Protein Expression and Purification 109 (2015) 62–69

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

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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ARTICLE INFO

Article history:
Received 10 December 2014
and in revised form 29 January 2015
Available online 7 February 2015

Keywords:
Japanese cedar pollen
T-cell epitope
Cry j 1
Cry j 2
Cholera toxin B subunit
Pollinosis

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.

Introduction

Japanese cedar (*Cryptomeria japonica*; CJ[2]) 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 designed

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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.

http://dx.doi.org/10.1016/j.pep.2015.02.001
1046-5928/

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 GM_1, which is found in membrane microdomains on the plasma membrane of host cells, and is able to cluster five GM_1 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).

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 cholerae* 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′-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*. 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

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	CGATCCAAAGCCCACCGCGCAATCCGCCAGTTTCATACGGTTCTG***ATTTGCCATACTAATTGCGG***
P2	Cry j 1-81-95	ACGGCCGTCGAACGTTTTATAGCCCGCAATATACATCGGCATTTT***CGATCCAAAGCCCAC***
P3	Cry j 1-106-125-1	GTTGCTCACACGTTTAATGAACACGCACGG***ACGGCCGTCGAACGTTTTAT***
P4	Cry j 1-106-125-2	GCCATACAGATGCAGGCCATGAATAATCAC***GTTGCTCACACGTTTAATGA***
P5	Cry j 1-212-224	CGGGCCGAACTGGTTGAACGCCACCGTCACTTTCATGCT***GCCATACAGATGCAGGCCAT***
P′1	Cry j 2-68-80	TTTCCAGCTCGCCGGGTTCTGATACGCCGCAATAATGCC***ATTTGCCATACTAATTGCGG***
P′2	Cry j 2-87-98	GCCCATCAGCGTGAAGCCCGTCAGTTTCGCGAACTG***TTTCCAGCTCGCCGGGTTCT***
P′3	Cry j 2-182-200-1	ATGGAAGTTTTTGCTCGCGAAAATATCAAT***GCCCATCAGCGTGAAGCCCG***
P′4	Cry j 2-182-200-2	GCCCGTGCCAATCGTGTTTTTCTGCAG***ATGGAAGTTTTTGCTCGCGA***
P′5	Cry j 2-236-250	GAATTTCGCGCCGTTCACATGCACATAGCTCACTTCCGCACGGCT***GCCCGTGCCAATCGT***
P′6	Cry j 2-336-365-1	GCTCGTCAGTTTCAGGCTAATATCGCTCAGTTTAATATCTTTGCA***GAATTTCGCGCCGTT***
P′7	Cry j 2-336-365-2	GAAATAGCCGTTCGCGTTATCGTTCAGGCAGCTCGCAATTTTGCC***GCTCGTCAGTTTCAG***

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

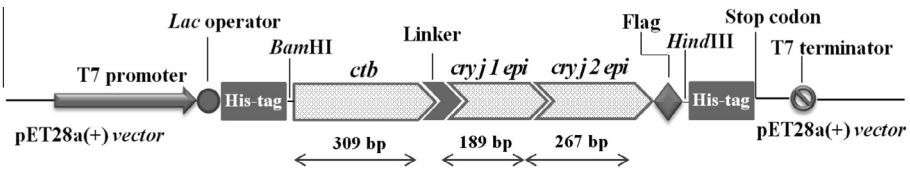

Fig. 1. Schematic diagram of the fusion gene in the pET28a(+) vector.