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Allergenicity of Native/Recombinant Tropomyosin, Per a 7, of American Cockroach (CR), *Periplaneta americana*, among CR Allergic Thais

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SUMMARY In this study, native tropomyosin (Per a 7) of American cockroach (CR), *Periplaneta americana*, caught in Thailand was purified. Also, gene sequence encoding full length tropomyosin of the CR was PCR amplified by using degenerate primers designed from gene sequences coding for *P. americana* tropomyosin of the database (Per a 7.0101 and Per a 7.0102; accession no.Y14854 and AF106961, respectively). Amino acid sequence deduced from the nucleotide sequence encoding *P. americana* tropomyosin of this study (GenBank accession no. FJ976895) had 98.59% identity with the sequences of Per a 7.0101 and Per a 7.0102 and was 97.18% identical to the Bla g 7 sequence of German cockroach, *Blatella germanica* (accession no. AF260897)**.** The native and recombinant tropomyosins (~34 kDa) were used as antigens in sandwich ELISA for detecting specific IgE in serum samples of 14 consented allergic patients who were positive by skin test to crude CR extract in comparison to 5 individuals who were skin test negative. It was found that 8 (57%) and 6 (43%) of the CR allergic patients gave positive IgE binding results to the native and the recombinant proteins, respectively, while none of the non-allergic counterparts was positive. Results of immunoblotting conformed to the ELISA results. Tropomyosin extracted from the *P. americana* caught in Thailand has potential as standard *P. americana* allergen in clinical monitoring of the allergic Thai patients.

Cockroach (CR) is one of the most common causes of indoor allergens in Thailand.[1] The incidence of CR allergy among the allergic Thais as determined by skin test using crude CR extract ranged from 44-77.5%.[1-3] American cockroach, *Periplanata americana* is predominant CR species in the country.[4,5] This species of insect generates a plethora of allergens in human dwellings that set a cascade of sensitization and subsequent allergic morbidity upon the allergen re-exposure.[6] CR allergic manifestations range from mild such as skin rashes, itch, rhinitis, and conjunctivitis, to the most severe, *i.e.* asthmatic attack that needs immediate resuscitation. Among the CR allergenic substances reported, two *P. americana* allergens, *i.e.* Per a 1.0105 (AY259514) which is a variant of the Per a 1 and the recently recognized Per a 9 (arginine kinase) were the major CR aller-

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gens among the allergic Thais as they bound to specific IgE in the sera of all CR allergic patients tested.[7,8] Another allergen of *P. americana*, *i.e.* tropomyosin or Per a 7, which has been recognized as a pan-invetebrate allergen, has been shown to be an important CR allergen among allergic patients.[9] In this study, native and recombinant tropomyosins of *P. americana* were tested for serum IgE binding frequency by IgE-ELISA and IgE-immunoblotting in order to determine allergenic potential of this insect allergen among the CR allergic Thai patients.

MATERIALS AND METHODS

Preparation of native tropomyosin of American cockroach, *P. americana*

Live adult CR were trapped, entomologically identified, and only the *P. americana* were kept at -70°C. Whole body of the frozen CR was ground in liquid nitrogen using a homogenizer (IKA, Germany). Native tropomyosin was extracted from the CR powder by using ammonium sulfate precipitation as described previously.[10]

P. americana cDNA preparation and amplification of full-length tropomyosin coding sequence

Total RNA of the CR was prepared from the CR powder using TRIzol reagent (Invitrogen, USA). Purity of the RNA was determined by measuring optical densities (OD) of the preparation at absorbance (*A*) 260 and 280 nm. The integrity of the total RNA was checked by agarose gel electrophoresis, ethidium bromide staining and UV transillumination (UVP BioDoc-It™ Imaging System, USA). Complementary DNA was synthesized from the total RNA by using a RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, USA) under the RNase-free condition. The cDNA was used as a template for PCR amplification of the tropomyosin coding DNA sequence.

Forward (F) and reverse (R) nucleotide primers for the PCR amplification of *P. americana* CR tropomyosin gene sequence were designed from the DNA sequences coding for tropomyosin of the GenBank database (accession no. Y14854 and AF106961). Restriction sites of *Eco*RV and *Hin*dIII were introduced to the 5' ends of the F and the R primer sequences (underlined), respectively. The F sequence was 5'-CCGGATATCATGGACGCK (G/T)ATCAAGAAGA-3', and the R sequence was 5'-CGAAAGCTTGTTR(A/G)CCAATAAGTTCGG T-3'. Tropomyosin encoding DNA sequence was PCR amplified in a Thermo Cycler (Eppendorf, Germany). The PCR reaction mixture (25 µl) consisted of: 1 µl cDNA, 0.5 µl containing 10 µM each of the F and R primers, 2.5 µl of 10x buffer, 3 µl of 25 mM $MgCl_2$, 2 µl of 2.5 mM dNTP, 0.2 µl of 5 units/µl *Taq* DNA polymerase (Fermentas), and ultrapure distilled water (UDW). After 4 minutes of an initial denaturation at 94°C, 35 cycles of PCR reaction were performed. Each cycle was 30 seconds at 94°C, 30 seconds at 55°C, and 40 seconds at 72°C and followed by final extension at 72°C for 7 minutes. The PCR product was visualized after 1% agarose gel electrophoresis and ethidium bromide staining using the UV transilluminator.

Cloning of the tropomyosin gene sequences

The tropomyosin DNA amplicon was purified from the agarose gel by using the SiMax™ PCR Products/Agarose Gel Purification Kit (SBS Genetech, China). The sequences were ligated to pKRX-T vector (SBS Genetech, China) *via* the overhang T of the vector and the A of the DNA amplicon. The ligation mixture contained 1 µl of 10× T4 DNA Ligase buffer, 1 µl of pKRX-T vector (30 ng), 3 µl of tropomyosin DNA (~150 ng), 1 µl of T4 DNA ligase (3 Weiss units/µl), and sterile UDW to a volume of 10 µl. The mixture was kept at 16°C overnight. The recombinant plasmid was cloned into competent JM109 *E. coli* cells by using a chemical transformation protocol. White colonies of the transformed *E. coli* on a Luria-Bertani (LB) agar plate containing 100 µg ampicillin/ml, 1 mM IPTG and 5% X-Gal incubated at 37°C overnight were randomly picked, individually inoculated into 5 ml of LB–ampicillin (100 µg/ml) broth and incubated at 37°C with shaking (150 rpm) overnight. The bacterial cells were harvested by centrifugation and the plasmids in each *E. coli* clone were extracted as the following: cell pellet in each centrifuge tube were added with 200 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) containing RNase A (20 µg/ml). The contents were mixed by

inverting the tube and 400 µl of solution II (0.1 N NaOH, 1% SDS) was added and the preparation was placed on ice for 5 minutes. The mixture was added with 300 µl of solution III (3 M potassium acetate pH 5.2), kept on ice for 5 minutes and centrifuged at 12,000 × *g* for 5 minutes. The plasmids in the pellet were air-dried. The DNA was doubly digested with *Eco*RV and *Hin*dIII endonucleases and subjected to 1% agarose gel electrophoresis, ethidium bromide staining and UV transillumination in order to ensure that the plasmids contained DNA insert of correct size. The DNA encoding tropomyosin was verified by sequencing.

The tropomyosin DNA with correct nucleotide sequence was extracted from the JM109 *E. coli* and subcloned into a protein expression vector, pET20b(+), which had the 6x His-tag at the C-terminus as a fusion peptide (Novagen, Germany). Briefly, the recombinant plasmid from the cloning vector and the pET20b(+) plasmid were individually digested with *Eco*RV and *Hin*dIII restriction endonucleases as mentioned above and subjected to 1% agarose gel electrophoresis, ethidium bromide staining and UV transillumination to ensure the appropriate digestion. The tropomyosin DNA fragments and the linear pET20b(+) DNA were individually cut from the agarose gel and purified by using the Si-Max™ PCR Products/Agarose Gel Purification Kit. They were then ligated and introduced into competent BL21(DE3)pLysS *E. coli* cells by chemically transformation method. Colonies of the transformed *E. coli* were randomly picked for recombinant plasmid extraction and tropomyosin gene amplification in order to screen for the appropriate transformed *E. coli* clones carrying the *tropomyosin*-pET20b(+) recombinant plasmids.

Preparation of the recombinant tropomyosin

A colony of the selected transformed BL21(DE3)pLysS *E. coli* on an overnight LB-ampicillin agar plate was inoculated into 5 ml of LB-ampicillin broth and incubated at 37°C with shaking at 250 rpm overnight. One ml of the culture was inoculated into 50 ml of fresh LB-ampicillin broth and incubated at 37°C with shaking at 250 rpm until the optical density (OD) at A_{600nm} was 0.6. IPTG was added to the final concentration of 1 mM and the culture was incubated further for 4 hours. The bacterial cells were harvested by centrifugation at 4,000 × *g* at 4°C for 20 minutes. The cells were lysed in a lysis buffer by sonication at 20 kHz, 2 minutes pulse-on, 3 minutes pulse-off. Cell debris was removed by centrifugation at 12,000 × *g* at 4°C for 20 minutes. The supernatant (cell lysate) was collected. The presence of recombinant protein in the lysate was checked by SDS-PAGE and Western blot analysis using anti-6x His-HRP conjugate and peroxidase substrate. The recombinant protein was purified from the *E. coli* lysate using a pre-equilibrated Nickel column (Pro-Bond™, Invitrogen).

Protein identification by LC/MS-MS

Tropomyosin was verified by LC/MS-MS according to the method described previously.[11] For the data analysis, the ion spectra of the peptides generated by mass spectrometry were interpreted using the Turbo SEQUEST algorithm in the Bio-Works™3.1SR1 software package (Thermo Electron) and the nr.fasta database. The protein search parameter included a precursor peptide mass tolerance of ±1.25 amu, a fragment mass tolerance of ±0.4 amu, methionine (M) oxidation, and threonine (T) or serine (S) phosphorylation. For the tryptic status requirement, at least one end of the peptide had to be a tryptic site. The identified peptides were further evaluated using charge state *versus* cross-correlation numbers (X_{corr}). The criteria for a positive identification of the peptides were X_{corr} >1.5 for singly charged ions, X_{corr} > 2.0 for doubly charged ions, and X_{corr} >2.5 for triply charged ions. A delta correlation (ΔC_n) > 0.08 was used as cut-off for peptide acceptance. The minimum of one peptide per protein was specified by the software.

Human subjects and serum samples

Sera were collected individually from 14 CR allergic Thai patients of the Department of Oto-rhino-laryngology, Faculty of Medicine Siriraj Hospital, Mahidol University, in Bangkok, Thailand. These subjects were positive by skin prick test to crude extract of *P. americana*. Sera of 5 individuals who gave negative skin prick test to the CR extract were used as non-allergic controls. This part of work was approved by the Ethical Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University (COA no. SI268/2008).

IgE ELISA

The IgE ELISA was performed for determining the binding frequencies of the native and the recombinant tropomyosin proteins to IgE in the serum samples. The assay was performed as described previously.[8] Briefly, native or recombinant tropomyosin (5 µg/ml carbonate-bicarbonate buffer pH 9.6) was added to each well (100 µl) in a microtiter plate (Costar). The plate was incubated at 37 °C until dried. All antigen coated wells were washed with PBS containing 0.5% Tween-20 (PBST), added individually with 200 µl of a blocking solution (1% bovine serum albumin in PBS), and the plate was incubated at 37 °C for 1 hour. After washing thoroughly with PBST, 100 µl of 1:10 diluted serum were added to each antigen well and the plate was incubated for 3 hours. Wells added with only the serum diluent served as blank wells. After incubation and all wells were washed as above, 100 µl of mouse anti-human IgE-biotin conjugate (Southern Biotech Associates Inc., USA; diluted 1:1,000 in PBST) was added appropriately. The plate was incubated for 2 hours, washed with PBST, and each well was added with 100 µl of streptavidin-horseradish peroxidase conjugate (DakoCytomation) diluted 1:1,000 with PBST. After further incubating at 37°C for 30 minutes, the plate was washed as above and freshly prepared substrate solution (*o*-phenylenediamine; Zymed Laboratory) in citrate-phosphate buffer pH 5.0 containing 0.03% hydrogen peroxide was added to each well (100 µl per well). The plate was kept in the dark for 25°C for 30 minutes, then the enzymatic reaction was stopped by adding 50 ml of 4 N H_2SO_4 into each well. Optical density (OD) of the content in each well was determined at A_{492nm} using an ELISA reader (Multiscan *EX*, Labsystem, Helsinki, Finland) against the blank. The cut-off OD between positive and negative IgE ELISA was mean of OD of non-allergic subjects + 2 standard deviations.

IgE-immunoblotting

Tropomyosin preparation was subjected to SDS-PAGE and transblotted onto a nitrocellulose membrane (NC). The NC strips blotted with SDS-PAGE separated-protein were allowed to react with individual human sera diluted 1:10 in Tris buffered saline (TBS) containing 0.5% Tween-20 (TBS-T) and kept at 4°C overnight. The strips were washed with the TBS-T and incubated with appropriately diluted mouse anti-human IgE-biotin conjugate (Zymed Laboratory, USA) and kept at 25°C on a rotating platform for 3 hours. The proteins bound by specific IgE in the human sera were revealed by using streptavidin-alkaline phosphatase conjugate (DakoCytomation, Denmark) and BCIP-NBT substrate (KPL, USA).

RESULTS

The PCR amplicon of the gene sequence encoding full length *P. americana* tropomyosin (867 bp) is shown in Fig. 1. The nucleotide and deduced amino acid sequences of the amplified tropomyosin gene (deposited as GenBank accession no. FJ976895) are shown in Fig. 2. Pair-wise alignment generated by Clustal W revealed that the deduced amino acid sequence of the *P. americana* tropomyosin of this study had 98.59% identity with the sequences of Per a 7.0101 (accession no. Y14854) and Per a 7.0102 (accession no. AF106961) and was 97.18% identical to the Bla g 7 sequence of German cockroach, *Blatella germanica* (accession no. AF260897) (Fig. 3). There were 4 amino acid differences between the recombinant tropomyosin and Per

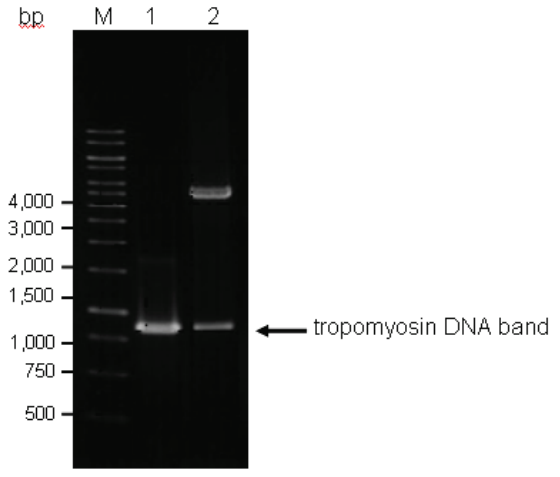

Fig. 1 PCR amplicon of *P. americana* DNA coding for full length tropomyosin (867 bp) (lane 1). Lane 2, recombinant pET20b(+) plasmids with tropomyosin gene sequence insert doubly digested with *Eco*RV and *Hin*dIII. Lane M, 1 kb DNA marker. Numbers at the left are DNA sizes in bp.

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atggatgctatcaagaagaagatgcaggcgatgaagctggagaaggacaacgcgatggac   60
 M  D  A  I  K  K  K  M  Q  A  M  K  L  E  K  D  N  A  M  D
cgcgcccttctctgcgagcagcaggcccgcgacgccaaccttagggccgagaaggctgag  120
 R  A  L  L  C  E  Q  Q  A  R  D  A  N  L  R  A  E  K  A  E
gaggaggcgcgctccctgcagaagaagacccagcagattgagaacgatctggatcagacc  180
 E  E  A  R  S  L  Q  K  K  T  Q  Q  I  E  N  D  L  D  Q  T
atggagcagctgatgcaggtcaacgccaagctggacgagaaggacaaggccctccagaac  240
 M  E  Q  L  M  Q  V  N  A  K  L  D  E  K  D  K  A  L  Q  N
gctgagagtgaagttgctgccctgaaccgccgtatccagctgctagaggaggacctcgag  300
 A  E  S  E  V  A  A  L  N  R  R  I  Q  L  L  E  E  D  L  E
aggtctgaggaacgattggccactgccactgccaaactggccgaggcttcacaggctgac  360
 R  S  E  E  R  L  A  T  A  T  A  K  L  A  E  A  S  Q  A  D
gatgaatccgagcgagcccgtaagattcttgaatccaagggcctggccgatgaagaacgc  420
 D  E  S  E  R  A  R  K  I  L  E  S  K  G  L  A  D  E  E  R
atggacgcactggagaaccagctgaaggaggccaggttcatggctgaagaagcagacaaa  480
 M  D  A  L  E  N  Q  L  K  E  A  R  F  M  A  E  E  A  D  K
aaatatgatgaggttgcacgtaagttggccatggttgaggccgacttggagagagcagag  540
 K  Y  D  E  V  A  R  K  L  A  M  V  E  A  D  L  E  R  A  E
gaacgtgccgagagtggtgaatccaaaattgtagagcttgaggaggagctccgtgttgtc  600
 E  R  A  E  S  G  E  S  K  I  V  E  L  E  E  E  L  R  V  V
ggcaacaacctgaagtcccttgaggtgtctgaggagaaggccaacctgcgtgaagaagag  660
 G  N  N  L  K  S  L  E  V  S  E  E  K  A  N  L  R  E  E  E
tacaagcagcagattaagaccttgactaccaggctaaaggaggctgaggcccgtgctgag  720
 Y  K  Q  Q  I  K  T  L  T  T  R  L  K  E  A  E  A  R  A  E
ttcgctgagaggtccgtgcagaagctgcagaaggaggttgacaggcttgaggatgaattg  780
 F  A  E  R  S  V  Q  K  L  Q  K  E  V  D  R  L  E  D  E  L
gtacacgagaaggagaagtacaagttcatttgtgatgatcttgatatgactttcaccgaa  840
 V  H  E  K  E  K  Y  K  F  I  C  D  D  L  D  M  T  F  T  E
cttgttggttactag                                               855
 L  V  G  Y  -
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Fig. 2 Nucleotide and deduced amino acid sequences of American cockroach, *P. americana*, tropomyosin of this study.

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Tropomyosin      MDAIKKKMQAMKLEKDNAMDRALLCEQQARDANLRAEKAEEEARSLQKKTQQIENDLDQT 60
Per a 7.0101     ********************C****************************I********** 60
Per a 7.0102     ************************************************I********** 60
Bla g 7          ********************************I***************I********** 60

Tropomyosin      MEQLMQVNAKLDEKDKALQNAESEVAALNRRIQLLEEDLERSEERLATATAKLAEASQAD 120
Per a 7.0101     ************************************************************V 120
Per a 7.0102     ************************************************************A 120
Bla g 7          ************************************************************A 120

Tropomyosin      DESERARKILESKGLADEERMDALENQLKEARFMAEEADKKYDEVARKLAMVEADLERAE 180
Per a 7.0101     ************************************************************ 180
Per a 7.0102     ************************************************************ 180
Bla g 7          ************************************************************ 180

Tropomyosin      ERAESGESKIVELEEELRVVGNNLKSLEVSEEKANLREEEYKQQIKTLTTRLKEAEARAE 240
Per a 7.0101     ************************************************************ 240
Per a 7.0102     ************************************************************ 240
Bla g 7          ****T*******************************************N*********** 240

Tropomyosin      FAERSVQKLQKEVDRLEDELVHEKEKYKFICDDLDMTFTELVGY 284
Per a 7.0101     ******************************************A** 284
Per a 7.0102     ******************************************I*N 284
Bla g 7          ****************************Y************I*N 284
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Fig. 3 Comparison of amino acid sequence of *P. americana* tropomyosin of this study (accession no. FJ976895) with the amino acid sequences of Per a 7.0101 (Y14854), Per a 7.0102 (AF106961) and Bla g 7 of German cockroach, *Blatella germanica* (AF260897). The alignment was generated by Clustal W2.

a 7.0101 / Per a 7.0102, *i.e.* R21C, T50I, D120V and V282A/T50I, D120A, V282I and Y284N. There were 8 amino acid differences between the recombinant tropomyosin and Bla g 7, *i.e.* L34I, T50I,

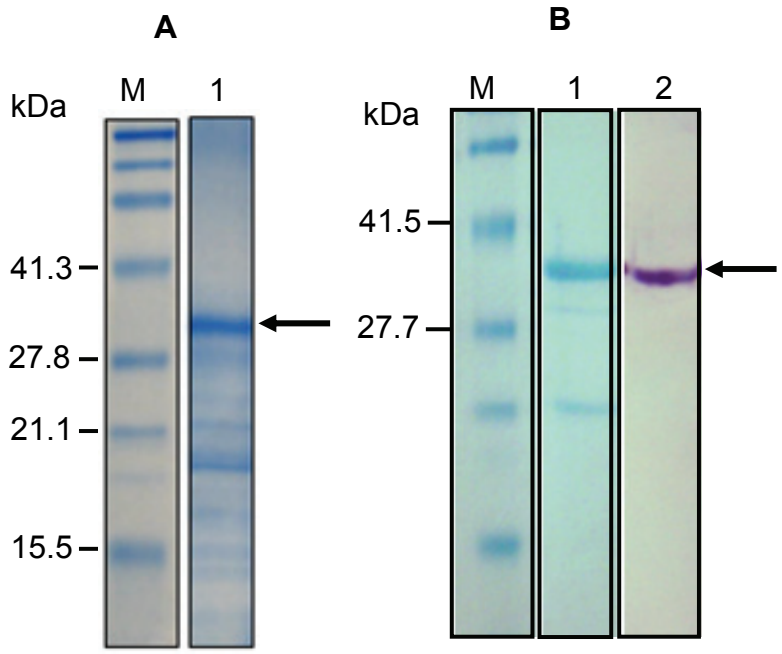

Fig. 4 SDS-PAGE patterns of the native tropomyosin (lane 1 of A; indicated by arrow) and recombinant tropomyosin (lane 1 of B; indicated by arrow). Lane 2 of B is Western blot pattern of SDS-PAGE separated-recombinant tropomyosin probed with mouse monoclonal anti-6x His antibody. Lanes M of both blocks, standard molecular weight proteins. Numbers at the left of both blocks are molecular masses of proteins.

D120A, S185T, T229N, F269Y V282I and Y284N. The native tropomyosin purified from *P. americana* extract and the recombinant tropomyosin purified from lysate of a selected BL21(DE3)pLysS *E. coli* clone carrying *tropomyosin*-pET20b(+) recombinant plasmids are shown in Figs. 4A and 4B, respectively. Both proteins are ~34 kDa. Peptide sequences generated from the recombinant *P. americana* tropomyosin by LC/MS-MS matched with peptide sequences of *P. americana* tropomyosin of the database (accession no. 14423957) (Table 1).

The native and recombinant tropomyosins were used as antigens in the IgE ELISA for detecting IgE in serum samples of 14 consented allergic patients who were positive by skin test to crude CR extract in comparison to 5 negative skin test individuals. It was found that IgE in sera of 8 (57%) and 6 (43%) of the 14 CR allergic patients bound to native and recombinant tropomyosin proteins, respectively, while none of the non-allergic counterparts was IgE ELISA positive (Fig. 5). IgE immunoblot results performed by using recombinant tropomyosin conformed to the IgE ELISA results as shown in Fig. 6.

DISCUSSION

Tropomyosin derived from various invertebrates including insects (*e.g.* cockroaches, mosquitoes), arachnids (*e.g.* house dust mites) and crustacean (*e.g.* shrimp) is known as a pan-invertebrate aero- or food allergen among humans.[12] Tropomyosins share ~80% amino acid identity among inveterbrates but they were less identical to the vertebrate tropomyosins.[10] Within a species several isoforms of the protein exist.[13] In this study, gene sequence encoding full length recombinant tropomyosin of *P. americana* caught in Thailand was amplified, using primers designed from gene sequences of Per a 7.0101 and Per a 7.0102 deposited in the GenBank database (accession nos. Y14854 and AF106961, respectively). Because the sequences of the Per a 7.0101 and Per a 7.0102 were different at one base pair located within the forward and reverse primer binding sites, thus degenerate nucleotides were used for amplification of the particular nucleotides. The nucleotide sequence coding for *P. americana* tropomyosin of this study has been deposited in the

Table 1 Peptide sequences generated from the recombinant *P. americana* tropomyosin by LC/MS-MS that matched with the peptides of a tropmyosin of the database (tropomyosin, accession no. 14423957)

	Accesssion no.	X_{corr} (charge)
Orthologous protein		
Tropomyosin (Per a 7)	14423957	188.27
Matched peptides		
R.FM*AEEADKK.Y		2.84
K.GLADEER.M		1.80
R.FM*AEEADK.K		2.08
R.FM*AEEADK.K		3.02
R.FMAEEADKK.Y		2.68
K.ANLREEEYK.Q		2.26
R.FM*AEEADKKYDEVAR.K		2.77
R.FM*AEEADKKYDEVAR.K		2.88
R.FM*AEEADKKYDEVAR.K		4.07
R.FM*AEEADKKYDEVAR.K		4.17
R.LEDELVHEK.E		3.09
R.FMAEEADKKYDEVAR.K		5.49
R.FMAEEADKKYDEVAR.K		5.46
K.EVDRLEDELVHEKEK.Y		4.07
K.EVDRLEDELVHEKEK.Y		4.03
R.MDALENQLK.E		3.07
K.GLADEERM*DALENQLK.E		3.30
K.GLADEERM*DALENQLK.E		3.76
K.EVDRLEDELVHEK.E		3.43
K.GLADEERM*DALENQLK.E		3.48
K.EVDRLEDELVHEK.E		3.57
K.GLADEERM*DALENQLK.E		3.81
K.ALQNAESEVAALNR.R		4.54
K.ALQNAESEVAALNR.R		3.36
K.EVDRLEDELVHEK.E		2.65
K.DKALQNAESEVAALNR.R		2.71
K.DKALQNAESEVAALNR.R		2.68
R.RIQLLEEDLERSEER.L		2.64
R.RIQLLEEDLERSEER.L		3.09
K.DKALQNAESEVAALNR.R		4.83
K.GLADEERMDALENQLK.E		2.58
R.RIQLLEEDLER.S		3.97
K.GLADEERMDALENQLK.E		2.80
R.RIQLLEEDLER.S		3.81
R.RIQLLEEDLER.S		3.39
R.RIQLLEEDLER.S		3.33
K.GLADEERMDALENQLK.E		3.46
K.GLADEERMDALENQLK.E		3.21
R.IQLLEEDLERSEER.L		3.54
R.IQLLEEDLERSEER.L		3.16
R.IQLLEEDLER.S		3.25
K.IVELEEELRVVGNNLK.S		3.58
K.IVELEEELRVVGNNLK.S		3.06

*Indicates site of the peptide with post-translational modification.

GenBank (accession no. FJ976895). Amino acid sequence deduced from the PCR amplified tropomyosin coding sequence showed 4 amino acid differences with the Per a 7.0101 and Per a 7.0102. Nevertheless, the differences were either at different positions or different substitutions at the same site (R21C, T50I, D120V and V282A for the recombinant tropomyosin *versus* the Per a 7.0101 and T50I, D120A, V282I and Y284N for the recombinant tropomyosin *versus* the Per a 7.0102). Thus, it is possible that the

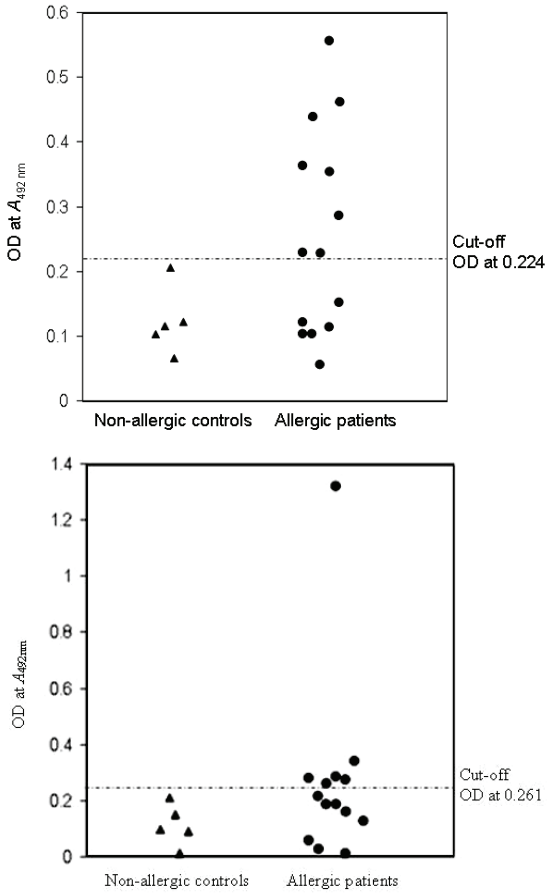

Fig. 5 Scatter plots of sandwich ELISA results for detecting frequencies of binding of native *P. americana* tropomyosin (upper) and recombinant *P. americana* tropomyosin (lower) to specific IgE in sera of 14 allergic patients (●) and 5 normal controls (▲). Cut-off OD between positive and negative IgE ELISA was mean of OD of non-allergic subjects + 2 standard deviations which was 0.224 for native tropomyosin and 0.261 for recombinant tropomyosin.

tropomyosin of the American cockroach caught in Thailand might represent another tropomyosin isoform.

Specific human IgE antibody incited by tropomyosin of one species cross reacts significantly with the allergen from another.[14] Moreover, different isoforms of the same species had different degrees of allergenicity/IgE-binding frequencies.[15] Several studies have shown that native tropomyosins of various species bound specific IgE of allergic patients several fold more frequent than the recombinant counterpart.[16-17] Nevertheless, for American cockroaches, IgE binding of native and recombinant tropomyosins, *i.e.* Per a 7, bound IgE of the allergic French patients at equal frequency, *i.e.* 41 %. In this study, the native tropomyosin had 57% IgE binding frequency compared to 43% of the recombinant protein which conformed to the notion that native protein has more allergenicity than the recombinant counterpart. Reasons have been given to explain these allergenic differences between the native and the recombinant proteins including a small change in amino acid sequence, post translational modifications, *i.e.* acetylation, phosphorylation, glycosylation of the former and inappropriate peptide folding of the latter.[15] Because data in the literature have reported significant degrees of amino acid homologies of *P. americana* tropomyosin with tropomyosins of other CR species and other invertebrates including *P. fuliginosa, B. germanica*, house dust mites, and shrimp,[18] it is worthwhile investigating further the source(s) of the allergenic tropomyosin that induced the specific IgE production in the allergic Thai patients. The finding that native tropomyosin of *P. americana* bound to 57% of the CR allergic Thais indicates that *P. americana* tropomyosin is another major allergen among CR allergic Thais.

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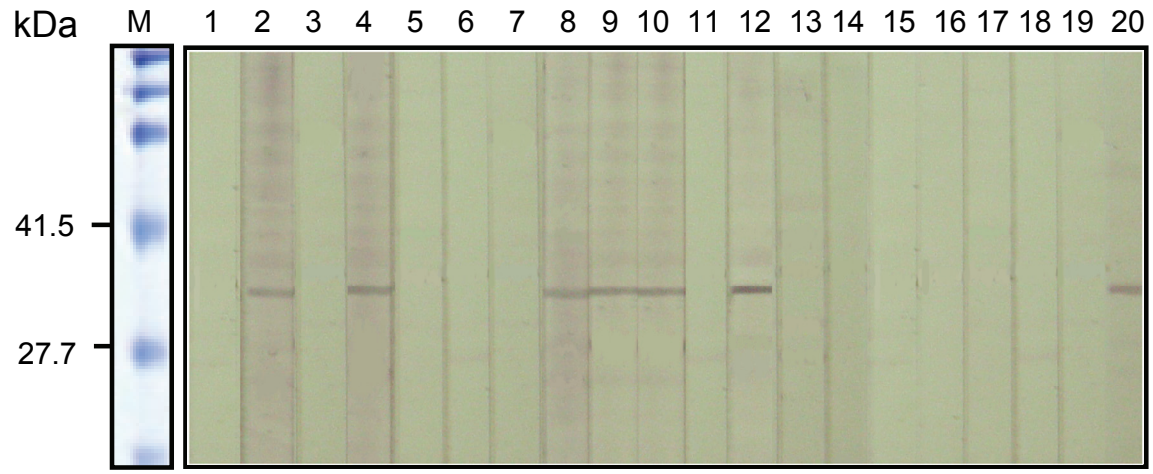

Fig. 6 IgE-immunoblotting of the SDS-PAGE-separated-recombinant tropomyosin probed with individual sera of the 14 CR allergic patients (lanes 1-14) and 5 non-allergic counterparts (lanes 15-19). Lane 20 is SDS-PAGE-separated recombinant tropomyosin probed with mouse anti-6x His monoclonal antibody (band at ~34 kDa) which served as positive control. Sera of 6 of 14 CR allergic patients (43%) had IgE that bound to the recombinant tropomyosin of *P. americana* (nos. 2, 4, 8-10 and 12).

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