Parasitol Res (2007) 100:1233–1241
DOI 10.1007/s00436-006-0396-4

ORIGINAL PAPER

# Molecular cloning and expression of two new allergens from *Anisakis simplex*

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Received: 13 October 2006 / Accepted: 16 November 2006 / Published online: 19 December 2006


**Abstract** The nematode *Anisakis simplex* is a marine parasite that causes allergy as well as anisakiasis. Although five *Anisakis* allergens have already been identified, immunoblotting studies suggested that unidentified allergens still exist. In this study, an expression cDNA library constructed from *A. simplex* was subjected to immunoscreening using an *Anisakis*-allergic patient serum, and two positive clones coding for allergens (named Ani s 5 and 6) were obtained. Ani s 5 (152 amino acid residues) is homologous with nematode proteins belonging to the SXP/RAL-2 protein family and Ani s 6 (84 amino acid residues) with serine protease inhibitors from various animals. Of the 28 patient sera examined, seven and five reacted to recombinant Ani s 5 and 6 expressed in *Escherichia coli*, respectively. By inhibition immunoblotting experiments using the recombinant allergens as inhibitors, natural Ani s 5 could be identified as a 15-kDa protein in the crude extract of *A. simplex* but natural Ani s 6 could not be identified probably due to its low expression. In conclusion, Ani s 5 and 6 are new allergens of *A. simplex* that are specific to some *Anisakis*-allergic patients.

## Introduction

The nematode *Anisakis simplex* is a representative marine parasite that invades fish, cephalopods, crustaceans, and sea mammals. When raw or undercooked seafoods with the third-stage larvae of *A. simplex* are ingested, the parasites occasionally penetrate a gastroduodenal mucosa, causing a disease known as anisakiasis (Sakanari and Mckerrow 1989). Although clinical manifestations of anisakiasis are nausea, vomiting, and diarrhea, allergic reactions mediated by immunoglobulin E (IgE) antibodies, such as urticaria, angioedema, and anaphylaxis, are also induced in individuals previously sensitized by *A. simplex* (Audicana et al. 1995; Del Pozo et al. 1997; Fernández de Corres et al. 1996; Montoro et al. 1997; Moreno-Ancillo et al. 1997).

Diagnosis of *A. simplex* allergy is currently performed based on the IgE reactivity to a somatic crude extract of *A. simplex* using CAP-RAST (capsulated hydrophilic carrier polymer-radioallergosorbent test) and skin tests. However, subclinical subjects are often misdiagnosed to be false positive probably because some proteins in the crude extract of *A. simplex* nonspecifically react with IgE (García et al. 1997; Moneo et al. 1997). To avoid misdiagnosis, it is desirable to use purified or recombinant allergens specific for *Anisakis*-allergic patients in the diagnostic assays. For this purpose, accumulation of information on *A. simplex* allergens is requisite.

Four classes of proteins, a secretary protein of 21 kDa (Ani s 1) (Moneo et al. 2000a; Shimakura et al. 2004), a paramyosin of 100 kDa (Ani s 2) (Pérez-Pérez et al. 2000), a troponin-like protein of 21 kDa (Arrieta et al. 2000), and a thermostable 9-kDa protein (Ani s 4) (Moneo et al. 2005), have so far been identified as allergens of *A. simplex*. Also, tropomyosin of 41 kDa could be one of *A. simplex* allergens (Asturias et al. 2000a,b). However, several IgE-reactive proteins differing from the known allergens have been observed in many studies using immunoblot analysis or crossed immunoelectrophoresis analysis (Moneo et al. 2000b; Arlian et al. 2003; Baeza et al. 2004). This situation prompted us to identify unrecognized allergens of *A.*

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*simplex* by immunoscreening of an expression cDNA library constructed from *A. simplex*. We report in this study the cDNA cloning and expression in *Escherichia coli* of two new *Anisakis* allergens (named Ani s 5 and 6).

## Materials and methods

### Parasite

Third-stage larvae of *A. simplex* were collected from the surface of hepatopancreas of walleye pollack (*Theragra chalcogramma*) and immediately used for preparation of crude extract. For molecular cloning experiments, some of the larvae were frozen in liquid nitrogen and kept at −80°C until used.

### Human sera

Sera were obtained from 28 patients with clinical histories of allergic reactions, such as urticaria and anaphylaxis, after eating raw or cooked fish. These patients were all diagnosed to be allergic not to fish but to *A. simplex* based on the determined CAP-RAST classes of 2–6 against *A. simplex*. In this study, sera from 14 healthy subjects were used as controls.

### Immunoscreening

The λ ZipLox expression cDNA library used was the same as constructed in our previous paper (Shimakura et al. 2004). *E. coli* Y1090 (ZL) infected with the cDNA library was cultured on an LB agar plate at 42°C for 4 h, and a nitrocellulose membrane presoaked in 10 mM isopropyl-β-D-thiogalactoside (IPTG) was then overlaid on the plate and incubated at 37°C for 4 h. After being washed with TBST (50 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl and 0.05% Tween 20), the membrane was blocked with 3% bovine serum albumin in TBST at 4°C overnight. The membrane was again washed with TBST and reacted with the patient serum (diluted 1:50) at 37°C for 3 h, followed by peroxidase-conjugated goat anti-human IgE antibody (1 μg/ml; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at 37°C for 2 h. Enzyme reaction was performed using substrate solution containing 0.03% 4-chloro-1-naphtol, 10 mM imidazole, and 0.017% $H_2O_2$. Plaques corresponding to the positive signals were individually picked up and subjected to subcloning.

### Subcloning and DNA sequencing

*E. coli* DH10B (ZIP) was infected with each positive λ ZipLox clone and plated on LB agar containing 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 0.01% ampicillin, and 2 mM IPTG. After incubation at 37°C overnight, each white colony was picked up and cultured in LB medium containing 0.005% ampicillin at 37°C overnight. The plasmid DNA was extracted from the bacterial suspension using a Quantum Prep Plasmid Mini Prep Kit (Bio–Rad Laboratories, Hercules, CA, USA) and sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

### Expression and purification of recombinant allergens

Ani s 5 was expressed in *E. coli* as a glutathione-*S*-transferase (GST)-fusion protein using the pGEX-6P-3 vector (Amersham Biosciences, Piscataway, NJ, USA). A cDNA corresponding to mature Ani s 5, with addition of *Bam*HI and *Eco*RI restriction sites at 5′ and 3′ ends, respectively, was amplified by polymerase chain reaction (PCR) using the isolated clone as a template. The PCR product and the expression vector were digested with *Bam*HI and *Eco*RI and ligated using a DNA Ligation Kit (Takara, Otsu, Japan). *E. coli* JM109 was transformed with the ligated product and cultured on LB agar containing 0.005% ampicillin at 37°C overnight. A single colony was selected and grown in 500 ml of LB medium containing 0.005% ampicillin at 37°C until the absorbance at 600 nm reached 0.7. The culture was then added with IPTG at a concentration of 1 mM and further incubated for 3 h. Bacteria were harvested by centrifugation and resuspended in 25 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. After the bacterial suspension was sonicated and centrifuged, the GST-fusion protein recovered in the supernatant was purified by affinity chromatography on a Glutathione Sepharose 4B column (Amersham Biosciences) according to the manufacturer's instructions and digested with 160 U of PreScission Protease (Amersham Biosciences) at 4°C for 16 h. The digest was applied to the Glutathione Sepharose 4B column, and the GST-free recombinant Ani s 5 was obtained in the flow-through fraction. In the case of Ani s 6, the GST-fusion protein expressed in *E. coli* using the pGEX-6P-3 vector was obtained as an inclusion body and could not be purified by affinity chromatography. Therefore, Ani s 6 was expressed as a His-tagged protein using the pQE-30 Xa vector (Qiagen, Hilden, Germany). A cDNA corresponding to mature Ani s 6, with the addition of *Stu*I and *Hin*dIII restriction sites at 5′ and 3′ ends, respectively, was amplified by PCR. The PCR product and the expression vector were digested with *Stu*I and *Hin*dIII and ligated as described above. Subsequent expression procedures, including transformation of *E. coli* JM109, culturing on LB

agar, selection and growing of a colony, induction of protein expression by IPTG, and harvesting and suspension of bacteria, were the same as adopted for Ani s 5. Then, the bacterial suspension (25 ml) was digested with 0.2 mg/ml lysozyme at 4°C for 1 h. After centrifugation, the precipitate containing the His-tagged Ani s 6 was dissolved in 25 ml of 20 mM phosphate buffer (pH 7.4) containing 500 mM NaCl, 10 mM imidazole, 6 M guanidine hydrochloride, and 5 mM 2-mercaptoethanol. The His-tagged protein was purified by affinity chromatography on a HisTrap Chelating HP column (Amersham Biosciences) as recommended by the manufacturer and dialyzed against 20 mM Tris–HCl buffer (pH 8.0) containing 400 mM L-arginine and 50 mM NaCl for refolding and then against the same buffer devoid of L-arginine.

Protein concentrations of the recombinant allergens were estimated by the method of Gill and von Hippel (1989), using an extinction coefficient $\left(E_M^{280}\right)$ of 8,250 $M^{-1}$ $cm^{-1}$ for Ani s 5 and 6,320 $M^{-1}$ $cm^{-1}$ for Ani s 6, calculated from the number of tyrosine, tryptophan, and cysteine residues.

## Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a PhastSystem apparatus (Amersham Biosciences). Each sample was dissolved in 62.5 mM Tris–HCl buffer (pH 6.8) containing 2% SDS, 100 mM dithiothreitol, and 6 M urea, heated at 70°C for 10 min, and run on a PhastGel Gradient 8–25 gel (Amersham Biosciences). Precision Plus Protein Standards (Bio–Rad Laboratories) were used as references. Proteins were detected by staining with Coomassie Brilliant Blue R-250.

## Enzyme-linked immunosorbent assay

IgE reactivity of the recombinant allergens was examined by fluorescence enzyme-linked immunosorbent assay (ELISA), essentially as reported previously (Hamada et al. 2004). In brief, each sample (50 ng) coated on a 96-well microtiter plate [Type H (black); Sumitomo Bakelite, Tokyo, Japan] was reacted successively with patient or control serum (diluted 1:200) and β-galactosidase-conju-

(a)

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GAGGCTTGGTAGTTTTGAAGGCAAGTAGACTTCCGAAAACAATGAAAACTCTGATCGTAGCCGCCTTGTTTTGCACCATCGGTATGGCG  89
                                              M  K  T  L  I  V  A  A  L  F  C  T  I  G  M  A   16

TTAGCCGACGATACTCCCCCTCCACCACCATTCTTGGCCGGTGCACCACAAGACGTCGTGAAGGCTTTCTTCGAGTTGTTGAAAAAAGAC 179
 L  A  D  D  T  P  P  P  P  P  F  L  A  G  A  P  Q  D  V  V  K  A  F  F  E  L  L  K  K  D   46

GAGACAAAAACTGATCCTGAAATCGAGAAGGACCTCGATGCATGGGTAGACACTCTTGGCGGTGATTACAAGGCGAAGTTCGAGACGTTC 269
 E  T  K  T  D  P  E  I  E  K  D  L  D  A  W  V  D  T  L  G  G  D  Y  K  A  K  F  E  T  F   76

AAGAAAGAAATGAAAGCAAAAGAGGCTGAATTGGCGAAGGCTCATGAGGAGGCAGTCGCTAAGATGACTCCGGAAGCCAAGAAGGCTGAT 359
 K  K  E  M  K  A  K  E  A  E  L  A  K  A  H  E  E  A  V  A  K  M  T  P  E  A  K  K  A  D  106

GCTGAGCTGAGCAAAATTGCAGAAGATGACAGCCTGAATGGCATCCAGAAGGCTCAGAAGATCCAGGCAATCTACAAAACGCTGCCGCAA 449
 A  E  L  S  K  I  A  E  D  D  S  L  N  G  I  Q  K  A  Q  K  I  Q  A  I  Y  K  T  L  P  Q  136

TCCGTTAAAGACGAACTCGAGAAGGGTATCGGTCCGGCTGTGCCTCAATAATAAGTGCCATCCATCTCCCTCGCCGTCCACCTTCACTTA 539
 S  V  K  D  E  L  E  K  G  I  G  P  A  V  P  Q  *                                        152

ACGAACAAACAAGATGAGATTCACCGTCACCTATGGAATAAACCGTTCATTTTTTCCGTCGTTACGATCGTCGTTCTCAGTGCTGTGTAA 629
TATTTCATTAAGATATTTGATGTAATTAAAGCGTTTCAACGCCTCCAAAAAAAAAAAAAAAAA                            691
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(b)

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GTTTGAGCACACTGACACGATCGCGAATCGGCAATATGTTCCAATCAACATTCTTTCTGGTATTGATGGTTTGCGTGGCAACTGCCCGA  89
                                              M  F  Q  S  T  F  F  L  V  L  M  V  C  V  A  T  A  R   18

TTCGCAAATAAAGATCACTGTCCACCTAACGAAGAATATAACGAATGTGGAAATCCGTGTCAGGAAAAGTGTGATAATGGAGAACCGGTA 179
 F  A  N  K  D  H  C  P  P  N  E  E  Y  N  E  C  G  N  P  C  Q  E  K  C  D  N  G  E  P  V   48

ATCTGCACCTATCAATGTGAACATCGGTGCTTCTGCAAACAAGGCTACGTTCGACTTACAGAGGACGGTGAATGCGTTCCTGAAGAATTT 269
 I  C  T  Y  Q  C  E  H  R  C  F  C  K  Q  G  Y  V  R  L  T  E  D  G  E  C  V  P  E  E  F   78

TGCAAACCAATCCATTATTGATGATATGATTGGGTCGAAATCCTTTGAAGTTGGGTTACTAAACTTCTACTACCAATAATCGATTCTGTT 359
 C  K  P  I  H  Y  *                                                                        84

ATTCCTATCCATCAACAGTTATTCCCTCATTTATGACTTTCAAATTAAGCAACCGTGATTGATTTTTTCAAACTAACAATATTGATCACC 449
ATGGAAGATCAGCAGTCCATGTCATTGAAGCAGTATGCGAATAAACGATTTTGCACAGAAAAAAAAAAAAAAA                  522
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**Fig. 1** Nucleotide sequences of cDNA encoding Ani s 5 (**a**) and 6 (**b**). The deduced amino acid sequences are denoted below the nucleotide sequences. In-frame stop codons are indicated by *asterisks*. The predicted signal peptides are *underlined*. The nucleotide sequences of Ani s 5 and 6 cDNAs have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers of AB274998 and AB274999, respectively

gated goat anti-human IgE antibody (0.25 μg/ml; American Qualex, San Clement, CA, USA). Enzyme reaction was performed using 0.1 mg/ml 4-methylumbelliferyl-β-D-galactoside. Fluorescence units were determined on a SPECTRAmax GEMINI XS (Molecular Devices, Tokyo, Japan) with excitation and emission wavelengths at 367 and 453 nm, respectively.

IgE reactivity of the His-tag (22 amino acid residues) contained at the N terminus of recombinant Ani s 6 was also evaluated by fluorescence ELISA using a Nunc Immobilizer Amino plate for peptide (Nalge Nunc International, Rochester, NY, USA). The His-tag peptide was synthesized with a PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan) by the 9-fluorenylmethyloxycarbonyl strategy using benzotriazol-1-yl-oxy-*tris*-pyrrolidino-phosphonium hexafluorophosphate, *N*-methylmorpholine, and *N*-hydroxybenzotriazole according to the manufacturer's instructions.

## Protease inhibition assay

Recombinant Ani s 6 was tested for inhibitory activity against two kinds of serine proteases, trypsin (Merck, Darmstadt, Germany) and α-chymotrypsin (Merck), by the method of Ellis (1990). The substrates used were *N*-

(a)

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Ani s 5           MKTLIVAALFCTIGMALADDTPPP              PPFLAGAPQDVVKAFFELLKKDE  47
AS16              ..Y..TVTCLFVLALVEGQTPSRV              ....V...ESA..D....I....  47
WB14              ..YF.FLSIGLIA.ALAQREAQI.          QSDI....S...NH...Q..D..RA..  51
SXP       VTSSLNLTK..YF.FLSIGLIA.ALAQREAQI.          QSDI....S...NH...Q..D..RA..  60
IHA               ..FV.LLTIGLLVVA.IPQRRQQQQQQQQQQRDEREI....E...PS.IDE.YN...T..  61

Ani s 5   TKTDPEIEKDLDAWVDTLGGDYKAKFETFKKEMKAKEAELAKAHEEAVAKMTPEAKKADAELSKIAEDDS 117
AS16      E.......A.I..F.AK..A..TN..KA..A.L..H...YE...AA.I..FS.A..E...K.TA....AK 117
WB14      S....QT.A.IE.FMRR...V.Q.R..Q..Q...KQK.QYD.V.QA.LSRFS.A.RQ...RM.A...SKQ 121
SXP       S....QT.A.IE.FMRR...V.Q.R..Q..Q...KQF.QYD.V.QA.LSRFS.A.RQ...RM.A...SKQ 130
IHA       N...QQT.A.VE.FINR...S..VR.TQ.ME.V.KAR.DYERI.QQ...RFS.A..D...RM.A..DSPH 131

Ani s 5   LNGIQKAQKIQAIYKTLPQSVKDELEKGIGPAVPQ                                   152
AS16      ......R...KETMES..KE.R.....A.AGGA                                     150
WB14      .T.K..TEQ.K..MDS.SE..RK.ILE.FNSK                                      153
SXP       .TVK..TEQ.K..MDS.SE..RK.ILE.FNSKFCVISVLLNVTIKIFSKWRKNHMRQKSNK         191
IHA       .TTR..S.Q....MDS.SE..RR.IINALS.QE                                     164
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(b)

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Ani s 6           MFQSTFFLVLMVCVATARFA   NKDHCPPNEEYNECGNPCQEKCDNG   EPVICTYQCE  55
Ixodidin                          Q.GS   RGQR.G.G.VF.Q..SA.PRV.GRP   PAQA..L..V  39
SPI                MRAI.A.LIVAIF.FLGVS  A.ANK.GE..I.QR..TA.ERT.S..EEWNKP .KQP.V  57
PrInh6                      LDHSVEAQS   RGRG.GQ...FTQ..SA.EPS.NRP   RAQA..L..I  44
Api m 6   MSRLVLASFLLLAV.SMLVGGFGGFGG.GGLGGRGK..S..IFSR.DGR..RF.P.  VVPKPL.IKI.A  68

Ani s 6   HRCFCKQGYVRLTEDGECVPEEFCKPIHY                                          84
Ixodidin  SG...RR..I. .QR.G.I..RQ.HQR                                          65
SPI       DK...QE.FL. DGN.N..RAWR.N.NL                                          84
PrInh6    VG.Q.R..FL. NSS.R..TPRE.RR                                            69
Api m 6   PG.V.RL..L. NKKKV...RSK.L.G                                          94
```

**Fig. 2** Amino acid sequence alignment of Ani s 5 with four members of the SXP/RAL-2 protein family (**a**) and Ani s 6 with four proteins containing a trypsin inhibitor-like, cysteine-rich domain (**b**). **a** Proteins: AS16, AS16 protein from *Ascaris suum* (GenBank accession number AB089179); WB14, antigen WB14 from *Wuchereria bancrofti* (GenBank AF063940); SXP, SXP antigen from *Wuchereria bancrofti* (GenBank AF098861); IHA, immunodominant hypodermal antigen from *Onchocerca volvulus* (GenBank U00693). The DUF148 domain is *shaded*. **b** Proteins: ixodidin, chymotrypsin-elastase inhibitor ixodidin from *Boophilus microplus* (Swiss-Prot accession number P83516); SPI, putative salivary secreted serine protease inhibitor from *Anopheles stephensi* (GenBank AY162234); PrInh6, immune reactive putative protease inhibitor PrInh6 from *Glossina morsitans morsitans* (GenBank AF368912); Api m 6, allergen from *Apis mellifera* (GenBank DQ384991). The trypsin inhibitor-like, cysteine-rich domain is *shaded*. *Dots* represent the residues identical with the sequence of Ani s 5 or 6

benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA; Sigma-Aldrich, Saint Louis, MO, USA) for trypsin and *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA; Sigma-Aldrich) for α-chymotrypsin. The assay solution, containing 30 μl of protease solution (10 U/ml trypsin or α-chymotrypsin), 30 μl of recombinant Ani s 6 solution (0.3–100 μg/ml), and 140 μl of 100 mM Tris–HCl (pH 7.5) containing 10 mM NaCl, was incubated at 37°C for 10 min in a 96-well microtiter plate. To estimate the residual enzyme activity, the reaction mixture was added with 50 μl of substrate solution (2.5 mM BAPNA in dimethylsulfoxide or 2.5 mM BTPNA in acetone), incubated at 37°C for 30 min, and measured for absorbance at 405 nm using a reagent blank.

### Immunoblotting

The third-stage larvae of *A. simplex* were homogenized with 10 volumes of 10 mM phosphate buffer (pH 7.0) containing 150 mM NaCl. After centrifugation, the supernatant (crude extract) was subjected to immunoblotting, which was performed as reported previously (Kobayashi et al. 2006). Briefly, the proteins separated by SDS-PAGE were electrotransferred from the gel to a polyvinyliden difluoride membrane, which was successively reacted with patient serum (diluted 1:200) and peroxidase-conjugated goat anti-human IgE antibody (0.1 μg/ml; Kirkegaard & Perry Laboratories). Antigen-antibody binding was visualized using an ECL Plus Western Blotting System (Amersham Biosciences). For inhibition immunoblotting, each patient serum (diluted 1:100) was preincubated with an equal volume of inhibitor (recombinant Ani s 5 or 6) solution (20 μg/ml) at 37°C for 1 h and used as a primary antibody.

## Results

### Primary structures of Ani s 5 and 6

The expression cDNA library was screened using one patient serum (patient 13 serum), which was selected because of the sufficient volume available. Of approximately $10^5$ phages screened, ten clones reactive with serum IgE were isolated. Although eight of these clones were judged to be false positive because their nucleotide sequences had no open reading frame, the remaining two clones were shown to include a full-length cDNA.

One clone (corresponding to Ani s 5) had an insert cDNA of 691 bp (Fig. 1a). An open reading frame is composed of 456 bp, which encodes 152 amino acid residues. The N-terminal segment (up to the 18th residue) was predicted to be a signal peptide by the SignalP analysis (Bendtsen et al. 2004), indicating that the mature Ani s 5 corresponds to the segment 19–152 (134 residues). A homology search by the BLAST program (Altschul et al. 1990) revealed that Ani s 5 is a member of the SXP/RAL-2 protein family including proteins from various species of nematodes, such as the AS16 protein from *Ascaris suum*, the antigen WB14 and SXP antigen from *Wuchereria bancrofti*, and the immunodominant hypodermal antigen from *Onchocerca volvulus* (Fig. 2a). The sequence identities are 57% with the AS16 protein, 46% with the antigen WB14, and 45% with the SXP antigen and the immunodominant hypodermal antigen. The DUF148 domain commonly contained in the SXP/RAL-2 protein family was also detected at positions 25–147 of Ani s 5 as searched by the Pfam program (Finn et al. 2006).

The other clone (corresponding to Ani s 6) contained an insert cDNA of 522 bp (Fig. 1b). An open reading frame of

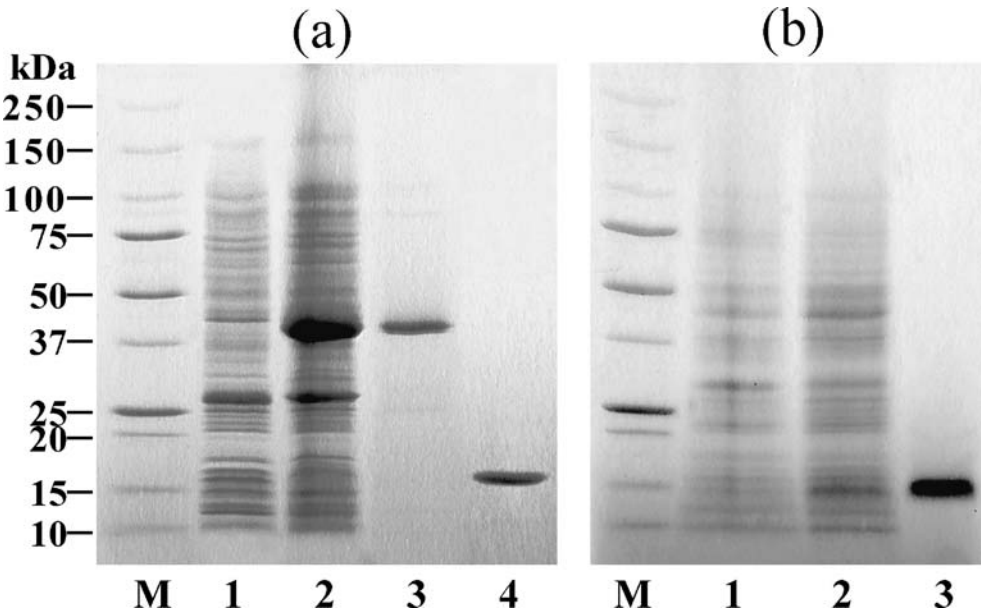


**Fig. 3** SDS-PAGE of recombinant Ani s 5 (**a**) and 6 (**b**). **a** *Lanes M* Molecular weight marker proteins, *1* lysate of *E. coli* not induced by IPTG, *2* lysate of *E. coli* induced by IPTG, *3* purified GST-fusion Ani s 5, *4* purified GST-free Ani s 5. **b** *Lanes M* Molecular weight marker proteins, *1* lysate of *E. coli* not induced by IPTG, *2* lysate of *E. coli* induced by IPTG, *3* purified His-tagged Ani s 6

252 bp encodes 84 amino acid residues, whose N-terminal segment (up to the 22nd residue) was predicted to be a signal peptide. Thus, the mature Ani s 6 was assumed to correspond to the segment 23–84 (62 residues). A BLAST search proved that Ani s 6 has high sequence identity with serine protease inhibitors from various animals as follows: 36% identity with the chymotrypsin-elastase inhibitor ixodidin from the southern cattle tick *Boophilus microplus*, 30% with the putative salivary secreted serine protease inhibitor from the African malaria mosquito *Anopheles stephensi* and the immune reactive putative protease inhibitor PrInh6 from the tsetse fly *Glossina morsitans morsitans*, and 29% with the honey allergen Api m 6 from the honeybee *Apis mellifera* (Fig. 2b). In common with the above serine protease inhibitors, Ani s 6 was found to include a trypsin inhibitor-like, cysteine-rich domain at positions 25–79 by the Pfam analysis.

## Expression and purification of recombinant allergens

When analyzed by SDS-PAGE, the GST-fusion Ani s 5 (41 kDa) was recognized in the lysate of *E. coli* induced by IPTG (lane 2 in Fig. 3a) but not in that of noninduced bacteria (lane 1 in Fig. 3a). The GST-fusion Ani s 5 was purified by affinity chromatography on a Glutathione Sepharose 4B column (lane 3 in Fig. 3a). After digestion of the GST-fusion Ani s 5 with PreScission Protease, followed by passing the digest through the same affinity

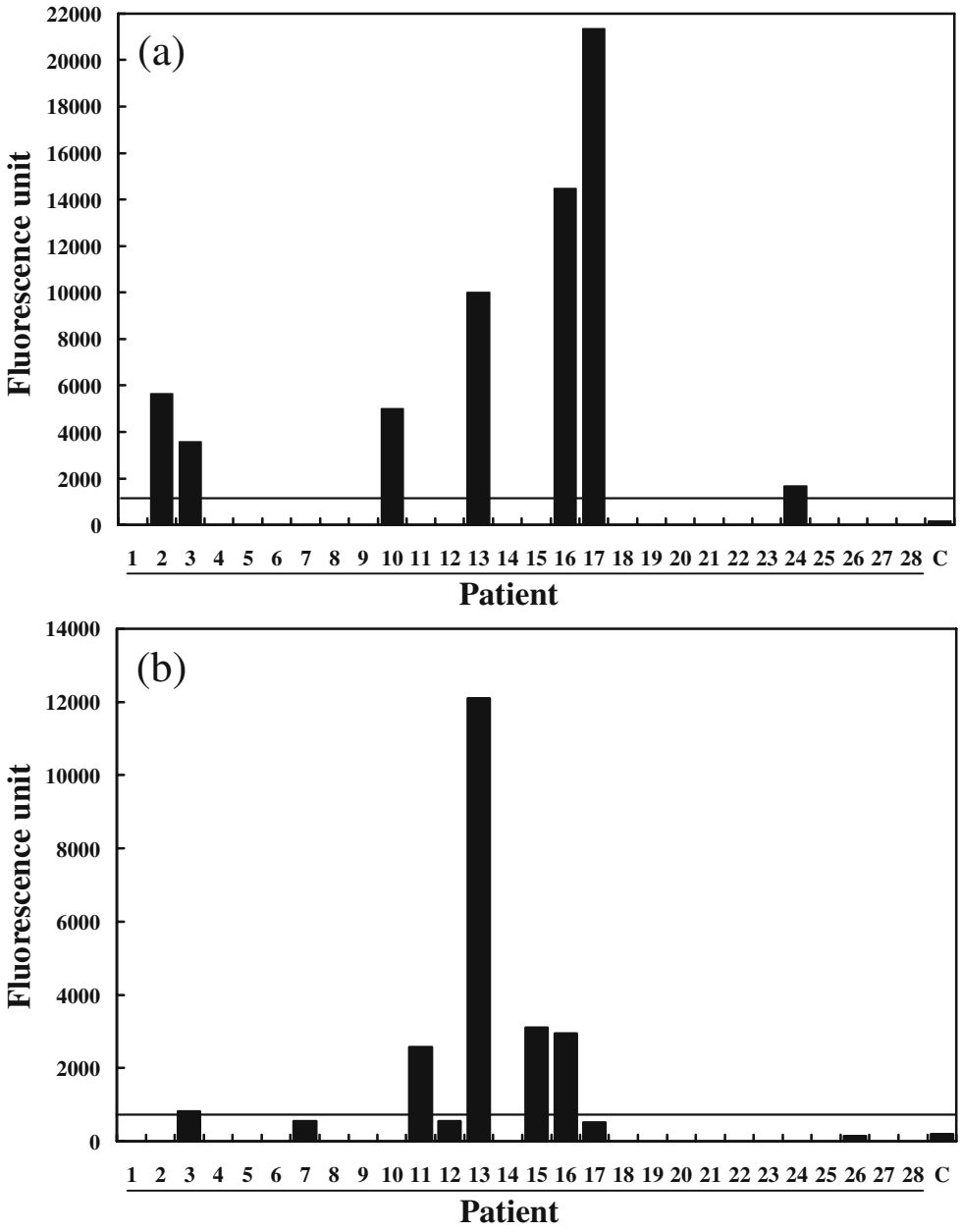


**Fig. 4** Analysis of IgE reactivity of recombinant Ani s 5 (**a**) and 6 (**b**) by fluorescence ELISA using 28 patient sera. Control data (denoted by *C*) obtained with 14 healthy subjects were averaged, and the mean + 2SD is represented by the *horizontal line in each figure*. The values above this line were judged to be positive

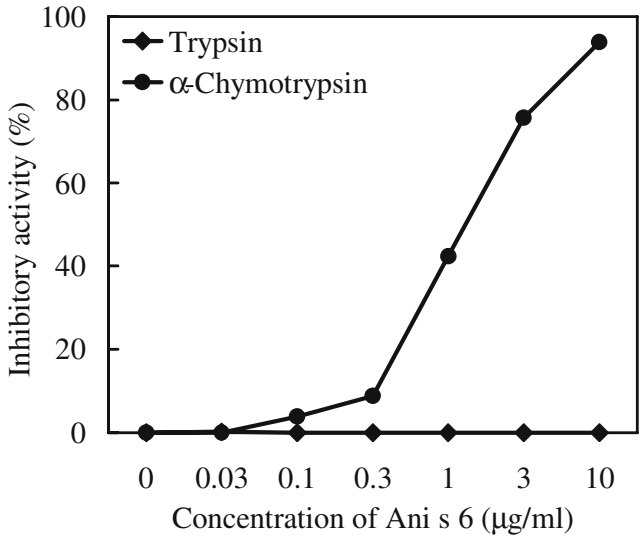


**Fig. 5** Inhibition activity of recombinant Ani s 6 against trypsin and α-chymotrypsin

column, the GST-free recombinant Ani s 5 of 15 kDa was obtained in a pure state (lane 4 in Fig. 3a). The yield of the recombinant Ani s 5 was 11.8 μg/ml culture medium. On the other hand, the His-tagged Ani s 6 was detected only in the lysate of *E. coli* induced by IPTG (lane 2 in Fig. 3b). After affinity chromatography on HisTrap chelating HP, homogeneous His-tagged Ani s 6 (lane 3 in Fig. 3b) was obtained with a yield of 5.5 μg/ml culture medium. We have no explanations for the reason why the His-tagged Ani s 6 behaved as a significantly larger molecule (15 kDa) in SDS-PAGE than the calculated molecular mass (9.5 kDa).

## Properties of recombinant allergens

Analysis by fluorescence ELISA showed that seven (25%) and five (18%) of the 28 patient sera were reactive to the recombinant Ani s 5 and 6, respectively (Fig. 4). None of the five patient sera recognizing recombinant Ani s 6 reacted to the synthesized His-tag peptide (data not shown), implying that the His-tag portion is not implicated in the IgE binding of the recombinant Ani s 6.

Because Ani s 6 shows high sequence identity with serine protease inhibitors from various animals as described above, the recombinant Ani s 6 was evaluated for inhibitory activity against two serine proteases, trypsin, and α-chymotrypsin. As shown in Fig. 5, the recombinant Ani s 6 inhibited the activity of α-chymotrypsin in a concentration-dependent manner but exhibited no inhibition against trypsin.

## Identification of Ani s 5 in the crude extract

A number of proteins were observed in the crude extract of *A. simplex* as analyzed by SDS-PAGE (lane 2 in Fig. 6a), and many of them were shown to be IgE reactive by immunoblotting using six patient sera reacting to the recombinant Ani s 5 (lane 2 in Fig. 6b). Importantly, all the patient sera reacted to a 15-kDa protein that is comparable in molecular mass to Ani s 5. Moreover, preincubation with the recombinant Ani s 5 abolished the reactivity of the patient sera to the 15-kDa protein as well as to the recombinant Ani s 5, except for the case of patient 13 serum. These results led us to conclude that the 15-kDa protein in the crude extract of *A. simplex* is the natural Ani s 5. In this study, however, natural Ani s 6 could not be identified in the crude extract because any protein bands reactive with the five patient sera recognizing the recombinant Ani s 6 did not disappear after preincubation of the sera with the recombinant Ani s 6.

## Discussion

In this study, two clones encoding allergens (Ani s 5 and 6) were isolated from the expression cDNA library con-

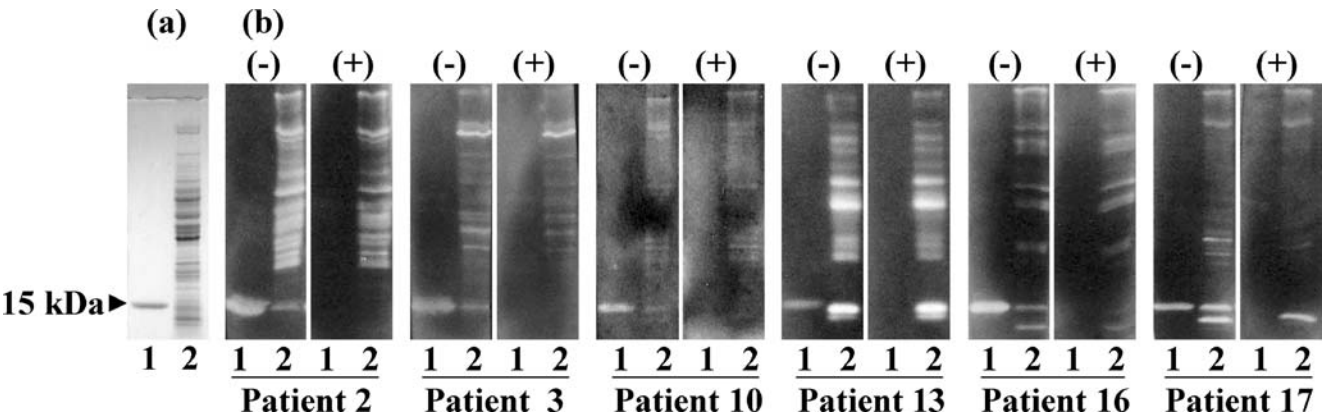


**Fig. 6** Analysis of the crude extract from *Anisakis simplex* by SDS-PAGE (**a**), immunoblotting (−), and inhibition immunoblotting (+) (**b**). *Lanes 1* Recombinant Ani s 5, *2* crude extract from *A. simplex*. In inhibition immunoblotting, each patient serum was preincubated with an equal volume of recombinant Ani s 5 solution (20 μg/ml) and used as a primary antibody

structed from the third-stage larvae of *A. simplex* and their nucleotide sequences elucidated. The deduced amino acid sequences showed that Ani s 5 is composed of 152 amino acid residues and Ani s 6 of 84 residues and that both allergens are distinct from the known *A. simplex* allergens (Arrieta et al. 2000; Asturias et al. 2000a; Moneo et al. 2000a, 2005; Pérez-Pérez et al. 2000; Shimakura et al. 2004). Although both Ani s 5 and 6 were not major allergens of *A. simplex*, they were established to be IgE reactive by fluorescence ELISA using recombinant preparations expressed in *E. coli*. In addition, the 15-kDa protein in the crude extract of *A. simplex* was identified as natural Ani s 5 by inhibition immunoblotting, while identification of natural Ani s 6 was unsuccessful probably due to the low expression of Ani s 6 in *A. simplex*. Taken together, at least Ani s 5 is an important allergen for some *Anisakis*-allergic patients and hence, its recombinant preparation could be a useful tool in future accurate diagnosis of *A. simplex* allergy.

Ani s 5 is highly homologous with nematode proteins belonging to the SXP/RAL-2 protein family. It is worth mentioning that some proteins of the SXP/RAL-2 protein family, such as the AS16 protein from *A. suum* (a gastrointestinal parasite distributing in both human and swine) (Tsuji et al. 2003) and the SXP/RAL-2 protein from *Meloidogyne incognita* (a major parasite of plant) (Tytgat et al. 2005), have been demonstrated or suggested to be secreted to the outside of the parasite. We thus assume that Ani s 5 is secreted in the human gastrointestinal tract from the third-stage larvae of *A. simplex* ingested with fresh or undercooked seafood and that the secreted Ani s 5 stimulates human immune responses leading to the production of IgE antibodies against Ani s 5.

On the other hand, Ani s 6 shares high sequence identity with serine protease inhibitors from various animals. In accordance with the sequence identity, the recombinant Ani s 6 was demonstrated to be inhibitory against α-chymotrypsin but not against trypsin. Although the mature Ani s 6 contains as many as ten Cys residues probably involved in the formation of five disulfide bridges, the fact that the recombinant Ani s 6 displays a significant inhibitory activity against α-chymotrypsin supports that it was correctly refolded in the presence of L-arginine. A number of protease inhibitors, such as Api m 6 from honeybee *A. mellifera* (Kettner et al. 2001) and Fel d 3 from cat *Felis domesticus* (Ichikawa et al. 2001), have so far been identified as allergens. To our knowledge, however, Ani s 6 is the first protease inhibitor experimentally identified as an allergen in nematodes. It should be noted that protease inhibitors from the intestinal parasitic nematodes are assumed to be secreted out of their body for protection from the hostile proteolytic environment (Maizels et al. 2001; Zang and Maizels 2001; Hartmann and Lucius 2003). Possibly, Ani s 6, like Ani s 5, is secreted from *A. simplex* invading the human gastrointestinal tract, being easily recognized by human.

Immunoscreening of an expression cDNA library using patient sera is an effective technique to identify allergens from biological samples. Previously, this technique has been successfully used in identifying Ani s 2 (Pérez-Pérez et al. 2000) and the troponin-like protein (Arrieta et al. 2000) as *Anisakis* allergens. However, only one allergen was identified in each previous study. Similarly, only two allergens were found in our study although they were different from the known *Anisakis* allergens. The fact that different allergens are found by immunoscreening using different patient sera conforms well to the generalization for *A. simplex* allergy that allergens significantly vary from patient to patient (Moneo et al. 2000b; Arlian et al. 2003; Baeza et al. 2004). Therefore, future immunoscreening using different patient sera will discover more unidentified allergens in *A. simplex*.

**Acknowledgments** We are grateful to Dr. T. Shirai, Department of Internal Medicine, Fujinomiya City Central Hospital, and Dr. S. Kasuya, Faculty of Regional Studies, Gifu University, for providing sera from *Anisakis*-allergic patients. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and a grant from the Ministry of Health, Labor and Welfare of Japan. All experiments were performed according to the current laws of Japan.

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