

Molecular Characterization of American Cockroach Tropomyosin (*Periplaneta americana* Allergen 7), a Cross-Reactive Allergen¹

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Inhalation of allergens produced by the American cockroach (*Periplaneta americana*) induces IgE Ab production and the development of asthma in genetically predisposed individuals. The cloning and expression in *Escherichia coli* of *P. americana* tropomyosin allergen have been achieved. The protein shares high homology with other arthropod tropomyosins (80% identity) but less homology with vertebrate ones (50% identity). The recombinant allergen was produced in *E. coli* as a nonfusion protein with a yield of 9 mg/l of bacterial culture. Both natural and recombinant tropomyosins were purified by isoelectric precipitation. *P. americana* allergen 1 (Per a 1) and Per a 7 (tropomyosin) are to date the only cross-reacting allergens found in cockroaches. ELISA and Western blot inhibition experiments, using natural and recombinant purified tropomyosins from shrimp and cockroach, showed that tropomyosin induced cross-reactivity of IgE from patients allergic to these allergens, suggesting that this molecule could be a common allergen among invertebrates. *The Journal of Immunology*, 1999, 162: 4342–4348.

The prevalence of allergic diseases in most human populations is steadily increasing. Inhalation of environmental allergens, derived from pollens, dust mites, animal danders, insects, and fungi is the most frequent cause of IgE Ab responses in humans. Cockroaches, house flies, and moths are the most common insects involved in arthropod inhalation allergy (1). Infestations of houses with cockroaches (CR)³ result in the accumulation of high levels of potent allergens, which induce IgE Ab responses and subsequent development of asthma in genetically predisposed individuals. In urban areas of Spain, the prevalence of CR sensitivity among patients with asthma is 26% (2), although this problem is particularly acute in the United States, where up to 60% of patients with asthma are allergic to CR (3). The principal domiciliary CR are *Blattella germanica* (German cockroach) and *Periplaneta americana* (American cockroach). Over the last few years, several allergens from the German cockroach have been cloned: Bla g 1, a protein of unknown function that shares significant homology with Bla g Bd90K (4, 5); Bla g 2, an aspartic protease (6); Bla g 4, a calcium binding protein (7); Bla g 5, a glutathione S-transferase (GST) (8); and Bla g 6, troponin C (9). In contrast, the molecular nature of the American cockroach allergens is poorly understood. Five proteins of 78, 72, 45, 32, and 28 kDa from the American cockroach extract have been identified as major

allergens, having a prevalence of >80% (10). An allergen of 29 kDa, with an incidence of 100% among CR-allergic patients and a pI of 3.5, has been isolated from *P. americana* and named Per a 1 (11). Only Per a 1 and the 72–78 kDa allergen, Per a 3, have recently been cloned, and show homology to Bla g 1 (12, 13) and insect hemolymph proteins (14–16), respectively.

Very often the sensitization to CR extends to all arthropods (insects, arachnids, and crustaceans) and sometimes also to other invertebrates (17–20). Tropomyosins are a family of closely related proteins present in muscle and nonmuscle cells and in association with the troponin complex play a central role in the actin-linked calcium regulatory system of muscle contraction (21). Tropomyosins have been described as relevant allergens in extracts from crustaceans, mites, insects, and mollusks (22–24) and could be involved in the high cross-reactivity found among these organisms (25, 26).

We report here the cloning of the tropomyosin from *P. americana* and its high level expression in *Escherichia coli* as a nonfusion protein. Ab binding reactivity of the recombinant allergen was compared with that of its natural counterpart, and recombinant allergen was used for studying the potential pan-allergen behavior of tropomyosins.

Materials and Methods

Purification of tropomyosins

Adult whole bodies of both sexes of *P. americana* were obtained from Dr. X. Bellés (Centro de Investigación y Desarrollo-Consejo Superior de Investigaciones Científicas, Barcelona, Spain). Atlantic shrimp (*Pandalus borealis*) was purchased from a local seafood store. Whole bodies of CR and peeled shrimp tails were snap-frozen, ground in a mortar, and conserved at –20°C until used. Preparation of acetone powder from CR and shrimp tails was performed as described by Smillie (21). Purification of tropomyosin from acetone powder was conducted by a modification of the isoelectric precipitation method (21). Briefly, 5 g of acetone powder was incubated in 50 ml of extraction buffer (1 M KCl and 0.5 mM DTT, pH 7.0) for 16 h at room temperature, and centrifuged at 5000 × *g* for 15 min. The precipitate was extracted one more time for 2 h. The pooled supernatants were cooled to 4°C, adjusted to pH 4.6, and stirred for 30 min. After centrifugation at the above conditions, the precipitate was dissolved in extraction buffer and centrifuged again. This isoelectric precipitation at pH

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Received for publication July 23, 1998. Accepted for publication January 8, 1999.

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¹ This work was supported in part by Bial-Arístegui and Grant 53-06-07 from the Plan Nacional de I+D (Farma III), Grant 94-0299 from the Ministerio de Industria y Energía, Spain, and Grant 346B04 from the Programa INTEK, Departamento de Industria, Agricultura y Pesca, Basque Government.

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³ Abbreviations used in this paper: CR, cockroach(es); pI, isoelectric point; PVDF, polyvinylidene difluoride; Per a 7, *Periplaneta americana* allergen 7; GST, glutathione S-transferase.

4.6 and dissolution of the precipitate in extraction buffer at pH 7.0 were repeated twice, the final precipitate was dissolved in 0.5 mM DTT, pH 7.0, and dialyzed against PBS. Further purification was obtained after precipitation with 35–60% ammonium sulfate saturation. The purified protein solution was dialyzed against 0.1× PBS and stored at -20°C . In a typical preparation, 13 mg of purified tropomyosin (0.08% yield) were obtained from 16 g of CR bodies, and 99 mg of purified tropomyosin (0.16% yield) were obtained from 61 g of shrimp tails.

Cloning of tropomyosin cDNA by PCR amplification

Poly(A)⁺-enriched mRNA was isolated from 100 mg of *P. americana* using the Quick Prep MicroRNA Purification Kit (Pharmacia, Uppsala, Sweden). First-strand cDNA was synthesized from 1 μg of poly(A)⁺-enriched mRNA using a first-strand synthesis kit (Pharmacia) and random hexadeoxynucleotides as primers. Degenerate oligodeoxynucleotide primers (Cruchem, Glasgow, U.K.) for cDNA amplification were designed according to previously reported invertebrate tropomyosin sequences (22, 27–29). The primers used were: TROF, 5'-CCGGGATCCCATATG GA(G/T)GC(C/T)ATCAAGAA(A/G)AA (sense); and TROR, 5'-CCGAATTC(T/C)A(A/G)TA(G/A/T)ACCAG(T/A/C)(A/C)A(G/A)(T/C)TCGG (antisense), where the slash between bases means that they were added at that particular position in equimolecular amounts during the synthesis of the primer. *Bam*HI, *Nde*I, and *Eco*RI restriction sites are underlined. Five microliters of the single-stranded cDNA were mixed with the described degenerate primers in standard PCR mixture and subjected to amplification with *Taq* DNA polymerase (Bioline, London, U.K.) in the following conditions. After denaturation at 94°C for 4 min, the sample was subjected to five cycles at 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min, followed by 30 cycles at 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min, and a final incubation at 72°C . A predominant band of about 870 bp was isolated from 1% agarose gels (GeneClean, BIO 101, La Jolla, CA). After an initial denaturation step at 94°C for 4 min, the cDNA fragment was reamplified with 30 cycles at 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min and a final incubation at 72°C for 6 min. The fragment was isolated from the gel and ligated to the pGEM-T vector (Promega, Madison, WI), and the resulting construction was designated pTP1. Recombinant DNA techniques were performed by standard methods (30).

Nucleotide sequencing

Nucleotide sequences were determined using the ABI PRISM Dye Terminator Cycle Sequencing Ready reaction kit and run on an ABI 373A DNA sequencer (both from Perkin-Elmer, Foster City, CA). Commercial and sequence-based primers were used. The analysis of the sequences was achieved using the GCG Program Package (Genetics Computer Group, Madison, WI) and the BLAST program (31).

Expression and purification of recombinant tropomyosin

Tropomyosin-encoding cDNA was cloned into the expression vector pKN172 (32) and expressed in *E. coli* BL21 (DE3) carrying the lacUV5 promoter and the gene for the T7 RNA polymerase (33). The lacUV5 promoter, induced by isopropyl-thio- β -galactoside (IPTG), directs transcription of the T7 RNA polymerase, which, in turn, transcribes target DNA cloned under control of the T7 promoter. Tropomyosin-encoding cDNA was transferred from pTP1 to pKN172 as a *Nde*I-*Eco*RI fragment. Competent *E. coli* BL21 (DE3) cells were transformed with the resulting construction. The cells were grown to an OD₆₀₀ of 0.6 in Luria Bertani broth containing 200 $\mu\text{g}/\text{ml}$ ampicillin. Isopropyl-thio- β -galactoside was then added to a final concentration of 0.6 mM, and incubation was continued for another 3 h. Cells were harvested by centrifugation, and the pellet was resuspended in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM EDTA containing 100 $\mu\text{g}/\text{ml}$ lysozyme and incubated for 15 min at 37°C . After mild sonication (six pulses of 10 s each), the recombinant tropomyosin was purified by a modification of the isoelectric precipitation method of Smillie (21) as described above.

Human and experimental sera

Twenty-nine household insect-allergic patients from economically depressed areas of Marseille, France (9 woman and 20 men; mean age, 32 yr; range, 6–62 yr) were selected. Patients suffered from asthma (19), allergic rhinitis (6), spasmodic cough (2), conjunctivitis (1), or atopic dermatitis (1). All of them demonstrated more than a class 1 score of serum-specific IgE to *P. americana* measured by the Hy-Tec specific IgE enzyme immunoassay (Hycor Biomedical, Irvine, CA), and positive skin prick test. A pool of sera from seafood allergic patients with a class 2 score of serum-specific IgE to shrimp was also used. Experimental and commercial rabbit polyclonal Abs against tropomyosins were employed to characterize re-

combinant protein. For the elaboration of experimental immunosera, immunization was performed in adult male White New Zealand rabbits and Swiss mice. Weekly injections of 20 μg of purified shrimp tropomyosin or 100 μg of purified CR tropomyosin, both with CFA, were given s.c., and after eight injections the animals were bled, and the serum was collected. Commercial immunosera against chicken tropomyosin were purchased from Sigma (St. Louis, MO).

Analytical methods

Determination of the protein concentration was performed according to the method of Bradford (34). Proteins were analyzed by SDS-PAGE under reducing conditions (35) and visualized by Coomassie Brilliant Blue R-250 (36). For immunoblotting experiments, proteins after SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA) (37). Membranes were incubated overnight at 4°C with allergic patient serum or at 37°C for 60 min with rabbit polyclonal serum against shrimp or chicken tropomyosin (diluted 1/1000 or 1/50, respectively). Immunodetection was performed as previously described using anti-rabbit IgG, anti-mouse IgG, or anti-human IgE Abs linked to horseradish peroxidase (38). Image analysis was performed using the Bio-Image system (Millipore). The shrimp tropomyosin used for immunization was isolated from shrimp extract by electroelution. Briefly, following SDS-PAGE, the gel was stained for a short period, just long enough to outline the bands, and the tropomyosin band (36 kDa) was cut, minced, placed in an elution tube, and eluted with a Bio-Rad model 422 electroeluter (Bio-Rad, Hercules, CA) at 10 mA/tube over 4 h.

Inhibition assays

For ELISA inhibition assays, microtiter plates (A/S, Nunc, Roskilde, Denmark) were coated with 200 ng/well of natural tropomyosins in 0.1 M bicarbonate buffer, pH 9.6, and blocked with blocking buffer (PBS supplemented with 0.05% Tween-20 and 1% BSA). One hundred-microliter aliquots of rabbit anti-shrimp (diluted 1/2500) or mice anti-CR (diluted 1/5000) tropomyosin serum or serum from allergic patients (diluted 1/2 or

ATG	GAT	GCT	ATC	AAG	AAG	AAG	ATG	CAG	GCG	ATG	AAG	CTG	GAG	AAG	45
M	D	A	I	K	K	K	M	Q	A	M	K	L	E	K	15
GAC	AAC	GCG	ATG	GAC	TGC	GCC	CTT	CTC	TGC	GAG	CAG	CAG	GCC	CGC	90
D	N	A	M	D	C	A	L	L	C	E	Q	Q	A	R	30
GAC	GCC	AAC	CTT	AGG	GCC	GAG	AAG	GCT	GAG	GAG	GAG	GCG	CGC	TCC	135
D	A	N	L	R	A	E	K	A	E	E	E	A	R	S	45
CTG	CAG	AAG	AAG	ATC	CAG	CAG	ATT	GAG	AAC	GAT	CTG	GAT	CAG	ACC	180
L	Q	K	K	I	Q	Q	I	E	N	D	L	D	Q	T	60
ATG	GAG	CAG	CTG	ATG	CAG	GTC	AAC	GCC	AAG	CTG	GAC	GAG	AAG	GAC	225
M	E	Q	L	M	Q	V	N	A	K	L	D	E	K	D	75
AAG	GCC	CTC	CAG	AAC	GCT	GAG	AGT	GAA	GTT	GCT	GCC	CTG	AAC	CGC	270
K	A	L	Q	N	A	E	S	E	V	A	A	L	N	R	90
CGT	ATC	CAG	CTG	CTA	GAG	GAC	GAC	CTC	GAG	AGG	TCT	GAG	GAA	CGA	315
R	I	Q	L	L	E	E	D	L	E	R	S	E	E	R	105
TTG	GCC	ACT	GCC	ACT	GCC	AAA	CTG	GCC	GAG	GCT	TCA	CAG	GCT	GTC	360
L	A	T	A	T	A	K	L	A	E	A	S	Q	A	V	120
GAT	GAA	TCC	GAG	GCA	GCC	CGT	AAG	ATT	CTT	GAA	TCC	AAG	GCC	GAT	405
D	E	S	E	R	A	R	K	I	L	E	S	K	G	L	135
GCG	GAT	GAA	GAA	GCG	ATG	GAC	GCA	CTG	GAG	AAC	CAG	CTG	AAG	GAG	450
A	D	E	E	R	M	D	A	L	E	N	C	Q	L	K	150
GCC	AGG	TTC	ATG	GCT	GAA	GAA	GCA	GAC	AAA	AAA	TAT	GAT	GAG	GTT	495
A	R	F	M	A	E	E	A	D	K	K	Y	D	E	V	165
GCA	CGT	AAG	TTG	GCC	ATG	GTT	GAG	GCC	GAC	TTG	GAG	AGA	GCA	GAG	540
A	R	K	L	A	M	V	E	A	D	L	E	R	A	E	180
GAA	CGT	GCC	GAG	AGT	GGT	GAA	TCC	AAA	ATT	GTA	GAG	CTT	GAG	GAG	585
E	R	A	E	S	G	E	S	K	I	V	E	L	E	E	195
GAG	CTC	CGT	GTT	GTC	GGC	AAC	AAC	CTG	AAG	TCC	CTT	GAG	GTG	TCT	630
E	L	R	V	V	G	N	N	L	K	S	L	E	V	S	210
GAG	GAG	AAG	GCC	AAC	CTG	CGT	GAA	GAA	GAG	TAC	AAG	CAG	CAG	ATT	675
E	E	K	A	N	L	R	E	E	E	Y	K	Q	Q	I	225
AAG	ACC	TTG	ACT	ACC	AGG	CTA	AAG	GAG	GCT	GAG	GCC	CGT	GCT	GAG	720
K	T	L	T	T	R	L	K	E	A	E	A	R	A	E	240
TTC	GCT	GAG	AGG	TCC	GTG	CAG	AAG	CTG	CAG	AAG	GAG	GTT	GAC	AGG	765
F	A	E	R	S	V	Q	K	L	Q	K	E	V	D	R	255
CTT	GAG	GAT	GAA	TTG	GTA	CAC	GAG	AAG	GAG	AAG	TAC	AAG	TTC	ATT	810
L	E	D	E	L	V	H	E	K	E	K	Y	K	F	I	270
TGT	GAT	GAT	CTT	GAT	ATG	ACT	TTC	ACC	GAA	CTT	GCT	GGT	TAC	TAA	855
C	D	D	L	D	M	T	F	T	E	L	A	G	Y	*	285

FIGURE 1. Complete nucleotide and amino acid deduced (in bold) sequences of Per a 7. Primers used for cDNA amplification are underlined. These sequence data are available from the GenBank/EBI databank under accession number Y14854.

	1				50		P9			100
CR	-MDAIKKMQ	AMKLEKDNAM	DCALLCEQQA	RDANLRAEKA	EEEEARSLOKK	IQQIENDLDQ	TMEQLMQVNA	KLDEKDKALQ	NAESEVAALN	RRIQLEEDL
fruitfly	-.E..N...	..VD..G..L	ER..V...E.	...T....	Q....	..TV..E...	..Q..A..TL.TG	..E..N....		
mite	---EFCGR.	I	.R.EIA..K.	S	V..A....	E...	VQ..SAA..T	..E..E....	T..GD....	I....
shrimp	L	..M..E..I	.R.EQM..KV	..VEETKN..L	...FNN....	FSNLQ..F.T	AN..G.TEAQT	..EASE.HVA	EL..DT..G.	
snail	V	..M..E..L	.R.EQL..KL	.ETEEAKA..I	..DDYN....	SI..T....N	..QT..QD..Q.	..YETTE.QIA	EH..Q.IQS.T	..K..SM....I
mussel		..I.....L	.R.DAA.EKV	..QITEKL..RV	...L.DT...	MT.TGD...K	AQ..D.SAATS	..E..E.TV.	E..A...S...	..MT....E.
nematode		ML..D.E..L	.R.EQA.ADK	KA.EE.SKQL	..D.LVA...	..LKGT..DE..K	YS..S.KDAQE	..ELA..KAT	D.....S...	V..E.
chicken	M.E.....	ML..D.E..L	.R.EQA.AEQ	KQ.EE.SKQL	..D.LAAM...	..LKGT..DE..K	YS..A.KDAQE	..ELAE.KAA	D..A...S...	V..E.
human	**	:	*	*	*	*	*	*	*	**
	101			E3	150	P6	E6		E2	200
CR	ERSEERLATA	TAKLAEASQA	VDESERARKI	LESKGLADEE	RMDALENQLK	EARFMAEAD	KKYDEVARKL	AMVEADLERA	EERAESGESK	IVELEELRV
fruitfly	GS.	S....	A.....	..NRA....	L....				Q..N.	
mite	KI.	E....S	A.....M..M	..HRSIT...	..EG.....	M...D.	R.....		T....	
shrimp	N..	..T.....	A.....M..V	..NRS.S...	L....		R.....		T....	
snail	QS.	..E..E..K.	A.....G..V	..RS...D.	LDG..A...	..KYI..D.E	R.....A...	..IT.V....	..A.L.AA.A.	..I.....K.
mussel	MK...YT.	AS..E..K.	A.....N..V	..NLNCGND.	..IDQ..K..T	..KWI....	..E.A....	..IT.V....	..A.L.AA.A.	VID...Q.T.
nematode	..A...KI.	..E..E..THN	V..V	M.NRS.Q...	..ANTV.A...	..QLL....	R.....		A..N.	
chicken	D.AQ.....	LQ...E..EK	A.....GM..V	I.NRAQK..	..K.EIQ..I...	..KHI....	R..E.A...	..VII.G....	LS...	CA.....KT
human	D.AQ.....	LQ...E..EK	A.....GM..V	I.NRA.K...	..K.ELQ..I...	..KHI....	R..E....	..VII.G...T	LA...	CS.....KN
	**	*	***	*	*	**	**	*	***	*
	201				250	E4			286	
CR	VGNNLKSLEV	SEEEKANLREE	EYKQIKTLT	TRLKEAEARA	EFAERSVQKL	QKEVDRLEDE	LVHEKEKYKF	ICDDLDMTFT	ELAGY-	
fruitfly		...S.Q...	..EN....N			D	..L...R..D	..G...TA.V	..ILKE	
mite		...QQ...	AHE...RIM.	..K.....		G....	S	..E...Q..A	..T..-	
shrimp		...Q...	A..E....	NK..A...			..N.....	..S.T.E..Q..S	..S..-	
snail	I	..QE.SQ..D	S.EET.RD.	Q...D..N.	TE...T.S.		..LA...R.S	..E...S..A		
mussel	..A.I.T.Q.	QNDQ.SQ..D	S.EET.RD.	N...D..N.	TE...T.S.	R.....	..LT...A	..E...A..A		
nematode		...LQ..D	S.EE..R.VS	S.....T.			R..T	..EE..S..Q	..S..-	
chicken	..T.....A	QA..YSQK.D	K.EEE..V.	DK.....T.	T.	E.SI.D...	..YAQ.LK..A	..EE..HALN	DMTSI-	
human	..T.....A	QA.EKYSQK.D	K.EEE..I.	DK.....T.	A.	E.TI.D...	..YAQ.LK..A	..EE..HALN	DMTSI-	
	*	*	*	*	*****	*	*	*	*	*

FIGURE 2. Comparison of Per a 7 with different tropomyosins: fruit-fly (*Drosophila melanogaster*, Swiss-Prot P06754), mite (*D. pteronyssinus*, GenBank Y14906), shrimp (*Metapenaeus ensis*, GenBank U08008), snail (*Biomphalaria glabrata*, Swiss-Prot P43689), mussel (*Mytilus edulis*, GenBank U40035), nematode (*Caenorhabditis elegans*, GenBank D38540), chicken (GenBank M32441), and human (skeletal, GenBank X04201). Points indicate the identities of residues with respect to the upper sequence. Residues conserved in >66% of the sequences are marked in bold typeface. Asterisks and double colon indicate residues conserved in all the tropomyosins or only in invertebrate tropomyosins, respectively. IgE-reactive shrimp tropomyosin peptides (24, 29) are marked.

1/4), previously incubated with different concentrations of natural and recombinant tropomyosins, were subsequently added and incubated for 1 h at 37°C. For immunoblot-inhibition studies, serum from household insect-allergic patients was incubated overnight at 4°C with 20 µg of purified inhibitor or BSA (negative control)/ml of sera. The preadsorbed sera were then used for immunoblot experiments.

Results

Amplification, cloning, and sequencing of *P. americana* tropomyosin cDNA

RT-PCR conducted on *P. americana* mRNA with the above-mentioned primers produced a single fragment of approximately 850 bp, which was cloned into pGEM-T and sequenced (Fig. 1). The sequence encoded a polypeptide of 284 amino acids with a predicted molecular mass of 32,776 Da and a pI of 4.69. No potential N-linked glycosylation sites (NXT/S) were identified. A periodic heptameric sequence constituting the α-helical turn and an extensive leucine zipper motif, both characteristic of tropomyosins, occur throughout the sequence. Sequence similarity searches using the BLAST program revealed that *P. americana* tropomyosin has the highest homology to insect tropomyosins from *Locusta migratoria* (89%) and *Drosophila melanogaster* (84%; Fig. 2). High homologies were also found in other arthropods such as crustaceans (shrimp, 84–81%) and arachnides (mites, 80%). The homology to other invertebrate tropomyosins decreased outside the phylum Arthropoda, i.e., nematodes (69%) or mollusks (69%) (22, 27–29, 39–41). Significant, but lower, homology was also found with vertebrate tropomyosins, including human (54% identity, 74% similarity). *P. americana* tropomyosin was designated Per a 7 in accordance with the International Union of Immunological Societies allergen nomenclature subcommittee (42).

Purification of natural *P. americana* tropomyosin and purification of recombinant protein

Natural *P. americana* tropomyosin was purified from whole body CR extract by isoelectric precipitation and ammonium sulfate fractionation. The protein gave a single homogeneous band on Coomassie blue-stained SDS-PAGE, with an apparent molecular mass of 37.1 kDa (Fig. 3A). The yield of natural allergen from CR extracts was 0.7% (w/w). Recombinant *P. americana* tropomyosin was expressed as a nonfusion protein in *E. coli* BL21 (DE3), using the pKN172 vector system, and purified by isoelectric precipitation

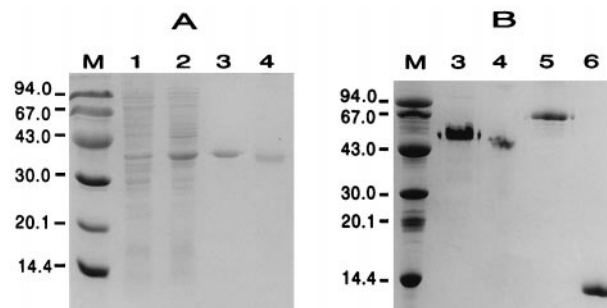


FIGURE 3. Purification of natural and recombinant *P. americana* tropomyosins. Coomassie-stained 12.5% SDS-PAGE (A) and 12.5% SDS-PAGE containing 6 M urea (B). Lane M, Molecular mass markers; lanes 1 and 2, total cell lysate from *E. coli* BL21 (DE3) containing pKN172 alone and pKN172 with tropomyosin-encoding cDNA, respectively; lane 3, purified rPer a 7; lane 4, purified natural Per a 7; lane 5, BSA; lane 6, cytochrome c.

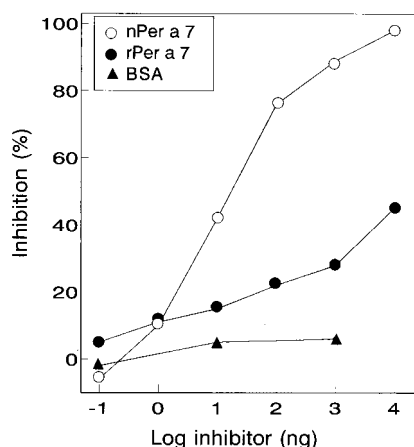


FIGURE 4. Competitive inhibition of the binding of CR tropomyosin-specific rabbit IgG polyclonal Abs to solid phase natural Per a 7 by natural (n) or recombinant (r) Per a 7. Control experiments were performed with BSA.

and ammonium sulfate fractionation. Repeated extraction of the first precipitate was necessary for improving Per a 7 recovery. The pure protein migrated as a 37.6-kDa band on SDS-PAGE (Fig. 3A). The yield of purified rPer a 7 was 9 mg/l of bacterial culture. In the presence of 6 M urea the mobility of tropomyosins from different sources is reduced compared with that of other proteins. This criteria was used to assess the identity and purity of tropomyosin preparations (21). The mobility of purified *P. americana* tropomyosins (natural and recombinant forms) indeed changed on SDS-PAGE containing 6 M urea, giving apparent molecular masses of 52 and 47.8 kDa, respectively, whereas BSA (67 kDa) and cytochrome c (12.5 kDa) migrated at the expected positions (Fig. 3B).

Comparison of Ab binding to natural and recombinant *P. americana* tropomyosins

The reactivity of natural and recombinant *P. americana* tropomyosins is higher to IgG rabbit Abs against shrimp tropomyosin than it is to those against chicken tropomyosin, as could be expected on the basis of amino acid homology (84% for shrimp and 54% for chicken tropomyosins; data not shown). The IgG binding specificity of natural and recombinant CR tropomyosins was assessed by ELISA-inhibition using CR tropomyosin-coated plates and antisera against CR and shrimp tropomyosins. Recombinant Per a 7 was able to inhibit IgG binding to natural tropomyosin up to 45% when sera against CR tropomyosin was used, while natural CR tropomyosin produced an inhibition of 95% (Fig. 4). Similar inhibition was found when ELISA-inhibition experiments were performed with sera against shrimp tropomyosin. No significant effect was detected when BSA was used as inhibitor. These results confirm that some epitopes from the natural tropomyosin are not present in its recombinant counterpart. The frequency of IgE binding to tropomyosin (from the extract and the purified recombinant protein) was compared by immunoblotting. IgE-reacting Abs to the purified tropomyosin were detected in 12 of 29 sera tested (41%). A representative Western blot including tropomyosin-positive sera is shown in Fig. 5. Seven of these sera gave a strong signal in the 37-kDa band, which was weaker in the other five sera. The former sera also reacted to bands of 33, 29, 21, and 18 kDa (probably tropomyosin degradation fragments) as well as to a 80-kDa band, which could be a dimeric form of the tropomyosin (Fig. 5B). These tropomyosin-positive sera also reacted with a 37-kDa

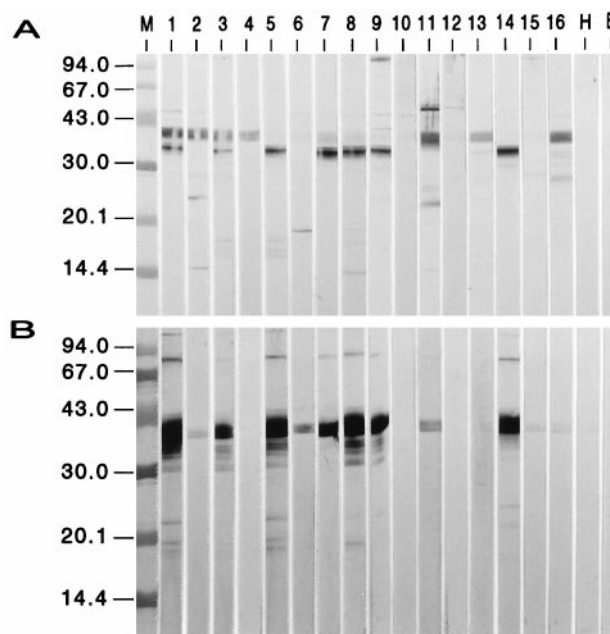


FIGURE 5. Immunological characterization of Per a 7. The IgE reactivity of sera from household-allergic patients with *P. americana* extract (A) and purified recombinant Per a 7 (B) is shown. Proteins transferred onto PVDF membranes were probed with sera from allergic patients to household insects (lanes 1–16), a serum pool from healthy individuals (lane H), and buffer alone (lane B). Molecular mass markers (lane M) were stained with Amido Black B.

band from the CR extract. Regardless of their reactivity to the 37-kDa band, almost all the sera in the CR extract reacted to a 40-kDa band (Fig. 5A).

Per a 7-like molecules as allergens in other arthropods

Inhibition experiments of IgE reactivities of tropomyosin-sensitive patients to other arthropod extracts by Per a 7 demonstrated that tropomyosins are important allergens in these species. Immunoblotting-inhibition experiments showed that both Per a 7 (natural and recombinant forms) were able to inhibit IgE binding to tropomyosin in *P. americana* and *Blatta orientalis*, and *Blattella germanica* extracts (Fig. 6). It is important to notice that almost all the IgE-binding bands (65, 52, 36, 27, and 20 kDa) detected in *B. orientalis* extract disappeared in the presence of tropomyosin. These IgE-reacting bands different from tropomyosin could be produced by tropomyosin aggregation or degradation processes. Nevertheless, natural tropomyosin produced stronger inhibition than the recombinant counterpart. When sera from seafood-allergic patients were used, both Per a 7 allergens (natural and recombinant forms) produced similar inhibition. The homologous allergen, shrimp tropomyosin, and Per a 7 also blocked IgE reactivity to the other allergens detected by the tested sera (Fig. 6C). In ELISA-inhibition experiments, Per a 7 allergens produced 50% inhibition of IgE binding to shrimp tropomyosin compared with 96% inhibition produced with shrimp tropomyosin (Fig. 7). This finding indicated that some common epitopes are present in these two tropomyosins.

Discussion

The CR allergen sequences and the production of recombinant allergens provide the basis for detailed studies of the immune response to these allergens, overcoming the problems related to variability of allergenic extracts. Since tropomyosins are a family of

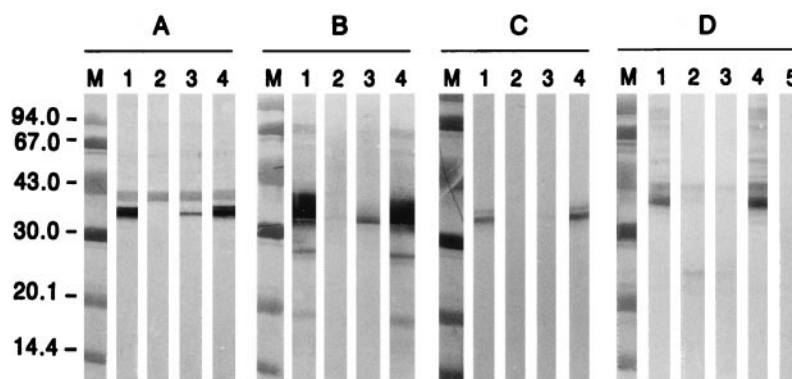


FIGURE 6. Western blot analysis of tropomyosin cross-reactivities. Protein extracts from *P. americana* (A), *B. orientalis* (B), *B. germanica* (C), and *P. borealis* (Atlantic shrimp; D) were separated by SDS-PAGE and transferred onto PVDF membranes. Inhibition was performed by preincubating household insect-allergic (A–C) and seafood-allergic (D) patients' serum pools with: lane 1, PBS buffer; lane 2, natural Per a 7; lane 3, recombinant Per a 7; lane 4, BSA; and lane 5, natural shrimp tropomyosin.

highly homologous proteins with both allergenic and nonallergenic effects, and the sequence of tropomyosins showed high identity to each other, tropomyosin is a good model to study the contribution of the primary structure to the allergenicity of proteins. A simple and efficient method has been employed to clone cDNA molecules encoding the translated region of a major allergen, tropomyosin, from *P. americana*, and to express this recombinant allergen in *E. coli* as a nonfusion protein. Previously reported recombinant tropomyosins from *Dermatophagoides farinae* and shrimp were expressed as fusion proteins with GST (22, 28); the disadvantage of this was that GST has been described as an allergen in CR and mites (8, 43). Furthermore, the yield of purified recombinant protein obtained in this work was 30-fold higher than the production of the *D. farinae* tropomyosin fused to GST (22). The differences between apparent and expected molecular masses of tropomyosins observed in the present work and others (18, 22, 24, 28, 29, 44) could be due to the particular structure of these proteins. Tropomyosins are elongated two-stranded proteins with a dimeric α -helical coiled structure along their entire length, which lent these particular migrating properties.

The IgG and IgE binding capacities of the recombinant tropomyosin were weaker than those of the natural protein, as rPer a 7 is able to inhibit IgG binding to natural CR tropomyosin to

only 50%, using experimental sera against CR tropomyosin and against shrimp tropomyosin as well. It is not unusual that a recombinant protein produced in prokaryotic cells has less biological activity than that of their natural counterpart (22). This fact could be due to improper folding of the recombinant protein produced in *E. coli* or to the existence of various isoforms of tropomyosin involved in the allergenic response. The production of the recombinant allergen in a more convenient host-vector system, such as insect cells using baculovirus-derived vectors, could solve the problems related to the improper folding of the expressed protein. On the other hand, the existence of distinct tropomyosin isoforms found in muscle, brain, or non-muscle cells is well known (45). We suppose that Per a 7, prepared in our laboratory, is one of the various tropomyosin isoforms present, which has some epitopes in common with the natural tropomyosins isoforms used for producing experimental sera, or with the tropomyosin isoforms that induce allergy. It is interesting to notice that in ELISA-inhibition experiments using sera from seafood allergic patients, both natural tropomyosin and rPer a 7 produced similar inhibition (Fig. 7). This effect that could indicate differences in the epitopes recognized by IgG and IgE Abs should be investigated in more detail.

Cross-reactivity between CR has been previously reported (11, 46), but analyses using cloned or purified CR allergens have shown that to date only Per a 1 and Bla g 1 share significant homology and antigenic cross-reactivity (4, 11, 12). *B. germanica* allergens Bla g 2, Bla g 4, and Bla g 5 are not present in *P. americana* extracts (6–8), and Per a 3 from *P. americana* is not detected in *B. germanica* (14). The Western blotting inhibition experiments described here showed that Per a 7 is the second cross-reacting allergen described in CR, as it is present in the two CR species tested (*B. germanica* and *B. orientalis*). It is interesting to notice that a doublet band of 36 and 40 kDa appeared in most of the patients' sera (Fig. 5), but with different intensities. This doublet was already detected at a frequency of 58.3% in sera from 12 atopic patients (10). This double band also appeared in extracts from other CR and shrimp extracts (Fig. 6), but in these cases the IgE reactivity of the larger component was inhibited by natural and recombinant CR tropomyosin, in contrast to that in the *P. americana* extract. Experiments involving isolation of this reactive protein are now in progress to understand these unexpected results. Preabsorption with tropomyosins removed IgE reactivity against other IgE-reactive bands in *B. orientalis* and *P. borealis*. This fact, reported previously in mollusca and crustacea extracts (18) could

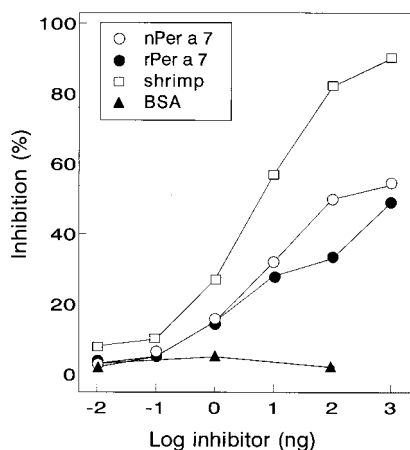


FIGURE 7. ELISA-inhibition of human IgE binding to shrimp tropomyosin-coated well by natural (n) and recombinant (r) Per a 7 and shrimp tropomyosin (shrimp). Control experiments were performed with BSA.

be due to the specific inhibitory effect to other tropomyosin isoforms, tropomyosin aggregates/degradation products, or other proteins that share common reactive epitopes with tropomyosin. IgE-reacting bands of 36–40 kDa, identified as tropomyosins, have been found in extracts from organisms such as crustaceans, insects, and mollusks, but not in vertebrates (chicken or mouse) (18, 25, 26, 47). Some of these species, belonging to the crustacean and mollusk groups, are important food sources in many parts of the world. The existence of cross-reactivity among these species may be explained by the close phylogenetic relationship between these Phyla, although different epitopes must exist among their tropomyosins, since Per a 7 could only produce a 50% inhibition of IgE binding to shrimp tropomyosin, while this protein produced a 95% inhibition (Fig. 7). Nevertheless, serological IgE reactivity does not always result in a clinical hypersensitivity reaction, thus rigorous clinical studies of the hypersensitivity reaction to different invertebrates should be performed among patients positive for tropomyosin. Recent works on shrimp tropomyosins reported six IgE-reactive peptides (24, 29) that showed different degrees of identity to the Per a 7 sequence. The sequence of the peptides E2, E3, and E6, obtained from *Penaeus aztecus* (brown shrimp) tropomyosin (Pen a 1), are highly homologous to Per a 7, with 100, 92, and 92% identical residues. The sequences of the peptides P6 and P9 from *Penaeus indicus* (Indian shrimp) tropomyosin have also high similarity, with 77 and 63% identical residues. The lack of allergenic cross-reactivity with tropomyosins from vertebrate sources suggests that some residues among these peptides may be the critical amino acids involved in binding to IgE. For example, the identities of P9, E4, and E3 to the corresponding regions of human tropomyosin are 30, 30, and 50%, respectively.

In conclusion, this paper described the primary structure of an allergenic tropomyosin from a previously unreported source and demonstrated that the heterologous expression system and purification procedures used are suitable for the production in high amounts of well-characterized and purified recombinant Per a 7. This recombinant allergen could allow more detailed immunological characterization of tropomyosins and establish the role of these proteins in the cross-reactivity among invertebrates.

Acknowledgments

We thank Dr. S. M. Prescott (University of Utah, Salt Lake City, UT) for critical reading of the manuscript, the Servicio de Secuenciación (Centro de Investigaciones Biológicas-Consejo Superior de Investigaciones Científicas, Madrid, Spain) for sequencing facilities, and Dr. R. Panzani (Centre des Recherches, Marseille, France) for providing human allergic sera.

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