

cDNA cloning and molecular identification of the major oyster allergen from the Pacific oyster *Crassostrea gigas*

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Summary

Background Shellfish is one of the most common food allergens. Despite the recent cloning and molecular identification of the major heat stable crustacean allergens in shrimp, lobster and crab, there have been no similar studies on molluscs to which a significant portion of populations allergic to shellfish are also hypersensitive. Recent biochemical evidence suggests that tropomyosin is also an allergen in molluscs, but data on the molecular cloning, nucleotide sequencing, expression and IgE binding to mollusc tropomyosin are lacking.

Objective This study was undertaken to clone, identify and determine the primary structure of a major IgE-reactive mollusc allergen in oyster at the DNA and protein level.

Methods We constructed an expression cDNA library from the Pacific oyster *Crassostrea gigas*. This library was screened for IgE binding clones using sera from 15 subjects with a well-documented history of type I hypersensitivity reactions to oysters. An IgE reactive clone was selected and sub-cloned into plasmids for nucleotide sequence determination and expression in *E. coli*.

Results We identified a 1.3-kb cDNA designated as Cra g 1.03. Expression of Cra g 1.03 in plasmid vector pGEX produced a 59-kDa recombinant fusion protein reactive to the IgE antibodies from patients with oyster allergies but not non-allergic controls. Cra g 1.03 has an open reading frame of 233 amino acids and demonstrates marked similarity in amino acid composition and peptide sequence with mollusc and crustacean tropomyosins. Absorption of oyster allergic sera with Cra g 1.03 totally removed IgE reactivity to oyster extract. Moreover, absorption of allergic sera with recombinant shrimp tropomyosin (Met e 1), lobster tropomyosin (Pan s 1) and crab tropomyosin (Cha f 1) removed most of the IgE reactivity to Cra g 1.03.

Conclusion Cra g 1.03 is the first oyster allergen identified at the molecular level. Nucleotide and amino acid comparison shows that this protein is the oyster tropomyosin.

Keywords: cDNA, Cra g 1.03, IgE, oyster allergen, tropomyosin

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Introduction

Seafood allergy is a serious food allergy, especially in coastal areas and fish processing communities where seafood is common in the diet [1]. It is estimated that more than 500 000 people in America, including consumers, fishermen, cooks and those involved in seafood processing, have seafood allergies.

The immediate allergic response following exposure to seafood is predominantly IgE-mediated type I hypersensitivity reactions, characterized by gastrointestinal disorders as well as cutaneous urticaria, angioedema, itchy throat and sometimes life-threatening and even fatal anaphylactic reactions.

Two invertebrate groups, Crustacea and Mollusca are generally referred to as 'shellfish' and are common constituents in the diet of many populations. With a global annual consumption of over 3 million tonnes, oysters constitute the most popular mollusc food item. The Pacific oyster *Crassos-*

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trea gigas makes up about half of oysters consumed in the world. Adverse reactions and/or anaphylaxis to molluscs such as abalone, limpets, squid and mussels have been frequently reported [2–4]. A heat-stable protein (Tod p 1) of 38 kDa has been partially purified from the squid *Todarodes pacificus* [5]. Based on amino acid sequence homology of Tod p 1 and the shrimp allergen Met e 1, it was suggested that the squid allergen may be tropomyosin.

To identify and characterize mollusc allergens, we have constructed a cDNA library from whole oyster (*Crassostrea gigas*) and screened it with sera from subjects with known adverse reactions upon ingestion of oysters. Ishikawa *et al.* [6] have chromatographically separated two allergenic proteins in oyster and designated them as Cra g 1 and Cra g 2. Interestingly, the amino acid compositions of Cra g 1 and Cra g 2 are similar to shrimp tropomyosins. Here, we report the molecular cloning and identification of the oyster allergen.

Materials and methods

Sera and cDNA clones

Sera from 15 subjects with documented clinical history of allergenic reactions to molluscs and crustaceans were obtained from the sera bank at the Division of Rheumatology/Allergy and Clinical Immunology at the University of California, Davis. All 15 subjects had clinical history of at least two or more symptoms, including pruritus, urticaria, angioedema and gastrointestinal disorders, within 1 hour after ingestion of oysters. In addition, sera from 10 normal volunteers without any previous history of shellfish allergies were used as controls. cDNA clones of the shrimp allergen Met e 1, lobster allergen Pan s 1 and crab allergen Cha f 1 have been described [7–9].

Pacific oyster extracts

Live Pacific oyster, *Crassostrea gigas*, was donated by Dr Dennis Hedgecock (Bodega Marine Laboratory, University of California, Davis, CA, USA). Whole oyster was homogenized in PBS, pH 7.2 with a polytron, mixed for 8 h in PBS containing PMSF plus (100 µg/mL) and aprotinin (1 µg/mL) (Roche Molecular Biochemicals, Indianapolis, IN, USA). The lysate was then centrifuged for 20 min at 10 000 g at 4 °C. The supernatant was assayed for its protein concentration [10] using the Bradford assay kit (Bio-Rad, Richmond, CA, USA) and stored at –20 °C.

Construction of cDNA library

Whole live oysters (*Crassostrea gigas*) were killed by opening up the shells. The oysters were cut into small pieces and frozen immediately in liquid nitrogen. Total RNA was isolated from the frozen tissues using Trizol reagent (Life

Technologies, Rockville, MD, USA). mRNA was then purified by oligo dT cellulose and cDNA synthesis was carried out using both oligo dT and random primers. The cDNA was then ligated into the EcoR I site of Lambda ZapII and packaged in λ phage (Stratagene, La Jolla, CA, USA).

Screening of *Crassostrea gigas* cDNA library

The *Crassostrea gigas* cDNA library was screened with pooled sera from patients ($n = 15$) with a clinical history of oyster allergies. Briefly, *E. coli* XL1 blue cells were infected with the *Crassostrea gigas* cDNA library at a density of 25 000–30 000 plaque-forming units per plate and incubated at 42 °C for 6–8 h until plaques formed. Plaques were overlaid with nitrocellulose filters saturated with 10 mM IPTG and incubated overnight at 37 °C. Thereafter, the nitrocellulose filters were lifted from the agar plate, blocked with 3% non-fat dry milk in PBS and incubated with pooled sera (1 : 10 dilution) from subjects with oyster allergies overnight at 4 °C. The filters were then washed with PBS containing 0.05% Tween four times, probed with ¹²⁵I-labelled antihuman IgE (Sanofi-Pasteur, Ohaska, MN, USA) at 4 °C overnight, washed as before and exposed to Kodak Bio-Max MS film. Positive plaques were subjected to two or more cycles of immunoscreening until homogeneity. Plaques that failed to demonstrate positive IgE reactivity upon secondary screening were considered negative and discarded. An IgE-reactive clone designated Cra g 1.03 was chosen. A single plaque was picked from the agar plate and eluted in phage support buffer (10 mM Tris pH 7.5, 10 mM MgCl₂). The Cra g 1.03 cDNA was recovered by *in vivo* excision from the λ phage. The size of the insert was determined by Eco R I digestion of the plasmid DNA and agarose gel electrophoresis.

Nucleotide sequence and amino acid sequence determination of Cra g 1.03

The sequence of the Cra g 1.03 cDNA was determined by cycle sequencing of the plasmid DNA with AmpliTaq DNA polymerase and dye-labelled terminator using the ABI Prism Dye terminator cycle sequencing ready reaction kit (Applied Biosystems Inc., Foster City, CA, USA) and sequencing primers T3 and T7. Nucleotide sequence was analysed with ABI Prism 310 Genetic Analyser (Applied Biosystems Inc.). In addition, amino acid sequence was deduced from an open reading frame of the Cra g 1.03 nucleotide sequence.

Expression of cDNA

The cDNA of the IgE reactive clone 1.03 was purified and ligated into the EcoR I site of plasmid expression vectors pGEX4T 1–3 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Transformants were selected on LB agar plates

containing 50 µg/mL ampicillin and colony immunoassay was performed [9] using sera from patients with oyster allergy. Positive colonies were identified and reconfirmed for the presence of inserts by miniprep DNA preparation and agarose gel electrophoresis.

Determination of molecular weight of Cra g 1.03

Clones expressing IgE binding proteins were grown in LB containing 50 µg/mL ampicillin at 37 °C overnight prior to induction by 1 mM IPTG for 4 h at 37 °C. The cell lysate was resolved on 8% SDS-PAGE which was then transferred onto nitrocellulose filter as described [7]. Pooled sera from patients with oyster allergy ($n = 15$, 1 : 10 dilution) were then used to probe the filters and IgE reactivity was determined using ^{125} I-labelled antihuman IgE (Sanofi-Pasteur Diagnostics). After washing and exposing to Kodak Bio-Max MS film, the position of the positive band was located. In addition, the molecular weight of the Cra g 1.03 peptide was determined from the deduced amino acid sequence.

Purification of Cra g 1.03 recombinant protein

A 500-mL overnight culture of a pGEX expressing clone of the Cra g 1.03 cDNA was induced with 1 mM IPTG and the cells were harvested. After which, the cells were suspended in 5 mL of lysis buffer (1% Triton X-100, 1% Tween 20, 10 mM dithiothreitol) and sonicated. The cell lysate was then centrifuged to pellet the debris and the supernatant was added to glutathione agarose (Sigma, St Louis, MO, USA). After

washing, the glutathione fusion protein was eluted as described [9].

Inhibition of IgE reactivity to oyster extract by recombinant Cra g 1.03

Total oyster extract was prepared as described above. Sera (at 1 : 10 dilution in PBS containing 3% milk powder) from subjects with oyster allergy were incubated for 8–12 h at 4 °C with 100 µg of recombinant Cra g 1.03 and in a final volume of 1 mL. In addition, identical reactions were set up using recombinant Met e 1, Pan s 1, Cha f 1 and an irrelevant antigen BCOADC-E2 [11] as controls. The absorbed sera were then tested for their IgE reactivities against oyster extract and recombinant Cra g 1.03 by immunoblotting.

Inhibition of IgE reactivity to Cra g 1.03 by Cra g 1.03 and other shellfish allergens

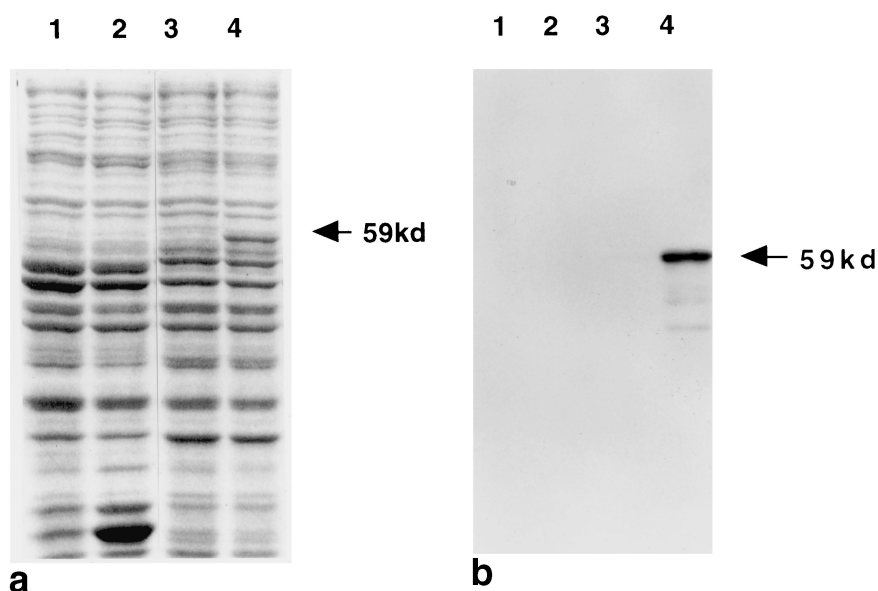
Sera from patients (1 : 10 dilution) with known IgE reactivity to oysters were incubated with recombinant proteins of shellfish allergens, including Cra g 1.03, Met e 1, Pan s 1 and Cha f 1 at 100 µg/mL in 3% milk in PBS at 4 °C overnight on a rocker. Absorbed sera were analysed for their reactivity to Cra g 1.03 by immunoblotting. Sera absorbed with an irrelevant antigen BCOADC-E2 were run in parallel as control.

Results

Screening of the Crassostrea gigas cDNA library

A *Crassostrea gigas* cDNA library was constructed in

Fig. 1. SDS-PAGE and IgE reactivity of sera from subjects with oyster allergy against recombinant fusion proteins. (a) Commassie blue stain of *E. coli* clone of uninduced and IPTG-induced negative control pGEX (lanes 1 and 2, respectively) and uninduced and IPTG-induced Cra g 1.03 cDNA in *E. coli* (lanes 3 and 4, respectively). (b) Immunoblot of an identical gel probed for IgE reactivity with sera from subjects with oyster allergy. Note the presence of a 59-kDa fusion protein band of the induced Cra g 1.03 clone (lane 4) but not in lanes 1–3.



	10	20	30	40	50	60	70	80	90	100	110
	-----	-----	-----	-----	-----	-NSARGFDTV	NEKYQECQTK	MEEAEKTA\$E	AEQEIQ\$SLNR	RIQLLEEDME	RSEERLQATAT
Cgi	MDAIKKKMVA	MKMEKENALD	RAEQLEQKLR	ETEEAKAKIE	DDYNSLQKKS	IQTENDL.NT	QTQL.DV.A.	Y.TT..QIA.	H.....T.	K.SM....IM	K...YT..A
Cvi	MDAIKKKMVA	MKMEKENALD	RAEQLEQKLR	ETEEAKAKIE	DDYNSLQKKS	IQTENDL.NT	QTQL.DV.A.	Y.TT..QIA.	H.....T.	K.SM....IM	K...YT..A
Med	MDAIKKKMVA	MKMEKENALD	RAEQLEQKLR	ETEEAKAKIE	DDYNSLQKKS	IQTENDL.NC	QTQL.DV.A.	Y.R...QIQ.	H.....T.	K.S....GIM	KA...FT..S
Pvi	MDAIKKKMVA	MKVDRENAQD	LAEQMEQKLR	DTETAKAKLE	EDFNDLQKKL	TTTENN..VA	QL..AN.	L.NSD.QITQ	L.SDVGA.Q.	L.....F.	K.A.T..S
Cni	MDAIKKKMVA	MKVDRENAQD	LAEQMEQKLR	DTETAKAKLE	EEFNELQKKL	TATENNY...	QL..AN.	L.NS..QITQ	L.SDVGA.Q.	LT.....Y.	...K.NST.
Chn	MDAIKKKMVA	MKVDRENAQD	LAEQMEQKLR	DTETAKAKLE	EEFNELQKKL	TTTENN..VA	QL..ANV.	L.NS..QITQ	L.SDVGA.Q.	L.....F.	...K.L.T.
Pye	MDAIKKKMVA	MKVDRENAQD	LAEQMEQKLR	DTETAKAKLE	EDFNDLQKCC	A.LEND..S.	..QV.VAMA.	L.TS..RVT.	M...VSGTT.	K.T.....L.	N.....S.
Hru	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DTEEQAKIE	EDLNNLQKCC	A.LEND..S.	..QV.VAMA.	L.TS..RVT.	M...VSGTT.	K.T.....L.	N.....S.
Hdi	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DTEEQAKIE	EDLNNLQKCC	A.LEND..S.	..QV.VAMA.	L.TS..RVT.	M...VSGTT.	K.T.....L.	N.....S.
Bgl-1	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	S.LOND...A	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Bgl-2	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	S.LOND...A	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Has	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Paz	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Men	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Men	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Ham	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Pst	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Cfe	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Ssa	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Xla	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Gga	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Ocu	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Mmu	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Hsa	MDAIKKKMVA	MKMEKENAID	RAEQMEQKLR	DVEETKNGLE	EEFNELQKCC	AILEND..SI	GLT.A...	L.AS..HVA.	L.SDTAG.	...L.....L.	...S.....S.
Cgi	EKLEASKAA	DESENRKVL	ENLNNA\$EER	TDVLEKQLTE	AKLIAE\$EADK	KYDEAARKLA	ITEVDL\$RAE	ARLEA\$EAKV	LELEBELKV	GNNMKSLEIS	EQEASQ\$EDS
Cvi	S.....	CGND..	I.Q.....	I.Q.....	W.....	E.....	E.....		ID...Q.T.	A.I.T.QVQ	NDQ.....
Med	S.....	CGND..	I.Q.....	I.Q.....	W.....	E.....	E.....		ID...Q.T.	A.I.T.QVQ	NDQ.....
Mga	G.....	SGND..	I.Q.....	I.Q.....	W.....	E.....	E.....		ID...Q.T.	A.I.T.QVQ	NDQ.....
Pvi	G.....	SGND..	I.Q.....	I.Q.....	W.....	E.....	E.....		ID...Q.T.	A.I.T.QVQ	NDQ.....
Cni	G.....	SGND..	I.Q.....	I.Q.....	W.....	E.....	E.....		ID...Q.T.	A.I.T.QVQ	NDQ.....
Chn	G.....	SGND..	I.Q.....	I.Q.....	W.....	E.....	E.....		ID...Q.T.	A.I.T.QVQ	NDQ.....
Pye	G.....	SGND..	I.Q.....	I.Q.....	W.....	E.....	E.....		ID...Q.T.	A.I.T.QVQ	NDQ.....
Hru	G.....	SGND..	I.Q.....	I.Q.....	W.....	E.....	E.....		ID...Q.T.	A.I.T.QVQ	NDQ.....
Bgl-1	G.....	SGND..	I.Q.....	I.Q.....	W.....	E.....	E.....		ID...Q.T.	A.I.T.QVQ	NDQ.....
Bgl-2	G.....	SGND..	I.Q.....	I.Q.....	W.....	E.....	E.....		ID...Q.T.	A.I.T.QVQ	NDQ.....
Paz	T.A..Q.	M.....	RSUSD.	M.A.N.K.	RFL.....R	V.....	MV.A.	E.A.TG.S.I	V.....R.	L...V.	EK.N..EA
Men	T.A..Q.	M.....	RSUSD.	M.A.N.K.	RFL.....R	V.....	MV.A.	E.A.TG.S.I	V.....R.	L...V.	EK.N..EA
Ham	T.A..Q.	M.....	RSUSD.	M.A.N.K.	RFL.....R	V.....	MV.A.	E.A.TG.S.I	V.....R.	L...V.	EK.N..EA
Pst	T.A..Q.	M.....	RSUSD.	M.A.N.K.	RFL.....R	V.....	MV.A.	E.A.TG.S.I	V.....R.	L...V.	EK.N..EA
Cfe	T.A..Q.	M.....	RSUSD.	M.A.N.K.	RFL.....R	V.....	MV.A.	E.A.TG.S.I	V.....R.	L...V.	EK.N..EA
Dfa	T.A..QS.	M.....	HR\$ITD.	M.G.N.K.	RFL.....R	V.....	MV.A.	E.A.TG.S.I	V.....R.	L...V.	EK.Q..EA
Ssa	T...E...	GM..I	RASKD..K	MELODI..K.	R.....R	E.V...V	I.S...T.	E.A.LS.G.C	S.....T.	T...L..AQ	AEKY..K.K
Xla	Q...TE..V	GM..I	RATKD..K	MVDQ..Q.R.	T...S.R	E.V...V	VI.G...S.	E.A.V..S.F	GD...NL	T...L..AQ	ADKY..SK.K
Gga	Q...E...	GM..I	RAQKD..K	MEIQ..I.K.	H...H..R	E.V...V	I.G...I	E.A.LS.S.C	A...T.	T...L..AQ	AEKY..K.K
Ocu	Q...E...	GM..I	SRAQKD..K	MEIQ..I.K.	H...H..D.R	E.V...V	I.S...I	E.A.LS.G.C	A...T.	T...L..AQ	AEKY..K.K
Mmu	Q...E...	GM..I	SRAQKD..K	MEIQ..I.K.	H...H..D.R	E.V...V	I.S...I	E.A.LS.G.C	A...T.	T...L..AQ	AEKY..K.K
Hsa	Q...E...	GM..I	SRAQKD..K	MEIQ..I.K.	H...H..D.R	E.V...V	I.S...I	E.A.LS.GQ.	RQ...Q	RIM	DQTL..A.MAA
											DKY..K.R

lambda Zap II from mRNA extracted from whole live oyster. The unamplified library contained 1.5×10^6 pfu/mL, with an average insert size of 1.4 kb. This library was screened for IgE reactive clones with sera from subjects with oyster allergy. A positive clone was designated as Cra g 1.03, according to the nomenclature proposed by Marsh *et al.* [12]. Cra g 1.03 reacted with sera from subjects with oyster allergy, but not with sera from normal volunteers. Upon *in vivo* excision and plasmid analysis, a 1.3-kb EcoR I fragment was identified.

Expression of Cra g 1.03 recombinant protein

The 1.3 kb Cra g 1.03 EcoR I fragment was expressed in pGEX 4T2. Lysate from this colony showed a 59-kDa induction band, whereas the control plasmid pGEX 4T-2 showed a 28-kDa band corresponding to glutathione

S-transferase. Immunoblot results showed that the IgE from oyster allergy subjects recognized the 59 kDa band in both induced clones (Fig. 1). Twelve out of fifteen sera from subjects with allergic reactions to oyster reacted to recombinant Cra g 1.03 and none of the normal sera reacted, showing that Cra g 1.03 is a major oyster allergen.

Nucleotide sequence, amino acid and molecular weight determination of Cra g 1.03

Nucleotide sequence analysis showed that Cra g 1.03 has significant similarity to tropomyosins from molluscs and crustaceans. The nucleotide sequence of the oyster (*Crassostrea gigas*) clone shared 94% identity with a partial sequence of tropomyosin gene of the eastern oyster *Crassostrea virginica*. The amino acid alignment of Cra g 1.03 and tropomyosins from different animals is shown in Fig. 2. The nucleotide sequence of Cra g 1.03 cDNA has an open reading frame of 233 amino acids encoding a peptide of 27 kDa. Cra g 1.03 is rich in glutamic acid, alanine, leucine, lysine and arginine, while it does not contain histidine and tryptophan.

Specificity of recombinant Cra g 1.03

Recombinant Cra g 1.03 was able to inhibit IgE reactivity against itself (59 kDa band) as well as a 38-kDa band of the oyster extract on immunoblot (Fig. 3). Alternatively, absorption of sera from oyster allergy subjects with recombinant proteins of the shrimp allergen Met e 1,

	230	240	250	260	270	280	% Identity
	YEETIRDLTQ	RLKDAENPAT	EAERTVSKIQ	KEVDRLDEL	LAEKERYKAI	SDELDQTFAE	AA
Cgi							94.0
Cvi							100
Med							74.7
Mga							67.8
Pvi							75.1
Cni							67.8
Cno							68.7
Pye							71.7
Hru							67.0
Hdi							68.1
Bgl-1							72.5
Bgl-2							77.7
Has							80.3
Paz							72.5
Men							69.2
Ham							71.4
Pst							69.7
Cfe							64.0
Dfa							63.1
Ssa							64.4
Xla							63.7
Gga							61.7
Ocu							60.0
Mmu							58.5
Has							56.9

Fig. 2. Alignment of deduced tropomyosin protein sequences from *Crassostrea gigas* (Pacific oyster, Cgi, GenBank accession no. AF239173), *C. virginica* (Eastern oyster, Cvi, AF093290), *Mytilus edulis* (Blue mussel, Med, U40035), *M. galloprovincialis* (Mussel, Mga, AB000907), *Perna viridis* (Green mussel, Pvi, AF216519), *Chlamys nipponensis* (Scallop, Cni, AB021681), *C. nobilis* (Scallop, Cno, AF216520), *Patinopecten yessoensis* (Scallop, Pye, AB000907), *Haliotis rufescens* (Red abalone, Hru, X75218), *H. diversicolor* (Small abalone, Hdi, AF216518), *Biomphalaria glabrata* (Snail, Bgl-1, M85199, and Bgl-2, M97554), *Helix aspersa* (Snail, Has, Y14855), *Penaeus aztecus* (Shrimp, Paz (23)), *Metapenaeus ensis* (Shrimp, Men, U08008), *Homarus americanus* (American lobster, Ham, AF034954), *Panulirus stimpsoni* (Spiny lobster, Pst, AF030063), *Charybdis feriatus* (Crab, Cfe, AF061783), *Dermatophagoides farinae* (House dust mite, Dfa, D17682), *Salmo salar* (Atlantic salmon, Ssa, L25609), *Xenopus laevis* (Frog, Xla, AF013612), *Gallus gallus* (Chicken, Gga, M36337), *Oryctolagus cuniculus* (Rabbit, Ocu, S78854), *Mus musculus* (Mouse, Mmu, AW258302) and *Homo sapiens* (Human, Hsa, M19267). The sequence of IgE-binding epitope in *Crassostrea gigas* proposed by Ishikawa *et al.* (18) is underlined.

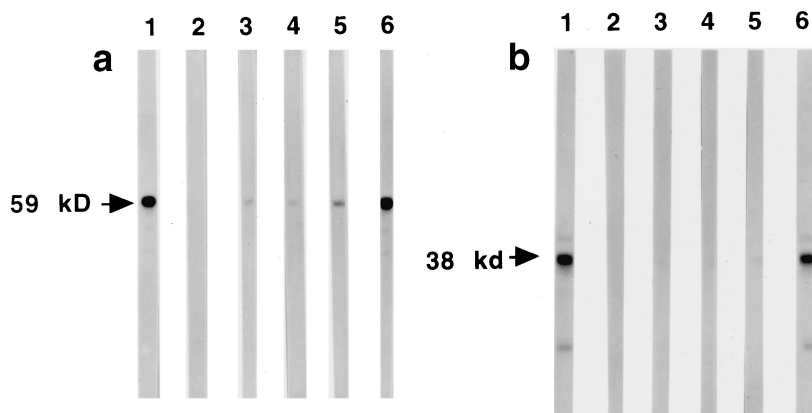


Fig. 3. IgE reactivity of oyster allergy sera against (a) purified recombinant Cra g 1.03 and (b) oyster extract. Sera from subject with oyster allergy were probed for their IgE reactivity against purified recombinant Cra g 1.03 protein either without absorption (lane 1) or absorbed with recombinant Cra g 1.03 (lane 2), absorbed with recombinant shrimp tropomyosin, Met e 1 (lane 3), recombinant lobster tropomyosin Pan s 1 (lane 4), recombinant crab tropomyosin Cha f 1 (lane 5) and recombinant BCOADC-E2 (lane 6). Note that the IgE reactivity to both recombinant Cra g 1.03 (59 kDa) and oyster tropomyosin from oyster extract (38 kDa) in lane 1 was removed completely by recombinant Cra g 1.03 tropomyosin (lane 2), only partially by recombinant either Met e 1, Pan s 1 or Cha f 1 (lanes 3–5), but not by recombinant BCOADC-E2 (lane 6).

lobster allergen Pan s 1 and the crab allergen Cha f 1 was able to remove most but not all of the IgE reactivity to either recombinant Cra g 1.03 or oyster extract. An irrelevant recombinant protein BCOADC-E2 failed to alter the IgE reactivity. These results strongly suggest that there is cross-reactivity between the crustacean allergens (Met e 1, Pan s 1 and Cha f 1) and the oyster allergen. In addition, there may be distinct IgE epitopes in Cra g 1.03 recognized by oyster allergy sera (Fig. 3).

Discussion

Although incidences of seafood allergy have been frequently reported, available information on seafood allergens is mostly limited to those of fish and crustaceans [13]. A number of crustacean allergens have been identified as the muscle protein tropomyosin; they include the shrimp species *Penaeus indicus* [14,15], *P. aztecus* [16] and *Metapenaeus ensis* [7], lobster species *Homarus americanus* and *Panulirus stimpsoni* [8] and crab species *Charybdis feriatus* [9]. Molluscs are also a major source of seafood; however, information on their allergens is relatively limited. This work reports the identification and characterization of mollusc allergens at the molecular level. We have chosen to study the Pacific oyster (*Crassostrea gigas*) because it is the most commonly consumed mollusc and is served in a variety of dishes throughout the world. Previous reports on the physical properties and biochemical properties of oyster allergens, such as molecular weight and amino acid composition, have pointed to tropomyosin as a candidate oyster allergen [6,17,18], but direct verification of its molecular identity is lacking.

Here, we report the identification and characterization of an oyster allergen, a 1.3-kb cDNA with an open reading frame of 233 amino acids, coined Cra g 1.03. This allergen is similar to the shrimp tropomyosins Met e 1 and Pen a 1, lobster tropomyosins Pan s 1 and Hom a 1 as well as the crab tropomyosin Cha f 1 in amino acid composition (data not shown). Although it is clear that the two previously identified allergens Cra g 1 and 2 [6,17] and the allergen identified in the present study represent oyster tropomyosin(s), it is difficult to tell whether the three allergens are identical or represent isoallergens or isoforms since the complete amino acid sequences of Cra g 1 and 2 are not available. Therefore the allergen identified in the present study was designated as Cra g 1.03, and the two other oyster allergens should be referred to as Cra g 1.01 and 1.02. Immunoblotting data demonstrated that 12/15 sera from subjects with allergic reactions upon ingestion of oyster had IgE reactivity to Cra g 1.03, whereas none of the normal control sera reacted. These data clearly demonstrate that Cra g 1.03 is the major oyster allergen.

The nucleotide sequence of the oyster (*Crassostrea gigas*) clone shared 94% identity with a partial sequence of tropomyosin gene of the eastern oyster *Crassostrea virginica* (Fig. 2). The deduced amino acid sequences of the two oysters are identical. When aligned with 11 tropomyosin sequences from different molluscs (including other bivalves and gastropods) available from the GenBank database, the nucleotide sequence of the clone exhibits 67.0–72.5% identity to these sequences. The identity of the deduced amino acid ranges from 71.7% to 82.0%. By sequence homology and IgE reactivity, we confirmed that the oyster allergen isolated from this study is tropomyosin.

The higher values of amino acid vs. nucleotide sequence comparison indicate that many of the nucleotide substitutions are synonymous. The average percentage of synonymous substitutions in the molluscs vs. *C. gigas* is 48.2%. The value increases to 52.6% if *C. virginica* (100% synonymous substitutions) is included in the comparison. When compared with tropomyosins from six species of arthropods, including five crustaceans (shrimp, lobster and crab) and the house dust mite *Dermatophagoides farinae*, where the allergic nature of this protein has been demonstrated [7–9,16,19], the nucleotide and amino acid sequences of the oyster clone exhibit 60–64% and 62.4–64.8% identity to the arthropods, respectively. On average, 33.4% of the substitutions are synonymous. When the sequences were compared with those from six selected vertebrates (salmon, frog, chicken, rabbit, mouse and human) from the GenBank database, the percentage identity of nucleotide and amino acid sequences ranged from 56.5% to 58.7% and 49.4% to 55.8%, respectively. On average, only 24.1% of the nucleotide substitutions are synonymous. It is apparent that both sequence identity and proportion of synonymous substitutions decrease when tropomyosins of more distantly related animals are compared.

Ishikawa *et al.* [6,17,18] proposed that two oyster allergens, Cra g 1 and Cra g 2, have a common IgE-binding epitope, a 14-mer peptide (IQLLEEDMERSEER) representing residues 92–105 of the complete amino acid sequence of tropomyosin (Fig. 2, underlined). A comparison of peptide sequences among different molluscs shows that nine polymorphic sites occur among the species. This value increases to 10 and 11 when arthropods and vertebrates are included in the comparison, respectively. Such analyses tend to indicate that the oyster epitope is species-specific. Ishikawa *et al.* [17,20] suggested that IgE-binding epitopes are distinct among different molluscs, and also different between crustaceans and molluscs. Interestingly, data from this work show that absorption of sera from oyster allergy subjects with recombinant Met e 1 and Pan s 1, as well as Cha f 1, were able to remove most but not all of the IgE reactivity to either recombinant Cra g 1.03 or oyster extract. These data suggest that cross-reactive as well as distinct IgE epitopes exist between the crustacean allergens (Met e 1, Pan s 1 and Cha f 1) and the oyster allergen, Cra g 1.03. It is interesting to note that these subjects had clinical histories of hypersensitivity to both crustaceans and molluscs. Further work in epitope mapping and inhibition studies are in progress to identify and distinguish between oyster-specific epitopes from cross-reactive shellfish epitopes.

Although allergenic cross-reactivity has been reported between crustaceans and molluscs [5,21,22] and evidence has suggested that tropomyosin is the allergen in both

molluscs and crustaceans, molecular data on the cross-reactive epitopes are not available. The availability of cDNA clones and complete amino acid sequence of shellfish tropomyosins will allow further dissection of the molecule and fine analysis of their IgE epitopes. Future work is directed towards mapping and comparison of IgE epitopes between mollusc and crustacean allergens and towards identifying critical amino acids that contribute to their specific allergenicity.

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Note

The nucleotide sequence data of *Cra g 1.03* (GenBank accession number AF239173) has been submitted to GenBank. New allergen data on *Cra g1.03* has been submitted to the WHO/IUIS Allergen Nomenclature Subcommittee.