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Native Troponin-T of the American cockroach (CR), *Periplaneta americana*, Binds to IgE in Sera of CR Allergic Thais

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SUMMARY The American cockroach, *Periplaneta americana*, is the predominant cockroach (CR) species in Thailand and a major source of indoor allergens second only to the house dust mite. The incidence of CR allergy among allergic Thai patients is increasing but basic information on the allergenic components is scarce. In this study a recombinant troponin-T was produced by using cDNA prepared from RNA of the *P. americana* as a template and PCR primers designed from the *P. americana* troponin-T sequence deposited in the GenBank database. The recombinant protein (*Mr* ~50) did not bind to IgE in the sera of 18 skin prick test positive CR allergic patients. Rabbit polyclonal antiserum (PAb) against the recombinant troponin-T was produced and used in preparing an affinity column for the purification of native troponin-T from the crude *P. americana* extract (*Mr* ~47). IgE-immunoblotting revealed that the native protein bound to IgE in 3 of the 18 (16.7%) patients. Our results imply that native *P. americana* troponin-T, but not its recombinant counterpart, is a minor allergen among the CR allergic Thais

Allergic diseases such as atopic dermatitis, rhinitis and asthma, have become a major public health problem worldwide.[1-3] People with severe allergic manifestations, *i.e.* bronchial asthma, have a poor quality of life and less choice of activities than those without the disease.[4] Sources of the allergens may stem from outdoor and indoor environments. The role of indoor allergens has become accentuated during the past few decades owing to the life style of urban people in closed apartments with inadequate fresh air ventilation. Among the indoor allergens, the house dust mite is regarded as the most important source.[5,6] The role of the cockroach (CR) as another important source of indoor allergens was recognized in 1964 when skin rashes appeared soon after a cockroach crawled over an allergic patient's skin.[7] Skin tests later in 1967 confirmed that the patient had a CR allergy which is a type-I, IgE-mediated, hypersensitivity.[8] People may be allergic not only to the insect's tissues and cast but also to their body secretions and excretions.[9] Presently several CR allergens have been recognized and characterized. These include: Per a 1,[10,11] Per a 3 or aryl phosphorin-hemocyanin,[12] Per a 6 or troponin-C (FT Chew, un-

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published data), Per a 7 or tropomyosin,[13] Per a 9 or arginine kinase,[14] and Per a 10 or serine protease,[15] of the American CR, *Periplaneta americana*, and Bla g 1,[16] Bla g 1(Bd90K),[17]Bla g 2 or aspartic protease,[18] Bla g 4 or lipocalin/calycin,[19] Bla g 5 or glutathione-S-transferase,[20] Bla g 6 or troponin-C,[21] Bla g 7 or tropomyosin,[22] and Bla g 8 or myosin light chain,[23] of the German CR, *Blattella germanica.*

In Thailand, the incidence of CR allergy among allergic Thais as examined by skin testing using crude CR extract ranged from 41-77.5%,[24,25] *P. americana* which is the predominant CR species in the Kingdom was found to be the most important source of CR allergens in the homes of CR allergic patients.[26] Among the American CR allergens, the Per a 1 variant, *i.e*. Per a 1.0105 (AY259514) and Per a 9 (arginine kinase) were found to react with IgE in the sera of all CR allergic Thais implying their role as the major *P. americana* allergens.[11,14] Recent findings, however, indicating that proteins involved in the muscle regulation of the CR*, i.e.* myosin light chain, tropomyosin and troponin-C are also CR allergens[13,22,23] led to this study investigating further the allergenic role of troponin-T, a major protein in the troponin-tropomyosin-actin complex.

MATERIALS AND METHODS

Production of recombinant troponin-T of American cockroach, *P. americana*

Adult cockroaches were trapped and only those entomologically identified as *P. americana* were kept at -70°C. The whole bodies of the frozen CR were ground in liquid nitrogen using a homogenizer (IKA, Germany). Total RNA was prepared from the CR powder using TRIzol Reagent (Invitrogen, USA) and reverse transcribed to cDNA by using a RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, USA) under RNase-free conditions. The cDNA was used as template for the PCR amplification of the troponin-T encoding gene.

Forward (F) and reverse (R) nucleotide primers for the PCR amplification of the *P. americana* CR troponin-T gene were designed from the DNA sequence encoding troponin-T of the GenBank database (Accession no. AF133520). Restriction sites of *Nco*I and *Xho*I were introduced to the 5' and 3' ends of the F and R sequences (underlined), respectively. The F sequence was 5'-ATCCATGGA-TATGTCC-GACGAGGAGGA GGAA-3', and the R sequence was 5'-ATCTCGAGTTCCTCCTTC-TTCTTCTC-3'. The troponin-T encoding DNA sequence was PCR amplified in a ThermoCycler (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 ultra-pure distilled water (UDW). After 5 minutes of an initial denaturation at 94°C, 30 cycles of PCR reaction were performed. Each cycle consisted of 30 seconds at 94°C, 30 seconds at 55°C, and 40 seconds at 72°C and was followed by a final extension at 72°C for 7 minutes. The PCR product was verified by 1% agarose gel electrophoresis, ethidium bromide staining and UV transillumination. The DNA amplicon was purified from the agarose gel by using the GENECLEAN II kit (Bio101, USA) and ligated to the *Nco*I- *Xho*I pre-cut pGEM®-T Easy vector (Promega, USA). The recombinant plasmids were cloned into competent JM109 *E. coli* cells and subcloned into a protein expression vector, pET-20b(+), which had the 6x-His-tag at the C-terminus as a fusion peptide. The recombinant pET-20b(+) plasmids were introduced into competent BL21(DE3)pLysS *E. coli* cells by electroporation.

For preparation of recombinant troponin-T, a colony of the selected transformed BL21(DE3)-pLysS *E. coli* grown overnight on an LB-ampicillin agar plate was inoculated into 5 ml LB-ampicillin broth and incubated overnight at 37°C with shaking at 250 rpm. One milliliter 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 A_{600nm} was 0.6. IPTG was added to the concentration of 1 mM and the culture was incubated further for 3 hours. The bacterial cells were harvested by centrifugation at 4,000 x *g* at 4°C for 20 minutes. The cells were lysed in a lysis buffer containing protease inhibitors by sonication (Roche Diagnostics GmBH, Germany) at 20 kHz, 2 minutes pulse-on, 3 minutes pulse-off. Cell debris was removed by centrifugation at 12,000 x *g* at 4°C for 20 minutes. The supernatant (cell lysate) was collected. The presence of recombinant troponin-T in the lysate was verified by SDS-

PAGE and Western blot analysis using anti-His-HRP and substrate. The recombinant troponin-T was purified from the *E. coli* lysate using a pre-equilibrated Nickel column (ProBond™, Invitrogen).

Human subjects and serum samples

Sera were collected individually from 18 CR-allergic Thai patients at the Department of Oto-Rhino-Laryngology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. These subjects were positive by skin prick test to crude extract of *P. americana*. The sera of 12 individuals who gave negative skin prick tests to the CR extract were used as non-allergic controls. The work was approved by the Ethical Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University, and verbal informed consents were obtained from all subjects.

SDS-PAGE, Western blot analysis (WB), and IgE-immunoblotting

SDS-PAGE, Western blot analysis and IgE-immunoblotting were performed as previously described.[14] A 4% stacking and 12% separating polyacrylamide gels were used in the SDS-PAGE which was run in a Mini-PROTEAN® 3 Cell (Bio-Rad, USA) following a method described previously.[28] The components resolved in the SDS-PAGE gel were electro-blotted onto a nitrocellulose membrane (NC) and the empty sites on the NC were blocked by 3% bovine serum albumin (BSA) in PBS. The NC blot was probed with the anti-His-HRP (Pierce, USA) and the target protein was revealed by using the HRP chromogenic substrate, *i.e.* 2,6-dichlorophenolindophenol. Alternatively, the NC blot was covered with the serum/rabbit polyclonal antibody (PAb) to recombinant troponin-T and the immune complexes on the NC were revealed by using appropriate enzyme labeled-anti-isotypic specific antibody and substrate.

For IgE-immunoblotting, the NC strips blotted with SDS-PAGE separated-CR proteins 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).

Two dimensional gel electrophoresis (2DE)

Two dimensional gel electrophoresis (2DE) of protein samples was performed as previously described.[29,30] For the first dimensional electrophoresis, a 7 cm-IPG strip and 0.5% NL pH 3-10 IPG buffer (Amersham Biosciences) were used. The electrophoresed-IPG strip was subjected to 12% SDS-PAGE and proteins in the gel were stained by Coomassie Brilliant Blue G-250. Gel plugs containing proteins of interest were excised from the stained gel and subjected to in-gel tryptic digestion and LC/MS-MS, respectively.[29,30] Protein orthologues were identified by comparing the peptide sequences of the CR proteins generated from the mass spectrometry with the sequences of the database.

Preparation of rabbit anti-recombinant troponin-T polyclonal antibody (PAb)

All animal manipulations in this study followed the guidelines of the National Research Council of Thailand and were approved by the Ethical Committee of the Faculty of Allied Health Sciences, Thammasat University, Thailand. A New Zealand White rabbit weighing ~1.5 kg, purchased from the National Laboratory Animal Center, Mahidol University, Salaya Campus, Nakhon-pathom province, was intramuscularly immunized with the recombinant troponin-T. To prepare the immunogen, recombinant troponin-T, purified from the transformed *E. coli* lysate by a Nickel column, was subjected to SDS-PAGE. Gel strips containing troponin-T (detected by the anti-His-HRP and substrate) were cut and minced in a small volume of PBS. The preparation was mixed with an equal volume of Freund's complete adjuvant and the mixture was injected intramuscularly into both thighs of the animal. A booster dose was given using the same immunogen mixed with Freund's incomplete adjuvant. The rabbit was test-bled seven days after the second immunization and the indirect ELISA antibody titer was de-

termined against the homologous antigen.[31] The rabbit was bled *via* heart puncture under ether anesthesia. Immune serum was collected from the blood, immunoglobulins were precipitated from the serum using 40% saturation of ammonium sulfate, and the IgG fraction was prepared using a protein-A-agarose column (Affi-Gel, Bio-Rad Laboratories, CA, USA).

Preparation of native troponin-T from *P. americana*

The rabbit anti-recombinant troponin-T IgG was coupled to CNBr-activated Sepharose™ (Amersham Biosciences, Uppsala, Sweden) and the resin was used for preparing native troponin-T from the crude CR extract. One hundred micrograms of the *P. americana* extract in 8 ml of PBS were added to a tube containing rabbit IgG anti-recombinant troponin-T-CNBr-activated Sepharose resin. The tube was placed on an end-over-end mixer at 4°C overnight. The fluid was decanted and the resin was washed with PBS until there was no detectable absorbance at A_{280nm} in the wash fluid. The resin was packed into a column and the resin bound CR antigen was eluted from the affinity column by using 0.1 M glycine-HCl, pH 2.0. Fractions of 1.0 ml were collected and immediately neutralized by adding 150 µl of 1 M Tris-HCl pH 8.5. The optical density (OD) of each fraction at A_{280nm} was determined and only the fractions with OD above the blank (Tris-HCl buffer) were subjected to SDS-PAGE and Western blot analysis for detecting the native CR troponin-T. Subsequently, fractions containing the troponin-T were pooled and concentrated by lyophilization.

RESULTS

Total RNA extracted from the *P. americana* and the troponin-T DNA amplicon (1,200 bp) are shown in Fig. 1A and 1B, respectively. Fig. 1C shows troponin-T DNA amplified from *Nco*I and *Xho*I doubly digested recombinant pET-20b(+) plasmids extracted from a transformed BL21(DE3)-pLysS *E. coli* clone. The troponin-T DNA was extracted from the gel and sequenced. Pair-wise alignment between the nucleotide and deduced amino acid sequences of the troponin-T constructed in this study with the respective sequences of *P. americana* troponin-T of the database (GenBank Accession no. AF133520) by Clustal W using the BLOUM62 scoring matrix revealed 99% homology (Fig. 2).

Fig. 3A shows SDS-PAGE-separated patterns of fractions containing recombinant troponin-T purified from the whole cell homogenate of the transformed *E. coli* grown under IPTG induction by using a nickel column and batch elution with a 50-250 mM imidazole solution. The respective Western blot patterns revealed by the anti-His-HRP and the enzyme chromogenic substrate are shown in Fig. 3B. The recombinant troponin-T with a relative molecular mass (*Mr*) ~50 was clearly seen in fractions eluted by 50-200 mM imidazole with some protein

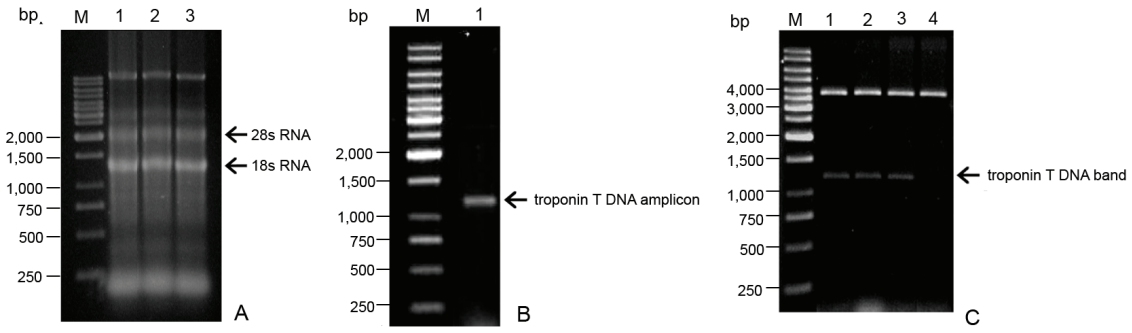

Fig. 1 (A) Total RNA extracted from *P. americana* (lanes 1-3); (B) troponin-T gene of 1,200 bp amplified from the cDNA prepared from the total RNA; and (C) *troponin-T*-recombinant pET-20b(+) plasmids doubly digested with *Not*I and *Xho*I (lanes 1-3) and the original pET-20b(+) vector similarly digested by the endonucleases (lane 4). Lanes M of all blocks are 1 kb DNA marker. Numbers at the left of all blocks are DNA sizes in bp.

degradations at the lower *Mr* (Fig. 3A and 3B).

Fig. 4A illustrates protein spots of troponin-T fractions eluted with 150 mM imidazole after 2DE and Coomassie Brilliant Blue G-250 staining. The prominent protein in the spot located at *Mr* ~46-50 (the expected location of recombinant troponin-T) was subjected to LC/MS-MS to generate a peptide mass map and the prominent mass peaks (Fig. 4B) were chosen for the database search for their orthologues. The protein was confirmed as the *P. americana* troponin-T.

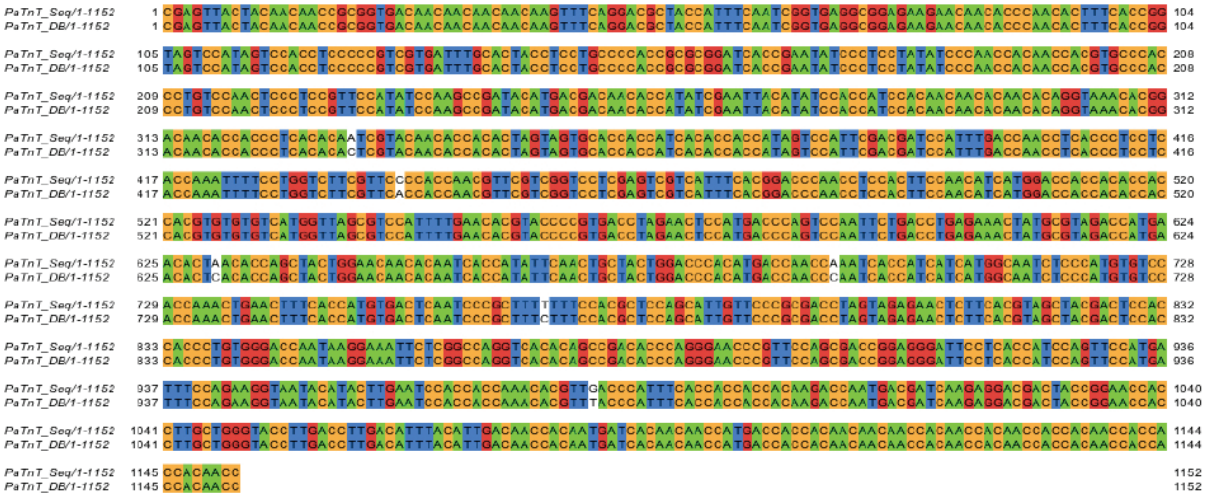

Fig. 2 Pair-wise alignment between nucleotide sequence of *P. americana* troponin-T of the database (GenBank Accession Number AF133520) and that of the recombinant *P. americana* troponin-T of this study. They showed 99% identity. The alignment was generated by Clustal W using the BLOSUM62 scoring matrix. The graphic program was performed under JalView version 2.2 software.

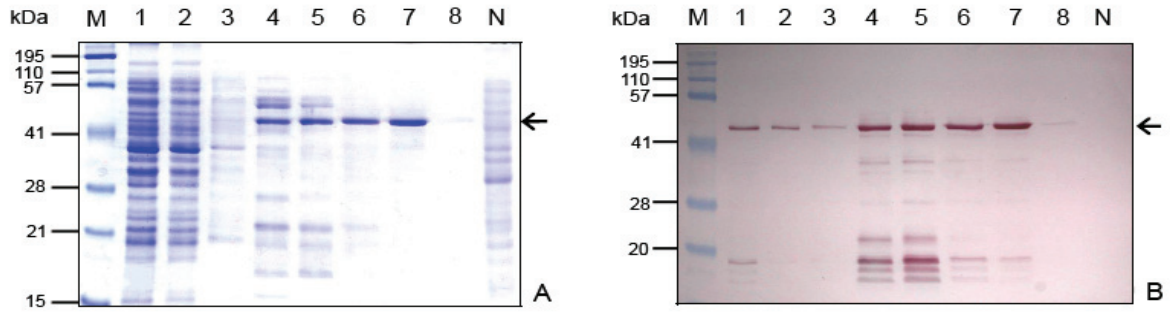

Fig. 3 (A) Fractions eluted from a Nickel column loaded with lysate of transformed *E. coli* cells harboring *troponin-T*-pET-20b(+) plasmids after IPTG induction, stained by Coomassie Brilliant Blue G-250. Lane M, Pre-stained protein marker; lanes 1-3 are proteins in the *E. coli* lysate, unbound materials, and washed fluid, respectively. Lanes 4-8 are fractions eluted with 50-250 mM imidazole, respectively. (B) Western blot patterns of A probed with anti-His-HRP. Lanes N of A and B are lysates of *E. coli* harboring empty pET-20b(+) plasmids as negative controls. Recombinant troponin-T (*Mr*~50) was contained in fractions eluted with 50-200 mM imidazole with some degradation seen at the smaller *Mr*.

Native troponin-T of *P. americana* was purified by loading the crude CR extract to the rabbit anti-recombinant troponin-T-affinity column. After the flow-through proteins were removed by washing, the bound protein, *i.e.* native troponin-T was eluted.

The IgE-binding capacity of the recombinant and native troponin-T proteins was investigated by IgE-immunoblotting using individual serum samples of the 18 CR allergic patients to probe the SDS-PAGE separated-proteins. No IgE reactive band was found when all sera were allowed to react with the recombinant troponin-T (data not shown). Nevertheless, sera of three CR allergic patients (no. 6, 13 and 14) gave IgE reactive bands to the native troponin-T. None of the 12 serum samples of the non-CR allergic controls gave IgE-positive bands (Fig. 5).

DISCUSSION

Recently tropomyosin and troponin-C which are involved in the regulation of muscle contractions of cockroaches (CR) were reported as human allergens.[32,33] Thus, in this study we elaborated further the allergic role of troponin-T, which is another member of the troponin-tropomyosin-actin complex,

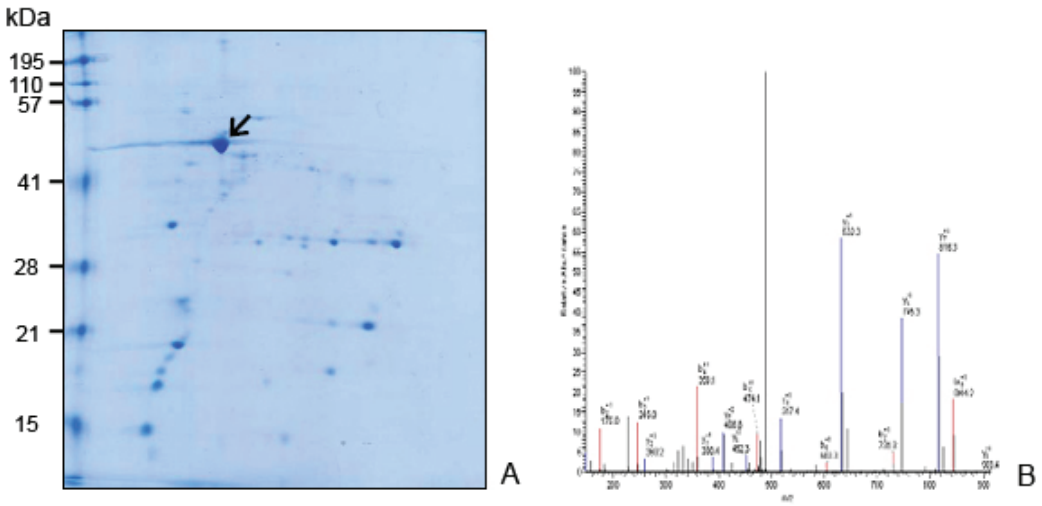

Fig. 4 (A) Proteins contained in the 150 mM imidazole eluted fraction after 2DE and Coomassie Briliant Blue G-250 staining. The arrow indicates a protein spot (*Mr*~46-50) which was subjected to LC/MS-MS using model Finnigan LTQ Linear Ion Trap to generate peptide mass fingerprints as seen in (B). The orthologous protein of the database highest matched with the peptide sequences was the *P. americana* troponin-T.

Fig. 5 IgE-immunoblotting of the SDS-PAGE-separated-native troponin-T probed with individual sera of the 18 CR allergic patients (lanes 1-18) and 4 non-allergic counterparts (lanes 19-22). Lanes B and 23, and lane 24 are SDS-PAGE-separated native troponin-T reacted with serum diluent, and anti-recombinant troponin-T polyclonal antibody, respectively.

among CR allergic Thai patients. The gene encoding *P. americana* troponin-T was amplified from cDNA of the American cockroaches using a pair of oligonucleotide primers designed from the only one DNA sequence of the *P. americana* troponin-T deposited in the database (GenBank, Accession number AF 133520). The 1,153 bp-troponin-T gene was successfully amplified from the cDNA of *P. americana* samples and ligated to a pET-20b(+) expression vector containing a strong bacteriophage T7 promoter. The recombinant troponin-T was expressed in BL21(DE3)pLysS competent *E. coli.* The nucleotide and the deduced amino acid sequences of the recombinant troponin-T produced in this study had 99% identity to those of the *P. americana* troponin-T deposited in the database. The calculated molecular mass of the complete sequence of the troponin-T gene of the database was 45.9 kDa and an isoelectric point (pI) was 4.82. In this study, the recombinant His-tagged troponin-T which was found in the soluble part of the transformed *E. coli* had a relative molecular mass (*Mr*) of ~50 which is slightly larger than the database calculated size but not unexpected as the recombinant troponin-T contains also the restriction endonuclase sites and 6xHis-tag. Nevertheless, the CR troponin-T is markedly larger than the troponin-T subunit of a vertebrate striated muscle which is only 37 kDa.[34] Since the molecular mass of the troponin complex in the insect flight muscle, *i.e.* 148 kDa,[35] is approximately two times greater than in the vertebrate muscle, *i.e.* 79 kDa,[36] it is possible that the cockroach troponin-T might possess a larger molecular mass than the vertebrate troponin-T.

The recombinant CR troponin-T was successfully purified from the Nickel column by 50-200 mM imidazole batch elution. The recombinant troponin-T eluted in a 150 mM fraction was revealed by 2DE to be also *Mr* ~50 kDa and pI ~5 and it was confirmed by LC/MS-MS to be troponin-T of *P. americana*.

None of the 18 CR allergic Thai patients who gave positive skin prick test results to *P. americana* crude extract had IgE that bound to the recombinant troponin-T. This can be interpreted in several ways. The IgE-immunoblotting used for detecting the specific IgE to the recombinant troponin T might not be sensitive enough to detect such minute amounts of the IgE in the patients' sera at the serum dilution used in the test, *i.e.,* 1:10 (a higher concentration of the sera was not possible because of the limited volume of samples available). A more sensitive IgE detection assay such as the inhibition ELISA[37] or a fluorescence multiplex assay system which detects IgE binding to a minor allergen[33] should be performed. Another reason for the negative IgE-immunoblotting results could be that there might have been no allergenic epitopes on the recombinant troponin-T as a result of nucleotide substitution during the gene amplification by PCR using the *Taq* polymerase and/or at the step of the recombinant plasmid segregation by *E. coli* cells. In this study, the troponin-T encoding DNA obtained from the selected transformed *E. coli* clone showed 99% sequence homology with the *P. americana* sequence of the database implying some degree of nucleotide replacement during the gene amplification. Moreover, the number of allergic patients used for verification of the IgE binding activity of the recombinant troponin-T could also be a factor. Regulatory proteins of the muscle contraction apparatus, *e.g.* tropomyosin and troponin-C, of the *P. americana* are minor CR allergens, *i.e.* less than 50% of CR allergic patients had specific IgE to these allergenic proteins.[38] Specific IgE to the CR tropomyosin was found in 41% of the CR allergic patients.[13] Bla g 6 (troponin-C) was reported as a minor CR allergen with a prevalence of IgE binding to recombinant Bla g 6 of 14% among of 104 patients with cockroach allergy.[33] Recombinant Tyr p 13, the fatty acid binding protein of the storage mite, *Tyrophagus putrescentiae*, is another example of a minor antigen which could be detected in only 5 of 78 (6.4%) allergic sera tested. Only eighteen CR allergic patients were included for testing the IgE binding activity of the recombinant troponin-T in this study. The number of samples might have been too small to reveal a positive IgE reactivity of the recombinant troponin-T.

There are many troponin-T isoforms in striated and cardiac muscles and different isoforms play different roles in the regulation of the muscle contraction.[39-40] However, the isoforms of *P. americana* troponin-T have never been reported. Isoforms of protein differ from each other in numbers and compositions of amino acid sequence resulting in a difference in their electrophoretic mobility.[39] The presence of protein isoforms can be identified by the migration pattern of the purified protein on SDS-PAGE

or 2DE. The recombinant protein produced from a sequence of a particular isoform would not represent other native isoforms. The allergenic epitope may also be absent on that recombinant counterpart of the selected isoform; consequently, the recombinant protein would be unable to bind to IgE in the allergic patients' serum elicited by the natural proteins. Therefore, using recombinant allergen as an antigen for detecting a specific IgE activity may not be appropriate. This is exemplified by the work of Wang and his colleagues who tested the skin reactivity of patients using natural Per a 3 compared to recombinant Per a 3. They found a marked difference in the percentage of positive reactions, 83% for natural Per a 3 *versus* only 47% for recombinant Per a 3.[41] Thus, to pinpoint the allergenic role of troponin T, a native troponin-T is needed for testing IgE reactivity in an appropriate number of allergic sera.

In this study, native *P. americana* troponin-T was purified by using an anti-troponin-T affinity column. The results of the IgE-immunoblotting have shown that 3 of 18 (16.7%) CR allergic patients' sera gave a positive reaction to the native troponin-T preparation implying the role of this muscular regulated protein component as a minor CR allergen among the CR allergic Thais.

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