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Boiling is an Efficient Way to Purify Recombinant Allergen Lit v 1 from Whiteleg Shrimp (*Litopenaeus vannamei*)

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Abstract

Background: The whiteleg shrimp *(Litopenaeus vannamei)* allergen protein Lit v 1 is tropomyosin with a double-stranded alpha-helical coiled-coil structure, which is so stable that the native structure can be easily restored even after heating.

Methods: Recombinant Lit v 1 was expressed in *Escherichia coli* (*E. coli)* and *E. coli* were lysed by ultra sonication or boiling. Recombinant Lit v 1 was purified by affinity chromatography. The structure of the recombinant Lit v 1 was analyzed by circular dichroism while the IgE binding ability of Lit v 1 was examined by Western blotting and inhibition ELISA.

Results: Recombinant Lit v 1 was expressed efficiently in *E. coli*. The double-stranded alpha-helical coiled-coil structure of Lit v 1 can be easily restored even after boiling. Furthermore, boiling did not change the IgE binding ability of Lit v 1.

Conclusions: Our results suggest that boiling is a convenient and sufficient method to isolate extremely thermo stable recombinant proteins such as tropomyosin from *E. coli*.

Keywords: ***Litopenaeus vannamei;*** **Allergen; Tropomyosin; Boiling**

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Introduction

The white leg shrimp *Litopenaeus vannamei*, also known as Pacific white shrimp, is a variety of prawn in the eastern Pacific Ocean that is commonly farmed or caught for food [1]. Shrimp allergy is a relatively common and long-lasting disorder, whose symptoms range from local itching to anaphylaxis [2]. Allergen avoidance as the first treatment for food allergy does not work well due to allergen contamination in foods [3]. Specific immunotherapy (SIT) is the only method able to change the natural course of the disease but its therapeutic effects depend on the quality of allergen vaccines [4-6]. The currently available allergen vaccines are native allergen extracts that contain multiple components. Therefore, it is difficult to control the quality and standardization of the native allergen extracts, making them inefficient and unsafe for SIT [7,8]. With the development of recombinant DNA technology, recombinant allergens and engineered hypoallergenic proteins can be produced in large quantities that can be used for diagnosis of allergy and SIT, respectively [9-12].

Tropomyosin is the major allergen in shellfish such as shrimp [13]. Invertebrate tropomyosins share a high degree of sequence homology, a reason for antibody cross-reactivity [14,15]. In contrast, vertebrate tropomyosins have rarely been identified as allergens. Hence, invertebrate tropomyosin is commonly regarded as a pan-allergen [14]. Isoforms have been identified in several shrimp species, including *Penaeus aztecus* (Pen a 1) and *Litopenaeus vanamei* (Lit v 1) [16,17]. Previous studies have demonstrated that Pen a 1 has five IgE-binding regions and that there is a high degree of similarity between Pen a 1 IgE-binding regions and homologous sequences in invertebrate tropomyosin suggested a structural basis for cross-reactivity of allergenic tropomyosin [18,19]. When the critical amino acids in the allergenic IgE-binding regions were replaced with their counterparts found in non-allergenic tropomyosin, the allergenic potency of the Pen a 1 mutant VR9-1 was greatly decreased despite the fact that the protein structure was unchanged [11]. Similarly, two hypoallergenic derivatives of another shrimp protein Met e 1 were constructed by site-directed mutagenesis and epitope deletion. These Met e 1 derivatives exhibited a marked reduction in IgE reactivity and allergenicity, as well as the ability to induce blocking IgG antibodies [20].

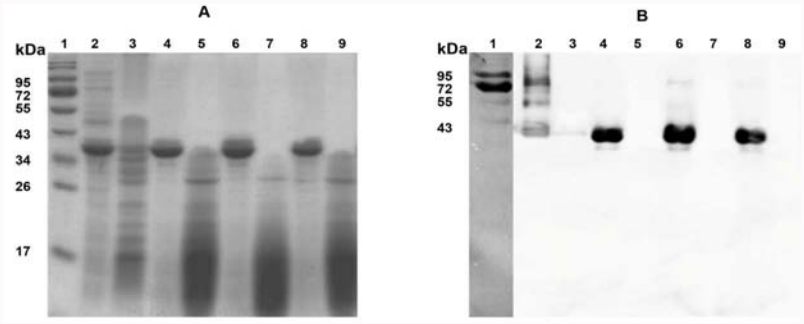

Figure 1: SDS–PAGE and Western blotting analysis of Lit v 1. The samples were fractionated on a 12% SDS–PAGE gel (A) and analyzed by Western blotting (B). (A and B) Lane 1: Protein molecular weight markers, 2: the ultrasonicated sample supernatant, 3: the ultrasonicated sample pellet, 4: the 10 min-boiled sample supernatant, 5: the 10min-boiled sample pellet, 6: the 20min-boiled sample supernatant, 7: the 20min-boiled sample pellet, 8: the 30min-boiled sample supernatant, 9: the 30min-boiled sample pellet.

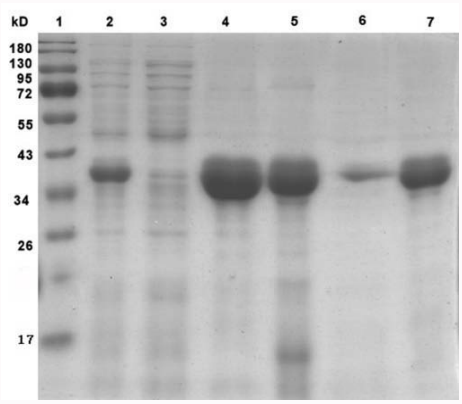

Figure 2: SDS-PADE analysis of purified Lit v 1. Lane 1: Protein molecular weight markers, 2: the supernatant of the ultrasonicated sample (before purification), 3: the flow-through peak from the ultrasonicated sample, 4: the elution peak of the ultrasonicated sample 5: the supernatant of the boiled sample (before purification), 6: the flow-through peak of the boiled sample, 7: the elution peak of the boiled sample.

Figure 3: The three-dimensional structure of Lit v 1.

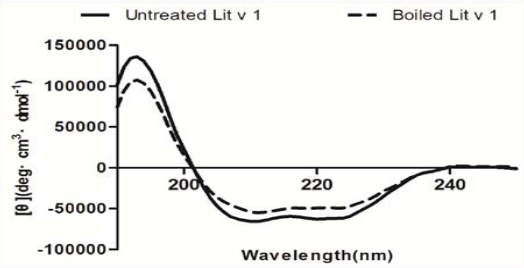

Figure 4: Far-UV CD spectra of the ultrasonicated and the boiled Lit v 1.

E. coli is the most widely used host for recombinant protein expression due to convenience and low cost: it is easy to genetically manipulate and grow *E. coli* quickly in inexpensive media [21]. *E. coli* can be easily lysed by several methods including sonication, shearing, and freeze–thaw cycles, all of which require specific equipment or take a considerable amount of time [21]. Tropomyosin is a coiled-coil protein and consists of multiple domains with a diverse range of stability in the coiled-coil form [22,23]. Increasing evidence has shown that tropomyosins from different species are extremely thermostable [24-26]. Here, we demonstrate that boiling is a simple and effective way to isolate recombinant shrimp tropomyosin Lit v 1 from *E. coli*.

Materials and Methods

Cloning and expression of Lit v 1

The cloning and expression of Lit v 1 was performed as described [27]. The Lit v 1 cDNA was inserted in pET-44a after digestion with *Nde I* and *Pst I*, and then the construct was transferred into *E. coli* JM109 in our laboratory. Transformants were identified by colony PCR. The positive plasmids were transferred into the *E. coli* Rosetta strain. The transformed Rosetta cells were inoculated in LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl) supplemented with 50 μg/mL ampicillin, and cultured at 37°C for 12 h with agitation. Subsequently, 0.1% of the culture was transferred into fresh medium containing 0.4% glucose, and cultured at 37°C, 200 rpm. When the optical density (OD) at 600 nM reached about 0.6~0.8, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM. The cells were collected after an additional 4 h incubation.

Lysis of bacteria

After induction by IPTG, 1 mL of the bacterial culture was transferred to each of 12 tubes. After centrifugation, the supernatant was removed and the pellet was resuspended in 150 μL of PBS. The samples were boiled for various time periods, after which they were immediately placed on ice. The supernatant and the precipitate were separated by centrifugation. The remaining bulk of the bacterial culture was centrifuged and the pelleted bacterial cells were resuspended in a volume of PBS equal to 10% of the original bacterial liquid volume. This suspension was sonicated on ice (50% intensity, ultrasound 5 sec, stop 5 sec, then ultrasound for 30 min), followed by centrifugation. The supernatant and pellet were collected after centrifugation and analyzed by SDS–PAGE and Western blotting (Figure 1).

Protein concentration

The bicinchoninic acid method (BCA, Pierce® BCA Protein Assay Kit, Thermo Scientific, USA) was used to determine the protein concentration according to manufacturer's instructions. After dilution of the standard, 10 μL of sample was added to wells of a 96-well plate. Standard working reagent (SWR) was freshly prepared

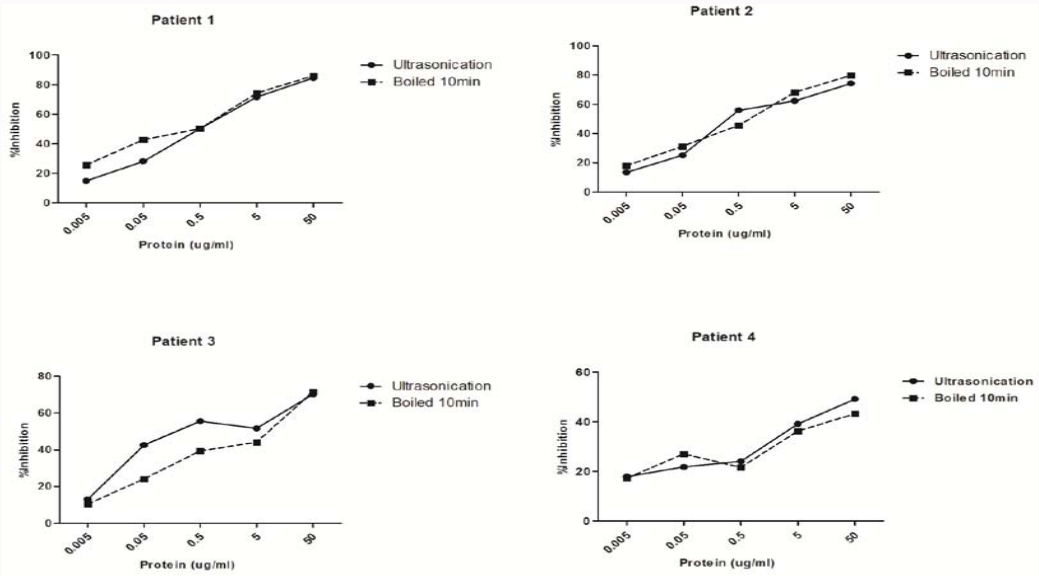

Figure 5: IgE-binding affinity of the ultrasonicated or boiled sample was compared using inhibition ELISA with serum from shrimp allergic patients.

just before use by mixing Reagent B with Reagent A by the ratio of 1:50, 200 µL of which was added per well. After incubation at 37°C for 30 min, the absorbance was measured at 562 nm, against the blank, using Varioskan Flash (Thermo Scientific, USA).

SDS-PAGE and western blotting analysis

The protein samples were mixed with the loading buffer, boiled for 10 min, centrifuged at 10,000 g for 5 min and subjected to 12% SDS–PAGE [28]. The gel was stained with Coomassie bright blue or transferred to a PVDF membrane for Western blotting. The membrane was blocked in 5% skim milk with PBST (PBS buffer containing 0.2% Tween 20 (V/V)) for 2 h, then incubated with anti-Strep II antibody (1:1000) overnight at 4°C. After washing with PBST buffer three times and incubation with HRP-conjugated horse anti-mouse IgG antibody (1:4000) at room temperature for 1 h, the membrane was analyzed using Image Quant LAS 4000 mini (GE-Healthcare, USA).

Purification of Lit v 1

Purification of Lit v 1 from the soluble fraction was performed as described previously [27]. Purification was carried out at room temperature on a HisTrap™ HP column (1 mL, GE-Healthcare, USA) hooked up to a GE AKTA pure FPLC system (GE-HealthCare, USA). After pre-equilibration with PBS, the sample was loaded onto the column. After washing with PBS, the protein was eluted with PBS containing 500 mM desthiobiotin. Purity was monitored by SDS-PAGE (Figure 2).

Protein homology modeling

The three-dimensional structure of Lit v 1 protein was obtained by SWISS-MODEL (http://swissmodel.expasy.org/) homology modeling (Figure 3).

Circular Dichroism (CD)

The CD spectral measurement was performed on MOS-500 spectropolarimeter (Bio-Logic, Grenoble, France), with three accumulations. The secondary structure of Lit v 1 (195–250 nm) was recorded at the far UV range using the protein concentration of 0.01 mg/mL in water at room temperature. The spectrum of water was recorded as the baseline under the same condition. The protein spectra were calculated after subtracting the baseline before further analysis [29]. Using the Dicroprot program, the data were analyzed by K2D (a neural network method) [30] (Figure 4).

Competitive inhibition ELISA

The competitive IgE binding assay used to assess IgE binding activity was performed as described previously [31,32] with some modifications. Stripwell™ Microplates (Costar, USA) were coated overnight at 4°C with 100 µL of ultrasonicated protein (20 ug/mL) in carbonate buffer (35 mM $NaHCO_3$, 10 mM $NaCO_3$, pH9.6) and blocked at RT (room temperature) for 2 h in 0.1% PBST containing 5% BSA. After washing with 0.1% PBST, wells were incubated for 2h at RT with patient serum mixed with various concentrations of the sonicated or the boiled Lit v 1. BSA (0.1%) was used as the negative control. After washing, the plates were incubated for 1 h at 37°C with mouse anti-human IgE (1:2000) (Abcam) followed by horse anti-mouse IgG-HRP (1:4000) (Abcam). Color development was performed using TMB substrate (Cell Signaling Technology, USA), and the OD at 450 nm was measured using Varioskan Flash (Thermo Scientific, USA).

Results

The effect of ultrasonication or boiling on bacterial lysis

The efficiency of bacterial lysis by ultrasonication or boiling of the recombinant allergen Lit v 1-expressing *E. coli* was somewhat different (Figure 1). Both methods were able to lyse the bacteria equally well. But the purity of Lit v 1 in the supernatant after boiling was significantly better than that after ultrasonication. As shown in Figure 1, 10-min boiling was sufficient to lyse the bacteria.

Purification of Lit v 1

The supernatants of either the ultrasonicated or boiled samples were pooled for purification by HisTrap™ HP column chromatography. The purity of the elution peak from the ultrasonicated and 10 min-boiled samples (Figure 2. lanes 4,7) was quite similar.

The secondary and three-dimensional structure of Lit v 1

The 3-D structure obtained by SWISS-MODEL homology modeling showed that the protein contains an alpha helix but no

other secondary structure (Figure 3). The secondary structure of the ultrasonicated and boiled Lit v 1 was almost identical: two negative bands of comparable magnitude at 222 nm and 208 nm and a stronger positive band near 190 nm, the most distinctive and strongest CD spectrum of the a-helix (Figure 4). The result of the software analysis was 100% α-helix, which is in line with the SWISS-MODEL homology modeling.

IgE binding activity of Lit v 1

For the inhibition ELISA, the wells were coated with the ultrasonicated Lit v 1 and the serum sample mixed with either the ultrasonicated or boiled sample as a competitive inhibitor before being added to the Lit v 1 coated wells. Each showed a similar binding capacity to the specific IgE from different shrimp allergic patients (Figure 5), suggesting boiling did not change the allergenicity of Lit v 1.

Discussion

Invertebrate tropomyosin is a well-known pan-allergen [14]. Increasing evidence shows that there are allergenic tropomyosins in both invertebrates and vertebrates [33]. Cross-reactivity among allergenic tropomyosins exists due to their high sequence identity and similarity, especially in allergen epitopes [14,19,34]. Tropomyosin has a conserved structure and high thermostability [24]. Recombinant allergens are a good choice for producing standardized allergen vaccines on a large scale[9]. Furthermore, engineered hypoallergenic allergen vaccines can be engineered once the allergen specific epitopes of allergenic tropomyosin have been identified [17].

Despite the development of eukaryotic expression systems, the *E. coli* expression system remains the most widely used system for production of recombinant tropomyosin because the highly conserved proteins are ubiquitously expressed in most eukaryotes and it is challenging to separate and isolate a recombinant tropomyosin from the host's tropomyosin due to their conserved structures [2]. However, as a host for recombinant protein expression, *E. coli* still has some shortcomings. On one hand, the reducing environment of the *E. coli* cytoplasm is hostile to disulfide bond formation between two cysteines in an expressed protein [35,36]. On the other hand, *E. coli* does not have the ability to glycosylate proteins. Nevertheless, there are no cysteine residues in the shrimp tropomyosin Lit v 1 and recombinant Lit v 1 retains its antigenicity and allergenicity without sugar chains (Figures 4,5). Collectively, *E. coli* as an expression host remains the best choice for the production of recombinant Lit v 1.

Recombinant proteins can be expressed either as a soluble form or as inclusion bodies in *E. coli* where it can be easily extracted by several methods including sonication, shearing, or the action of freeze–thaw cycles, with sonication being the most used method to disrupt *E. coli* [21]. These methods need special equipment or take considerable time. Here, we demonstrate that boiling is a simple, fast, and efficient method to isolate the shrimp allergen Lit v 1 from *E. coli*. Furthermore, the crude extract of recombinant Lit v 1 produced after ultrasonication contained numerous bacterial proteins while boiling eliminated most of them, especially the high molecular weight proteins (Figure 1). Boiling did not alter the structure of the recombinant Lit v 1 that is essential for its thermostability (Figure 4) [25]. The ELISA results demonstrated that the boiled recombinant Lit v 1 has a strong IgE binding affinity, which is in accordance with the previous work (Figure 5) [24]. Thus, our results suggest that boiling is a convenient and sufficient method to isolate extremely thermostable recombinant proteins such as tropomyosin.

Acknowledgments

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