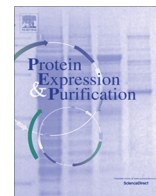




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Expression of recombinant T-cell epitopes of major Japanese cedar pollen allergens fused with cholera toxin B subunit in *Escherichia coli*

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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, cry j 1 epi and cry j 2 epi were linked and then fused to the CTB gene in tandem to construct a fusion gene, ctb-linker-cry j 1 epi- cry j 2 epi-flag. The fusion gene was introduced into a pET-28a(+) vector and expressed in *Escherichia coli* BL21(DE3). 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 protein also proved to be antigenic against anti-Cry j 1 and anti-Cry j 2 antibodies. Expression of the recombinant protein induced with 1 mM IPTG reached a maximum in 3–5 h, and recovery of the affinity-purified recombinant protein was approximately 120 mg/L of culture medium. The present study indicates that production of sufficient amounts of recombinant protein with antigenic epitopes may be possible by recombinant techniques using *E. coli* or other bacterial strains for protein expression.

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Introduction

Japanese cedar (*Cryptomeria japonica*; CJ²) 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]; therefore, CJ pollinosis is considered to be a national affliction. 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, which lack epitopes reactive to IgE. The application of peptides containing only T-cell epitopes that induce T-cell tolerance is a safe treatment strategy to control allergies [9,10]. However, a major obstacle to this approach is the diversity of major histocompatibility complex (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

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² Abbreviations used: CJ, *Cryptomeria japonica*; CTB, cholera toxin B subunit; MHC, major histocompatibility complex; HLA, human leukocyte antigen; IPTG, isopropyl β-D-1-thiogalactopyranoside.

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 B subunit (CTB) has been used as an efficient carrier molecule to generate mucosal immune responses and induce T-cell tolerance to antigens via linkage [22–24]. CTB binds with high affinity to the ganglioside GM₁, which is found in membrane microdomains on the plasma membrane of host cells, and is able to cluster five GM₁ molecules at once [25]. Owing to this property, CTB can be employed as an adjuvant and transporter for effective delivery of antigens as a mucosal vaccine with reduced toxicity and high efficacy [24]. In this study, genes encoding major T-cell epitopes from Cry j 1 and Cry j 2 were fused to the CTB gene (*ctb*) in tandem by overlap extension PCR. Then, the fusion gene was inserted 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.

Materials and methods

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 isopropyl β-D-1-thiogalactopyranoside (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).

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

Primer	Position in Cry j antigen	Sequence* (5'–3')
P1	Cry j 1-16-30	CGATCCAAAGCCACCGCGCAATCCGCCAGTTTCATACGGTTCTG ATTGCCATACTAATTGCGG
P2	Cry j 1-81-95	ACGGCCGTCGAACGTTTTATAGCCCGCAATATACATCGGCATTTT CGATCCAAAGCCAC
P3	Cry j 1-106-125-1	GTTGCTCACACGTTTAATGAACACGCACGG ACGGCCGTCGAACGTTTTAT
P4	Cry j 1-106-125-2	GCCATACAGATGCAGCCATGAATAATCAG TGTGCTCACACGTTAATGA
P5	Cry j 1-212-224	CGGGCCGAAGTGGTGAACGCCACCGTCACCTTCATGCT GCCATACAGATGCAGGCCAT
P'1	Cry j 2-68-80	TTTCCAGCTCGCCGGTCTTGATACGCCGAATAATGCC ATTGCCATACTAATTGCGG
P'2	Cry j 2-87-98	GCCATCAGCGTGAAGCCCGTCAGTTTCGCGAAGT TTTCCAGCTCGCCGGTCT
P'3	Cry j 2-182-200-1	ATGGAAGTTTTGCTCGCGAAAATATCAAT GCCATCAGCGTGAAGCCCG
P'4	Cry j 2-182-200-2	GCCCGTGCCAATCGTGTTTTCTG CAGATGGAAGTTTTGCTCGCGA
P'5	Cry j 2-236-250	GAATTCGCGCGGTTACATGCACATAGCTCACTTCCGACGGCT GCCCGTGCCAATCGT
P'6	Cry j 2-336-365-1	GCTCGTCAAGTTTACAGGCTAATATCGCTCAGTTTAATATCTT GAGAAATTTGCGCCGCTT
P'7	Cry j 2-336-365-2	GAAATAGCCGTTCGCGTTATCGTTCAGGCAGCTCGCAATTT GCCGCTCGTCAGTTTCAG

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

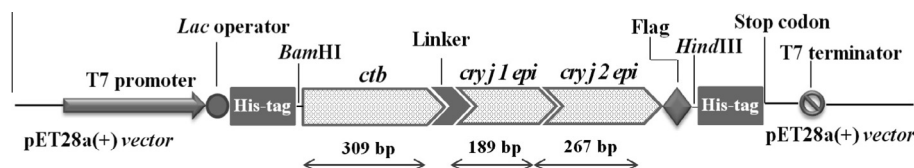


Fig. 1. Schematic diagram of the fusion gene in the pET28a(+) vector.

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 forward primer *ctb*-F (5'-ACACCTCAAAATATTACTGATTGT-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*. The sequences and reaction order of the reverse primers are shown in Table 1. The reverse primers were designed based on codon usage in *E. coli* (<http://www.kazusa.or.jp/codon>, Nov. 29 2014). DNA constructs *cry j 1 epi* and *cry j 2 epi* amplified by PCR were employed to construct the fusion gene. All PCRs were performed with fidelity KOD-Plus DNA polymerase (Toyobo, Osaka, Japan).

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. 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 [26] as depicted in Fig. 2. Primers used for the construction are shown in Fig. 2 and Table 2.

First, the *BamHI* site was linked to the 5' end of the *ctb* gene. Then, the *linker* and 5' end sequence of *cry j 1 epi* (overlap *cry j 1 epi*) were also linked to the 3' end of the *ctb* gene (Fig. 2A). Second, the 5' end sequence of *cry j 2 epi* (overlap *cry j 2 epi*) was added to the 3' end of the *cry j 1 epi* gene (Fig. 2B). Third, the 3' end sequence of *cry j 1 epi* (overlap *cry j 1 epi*) was linked to the 5' end of the *cry j 2 epi* gene, and then the sequence of *flag-HindIII* was linked to the 3' end of the *cry j 2 epi* gene by stepwise PCR (Fig. 2C).

Finally, the three DNA constructs shown in Fig. 2 were linked by overlap extension PCR to construct the fusion gene *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII*. First, the two PCR products

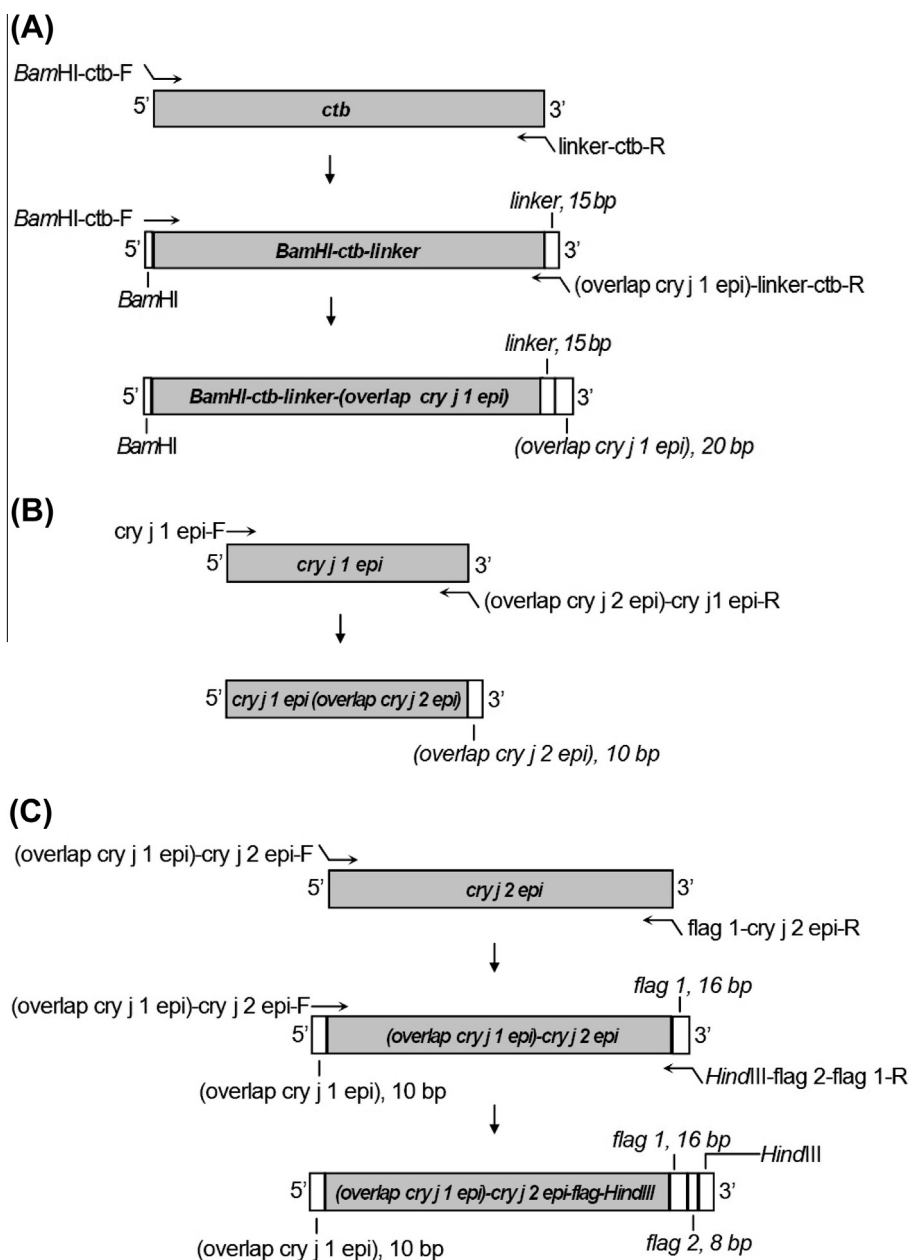


Fig. 2. Construction of DNA templates for overlap extension PCR. Three DNA constructs for overlap extension PCR were prepared as described in the Materials and Methods. (A) Construction of *BamHI-ctb-linker-(overlap cry j 1 epi)*. (B) Construction of *cry j 1 epi-(overlap cry j 2 epi)*. (C) Construction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

Table 2
Primers used to construct the fusion gene *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII*.

Primer	Sequence (5'–3')	Bold letters
<i>BamHI-ctb-F</i>	GCGGGATCCACACCTCAAATATTACTGAT	<i>BamHI</i>
<i>Linker-ctb-R</i>	TCCGCTACCTCCGCCATTGGCATACTAATTGCGG	<i>linker</i>
<i>(overlap cry j 1 epi)-linker-ctb-R</i>	GCCAGTTTCATACGGTTCTGTCCGCTACCTCCGCCATTG	<i>overlap cry j 1 epi</i>
<i>Cry j1 epi-F</i>	CAGAACCGTATGAAACTGGCGGATT	
<i>(overlap cry j 2 epi)-cry j 1 epi-R</i>	CAATAATGCCCGGCCGAACCTGGTGAACG	<i>overlap cry j 2 epi</i>
<i>(overlap cry j 1 epi)-cry j 2 epi-F</i>	GTTCCGGCCCGGCATTATTGCGGCGTATCA	<i>overlap cry j 1 epi</i>
<i>Flag 1-cry j 2 epi-R</i>	CGTCGCTTTGTAGTCGAAATAGCCGTTCGCGTTA	<i>flag 1</i>
<i>HindIII-flag 2-flag 1-R</i>	GCGAAGCTTCTTGTCATCGTCGCTTTGTAGTC	<i>HindIII flag 2 (in italics)</i>

shown in Fig. 2A and B were mixed and subjected to an overlap-
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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
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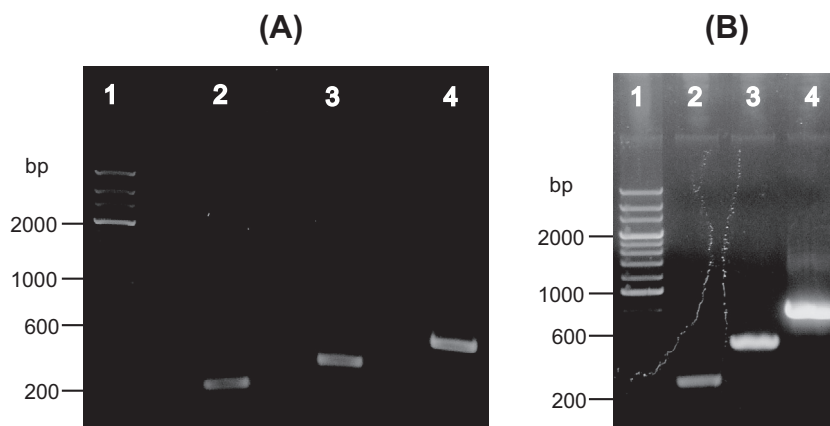


Fig. 3. Electrophoretic analysis of products acquired from overlap extension PCR. (A) PCR products in the construction of *BamHI-ctb-linker-cry j 1 epi-(overlap cry j 2 epi)*: Lane 1, 200 bp marker; Lane 2, *cry j 1 epi-(overlap cry j 2 epi)* (199 bp); Lane 3, *BamHI-ctb-linker-(overlap cry j 1 epi)* (353 bp); Lane 4, *BamHI-ctb-linker-cry j 1 epi-(overlap cry j 2 epi)* (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 j 1 epi-(overlap cry j 2 epi)* (532 bp); Lane 4, the final product of *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* (822 bp).

reaction was carried out in the same manner to generate *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* (Fig. 3B).

Construction of the recombinant plasmid for *E. coli*

The pET28a(+) plasmid was used as an expression vector that provides two 6 × His-tag coding sequences at both N- and C-termini for one-step purification of expressed proteins. The fusion gene *BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* was 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. 1). The expression vector was constructed in *E. coli* strain JM109 and then transformed into *E. coli* strain BL21(DE3) for expression. DNA sequencing of the insert in the pET28a(+) vector was performed using the dye-terminator method by Bio Matrix Research (Nagareyama, Japan).

SDS–polyacrylamide gel electrophoresis (PAGE) and western blotting

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

Proteins resolved by SDS–PAGE were electroblotted 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 (Sigma–Aldrich Japan, Tokyo, Japan), goat anti-CTB antibody (List Biological Laboratories, Campbell, CA, USA), and rabbit anti-Cry j 1 and anti-Cry j 2 antibodies (Hayashibara, Okayama, Japan) were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology Japan, Tokyo, Japan) and horseradish peroxidase-conjugated anti-goat IgG (R & D Systems, Minneapolis, MN, USA) were used as secondary antibodies. Signals were detected by ECL detection reagents (GE Healthcare Japan). Cry j 1 and Cry j 2 antigens from Japanese cedar pollen were purchased from Hayashibara.

Expression and purification of antigen peptides

A single 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 OD₆₀₀ 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 determine the optimal conditions for protein expression. Cells were harvested by centrifugation, and bacterial pellets were lysed with HisTALON xTractor buffer containing DNase I 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.

Results

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 requiring 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 (human leukocyte antigen (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 3, 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. 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 3

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)		2
	346–360	DQ6	67 (28)	Cry j 2-336-365	
	351–365	ND*	61 (22)		

* Not determined.

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

TPQNITDLCAEYHNTQIHTLNDKIFSYESLAGKREMAITFKNGATFQVEVPGSQHIDSQKKAIE
RMKDTLRIAYLLEAKVEKLCVWNNKTPHAI AISMANGGGSGQNRMKLADCAVGFGS--KMPP
YIAGYKTFDGR--PCVFIKRVSNVHGLHLYG--SMKVTVAFNQFGP--GIIAAYQNPASWK--QFAKLT
GFTLMG--IDIFASKNFHLQKNTIGTG--SRAEVSYSYVHNGAKF--CKDIKLSDISLKLTSGLIASCLN
DNANGYFDYKDDDDK

Fig. 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, Cry j 2-236-250, Cry j 2-336-365, and Flag.

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. 1) was constructed by overlap extension PCR as described in the Materials and methods. To provide structural flexibility, a linker of 15 bp in length (aa sequence: GGGSG) [27,28] 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) [29] 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 sizes of resultant PCR products visualized on the agarose gel were in good agreement with the calculated sizes (Fig. 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 correctly orientated in the expression plasmid (Fig. 4). The nucleotide sequence has been deposited in the DDBJ under accession No. **AB978369**.

Expression and purification of the fusion antigen peptide

Next, we induced expression of the recombinant fusion antigen peptide in *E. coli* BL21(DE3) harboring the recombinant pET28a(+) vector. Although the DNA construct for the recombinant protein did not have a His-tag sequence for purification by an affinity column, pET28a(+) contains His-tag sequences in its multi-cloning site. Therefore, we expected that the recombinant protein was expressed as a peptide with His-tags at both N- and C-termini.

To achieve optimal expression of the recombinant protein, we examined conditions including the cell density for induction, IPTG

concentration, and growth phase for expression. The selected clone was cultured in LB medium with kanamycin at 37 °C until the OD₆₀₀ reached ~1 which was the most suitable cell density for expression. Protein expression was then induced with 1 mM IPTG, and the culture for expression was continued at 27 °C rather than 37 °C to prevent denaturation and degradation of the expressed protein. Bacterial growth and expression of the recombinant protein were monitored during incubation up to 20 h.

SDS-PAGE was carried out to analyze the expressed recombinant protein after 3 h of incubation with IPTG. As shown in Fig. 5A, a protein band corresponding to the expected mass (34.65 kDa) was detected in *E. coli* harboring the recombinant pET28a(+) vector (Lane a-1, Fig. 5A) but not in *E. coli* containing pET28a(+) without the DNA insert (Lane b-1, Fig. 5A). The expressed protein with high homogeneity was obtained from eluted fractions on the His-tag affinity column (Lanes a-2 and a-3, Fig. 5A).

Fig. 5B shows that the expression level of the fusion protein after 3 h of incubation did not depend on IPTG concentrations ranging from 0.1 to 3 mM. The time course of fusion protein production was examined further. As shown in Fig. 6, expression of the fusion protein increased markedly and reached a maximum in 3–5 h followed by a gradual decrease. An estimated expression level of >120 mg/L of culture medium was achieved by addition of 1 mM IPTG to the culture that had been incubated for 4 h at 37 °C (at OD₆₀₀ ~1).

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. 7A) and anti-CTB antibodies (Fig. 7B). This result demonstrated that the recombinant

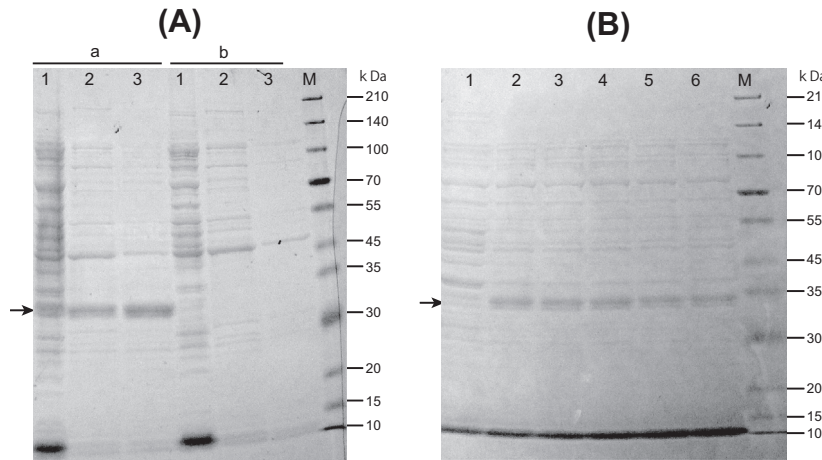


Fig. 5. SDS-PAGE analysis and purification of CTB-Linker-Cry j 1 epi-Cry j 2 epi-Flag. (A) Expression and purification of the recombinant antigen peptide: (a) *E. coli* BL21(DE3) harboring pET28a-BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII; (b) Negative control harboring pET28a(+) without the DNA insert. Lane 1, total intracellular protein; Lane 2, eluted fraction I from the His-tag column; 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, the culture was continued for 3 h at 27 °C. Arrows show the position of the recombinant peptide.

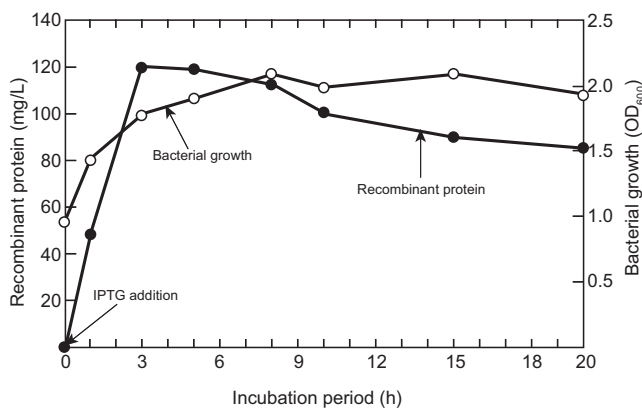


Fig. 6. Time course of recombinant protein production. *E. coli* BL21(DE3) harboring pET28a-BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII was inoculated at a ratio of 1:100 (vol: vol) into 60 ml LB medium and incubated at 37 °C for about 4 h up to an OD₆₀₀ value of 1. Then, protein expression 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.

antigen peptide Cry j 1 epi-Cry j 2 epi flanked by CTB and Flag was successfully expressed in *E. coli* BL21(DE3).

To examine the antigenicity of Cry j 1 and Cry j 2 epitopes in the recombinant peptide, western blotting was conducted using rabbit polyclonal anti-Cry j 1 and anti-Cry j 2 antibodies. Fig. 8 shows that the purified recombinant protein reacted against both anti-Cry j 1 and anti-Cry j 2 antibodies. Weak cross-reactivity was observed between the authentic Cry j 1 antigen and anti-Cry j 2 antibody (Lane 1, Fig. 8B). In addition, a band of ~45 kDa, which reacted with the anti-Cry j 1 antibody, was observed in the purified protein fractions from the His-tag column (Fig. 8A). However, this result was considered to be a false positive band because the band was also found in the His-tag column fractions from *E. coli* containing pET28a(+) without the DNA insert (Lane 8, Fig. 8A).

Discussion

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 [17,30,31]. 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

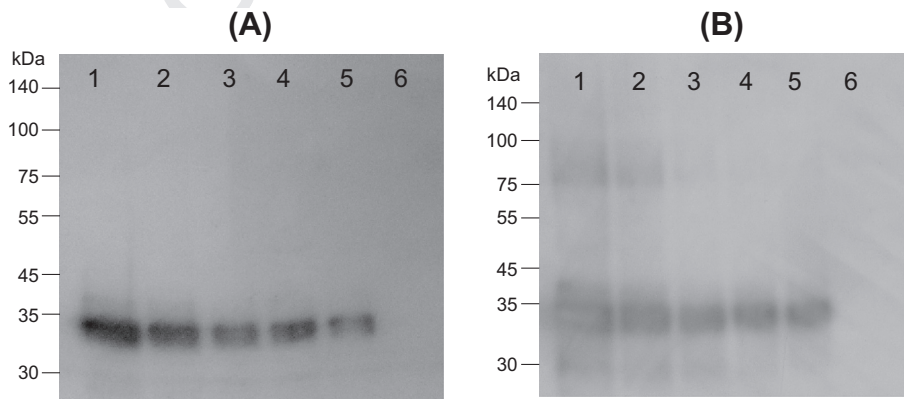


Fig. 7. Western blot analysis of the purified antigen peptide. (A) Western blot with an anti-Flag antibody. (B) Western blot with an anti-CTB antibody. Lanes 1–5, purified CTB-Linker-Cry j 1 epi- Cry j 2 epi-Flag (34.65 kDa). Lanes contained 2.5, 1.25, 0.85, 0.63, and 0.5 µg of the purified protein, respectively; Lane 6, His-tag column fraction from *E. coli* harboring pET28a(+) without the DNA insert (negative control).

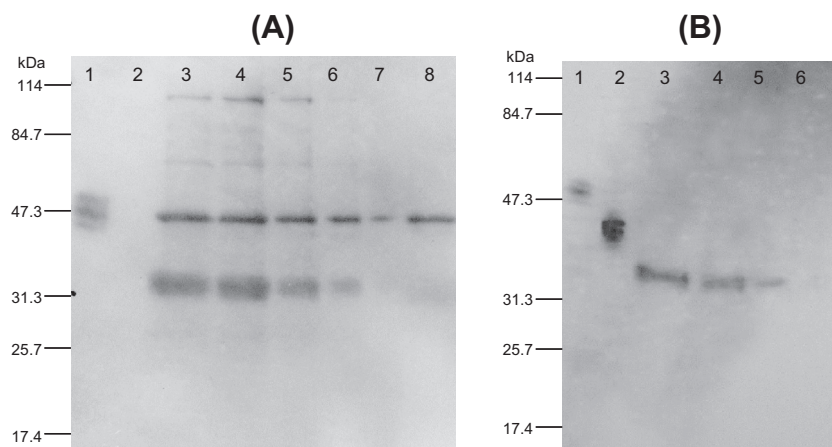


Fig. 8. Antigenicity of epitopes in the purified antigen peptide. (A) Western blot with the anti-Cry j 1 antibody. Lane 1, Cry j 1 antigen (0.075 μ g); Lane 2, Cry j 2 antigen (0.075 μ g); Lanes 3–7, purified recombinant protein (6.6, 4.9, 3.3, 1.6, and 0.33 μ g of the purified protein, respectively); Lane 8, His-tag column fraction from *E. coli* harboring pET28a(+) without the DNA insert (negative control). (B) Western blot with the anti-Cry j 2 antibody. Lane 1, Cry j 1 antigen (0.075 μ g); Lane 2, Cry j 2 antigen (0.075 μ g); Lanes 3–6, purified recombinant protein (1.44, 0.72, 0.36, and 0.12 μ g of the purified protein, respectively).

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 [32].

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 [33,34]. 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 this study, up to four major T-cell epitopes from Cry j 1 and six from Cry j 2 were selected based on previous studies. To apply CTB as an adjuvant and carrier, we designed a genetically engineered fusion peptide, CTB-Linker-Cry j 1 epi-Cry j 2 epi-Flag. Genes encoding epitopes from Cry j 1 and Cry j 2 were fused to *ctb* in tandem by PCR. The fusion gene CTB-Linker-Cry j 1 epi-Cry j 2 epi-Flag expressed in *E. coli* was purified to high homogeneity with a His-tag affinity column, providing approximately 120 mg/L of the recombinant protein in the culture. Reactivities to anti-CTB and anti-FLAG antibodies were confirmed by western blot analysis. The present study also demonstrated that the epitope domain of the recombinant protein retained antigenicity against anti-Cry j 1 and anti-Cry j 2 antibodies. A false band of ~45 kDa, which cross-reacted with the anti-Cry j 1 antibody, is considered to be a protein from the *E. coli* host because the negative control (*E. coli* containing pET28a(+)) without the insert also contained this protein band.

It is not yet known which effects the His-tag and FLAG tag have on the antigenicity of Cry j epitopes. Although we cannot exclude the possibility that these tags interrupt the folding of the antigen peptide through steric hindrance, the tags appeared to exert only a small effect on the antigenic properties of the epitopes. This is probably because the epitopes in the expressed protein are linear, in which the amino acid sequences are recognized by immune cells, and not conformational epitopes in which the conformation of each epitope is crucial for the antigenic properties. In addition, it is expected that each epitope would be presented to immune cells after the antigen peptide is digested into short peptide fragments, thereby removing any inhibitory effects of the tags. In fact, as shown in Fig. 8, western blotting with anti-Cry j 1 and anti-Cry j 2 antibodies indicated that the antigenicity of the epitopes was retained in the recombinant peptide even in the presence of His-tags and the FLAG tag. Although we confirmed the antigenicity of the epitopes by western blotting, enzyme-linked immunosorbent

assay (ELISA) using antibodies raised against the epitopes will be needed in the subsequent preclinical research to assay antigenicity selectively and quantitatively.

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 [35,36]. 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.

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