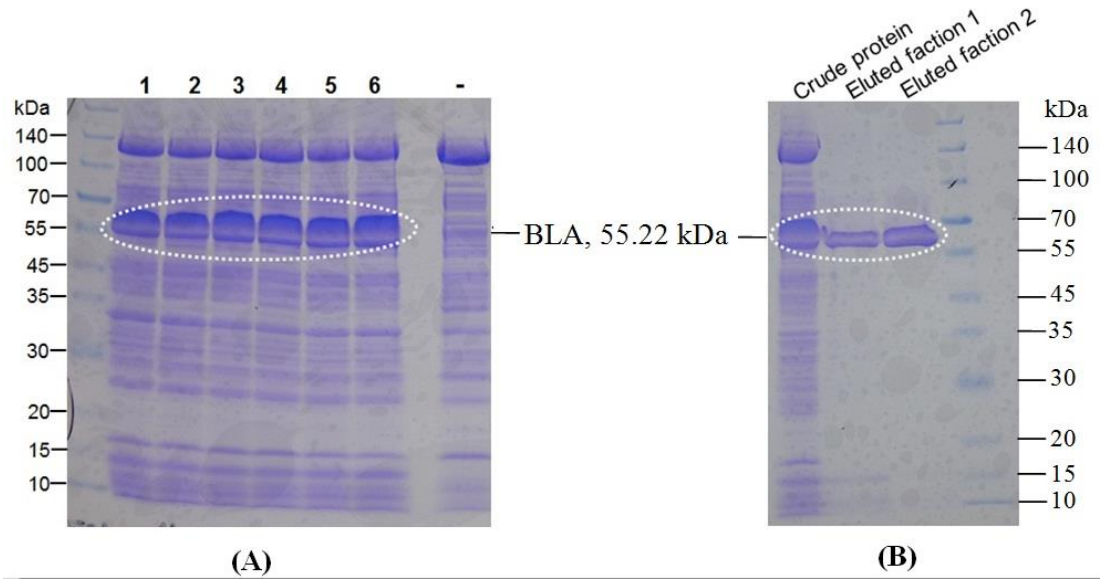

Fig. 3.8. Expression and purification of α-Amylase (BLA)

(A) Expression of BLA: (1~6), *B. choshinensis* harboring pNC-HisT-*BLA*; (-), Negative control harboring pNC-HisT vector without insert DNA. **(B)** Purification of BLA on His-tag affinity column.

3.3.2.3. Expression and purification of the BLA-linked fusion antigen peptide

The recombinant *B. choshinensis* harboring pNC-HisT-*BamHI-BLA-linker-ctb-linker-cry j1 epi-cry j2 epi-flag-stop-terminator-HindIII* was grown in TM and 2SY liquid media under varying culture conditions. SDS-PAGE was carried out on samples from the culture supernatants and the pellets for expressed protein analysis. Figure 3.9 shows that the BLA-linked fusion antigen peptide, BLA-Linker-CTB-Linker-Cry j1 epi-Cry j2 epi-FLAG with a molecular weight of 85.25 kDa was not visualized on the gel. However, a protein fragment with a molecular weight that corresponds to the size of BLA (55.22 kDa, positive control) was detected from the culture supernatant (Fig. 3.9). In addition, a single band with high homogeneity was obtained from eluted fractions on the His-tag affinity column (Fig. 3.10). These results suggest that the BLA-linked fusion antigen peptide could be expressed in the *B. choshinensis* host. However, the expressed recombinant protein was likely to be degraded by intra- or/and extra-cellular proteases, and the resultant cleaved fragment containing BLA (55.22 kDa) was secreted into culture supernatant, while the fragment of Linker-CTB-Linker-Cry j1 epi-Cry j2 epi-Flag (30.03 kDa) seemed to be

degraded completely.

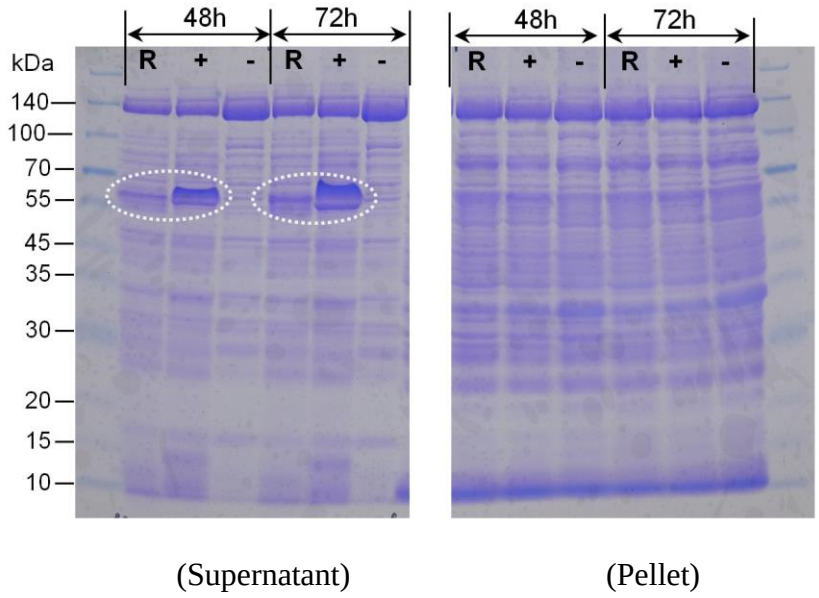

Fig. 3.9. Expression of the BLA-linked fusion antigen peptide (85.25 kDa)

(R), recombinant *B. choshinensis* harboring pNC-HisT-*BamHI-BLA-linker-ctb-linker-cry j1 epi-cry j2 epi-flag-stop-terminator-HindIII;* (+), Positive control, harboring pNC-HisT-BLA; (-), Negative control, harboring pNC-HisT vector without insert DNA.

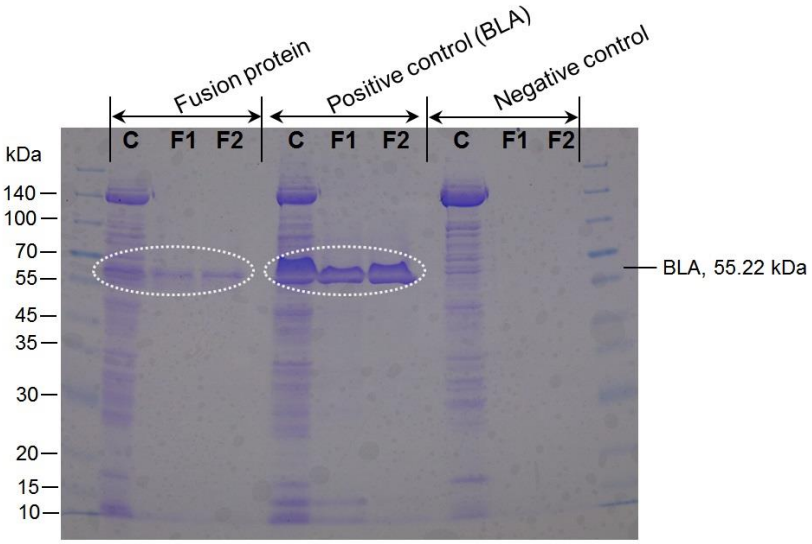

Fig. 3.10. Purification of the BLA-linked fusion antigen peptide (85.25 kDa)

(c) *Crude protein*; (F1) Eluted fraction I; (F2) Eluted fraction II.

To examine whether protease activity exists in the *B. choshinensis* host that might cause proteolytic degradation of the BLA-linked fusion antigen peptide, we added protease inhibitor cocktail for bacterial proteases (Sigma-Aldrich) during culture period to inactivate protease activities. SDS-PAGE was carried out on the samples from culture supernatants and pellets for the analysis of expressed protein. A band with a molecular weight corresponding to the size of BLA (55.22 kDa) was obtained from the culture supernatant (Fig. 3.11A). Furthermore, a band with a molecular weight corresponding to the size of Linker-CTB-Cry j1 epi-Cry j2 epi-Flag (30.03 kDa) was also visualized on SDS-PAGE from cell pellets (Fig. 3.11B).

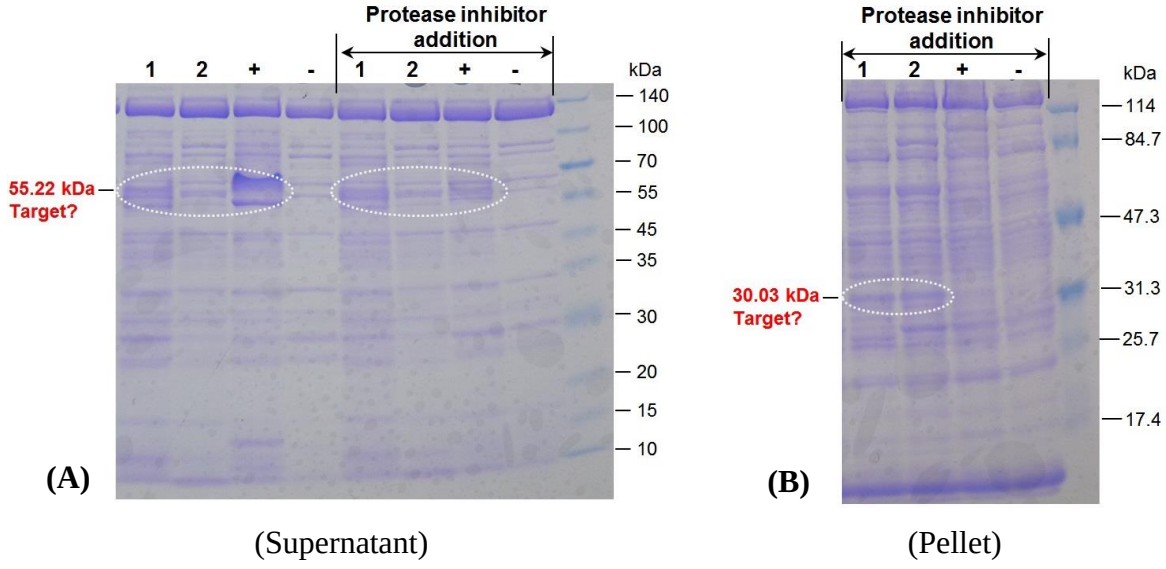

Fig. 3.11. Expression of the BLA-linked fusion antigen peptide in the presence of protease inhibitors

(1-2), *B. choshinensis* harboring pNC-HisT-*BamHI-BLA-linker-ctb-linker-cry j1 epi-cry j2 epi-flag-stop-terminator-HindIII;* (+), Positive control harboring pNC-HisT-*BLA-stop-terminator*; (-), Negative control harboring pNC-HisT vector without insert DNA.

3.3.3. Western blot analysis of the fusion antigen peptide

Western blotting was performed to confirm expression of the BLA-linked fusion antigen peptide in the presence of protease inhibitors. The BLA-linked fusion antigen peptides (85.25 kDa) and the degraded fragments (30.03 kDa) were recognized by anti-FLAG (Fig. 3.12A) and

anti-CTB antibodies (Fig. 3.12B). These results demonstrated that the recombinant antigen peptide BLA-Linker-CTB-Linker-Cry j 1 epi-Cry j 2 epi-FLAG was expressed in *B. choshinensis* host, and a part of the expressed recombinant proteins was degraded by proteases.

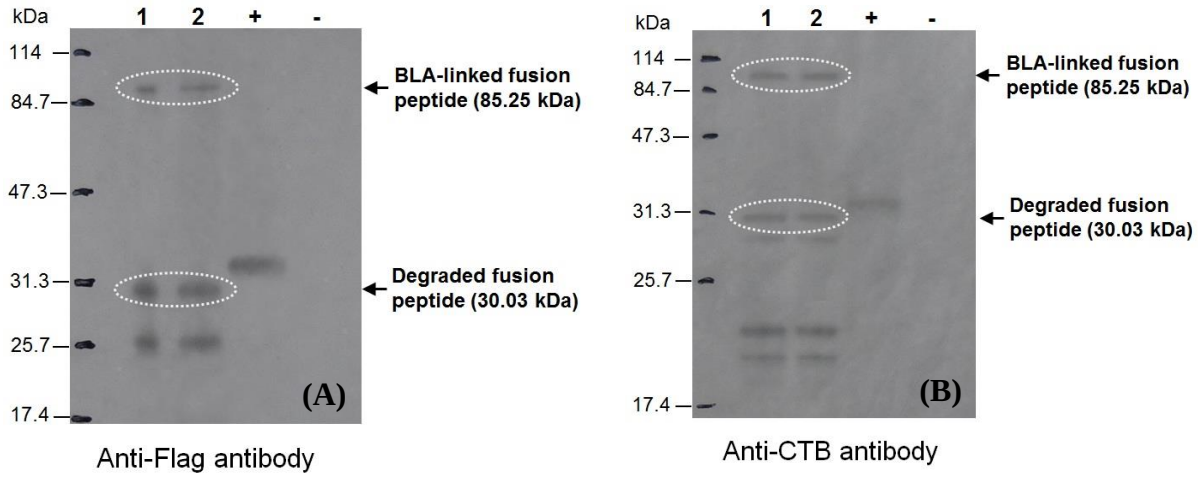

Fig. 3.12. Western blot analysis of expression of the BLA-linked fusion antigen peptide in the presence of protease inhibitors

(A) The primary antibody used was anti-FLAG antibody. **(B)** The primary antibody used was anti-cholera toxin B subunit antibody. (1), (2) *B. choshinensis* harboring pNC-HisT-*BamHI-BLA-linker-ctb-linker-cry j1 epi-cry j2 epi-flag-stop-terminator-HindIII* (pellet); (+) Positive control, purified CTB-linker-Cry j1 epi-Cry j2 epi-Flag from the recombinant *E .coli* BL21(DE3) (34.65 kDa); (-) Negative control, *B. choshinensis* harbouring pNC-HisT vector without the insert DNA.

3.4. Discussion

Expression of the fusion antigen peptide CTB-Linker-Cry j 1 epi-Cry j 2 epi-FLAG using *B. choshinensis* was investigated. The recombinant *B. choshinensis* harboring pNC-HisT-*BamHI-ctb-linker-cry j1 epi-cry j2 epi-flag-stop-terminator-HindIII* was grown in TM and 2SY liquid medium under varying culture conditions. SDS-PAGE was carried out on samples from the culture supernatants and the pellets for expressed protein analysis. However, the expression of the recombinant fusion antigen peptide with a molecular weight of 31.13 kDa was not detected by SDS-PAGE analysis. The experimental result suggested that there are some possible reasons of low-level expression or no production of the target protein. First, the secondary structure of mRNA may cause translation abnormalities due to the presence of a high-energy palindrome structure. Second, in *B. choshinensis*, the levels of production of eukaryotic proteins are generally much lower than those of bacterial proteins. One of the possible reasons is that the eukaryotic proteins synthesized are toxic to the bacterial cells and so the expressed products might inhibit growth of the host. Third, the antigen fusion protein may not be suitable for secretory production due to the interaction between the domains of expressed fusion antigen peptide, as the result forming a secondary structure of protein that might prevent the secretory production. Fourth, some proteases might be produced by the *B. choshinensis* host and caused proteolytic degradation of the fusion antigen peptide (Fig. 3.13).

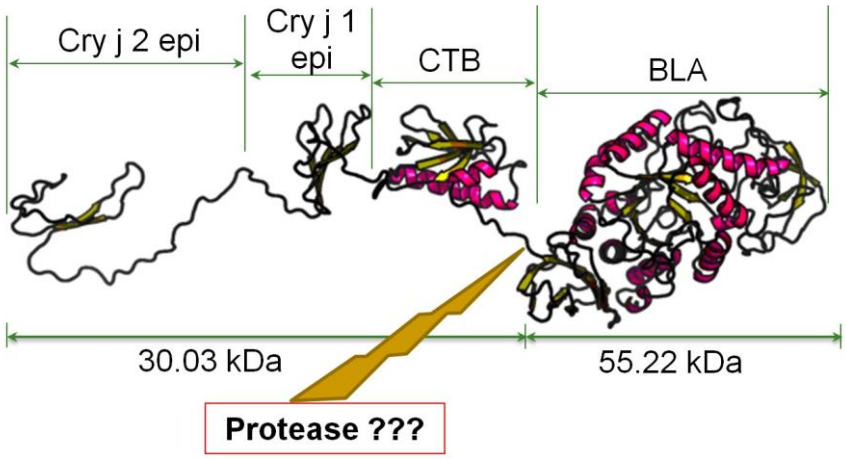

Fig. 3.13. Predicted structure of the fusion protein

BLA (55.22 kDa); Linker-CTB-Cry j1 epi-Cry j2 epi-Flag (30.03 kDa)

The fusion antigen peptide of BLA-Linker-CTB-Linker-Cry j 1 epi-Cry j 2 epi-Flag proved to be expressed in the *B. choshinensis* host using pNC-HisT vector. It was found that some proteases might be produced by the *B. choshinensis* host and caused proteolytic degradation of the BLA-linked fusion antigen peptide. The whole and degraded fusion antigen peptides could be obtained by adding protease inhibitors during culture period. Reactivities to anti-CTB and anti-FLAG antibodies of the BLA-linked and degraded fusion antigen peptides were confirmed by western blot analysis.

When the BLA-linked fusion protein was expressed, only BLA was secreted to the culture supernatant (Fig. 3.10), which suggests that other domains of fusion protein linked to BLA hindered the secretion of whole fusion protein and, therefore, proteolytic cleavage of the site between BLA and CTB might be necessary for the secretion of BLA (Fig. 3.13).

CHAPTER IV

Conclusions

- The recombinant fusion antigen peptide, CTB-Linker-Cry j 1 epi -Cry j 2 epi-Flag was successfully expressed in *E. coli* strain BL21(DE3) using pET28a(+) vector. The expressed recombinant antigen peptide was purified to a high homogeneity on the His-tag affinity column, giving the recovery of approximately 120 mg/L of culture. Reactivities to anti-CTB and anti-FLAG antibodies were confirmed by western blot analysis. Antigenicity of the purified recombinant antigen peptide against anti-Cry j 1 and anti-Cry j 2 antibodies was also confirmed.
- The BLA-linked fusion antigen peptide, BLA-Linker-CTB-Linker-Cry j 1 epi -Cry j 2 epi-Flag proved to be expressed in the *B. choshinensis* host using pNC-HisT vector. However, it was cleaved by proteolytic action and some of the resultant fragment containing BLA was secreted to the supernatant. The fusion antigen peptide that is not linked with BLA could not be expressed using the same expression system.
- Some proteases might be produced by the *B. choshinensis* host and caused proteolytic degradation of the integrated fusion antigen peptide.
- The BLA-linked and degraded fusion antigen peptides could be obtained by adding protease inhibitor during culture period. Reactivities to anti-CTB and anti-Flag antibodies of the BLA-linked and degraded fusion antigen peptides were confirmed by western blot analysis.
- The present study indicates that the production of sufficient amounts of recombinant proteins for immunotherapy is possible using *E. coli* for protein expression. Protein expression by other bacterial strains still remains to be investigated.

References

[1] Y. Okamoto, S. Horiguchi, H. Yamamoto, S. Yonekura, T. Hanazawa, Present situation of cedar pollinosis in Japan and its immune responses, Allergol. Int. 58 (2009) 155-162.

[2] Y. Kaneko, Y. Motohashi, H. Nakamura, T. Endo, A. Eboshida, Increasing prevalence of Japanese cedar pollinosis: a meta-regression analysis, Int. Arch. Allergy Immunol. 136 (2005) 365-371.

[3] H. Yasueda, Y. Yui, T. Shimazu, T. Shida, Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria japonica)* pollen, J. Allergy Clin. Immunol. 71 (1983) 77-86.

[4] T. Sone, N. Komiyama, K. Shimizu, T. Kusakabe, K. Morikubo, K. Kino, Cloning and sequencing of cDNA coding for *Cry j* I, a major allergen of Japanese cedar pollen, Biochem. Biophys. Res. Commun. 199 (1994) 619-625.

[5] M. Sakaguchi, S. Inouye, M. Taniai, S. Ando, M. Usui, T. Matuhashi, Identification of the second major allergen of Japanese cedar pollen, Allergy 45 (1990) 309-312.

[6] N. Komiyama, T. Sone, K. Shimizu, K. Morikubo, K. Kino, cDNA cloning and expression of *Cry* j II, the second major allergen of Japanese cedar pollen, Biochem. Biophys. Res. Commun. 201 (2) (1994) 1021-1028.

[7] M. Namba, M. Kurose, K. Torigoe, K. Hino, Y. Taniguchi, S. Fukuda, M. Usui, M. Kurimoto, Molecular cloning of the second major allergen, *Cry j* II, from Japanese cedar pollen, FEBS Lett. 353 (1994) 124-128.

[8] M. Hashimoto, H. Nigi, M. Sakaguchi, S. Inouye, K. Imaoka, H. Miyazawa, Y. Taniguchi, M. Kurimoto, H. Yasueda, T. Ogawa, Sensitivity to two major allergens (*Cry j* I and *Cry j* II) in patients with Japanese cedar (*Cryptomeria japonica*) pollinosis, Clin. Exp. Allergy 25 (1995) 848-852.

[9] F.R. Ali, M. Larché, Peptide-based immunotherapy: a novel strategy for allergic disease, Expert Rev. Vaccines 4 (2005) 881-889.

[10] M. Larché, Peptide immunotherapy for allergic diseases, Allergy 62 (2007) 325-331.

[11] C.A. Janeway, P. Travers, M. Walport, M.J. Shlomchik, The immune system in health and disease, in: Immunobiology, 5th ed., Garland Publishing, New York 2003 pp. 471-500.

[12] K. Hirahara, T. Tatsuta, T. Takatori, M. Ohtsuki, H. Kirinaka, J. Kawaguchi, N. Serizawa, Y. Taniguchi, S. Saito, M. Sakaguchi, S. Inouye, A. Shiraishi, Preclinical evaluation of an immunotherapeutic peptide comprising 7 T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen allergens, J. Allergy Clin. Immunol. 108 (2001) 94-100.

[13] S. Hashiguchi, K. Hino, Y. Taniguchi, M. Kurimoto, K. Fukuda, M. Ohyama, T. Fujiyoshi, S. Sonoda, Y. Nishimura, G. Yamada, K. Sugimura, Immunodominance of seven regions of a major allergen, Cry j 2, of Japanese cedar pollen for T-cell immunity, Allergy 51 (1996) 621-632.

[14] S. Saito, K. Hirahara, J. Kawaguchi, N. Serizawa, K. Hino, Y. Taniguchi, M. Kurimoto, M. Sakaguchi, S. Inouye, A. Shiraishi, Identification of T Cell determinants in Cry j 1 and Cry j 2 of size suitable for peptide immunotherapy against Japanese cedar pollinosis, Annu. Rep. Sankyo Res. Lab. 52 (2000) 49-58.

[15] T. Sone, K. Morikubo, M. Miyahara, N. Komiyama, K. Shimizu, H. Tsunoo, K. Kino, T cell epitopes in Japanese cedar (*Cryptomeria japonica*) pollen allergens: choice of major T cell epitopes in Cry j 1 and Cry j 2 toward design of the peptide-based immunotherapeutics for the management of Japanese cedar pollinosis, J. Immunol. 161 (1998) 448-457.

[16] T. Yoshitomi, K. Hirahara, J. Kawaguchi, N. Serizawa, Y. Taniguchi, S. Saito, M. Sakaguchi, S. Inouye, A. Shiraishi, Three T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen antigens, retain their immunogenicity and tolerogenicity in a linked peptide, Immunology 107 (2002) 517-522.

[17] H. Takagi, T. Hiroi, L. Yang, Y. Tada, Y. Yuki, K. Takamura, R. Ishimitsu, H. Kawauchi, H. Kiyono, F. Takaiwa, A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 17525-17530.

[18] F. Takaiwa, A rice-based edible vaccine expressing multiple T-cell epitopes to induce oral tolerance and inhibit allergy, Immunol. Allergy Clin. North Am. 27 (2007) 129-139.

[19] Y. Wakasa, H. Takagi, S. Hirose, L. Yang, M. Saeki, T. Nishimura, O. Kaminuma, T. Hiroi, F. Takaiwa, Oral immunotherapy with transgenic rice seed containing destructed Japanese cedar pollen allergens, Cry j 1 and Cry j 2, against Japanese cedar pollinosis, Plant Biotechnol. J. 11 (2013) 66-76.

[20] Y. Kawabe, Y. Hayashida, K. Numata, S. Harada, Y. Hayashida, A. Ito, M. Kamihira, Oral immunotherapy for pollen allergy using T-cell epitope-containing egg white derived from genetically manipulated chickens, PLoS One 7 (2012) e48512.

[21] K. Ohkouchi, S. Kawamoto, K. Tatsugawa, N. Yoshikawa, Y. Takaoka, S. Miyauchi, T. Aki, M. Yamashita, Y. Murooka, K. Ono, Prophylactic effect of *Lactobacillus* oral vaccine expressing a Japanese cedar pollen allergen, J. Biosci. Bioeng. 113 (4) (2012) 536-541.

[22] N.L.B. Wernick, D.J.-F. Chinnapen, J.A. Cho, W.I. Lencer, Cholera Toxin: An intracellular journey into the ctytosol by way of the endoplasmic reticulum, Toxins (Basel) 2 (2010) 310-325.

[23] Y. Fujinaga, A.A. Wolf, C. Rodigherio, H. Wheeler, B. Tsai, L. Allen, M.G. Jobling, T. Rapoport, R.K. Holmes, W.I. Lencer, Gangliosides that associate with lipid rafts mediate transport of cholera toxin from the plasma membrane to the ER, Mol. Biol. Cell 14 (2003) 4783-4793.

[24] R.G. Zhang, D.L. Scott, M.L. Westbrook, S. Nance, B.D. Spangler, G.G. Shipley, E.M. Westbrook, E.M. The three-dimensional crystal structure of cholera toxin, J. Mol. Biol. 251 (1995) 563-573.

[25] J. Holmgren, N. Lycke, C. Czerkinsky, Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems, Vaccine 11 (1993) 1179-1184.

[26] H.-Y. Wu, M. W. Russel, Induction of mucosal and systemic immune responses by intranasal immunization using recombinant cholera toxin B subunit as an adjuvant, Vaccine 16 (1997) 286-292.

[27] A. George-Chandy, K. Eriksson, M. Lebens, I. Nordstrom, E. Schon, J. Holmgren, Cholera toxin B subunit as a carrier molecule promotes antigen presentation and increases CD40 and CD86 expression on antigen-presenting cells, Infec. Immunol. 69 (9) (2001) 5716-5725.

[28] N.L.B. Wernick, D.J.-F. Chinnapen, J.A. Cho, W.I. Lencer, Cholera Toxin: An intracellular journey into the ctytosol by way of the endoplasmic reticulum, Toxins (Basel) 2 (2010) 310-325.

[29] A.M. Walmsley, C.J. Arntzen, Plant cell factories and mucosal vaccines, Curr. Opin. Biotechnol. 14 (2003) 145-150.

[30] G. Giddings, Transgenic plants as protein factories, Curr. Opin. Biotechnol. 12 (2001) 450-454.

[31] G. Lewis, I.W. Taylor, A.W. Nienow, C.J. Hewitt, The application of multi-parameter flow cytometry to the study of recombinant *Escherichia coli* batch fermentation process, J. Ind. Microbiol. Biotech. 31 (2004) 311-322.

[32] J. Bousquet, R. Lockey, H.-J. Malling, WHO panel members, Allergen immunotherapy: therapeutic vaccines for allergic diseases A WHO position paper, J. Allergy Clin. Immunol. 102 (1998) 558-562.

[33] B.M. Haselden, A.B. Kay, M. Larché, Peptide-mediated immune responses in specific immunotherapy, Int. Arch. Allergy Immunol. 122 (2000) 229-237.

[34] Y. Zou, Recombinantly Engineered Proteins Containing Cholera Toxin B Subunit as a Functional and Structural Element. Dissertation for Ph.D, Kochi University of Technology (2006).

[35] N. Amet, H.-F. Lee, W.-C. Shen, Insertion of the designed helical linker led to increased expression of tf-based fusion proteins, Pharm. Res. 26 (2009) 523-528.

[36] R. Arai, H. Ueda, A. Kitayama, N. Kamiya, T. Nagamune, Design of the linkers which effectively separate domains of a bifunctional fusion protein, Protein Eng. 14 (2001) 529-532.

[37] T.P. Hopp, K.S. Pricket, V.L. Price, R.T. Libby, C.J. March, D.P. Ceretti, D.L. Urdal, P.J. Conlon, A short polypeptide marker sequence useful for recombinant protein identification and purification, Nat. Biotechnol. 6 (1988) 1204-1210.

[38] A. Urban, S. Neukirchen, K.-E. Jaeger, A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR, Nucleic Acids Res. 25 (1997) 2227-2228.

[39] T. Murasugi, Y. Nakagami, T. Yoshitomi, K. Hirahara, M. Yamashita, Y. Taniguchi, M. Sakaguchi, K. Ito, Oral administration of a T cell epitope inhibits symptoms and reactions of allergic rhinitis in Japanese cedar pollen allergen-sensitized mice, Eur. J. Pharmacol. 510 (2005) 143-148.

[40] K. Okubo, M. Gotoh, S. Fujieda, M. Okano, H. Yoshida, H. Morikawa, K. Masuyama, Y. Okamoto, M. Kobayashi, A randomized double-blind comparative study of sublingual immunotherapy for cedar pollinosis, Allergol. Int. 57 (2008) 265-275.

[41] M. Mizukami, H. Hanagata, A. Miyauchi, *Brevibacillus* expression system: host-vector system for efficient production of secretory proteins, Curr. Pharm. Biotechnol. 11 (2010) 251-258.

[42] Y. Takimura, M. Kato, T. Ohta, H. Yamagata, S. Udaka, Secretion of human interleukin-2 in biologically active form by *Bacillus brevis* directly into culture medium, Biosci. Biotechnol. Biochem. 61 (1997) 1858-1861.

[43] H. Takagi, K. Kadowaki and S. Udaka, Screening and characterization of protein-hyperproducing bacteria without detectable exoprotease activity, Agric. Biol. Chem. 53 (1989) 691-699.

[44] K. Yashiro, J. W. Lowenthal, T. E. O'Neil, S. Ebisu, H. Takagi, High-Level protein production of recombinant chicken interferon-γ by *Brevibacillus choshinensis*, Protein Expr. Purif. 23 (2001) 113-120.

[45] M. Shahhoseini, A.A. Ziaee, N. Ghaemi, Expression and secretion of an alpha-amylase gene from a native strain of *Bacillus licheniformis* in *Escherichia coli* by T7 promoter and putative signal peptide of the gene, J. Appl. Microbiol. 95 (2003) 1250-1254.

[46] M. Shahhoseini, A.A. Ziaee, N. Ghaemi, Expression and secretion of an alpha-amylase gene from a native strain of *Bacillus licheniformis* in *Escherichia coli* by T7 promoter and putative signal peptide of the gene.

[47] D.D. Niu, M. Xu, J.S. Ma, Z.X. Wang, Cloning of the gene encoding a thermostable alpha-amylase from *Bacillus licheniformis* CICIM B0204 and functional identification of its promoter, Wei Sheng Wu Xue Bao 46 (4) (2006) 576-580.

[48] M. Sibakov, High expression of *Bacillus licheniformis* alpha-amylase with a *Bacillus* secretion vector, Eur. J. Biochem. 155 (1986) 577-581.

[49] Q.Z. Peng, W.C. Zhang, H.C. Zhu, The construction of shuttle vectors of *Brevibacillus brevis-Escherichia coli*, Wei Sheng Wu Xue Bao 18 (2002) 438-441.

[50] Y.S. Park, T. Dohjima, M. Okabe, Enhanced alpha-amylase production in recombinant *Bacillus brevis* by fed-batch culture with amino acid control, Biotechnol. Bioeng. 49 (1996) 36-44.

[51] T. Shiroza, Y. Shibata, M. Hayakawa, N. Shinozaki, K. Fukushima, S. Udaka, Y. Abiko, Construction of a chimeric shuttle plasmid via a heterodimer system: secretion of an scFv protein from *Bacillus brevis* cells capable of inhibiting hemagglutination, Biosci. Biotechnol. Biochem. 65 (2001) 389-395.

[52] N. Tsukagoshi, S. Iritani, T. Sasaki, T. Takemura, H. Ihara, Y. Idota, H. Yamagata, S. Udaka, Efficient synthesis and secretion of a thermophilic alpha-amylase by protein-producing *Bacillus brevis* 47 carrying the *Bacillus stearothermophilus* amylase gene, J. Bacteriol. 164 (1985) 1182-1187.

Acknowledgement

I would never have been able to finish my Ph.D study and research without the guidance of my supervisor, Prof. Keiichi Enomoto. I would like to express my sincerest gratitude to him for his excellent guidance, caring and providing me a great atmosphere for doing research during my study at Kochi University of Technology. In the past years, Prof. Enomoto showed great patience to help me improving my knowledge in microbiology, molecular biology and biochemistry. Special thanks to him for all he has done for me not only in my research, but also much help in my life in Japan.

I would like also thank to Associate Prof. Ariga Osamu and Associate Prof. Horisawa Sakae for their constructive comments and suggestions in my research and Prof. Kenzo Sato and Prof. Masaki Sazuka for their kind and excellent review in Ph.D. thesis defense.

I appreciate very much the staff of International Relations Center (IRC) of KUT for their professional work as well as their constant help to all the international students, which have make us easy to live here in Kochi.

I also want to express my thanks to all the students of Enomoto laboratory for their direct and indirect helps during my study.

Finally, I would like to express my gratitude to Kochi University of Technology for giving me such a good opportunity to study in Japan through Special Scholarship Program (SSP).

Appendix

I. Abbreviations

Name	***Abbr.***
Cholera Toxin B subunit	CTB
Allergen from the pollen of *Cryptomeria japonica*	Cry j
Bacillus licheniformis α-amylase	BLA
Immunoglobulin G	IgG
Immunoglobulin E	IgE
Major Histocompatibility Complex	MHC
Human Leukocyte Antigens	HLA
Escherichia coli	*E. coli*
Brevibacillus choshinensis	*B. choshinensis*
Deocyribonucleic Acid	DNA
Polymerase Chain Reaction	PCR
Base Pairs	bp
Kilodalton	kDa
Amino Acid	aa
Luria-Bertani	LB
Isopropyl-β-D-thiogalactopyranoside	IPTG
5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside	X-gal
Hexahistidine	His×6

Phosphate Buffered Saline	PBS
Bovine Serum Albumin	BSA
Polyvinylidene Difluoride	PVDF
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Ampicillin	Amp
Chloramphenicol	Cm
Kanamycin	Km
Neomycin	Nm

II. Reagents preparations

Name of medium, reagent	*Composition*	
LB medium (pH 7.2)	Tryptone	10 g
	Yeast extract	5 g
	NaCl	10 g
	Agar	15 g
	Distilled water	to 1000 ml
MT medium (pH 7.0)	Glucose	10 g
	Polypeptone	10 g
	Meat extract	5 g
	$FeSO_4$. $7H_2O$	10 mg
	$MnSO_4$. $4H_2O$	10 mg
	$MgCl_2$	1 mg
	Distilled water	to 1000 ml
TM medium (pH 7.0)	Glucose	10 g
	Polypeptone	10 g
	Meat extract	5 g
	Yeast extract	2 g
	$MnSO_4$. $4H_2O$	10 mg
	$ZnSO_4$. 7H2O	1 mg
	Distilled water	to 1000 ml

2SY medium	Glucose	20 g
	Bacto soytone (Becton Dickinson)	40 g
	Bacto yeast extract (Bacton Dickinson)	5 g
	$CaCl_2$. $2H_2O$	0.15 g
	Distilled water	to 1000 ml
SOC medium	Bacto tryptone	20 g
	Bacto yeat extract	5 g
	NaCl	0.5 g
	1M $MgSO_4$	10 ml
	1M $MgCl_2$	10 ml
	1M Glucose	20 ml
	Distilled water	to 1000 ml
Ampicillin stock solution 100 mg/ml (filter-sterilized, stored at -20 ^oC)	Ampicillin	5 g
	Distilled water	to 50 ml
Kanamycin stock solution 50 mg/ml (filter-sterilized, stored at -20 ^oC)	Kanamycin sulfate	2.5 g
	Distilled water	to 50 ml
Neomycin stock solution 10 mg/ml (filter-sterilized, stored at -20 ^oC)	Neomycin	0.5 g
	Distilled water	to 50 ml

50×TAE buffer	Tris base	242 g
	Acetic acid	57.1 ml
	EDTA (0.5 M)	100 ml
	Distilled water	to 1000 ml
1×TE buffer	Tris base	10 mM
	EDTA (pH 8.0)	1 mM
2× SDS-PAGE sample buffer	0.5 M Tris-HCl buffer (pH 6.8)	2 ml
	SDS solution (10 %)	4 ml
	B-mercaptoethanol	1.2 ml
	Glycerol	2 ml
	Distilled water	0.8 ml
	Bromophenol blue solution (1 %)	Some drops
10× SDS-PAGE running buffer	Tris base	15.15 g
	Glycine	72 g
	SDS	5 g
	Distilled water	to 500 ml
Destaining solution	Methanol	300 ml
	Acetic acid	100 ml
	Distilled water	600 ml
Staining solution (0.1 %)	Coomassie Blue R-250	0.3 g
	Destaining Solution	to 300 ml

Blotting buffer (stored at -20 ^{o}C)	Tris base	12 g
	Glycine	14.4 g
	Methanol	200 ml
	Distilled water	800 ml
10×TBS buffer, pH 7.6 (stored at -20 ^{o}C)	Tris base	60.6 g
	NaCl	80.6 g
	KCl	2 g
	Distilled water	1000 ml
TBS-T buffer (1 %) (stored at -20 ^{o}C)	Tween 20	1 ml
	1×TBS-T Buffer	1000 ml
Blocking Buffer (1 %)	Skim milk powder	1.5 g
	1×TBS-T buffer	30 ml