

Expression of tropomyosin from *Blattella germanica* as a recombinant non-fusion protein in *Pichia pastoris* and comparison of its IgE reactivity with its native counterpart

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Abstract

Tropomyosins derived from invertebrates are well-known pan allergens. However, the allergenicities of recombinant tropomyosins are variable. Here, we undertook to compare the IgE-binding reactivities of native and recombinant German cockroach tropomyosins. Native tropomyosin was purified by ammonium sulfate fractionation, hydroxyapatite column chromatography, and electroelution, and recombinant tropomyosin was expressed in *Pichia pastoris*. The allergenicities of the native and recombinant tropomyosins were compared by ELISA inhibition analysis. Native German cockroach tropomyosin showed 18% IgE-binding reactivity to German cockroach sensitized sera. Recombinant tropomyosin was produced without fusion protein and its N-terminus was blocked like that of the native counterpart. The IgE-binding reactivity of the recombinant was found to be comparable to that of native tropomyosin over the concentration range 1–1000 ng/ml by ELISA inhibition testing. Recombinant German cockroach tropomyosin expressed in *Pichia pastoris* showed better allergenicity than that expressed in *Escherichia coli*. Other factors in addition to the structural differences of native and recombinant proteins may also influence the IgE reactivities of tropomyosins.

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Allergen standardization is essential for diagnostic and immunotherapeutic purposes [1]. However, the protein contents of even commercially available natural allergen extracts are highly variable, and this is especially true in case of cockroach extract [2]. Moreover, recombinant allergens have been found to be useful for the identification and evaluation of important allergens from many different allergen sources, and microarray diagnostic assays using various recombinant allergens could simplify the future diagnosis of allergic diseases [3,4]. Thus, the genetic engineering of allergens could facilitate

the development of safe and effective therapeutic approaches [5–8].

Tropomyosin is an important respiratory allergen of the house dust mite, *Dermatophagoides farinae* [9], and of the cockroach, *Periplaneta americana* [10] (50–80.6% IgE-binding frequencies). However, recombinant tropomyosins expressed in *Escherichia coli* show low degrees of IgE-binding reactivity (5.6–29%) (*Dermatophagoides pteronyssinus*, [11]; *Anisakis simplex*, [12]; *Blomia tropicalis*, [13]; and *Lepidoglyphus destructor*, [14]). Specifically, the IgE reactivity of recombinant tropomyosin from *D. farinae* was found to be only up to 4% of that of its native counterpart [9]. Native tropomyosin is acetylated at its N-terminus [15], which is a major functional determinant [16]. Moreover, structurally unmodified

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and fully functional recombinant skeletal muscle α -tropomyosin, which was acetylated at the N-terminus, was produced in the methylotrophic yeast *P. pastoris* [17] and in *Saccharomyces cerevisiae* [18] at high level (20 mg/ml).

We cloned tropomyosin from the German cockroach, *Blattella germanica*, which is a dominant Korean species [19], and designated it as Bla g 7, according to the guidelines of the International Union of Immunological Societies Allergen Nomenclature Subcommittee. We previously produced recombinant Bla g 7 in *E. coli*, which showed low IgE-binding reactivity [20]. However, it is uncertain whether this low activity was due to structural differences between recombinant tropomyosin and native protein.

In the present study, we expressed recombinant German cockroach tropomyosin in *P. pastoris* as a means of producing protein with an allergenic activity comparable to that of its natural counterpart. The IgE-binding reactivities of the recombinant and native German cockroach tropomyosins were compared by ELISA inhibition testing.

Materials and methods

Purification of native Bla g 7

Cockroaches (60 g) were homogenized in 200 ml of 6 mM of 2-mercaptoethanol in a Waring blender at 4 °C. All subsequent steps were performed at 4 °C unless indicated otherwise. The homogenate was pressed through three layers of cheesecloth and mixed with 20 volumes (4 L) of ethanol. The solution was then allowed to stand for 1 h and centrifuged for 30 min at 3000 rpm. The supernatant was discarded and the sediment was resuspended in ether and air-dried. The dried residue was then extracted with gentle overhead stirring overnight in 1 M KCl, 10 mM Tris, 6 mM of 2-mercaptoethanol, 1 mg/ml of 1-phenyl-3-(2-thiazolyl)-2-thiourea (Sigma, St. Louis, MO, USA), 0.5 mM EDTA, and protease inhibitor set III (Calbiochem, San Diego, CA, USA), at pH 7.5. Insoluble residue was removed by centrifugation at 20,000g for 30 min, and the clear supernatant was fractionated with 40–55% saturated ammonium sulfate. The precipitate was then recovered by centrifugation at 30,000g for 30 min, extracted with 1 M KCl, 6 mM of 2-mercaptoethanol, and 10 mM phosphate, at pH 7.1, and then concentrated in the same buffer using stirred cell (Model 8200, Millipore, Bedford, MA, USA) in a N₂ atmosphere under a pressure of 20 PSI. The concentrated sample was then applied to a hydroxyapatite column to remove possible troponin contamination [21], which is another important German cockroach allergen [22]. Proteins were eluted with a linear gradient (10–250 mM phosphate in 1 M KCl and 6 mM of 2-mercaptoethanol, at

pH 7.1). Fractions of 2 ml were collected at a flow rate of 0.5 ml/min. The fraction containing tropomyosin was separated on 4–20% SDS-PAGE (Invitrogen, Carlsbad, CA, USA) and isolated by electroelution (Model 422 electro-eluter, Bio-Rad, Hercules, CA, USA).

Expression of Bla g 7 in *P. pastoris*

The open reading frame of Bla g 7 was obtained by RT-PCR and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) as described previously [20] (GenBank Accession No. AF260897). The coding region of Bla g 7 was amplified by PCR using cDNA cloned in pGEM-T Easy vector as template with the forward primer TmpF (5'-CGGGATCCACCATGGATGCCA TCAAG-3') and reverse primer TmpR (5'-CTTATT GGCAACTAAGCGGCCGCATTC-3'). The underlined sequences correspond to *Bam*HI and *Not*I sites, respectively, and TmpF primer contains an optimum translational initiation sequence (ACCATGG, [23,24]). The amplified cDNA was digested with *Bam*HI and *Not*I and subcloned into pPIC9 (Invitrogen). GS115 cells were transformed with *Stu*I-linearized plasmid using a *Pichia* EasyComp Kit (Invitrogen). His⁺ transformants were selected on RDB plates (1 M sorbitol, 1% dextrose, 4 × 10⁻⁵% biotin, 1.34% yeast nitrogen base without amino acids, and 0.005% each of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine). Genomic DNAs of individual colonies were isolated and analyzed by PCR using 5' *AOX* primer and 3' *AOX* primer (Invitrogen) to confirm the correct integration of cDNA into the *Pichia* genome, as recommended by the manufacturer. A clone with the correct integration was selected and cells were grown for 3 days at 30 °C, 250 rpm; 0.75% methanol (v/v) was added every 24 h. The harvested cells were resuspended in 1 M KCl, 10 mM Tris, 6 mM of 2-mercaptoethanol, 0.5 mM EDTA, and protease inhibitor set III (Calbiochem) at pH 7.5, lysed using a French presser, and subjected to ammonium sulfate fractionation, hydroxyapatite column chromatography, and electroelution as described above (purification of native Bla g 7).

Protein sequence analysis

Purified proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (0.45 μ m, Millipore) and subjected to Edman degradation using Procise 491HT protein sequencer (Applied Biosystems, USA) at the Korea Basic Science Institute to verify N-terminal blocking.

Serum samples

Sera from allergic patients with Uni-CAP values (Pharmacia, Uppsala, Sweden) higher than 0.7 kU/L to *B. germanica* (21 males and 13 females, mean age 29

years, ranging from 6 to 58 years) were used to assess the allergenicity of tropomyosin. Diagnosis was based on case history and skin testing, and followed the revised allergy nomenclature [25]. Ten healthy control sera, from individuals with no history of allergic symptoms and who were Uni-CAP negative to *B. germanica* and *D. farinae*, were used as negative controls for ELISA.

IgE-binding reactivity of native Bla g 7

Purified Bla g 7 (2.0 µg/ml) in coating buffer (0.1 M sodium carbonate, pH 9.6) was coated onto the microtiter plate (0.1 ml/well) and incubated overnight at 4 °C. The plate was then washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST) and blocked with 3% skim milk in PBS. The plate was then incubated with human sera (1:4 dilution) for 1 h. After washing, antibody was detected using biotinylated goat anti-human IgE (1:1000 dilution) (Vector, Burlingame, CA, USA) and streptavidin-peroxidase (1:1000 dilution) (Sigma). Signals were developed using 3,3',5,5'-tetramethyl-benzidine (TMB, KPL, Gaithersburg, MD, USA) as a substrate and optical density was determined at 450 nm using an automatic microplate reader (TECAN, Salzburg, Austria). The mean absorbance level plus five SDs of 10 healthy control serum samples was used as a cut-off value.

ELISA inhibition

Cockroach extract (20 µg/ml) in coating buffer was coated onto each well in duplicate and incubated at 4 °C overnight. After blocking with 3% skim milk, the wells were incubated with patient serum pool (1:4), which had been pre-incubated for 2 h at room temperature with solutions containing various concentrations of native protein, recombinant protein, or crude extract. Plates were processed as described for standard ELISA above. Results are presented as percent inhibition of the inhibitor free reaction.

SDS-PAGE and immunoblotting

Proteins were loaded in 5% polyacrylamide stacking gel above a 10% separating gel, and run with a discontinuous buffer system [26]. After electrophoresis, the stack-

ing gel was stained with 0.2% Coomassie Brilliant Blue R-250 or transferred electrophoretically to nitrocellulose membranes (OSMONICS, Westborough, MA, USA). Tropomyosin was immunodetected at every purification step using sera raised against *E. coli*-expressed recombinant (ER) tropomyosin and NBT/BCIP (Promega), as previously described.

Results

Purification of native tropomyosin and Pichia-expressed recombinant tropomyosin

Native tropomyosin was purified from the whole body extract of German cockroach by ammonium sulfate fractionation, hydroxyapatite column chromatography, and electroelution. The yield of native allergen was 0.08% (w/w) (Table 1). Recombinant tropomyosin was expressed as a non-fusion protein in *P. pastoris* and was purified using the same procedure as that used for native tropomyosin purification. The yield of purified recombinant tropomyosin was approximately 7.2 mg/L on yeast culture. We were unable to perform N-terminal sequencing of either the native or the recombinant tropomyosins, indicating that the N-termini were blocked as expected. Similar elution profile patterns were observed for native and recombinant tropomyosins on hydroxyapatite columns (Fig. 1). Both recombinant and native proteins were eluted by 111 mM phosphate. The purified proteins produced a single homogeneous band by Coomassie blue stained SDS-PAGE (Fig. 2). The native tropomyosin and the *Pichia*-expressed recombinant (PR) tropomyosin showed identical mobilities. The molecular weight of tropomyosin expressed from *E. coli* was much higher than that of the native or *Pichia*-expressed protein, because it contained an additional 37 amino acids (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRDPNWI) at the N-terminus.

Comparison of IgE binding to native and recombinant B. germanica tropomyosins

The frequency of IgE binding to tropomyosins was examined by ELISA. IgE-reacting antibody to the native tropomyosin was detected in six of 35 sera tested (18%)

Table 1
Purification of German cockroach tropomyosin

Step	Native tropomyosin		Recombinant tropomyosin	
	Total (mg)	Yield (%) ^a	Total (mg)	Yield (%) ^a
Crude extract/cell lysate	594	100	409	100
40–55% (NH ₄) ₂ SO ₄	93	16	165	40
Hydroxyapatite column	57	9.6	49	12
Electroelution	0.49	0.08	7.2	1.8

^a Yield of preparation.

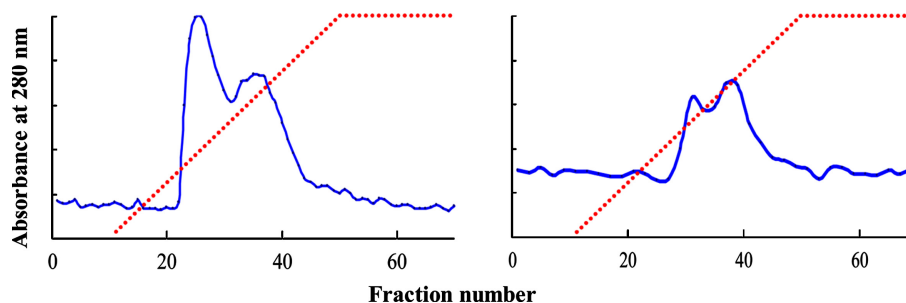


Fig. 1. Chromatographic purification of native tropomyosin (A) and *Pichia*-expressed recombinant tropomyosins (B), on a hydroxyapatite column. The dotted line indicates the concentration of elution buffer.

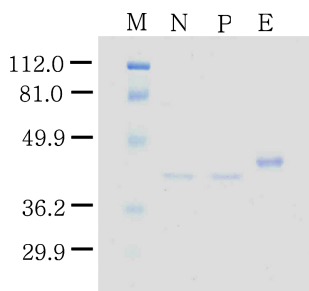


Fig. 2. SDS-PAGE of native and recombinant tropomyosins. M, molecular mass standard; N, native tropomyosin; P, *Pichia*-expressed recombinant tropomyosin; and E, *E. coli*-expressed recombinant tropomyosin.

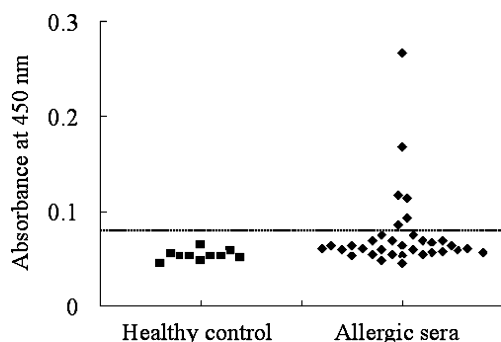


Fig. 3. IgE reactivity of human sera against native German cockroach tropomyosin. The dotted line indicates the cut-off value. ■, healthy control sera; ♦, German cockroach sensitized sera.

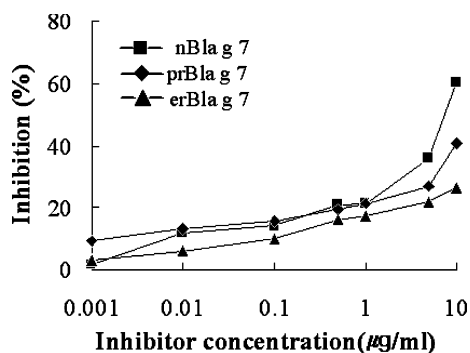


Fig. 4. Binding inhibition of IgE to German cockroach extract by native tropomyosin (■), *Pichia*-expressed recombinant tropomyosin (♦), and *E. coli*-expressed recombinant tropomyosin (▲).

(Fig. 3). In ELISA inhibition experiments, ER tropomyosin, PR tropomyosin, and native tropomyosin showed maximum inhibitions of 26.5, 40.8, and 60.5%, respectively, at an inhibitor concentration of 10 μg/ml. At inhibitor concentrations of 0.01–1 μg/ml, PR tropomyosin and native tropomyosin showed similar patterns of inhibition at inhibitor concentrations from 0.001 to 1 μg/ml. An abrupt increase in percentage inhibition was observed for native tropomyosin at inhibitor concentrations exceeding 1 μg/ml (Fig. 4).

Discussion

Various expression systems have been designed to produce recombinant allergens to overcome the clinical limitations of natural allergen extracts, i.e., stability, contamination, and variations in composition and quantity [27–30]. The methylotrophic yeast *P. pastoris* is one of several valuable tools, which are capable of performing post-translational modifications, such as, disulfide bond formation, glycosylation, acetylation, and protein folding [31]. For example, the biological activities of some allergens, such as of mite group 1 allergen, are critical for allergenicity [32]. Many researchers tried to produce enzymatically active recombinant mite group 1 allergens, and these efforts were eventually rewarded with *P. pastoris* [33,34].

Tropomyosin is one of those allergens for which molecular mimicry between allergen and endogenous host protein is believed to be important [35]. In the present study, recombinant tropomyosin was produced as a non-fusion protein in *P. pastoris* and its allergenicity was compared with its native counterpart. Moreover, phosphorylated tropomyosin is known to have a higher propensity for head-to-tail polymerization than the unphosphorylated form. These two forms of tropomyosin can be separated by anion exchange column chromatography, for example by using a Mono Q column with 9 M urea as eluant [36]. However, in the present study, this step was omitted because proteins purified using a hydroxyapatite column produced a single band on SDS-PAGE. Both native and PR tropomyosin were 40 kDa, by SDS-PAGE (Fig. 2), which is larger than that calculated from the nucleotide sequence (32.836 kDa).

Native tropomyosin had an IgE-binding frequency of 18%, which is in agreement with previous data on tropomyosins from *D. pteronyssinus* (5.6% [11]), *B. tropicalis* (29% [13]), and *L. destructor* (13% [14]) (Fig. 3). However, *D. farinae* tropomyosin showed a higher IgE-binding frequency than other tropomyosins known to be respiratory allergens [9]. It might be expected that food allergens (shellfish tropomyosins) and respiratory allergens (mite or cockroach tropomyosins) would elicit different immune responses. These observations suggest that the pathogenicity of a particular allergen might be determined by combinations of different genetic and environmental factors [37,38]. No proteolytic cockroach allergen has been studied to date, although the proteolytic activities of some allergens are believed to enhance their allergenicities [32]. Comparative modeling and experimental studies on Bla g 2, which shares sequence similarity with the aspartic proteases, led to speculation that key elements affecting the production of IgE antibodies appear to be the route of exposure, allergen dose, and the presence of genes that regulate the host's immune responses [39]. Age at the time of allergen exposure is also an important factor in allergic disease [40]. In addition, in a study using sera from 191 pairs of Australian twins, the genetic control of specific IgE responses to rye grass pollen allergens was found to be limited and to be determined substantially by environmental factors [41]. Tropomyosin may offer a good molecular model for the investigation of those factors affecting sensitization and the onset of allergic disorders.

The abrupt increase in the percentage inhibition of IgE binding above 1 µg/ml by native tropomyosin compared to recombinant tropomyosin implies the presence of isoforms with different IgE-binding activities. Multiple isoforms with few amino acid sequence variations may show cooperative IgE binding. In particular, natural isoforms of Bet v 1, the major birch pollen allergen, were found to have different IgE-binding reactivities [42]. Alternatively, IgE binding may be influenced by the aggregation of tropomyosin with other proteins, which is known to occur even in the absence of actin than in the presence (Fig. 4 [43]). Further investigations on polymorphism of tropomyosin will be required to confirm this observation.

In this study, we produced a recombinant German cockroach tropomyosin with improved allergenicity. The IgE-binding frequencies of native and PR tropomyosin in allergic sera were comparable to that shown by ER tropomyosin [20]. This recombinant tropomyosin may be useful for the diagnosis of tropomyosin-specific IgE and is important because of its high cross-reactivities [44]. The clinical relevance of this allergen remains to be evaluated in vivo on a large scale.

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