

## Large-Scale Production of Major House Dust Mite Allergen Der f 2 Mutant (C8/119S) in *Escherichia coli*

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**Hyposensitization, in which causative antigens of allergic diseases are injected, is the sole means of a radical cure for allergic diseases. Since the therapeutic allergens currently used are naturally extracted, producing preparations with a stable titer from such extracts is extremely difficult. There are several reports on the expression of recombinant mite allergens in *Escherichia coli* using inducers. The use of an inducer for industrial production will lead to high costs and, for therapeutic use, it must be removed in the purification process. C8/119S is a mutant of Der f 2, a major house dust mite allergen. The C8/119S gene was integrated downstream of the trp promoter to produce the expression plasmid (pWU11-C8/119S). Then, this expression plasmid was used to transform *E. coli* strain HB101 (pWU11-C8/119S/HB101). A recombinant *E. coli* pWU11-C8/119S/HB101 did not express C8/119S in a low-temperature culture (32°C), but C8/119S was induced to a high level of expression in a high-temperature culture (37°C). pWU11-C8/119S/HB101 proliferated when expression was induced by high temperature and an approximately 3-fold greater proliferation was obtained compared with the use of an inducer in a large-scale culture. The C8/119S protein was expressed as inclusion bodies and obtained by refolding and chromatography purifications. The immunological properties of C8/119S were assessed by western blotting. Western blotting demonstrated that purified C8/119S reacted with a monoclonal anti-Der f 2 antibody (18G8). pWU11-C8/119S/HB101 can be used as an easy, low cost expression system on a large scale. It is also advantageous for industrial production in that the addition of an inducer is not required to achieve expression of the mite allergen.**

[**Key words:** mite allergen, Der f 2, C8/119S, fermentation, *Escherichia coli*, thermal induction]

The house dust mite *Dermatophagoides farinae* is a major causative factor of various allergic diseases, such as allergic asthma, allergic rhinitis, allergic dermatitis, and allergic conjunctivitis (1). To treat allergic diseases, anti-allergic or anti-histaminic agents are generally used. This therapy, however, since it is symptomatic, is unable to radically cure allergic diseases. Hyposensitization, in which causative antigens of allergic diseases are injected, is believed to be the sole means of radical cure for allergic diseases. Hyposensitization is performed by injecting a small amount of causative allergens into a patient suffering from allergic disease to eliminate allergic reactions within the patient. The efficacy of this therapy using mite allergens is greater than 70% (2–6), but it involves the risk of inducing anaphylactic shock, a serious adverse reaction.

The therapeutic allergens currently used for this purpose are naturally extracted. Allergen preparations for use in the treatment of mite allergic diseases have been prepared from extracts of house dust mites. Extracts of house dust mites, however, contain not only major allergens but also very many

contaminants. Production of preparations with a stable titer from such extracts would thus be extremely difficult and there is always a risk of anaphylactic shock. Such preparations are also problematic in that their level of production is limited since their starting material is derived from a natural source.

In recent years, with the progress in the study of mite allergens, several major mite allergens (Der f 1, Der f 2, Der p 1, Der p 2, etc.) have already been identified (7–10) and the modification of major mite allergens aimed at the reducing anaphylactic shock has been performed (11–16).

C8/119S (14) is a mutant of Der f 2, a major house dust mite allergen. C8/119S is intended to reduce binding to IgE in atopic patients. It is expected that C8/119S has low risk of anaphylactic shock. A method for production of C8/119S has been reported, wherein *Escherichia coli* is transformed with an expression vector containing a mutant of Der f 2 in which two cysteine residues (Cys8, Cys119) in natural Der f 2 are replaced by serine residues, and the resulting transformants are cultured so that C8/119S is produced from the culture. However, even in that method, isopropyl-1-thio- $\beta$ -D-galactoside (IPTG) is used as an inducer for the expression of C8/119S, and approaches using an inducer are not prefera-

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(Funakoshi), and electrophoresis was performed under both non-reducing and reducing (with 2-mercaptoethanol) conditions. The gel was immersed in a transfer electrophoresis buffer (10 mM CAPS containing 10% methanol) for 5 min, and proteins were transferred to a polyvinylidene fluoride (PVDF; Millipore, Billerica, MA, USA) membrane using a transfer/Western electrophoresis transcription apparatus. The transferred PVDF membrane was soaked in 10 mM Tris buffer containing 2% bovine serum albumin, 0.1% Tween 20, and 0.5 M sodium chloride at 37°C for 2 h for blocking. Subsequently, 5 µg/ml of monoclonal anti-Der f 2 antibody (18G8) (18, 19) was added and reacted for 2 h at 37°C. After washing 5 times in 10 mM Tris buffer containing 0.1% Tween 20 and 0.5 M sodium chloride (TNT buffer), the PVDF membrane was immersed in HRP-labeled anti-mouse IgG antibody (Chemicon, Temecula, CA, USA) solution, and reacted for 2 h at 37°C. After washing 5 times in the TNT buffer, the C8/119S protein was detected by the addition of 0.05% 3,3-diaminobenzidine (DAB; Wako Pure Chemical Industries) solution.

**Gel filtration chromatography analysis** Purified C8/119S was analyzed by high performance liquid chromatography (HPLC) with a gel filtration column (G3000SW<sub>XL</sub>; Tosoh, Tokyo). Analytical conditions were as follows: mobile phase was 60% acetonitrile containing 0.1% TFA, flow rate was 1 ml/min, analysis time was 20 min, sample concentration was approximately 500 µg/ml, and the sample volume was 100 µl.

**Peptide mapping** Samples were diluted with a Tris-HCl buffer (pH 8.5) to obtain a concentration of 150 µg/ml and then treated with lysyl-endopeptidase (Wako Pure Chemical Industries). The resulting digested solution was directly used for analysis under non-reduced conditions. For analysis under reduced conditions, the solution was treated with the addition of dithiothreitol (DTT) (Wako Pure Chemical Industries). Digested peptides were analyzed by HPLC with a reverse-phase column (Vydac protein & peptide C18; Grace-Vydac, Columbia, MO, USA).

## RESULTS AND DISCUSSION

**Construction of recombinant *E. coli*** In order to select a suitable expression system for production on an industrial scale, two expression plasmids were constructed (Fig. 2A, B). Using these expression plasmids, four *E. coli* strains were transformed into eight types of production strains. Then the production strains were cultured at 32°C and 37°C to detect those exhibiting efficient thermal induction. At a temperature of 32°C, all of these strains expressed C8/119S at very low levels (Fig. 3A). During culture at 37°C, however, C8/119S was strongly expressed by strains JM109, HB101, and TB1 which were all transformed with the pWU11-C8/119S expression vector, in which the gene encoding C8/119S was located downstream of the *trp* promoter (Fig. 3B).

It is known that genes were expressed even when they were not induced in some expression system (20). But the LE392 strain has a *Trp* R<sup>55</sup> genotype and expresses the *trp* repressor, so the expression of C8/119S in the transformed LE392 was suppressed by the *trp* repressor. The other three strains JM109, HB101, and TB1 do not have the *Trp* R<sup>55</sup> genotype and could not express the *trp* repressor. Therefore, the strains JM109, HB101, and TB1 could express C8/119S without a chemical inducer, since these strains did not suppress expression of C8/119S at the high temperature where the biosynthesis activity level was high.

**Large-scale aerobic culture** We selected the pWU11-

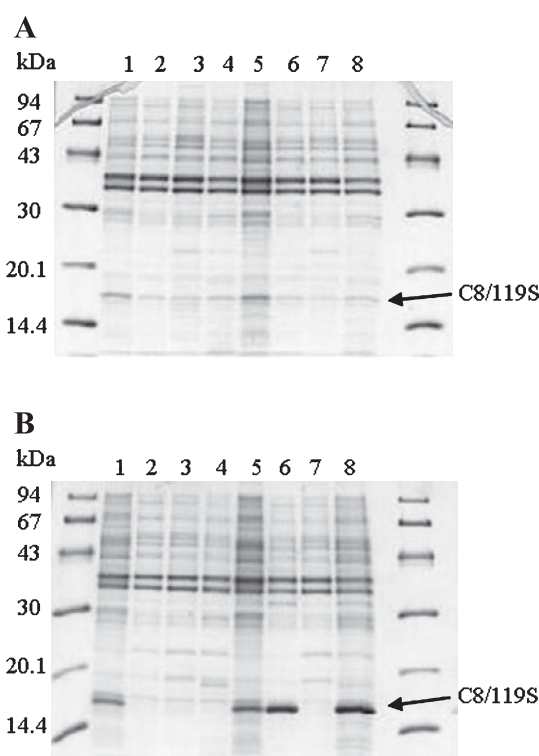


FIG. 3. (A) SDS-polyacrylamide gel electrophoresis of sonicated recombinant *E. coli* cells cultured at 32°C in the absence of inducers. Lane 1, pFLU11-C8/119S/JM109; lane 2, pFLU11-C8/119S/HB101; lane 3, pFLU11-C8/119S/LE392; lane 4, pFLU11-C8/119S/TB1; lane 5, pWU11-C8/119S/JM109; lane 6, pWU11-C8/119S/HB101; lane 7, pWU11-C8/119S/LE392; lane 8, pWU11-C8/119S/TB1. (B) SDS-polyacrylamide gel electrophoresis of sonicated recombinant *E. coli* cells cultured at 37°C in the absence of inducers. Lane 1, pFLU11-C8/119S/JM109; lane 2, pFLU11-C8/119S/HB101; lane 3, pFLU11-C8/119S/LE392; lane 4, pFLU11-C8/119S/TB1; lane 5, pWU11-C8/119S/JM109; lane 6, pWU11-C8/119S/HB101; lane 7, pWU11-C8/119S/LE392; lane 8, pWU11-C8/119S/TB1.

C8/119S/HB101 production strain, which is a combination of the *trp* promoter and HB101, and cultured it in a 300-l fermentor. Culture was stopped 12 h after the thermal induction of expression. At that time, turbidity exceeded 100 (Fig. 4A). Expression of C8/119S reached a level that could definitely be identified by electrophoresis at 3 h after increase in culture temperature. In addition, the amount of C8/119S expressed per *E. coli* cell was found to increase during the culture period (Fig. 4B). On the other hand, when expression was induced by the addition of IAA, final turbidity was approximately 30, and expression of C8/119S was identified by electrophoresis at 5 h after IAA induction (Fig. 4C).

### Purification and characterization of C8/119S

C8/119S accumulated in *E. coli* cells as inclusion bodies. *E. coli* were collected from the culture medium after repeated dilution and concentration using a micro filter (MF) (Asahi Kasei, Tokyo), after which the cells were lysed to recover the inclusion bodies. The amount of inclusion bodies recovered was 5–10 g/l in terms of wet weight.

These inclusion bodies were denatured with 8 M urea and dissolved, after which refolding was performed following re-

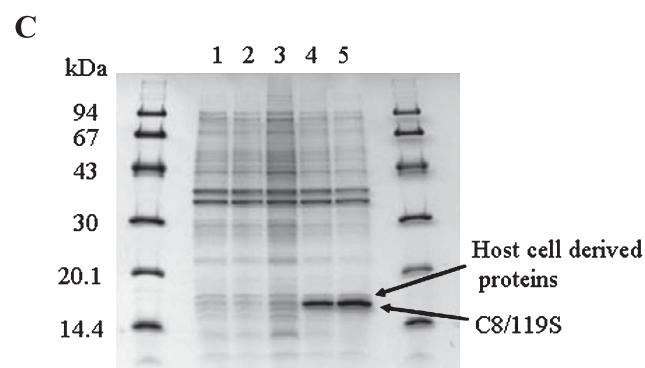
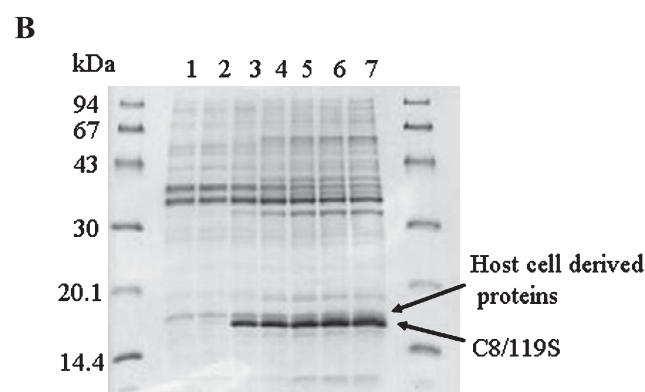
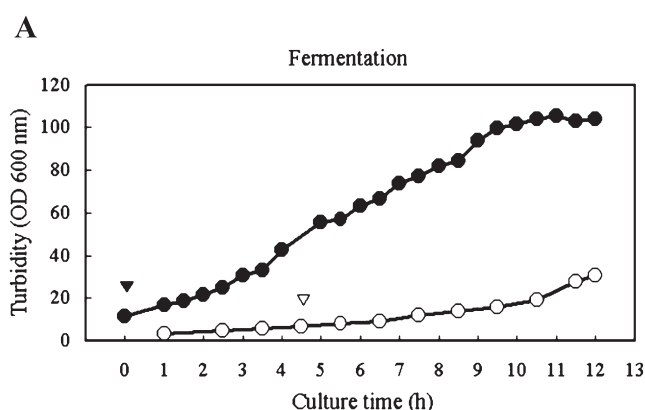


FIG. 4. (A) Proliferation curve of cells cultured in a 300-l scale fermentor. Closed circles show the proliferation curve of recombinant *E. coli* (pWU11-C8/119S/HB101) cultured at 37°C in the absence of an inducer (IAA). Open circles show the same recombinant cultured at 32°C with inducer. Closed and open triangles show the respective starting points of induction. (B) SDS-polyacrylamide gel electrophoresis of sonicate of recombinant *E. coli* cells cultured in a 300-l scale fermentor, a sample of which was tested with the passage of time. Lane 1, Before shifting to 37°C; lane 2, 1 h after temperature shift; lane 3, 3 h after temperature shift; lane 4, 5 h after temperature shift; lane 5, 7 h after temperature shift; lane 6, 9 h after temperature shift; lane 7, 12 h after temperature shift. (C) SDS-polyacrylamide gel electrophoresis of sonicate of recombinant *E. coli* cells cultured in a 300-l scale fermentor, a sample of which was tested with the passage of time. Lane 1, Before IAA induction; lane 2, 1 h after IAA induction; lane 3, 3 h after IAA induction; lane 4, 5 h after IAA induction; lane 5, 7.5 h after IAA induction.

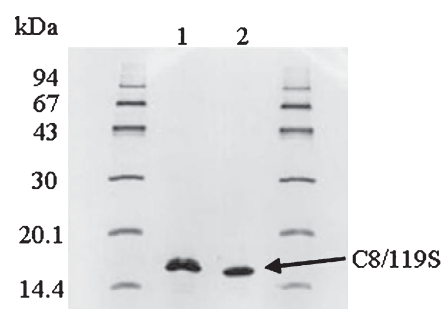


FIG. 5. SDS-polyacrylamide gel electrophoresis. Lane 1, Non-reductive treatment of a purified mutant major house dust mite allergen; lane 2, reductive treatment of a purified mutant major house dust mite allergen. The gel was silver-stained.

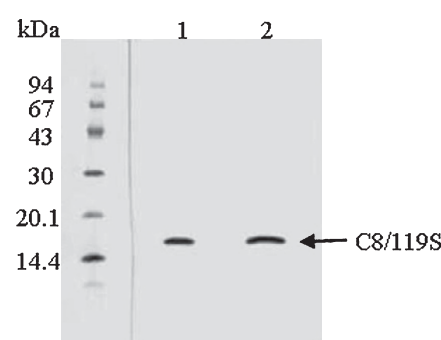


FIG. 6. Western blot analysis using anti-Der f 2 monoclonal antibody. Lane 1, Non-reductive treatment of a purified mutant major house dust mite allergen; lane 2, reductive treatment of a purified mutant major house dust mite allergen.

removal of the denaturing agent. After refolding, C8/119S was purified by anion-exchange chromatography, hydrophobic chromatography, and further anion-exchange chromatography. Purified C8/119S was displayed as a single band with a molecular weight of about 15 kDa in the SDS-PAGE analysis (Fig. 5). Reactivity of the purified C8/119S to the monoclonal anti-Der f 2 antibody 18G8 was demonstrated by Western blotting (Fig. 6). It was shown that 18G8 binds to C8/119S. The results of gel filtration chromatography analysis are shown in Fig. 7. The purity of the C8/119S monomer was over 99%.

The Der f 2 molecule has three disulfide bonds (21), while C8/119S is a mutant that lacks the Cys8-Cys119 disulfide bond. Therefore, the C8/119S molecule has two disulfide bonds (Fig. 1). In order to confirm that C8/119S had the intended disulfide bond structure, a peptide map analysis was conducted. A comparison of peptide map patterns under reduced and non-reduced conditions demonstrated two peaks of disulfide bond-related peptide fragments, peaks A and B (Fig. 8). These peaks were fractionated and identified by amino acid sequencing. The sequences of peaks A and B are shown in Fig. 9. We confirmed that C8/119S had the intended disulfide bond structure.

**Conclusion** In general, it is necessary to add inducers to express a target protein in *E. coli*. Such inducers have biological activity. For pharmaceutical production, it is preferable from the standpoint of safety that additives be com-



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