

# Molecular Cloning and Characterization of Tropomyosin, a Major Allergen of *Chironomus kiiensis*, a Dominant Species of Nonbiting Midges in Korea

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**Chironomids are widely and abundantly distributed in the vicinity of standing waters. Larvae of *Chironomus* and some other genera are known to contain hemoglobins, which have been described as a major allergen, and the adults that have no hemoglobins also have been reported to contain allergens. In this study, we tried to establish the role of chironomid allergy and characterize the allergen of *Chironomus kiiensis* adults. Skin tests using *C. kiiensis* adult extracts were performed on patients with allergic symptoms. A cDNA library of *C. kiiensis* adults was screened with *C. kiiensis* immune mouse sera to identify allergens, and results were confirmed using skin test-positive human sera. Recombinant allergen was expressed in *Escherichia coli* and purified by affinity chromatography using nickel-nitrilotriacetic acid agarose to investigate its allergenic properties. Out of 275 allergic patients 14.2% showed a positive reaction to *C. kiiensis* adult crude extracts in the skin test. The tropomyosin was cloned by immunoscreening and expressed in *Escherichia coli*. *C. kiiensis* tropomyosin has a high homology at the amino acid level with tropomyosins which were previously known to be allergens in various arthropods (*Periplaneta americana*, 86.3%; *Panulirus stimpson*, 78.9%; *Dermatophagoides pteronyssinus*, 76.5%). Specific immunoglobulin E antibodies reacting to recombinant tropomyosin were detected in 17 (81%) of 21 patients whose skin test results were positive. Cross-reactivity against house dust mites and other insects was noticed with mouse anti-recombinant tropomyosin immune serum. *C. kiiensis* adults were shown to be an important source of inhalant allergens in Korea. Molecular cloning of *C. kiiensis* tropomyosin was performed and IgE reactivity was demonstrated using skin test-positive human sera. Recombinant tropomyosin will be useful for further studies or clinical applications.**

Adults and larvae of nonbiting midges are known to have potent allergens that cause immunoglobulin E (IgE)-mediated asthma or, less frequently, allergic conjunctivitis. Allergy nomenclature in this paper follows the revised version (7). It has been reported that *Cladotanytarsus lewisi* occurred in enormous numbers in the Nile basins in Sudan and caused severe respiratory diseases in people living close to the Nile (10). Chironomid larvae were widely used as fish food in Germany and elicit occupational allergic diseases in a significant number of people who were exposed to these larvae (1, 15).

Hemoglobins, which are unique components in chironomid larvae among insects, have been identified as the most important allergen found in asthmatic patients (2, 3, 9, 18, 28). On the other hand, 40% of Japanese, Taiwanese, and Swedish sera from patients with IgE-mediated asthma showed positive reactions against crude extracts of a midge (*Cricotopus sylvestris*) extract which contained no hemoglobin (29). Up to the present, several clinical cases in which patients had positive skin tests or positive results for chironomid-specific IgE have been reported. Few studies on chironomid allergens except for he-

moglobin have been carried out, although a protein with a high molecular weight in the whole-body extract of chironomid adults has been suggested to be more allergenic than hemoglobin (16).

People are unlikely to inhale the hemoglobin of chironomid larvae, which live under water, but the dead debris of ephemeral chironomid adults, which often swarm and gather around lights, is more likely to contact persons and cause allergic diseases.

*Chironomus kiiensis* and *Chironomus flaviplumus* were reported to be the most prevalent species of chironomids in Korea out of about 50 recorded species to date (20). The latter emerges from eutrophic waters in urban areas, whereas the former is distributed mainly in rural areas, breeding in rice paddies, lakes, and other clean waters.

In the present study, IgE immunoblot analyses were performed using both human sera from individuals who had positive skin test reactions to the chironomid extract and the immunized BALB/c mouse sera in order to identify IgE-binding proteins in whole-body extracts of *C. kiiensis* adults, which represent the dominant species of nonbiting midges in Korea.

## MATERIALS AND METHODS

**Collection of *C. kiiensis* and preparation of the crude extract.** *C. kiiensis* adults were collected every three to four days per week from July to October in 1997 using three light-traps on rice paddies at Dokyang-gu, Koyang-si, Kyonggi-do,

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Korea. *C. kiiensis* adults were identified following the key for morphological identification and stored at  $-70^{\circ}\text{C}$  until used (20).

Frozen midges were defrosted, homogenized, and then extracted in 10 mM phosphate-buffered saline, pH 7.4, by incubation for 10 h at  $4^{\circ}\text{C}$ . The extracts were used for the enzyme-linked immunosorbent assay or immunoblot analysis. Lyophilized midges were extracted in the modified Coca solution (0.9% NaCl, 0.25%  $\text{NaHCO}_3$ , 0.04% phenol), and 1:40 (wt/vol) diluted extract at a final concentration of 1.5 mg/ml was used for the skin prick test (19). The tip of a 26-gauge needle was introduced through a drop of the extract into the superficial layers of the skin, which were gently lifted. A reaction was considered positive when a wheal with a diameter of 3 mm developed. Positive (histamine dihydrochloride [1 mg/ml]; Bayer) and negative (albumin-saline with phenol; Bayer) controls were applied in all tests.

**Skin prick test and collection of human sera.** The skin prick test using *C. kiiensis* crude extracts was performed on patients who came to the allergy clinic of the Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. A total of 275 subjects, which included females 10 to 75 years old ( $n = 138$ ; average age, 41 years) and males 15 to 69 years old ( $n = 147$ ; average age, 31 years), were enrolled in the study. All subjects underwent the skin prick test using extracts of *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blattella germanica*, *Periplaneta americana*, *C. kiiensis*, *Momomorium pharaonis*, *Drosophila melanogaster*, *Penicillium mix*, and *Aspergillus fumigatus* (Bencard Co., Gaggenau, Germany). Sera of the skin test-positive subjects were collected from September 1998 to February 1999 and kept frozen at  $-20^{\circ}\text{C}$ .

**Production of antisera against *C. kiiensis* adult crude extract in mice.** Three 6-week-old female BALB/c mice were immunized to obtain antiserum. Aluminum hydroxide gel (alum) was prepared as described previously (21). Twenty micrograms of the crude extract in phosphate-buffered saline was mixed with 1 ml of alum, and the mice received intraperitoneal injections with the crude extract-gel (0.1 ml, containing 2  $\mu\text{g}$  of the crude extract and about 14 mg of alum) at 0-, 4-, and 8-week intervals.

**Construction of a cDNA expression library.** The frozen sample of *C. kiiensis* after morphological identification was homogenized in liquid nitrogen and mRNA was purified by using the mRNA isolation kit (Stratagene, La Jolla, Calif.), and a cDNA expression library in the ZAP Express vector (Stratagene) was constructed using a ZAP Express cDNA Gigapack II gold cloning kit (Stratagene) according to the manufacturer's instruction. Immunoscreening was carried out by phage dot immunoblot analysis using a *pico*Blue immunoscreening kit (Stratagene) with a 1:500 dilution of serum from mice immunized with crude extract of *C. kiiensis* and alkaline phosphatase-labeled anti-mouse IgG1. Positive clones were rescreened until all plaques on the plate were positive. Selected clones in ZAP Express vector were excised *in vivo* with ExAssist helper phages (Stratagene).

**DNA sequencing and homology search.** DNA sequencing of plasmid purified from the transformed bacteria was performed by the dideoxy nucleotide chain termination method (26) using a Sequenase version 2.0 DNA sequencing kit (Amersham Life Science, Cleveland, Ohio). Analysis of the derived nucleotide and deduced amino acid sequences was performed using the NCBI/BLAST network service of the National Center for Biotechnology Information, Bethesda, Md. A homology search in GenBank was performed using the NCBI Blast program.

**Production of the recombinant tropomyosin.** Tropomyosin-encoding cDNA was subcloned into the *Bam*HI and *Xho*I sites of the expression vector pET-28a and expressed in *Escherichia coli* BL21(DE3). The cells were grown to an optical density at 600 nm of 0.6 in Luria-Bertani broth (23) containing kanamycin (50  $\mu\text{g}/\text{ml}$ ), and then expression was induced by the addition of isopropyl-thio- $\beta$ -galactoside to a final concentration of 1 mM. Incubation was continued for another 5 h, and the cells were harvested by centrifugation. Recombinant tropomyosin was purified by affinity column chromatography using Ni-NTA Agarose (QIAGEN, Valencia, Calif.) according to the manufacturer's manual.

**IgE immunoblot analysis.** Proteins after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were electrophoretically transferred onto the nitrocellulose membranes and reacted with 1:2-diluted human sera overnight at room temperature. After washing five times, the membranes were incubated with 1:1,000-diluted monoclonal (clone GE-1) anti-human IgE alkaline phosphatase conjugate (catalog no. A-3076; Sigma, St. Louis, Mo.) for 1 h at room temperature. Color was developed with 3-bromo-4-chloro-5-indolyl-phosphate (758 ng/ml) and nitroblue tetrazolium (379 ng/ml) in a solution containing 100 mM Tris-Cl (pH 9.5), 100 mM NaCl, and 5 mM  $\text{MgCl}_2$ .

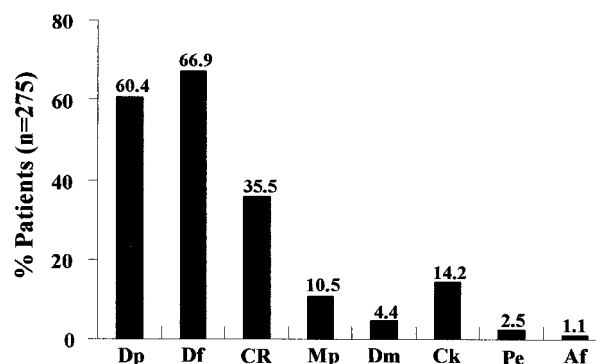


FIG. 1. Skin test reactivity to respiratory allergens among patients. Abbreviations: Dp, *D. pteronyssinus*; Df, *D. farinae*; CR, *P. americana* and *B. germanica*; Mp, *M. pharaonis*; Dm, *D. melanogaster*; Ck, *C. kiiensis*; Pe, *Penicillium*; Af, *A. fumigatus*.

## RESULTS

**Frequency of chironomid allergy among allergic patients in Korea.** Patients complaining of allergic asthma, allergic rhinitis, allergic urticaria, allergic conjunctivitis, throat irritation, atopic eczema/dermatitis syndrome, or itching underwent the skin prick test (Fig. 1). Thirty-nine of 275 subjects with the symptoms of allergic asthma, allergic rhinitis, allergic urticaria, or throat irritation showed a positive reaction to the *C. kiiensis* crude extract. As shown in Fig. 1, house dust mites are the most potent elicitor of allergic disease, and cockroaches are the second.

**Cloning of *C. kiiensis* tropomyosin.** Six strongly positive clones were identified by immunoscreening with serum from mice immunized with *C. kiiensis* crude extract, and all clones were found to have the identical cDNA sequence.

The cloned cDNA sequence contains an 855-bp open reading frame coding for 285 amino acids, with an estimated molecular mass of 32,531 Da and a pI of 4.58. No potential N-linked glycosylation sites were found (Fig. 2). As shown in Fig. 3, BLAST search showed that *C. kiiensis* tropomyosin has the highest homology with insect tropomyosins (*D. melanogaster*, 89.1%; *Periplaneta americana*, 86.3%). It also has high sequence identities with those of two other major arthropod groups that are known to be major allergens, such as *Panulirus stimpson* (Crustacea, 78.9%) and *D. pteronyssinus* (Chelicerata, 76.5%). This sequence, which was deposited in the GenBank under accession number AJ012184, was designated as Chi k 10, according to the allergen nomenclature of the World Health Organization and the International Union of Immunological Societies (13).

**Purification of recombinant tropomyosin.** The purified recombinant tropomyosin containing 34 more amino acids at the N terminus gave a band on Coomassie blue-stained SDS-polyacrylamide gels with an apparent molecular mass of 45 kDa. The size is larger than the one calculated from the nucleotide sequence. The mobility of the tropomyosin significantly reduced in the presence of 6 M urea, giving an apparent molecular mass of 67.6 kDa, whereas ovalbumin migrated at the expected position (Fig. 4). The final yield of the purified recombinant protein measured by Bradford assay (Bio-Rad, Hercules, Calif.) was approximately 2 mg/liter of *E. coli* culture.

1 cgcggatcagattcaagtttttacagtttgagtttaattgtgaaagtatcgaataa  
 61 atctgcccaactggatgccatcaagaaaagatgcaagcgtgaagcttgagaaggataa  
 M D A I K K K M Q A M K L E K D N  
 121 tgccttagatcgtgcccttctctgtgaaaatcaagctcgtgatgctaacctgctgctga  
 A L D R A L L C E N Q A R D A N L R A E  
 181 aaaggctgaagaagaagcccgacatgcaaaagaagatccaaactatgaaaacgatt  
 K A E E E A R T L Q K K I Q T I E N D L  
 241 ggatcaacacaaggacaagaactcttgcctcaagcaactcgaaggaaaggaaaagc  
 D Q T Q G Q E T L V N G K L E G K E K A  
 301 tctccaaaatgctgaatcagaagctgctgcttgaaccgtcgtatccaattggtgggaga  
 L Q N A E S E V A A L N R R I Q L L G E  
 361 agatcttgaccgttcagaagaacgttggcttcagcaacagcaaatgtcagaagcatc  
 D L D R S E E R L A S A T A K L S E A S  
 421 agctgctgctgatgagctgcaagctgcaagctaaaatcttgaagaacggctcactcgctga  
 A A A D E S E R A R K I L E N R S L A D  
 481 tgaagaacgtatggatgccctcgaagaatcaatgaaaggaagcagcttccttgcagaaga  
 E E R M D A L E N Q L K E A R F L A E E  
 541 agcagcaaaaaatcagatgaggttgcacgtaaaatggctatggttgaggtgatcttga  
 A D K K Y D E V A R K L A M V E A D L E  
 601 acgtgctgaagaacgtcagaggtggtgaagctaaaatgtgaaacttgaagaagaact  
 R A E E R A E A G E A K I V E L E E E L  
 661 ccgtgtttaggcaacaactgaaatcattagaagctcagaagaaaaggcaaaccaag  
 R V V G N N L K S L E V S E E K A N Q R  
 721 tgaagaagaatcaaaaaatcaaatcaaacccctcacaaccgcttgaaggagctgaagc  
 E E E Y K N Q I K T L T T R L K E A E A  
 781 ccgcgagaattcgtgaacgttccgttcagaatgtcagaaggaagtcgacagactga  
 R A E F A E R S V Q K L Q K E V D R L E  
 841 agacgaactcgttccgaaaaggaaaagtcagagaaaatggagacgactcagacaccg  
 D E L V S E K E K Y R E I G D D L D T A  
 901 atctgtggaacttactcctcaagaatgaacaaatctctatcgcaaatcaaatggaaatg  
 F V E L I L K E  
 961 agcacttatggatattttctatttctcaaaaaatcttattgttataaaatccgctc  
 1021 tttttacttactcaaaaaaacacacacacacacacacactcaatggatcacccctacaa  
 1081 atgatactttataatttctatttgaactatttactcaatttttaatttcaatccac  
 1141 atgtaattataacggttaagttatataaaaaatctcatatttcttaaacctgtgccc  
 1201 ttgaaactgaaacatcaacaaggaaacatcttttaattgaaactgcagatgataatt  
 1261 ttttaaaaacaactttagatcgaatttgcgtttttttgtttattattattattat  
 1321 gggtgaaacatcaattcaatgattgctgagctgagaatattttgcaaacatatttta  
 1381 tggaaataaagggaagaagtttattatgaacattttataaaaaaataaaaaa  
 1441 a

FIG. 2. Nucleotide and deduced amino acid sequences of *C. kiiensis* tropomyosin. The estimated molecular mass is 32.531 kDa, and no potential N-linked glycosylation sites were identified.

**IgE immunoblot analysis.** Immunoblot analysis was carried out to confirm the reactivity of the recombinant protein. IgE-reacting antibodies to the recombinant tropomyosin were detected in 17 (81%) of 21 sera tested (Fig. 5). Serum from the

nonallergic control was not reactive to the recombinant protein.

DISCUSSION

Two cases of asthmatic patients allergic to *Chironomus plumosus* and *Tokunagayusurika akamusi* extracts were reported in Korea (11). Twenty percent of the Korean patients with respiratory allergic symptoms were shown to react positively with chironomid extracts (19). Since the two species of chironomids that were used in these experiments are rarely found in Korea, the results indicate that their chironomid antigens contained materials cross-reactive to other chironomid species which are widely distributed in Korea. A high frequency of IgE antibody responses to chironomids was also demonstrated in Japanese patients with allergic asthma (14).

Chironomid allergy accounts for 14.2% of allergic subjects, which is more than the proportion of other allergic diseases that are caused by known indoor allergens except those caused by house dust mites and cockroaches. Large numbers of patients are likely to be found in the vicinity of standing waters where nonbiting midges emerge in large numbers. High levels of chironomid antigens in both air and soil were demonstrated, and a seasonal fluctuation in the air which peaked only in the autumn was also examined (12).

We have previously reported that the various bands of 110, 80, 73, 46, 40, 37, and 34 kDa of the chironomid adult extracts were found to react with skin test-positive human sera by IgE immunoblot analysis (31).

Change of allergenicity during metamorphosis was investigated. Mature adult extracts were shown to contain more substances that bound to the IgE of those chironomid allergic subjects (16, 17). Hemoglobin, known as a major allergen of chironomids (4, 27, 30), is known to be degraded during metamorphosis (17, 24). Galindo et al. have reported that subjects unexposed to midges could have IgE to chironomid hemoglobin (5) and hypersensitivity to these larvae without apparent contact with them (6). This led us to suppose that a different kind of allergen could be found in the adult. By using the IgG1

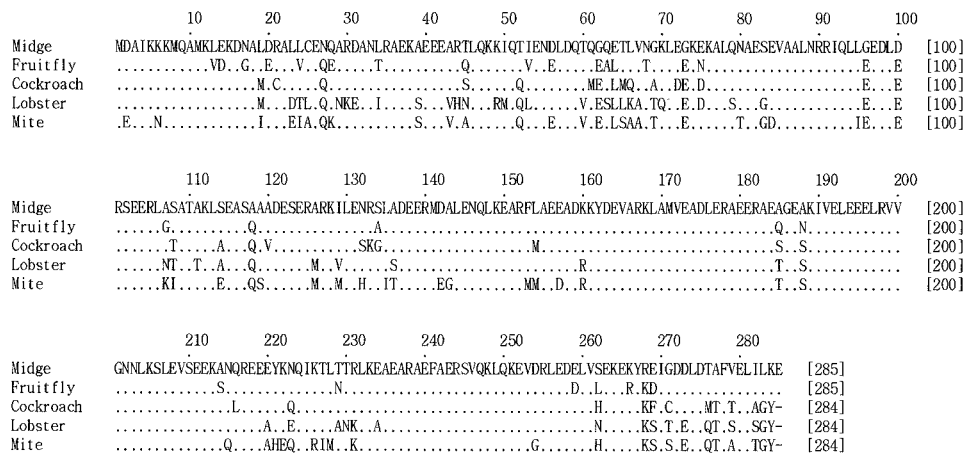


FIG. 3. Alignment of amino acid sequences of four arthropod tropomyosins: midge (*C. kiiensis*, accession number CAA09938), fruit fly (*D. melanogaster*, accession number P43689), cockroach (*P. americana*, accession number CAB38086), lobster (*P. stimpson*, accession number AF030063), and mite (*D. pteronyssinus*, accession number Y14906). A dot indicates amino acid identity with *C. kiiensis*.

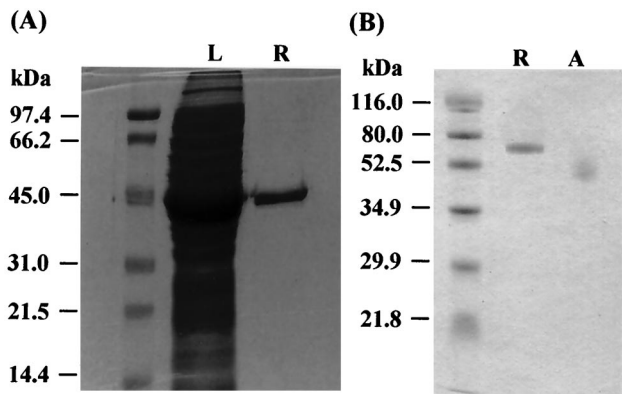


FIG. 4. Mass production of the recombinant allergen. (A) Recombinant tropomyosin was expressed in *E. coli* BL21(DE3) and purified by affinity column chromatography using Ni-NTA agarose. (B) Mobility of the recombinant allergen was tested in SDS-polyacrylamide gel containing 6 M urea. Lanes: L, lysate of *E. coli*; R, recombinant tropomyosin; A, ovalbumin.

of mouse immune serum, which is known to be produced by the influence of interleukin-4, tropomyosin was cloned from the cDNA library of chironomid adults. Recombinant protein was expressed in bacteria to test the allergenicity. The change in migration on electrophoresis in SDS gels containing 6 M urea is characteristic of tropomyosins (25). Tropomyosin-specific IgE was detected from 17 of 21 sera tested by immunoblotting, which indicated that tropomyosin is a major allergen of adult chironomid. Fifteen patients of 17 that were tropomyosin positive also had positive reactions to dust mites in the skin prick test. Of the 21 patients whose sera (19 patients were positive to dust mites) were used in IgE immunoblotting, only one patient had a positive reaction to *C. kiiensis* extract and negative reactions to mites and cockroaches. Five patients showed a stronger reaction (larger wheal size) to *C. kiiensis* than to mites and cockroaches in the skin test, implying the true sensitization of *C. kiiensis* tropomyosin. Only five subjects showed a stronger reaction to the mites and cockroaches. The other 10 persons showed similar reaction to the mites, cockroaches, and chironomid extract.

Tropomyosin, a kind of muscle protein, is a highly cross-reactive allergen which sensitizes individuals who are genetically predisposed to food or house dust allergy (22). It is

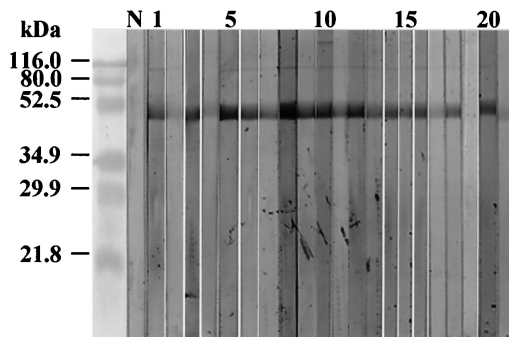


FIG. 5. IgE immunoblotting. Patients 2, 4, 17, 19, and 21 tested negative. Lanes: N, negative control; 1 to 21, sera of humans skin test positive to *C. kiiensis* extract.

suggested that tropomyosin is an invertebrate pan-allergen because of its high cross-reactivity. In the immunoblot experiments, the bands of 70 to 80 kDa are thought to be the dimers, and the bands of 30 to 50 are considered to be isoforms (data not shown). This pattern of tropomyosin bands is consistent with avian ones (8) and may implicate the potential cross-reactivity of other arthropods.

In conclusion, we have identified tropomyosin from *C. kiiensis* as an important adult nonbiting midge allergen. Tropomyosins of invertebrates share a high degree of sequence identity. Further investigations are necessary to examine the possibility of cross-sensitization between allergic disorders caused by mites and insects. This recombinant allergen will be useful for clinical applications and allow a more detailed immunological characterization to be made.

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