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Expression and Characterization of α-Methylacyl CoA Racemase from *Anisakis simplex* Larvae

Bong Jin Kim¹, Sun Mi Kim², Min Kyung Cho³, Hak Sun Yu³, Yong Seok Lee⁴, Hee Jae Cha⁵ and Meesun Ock^{5,*}

¹Departments of Internal Medicine, ⁵Parasitology and Genetics, Kosin University College of Medicine, Busan 602-703, Korea; ²Departments of Pharmacology and ³Parasitology, Pusan National University School of Medicine, Yangsan 626-870, Korea; ⁴Department of Parasitology, Inje University College of Medicine, Busan 614-735, Korea

Abstract: Larval excretory-secretory products of *Anisakis simplex* are known to cause allergic reactions in humans. A cDNA library of *A. simplex* 3rd-stage larvae (L3) was immunoscreened with polyclonal rabbit serum raised against *A. simplex* L3 excretory-secretory products to identify an antigen that elicits the immune response. One cDNA clone, designated as α-methylacyl CoA racemase (Amacr) contained a 1,412 bp cDNA transcript with a single open reading frame that encoded 418 amino acids. *A. simplex* Amacr showed a high degree of homology compared to Amacr orthologs from other species. Amacr mRNA was highly and constitutively expressed regardless of temperature (10-40°C) and time (24-48 hr). Immunohistochemical analysis revealed that Amacr was expressed mainly in the ventriculus of *A. simplex* larvae. The Amacr protein produced in large quantities from the ventriculus is probably responsible for many functions in the development and growth of *A. simplex* larvae.

Key words: Anisakis simplex L3, cDNA library, immunoscreening, excretory-secretory product, a-methylacyl CoA racemase

Anisakis simplex (Nematoda: Anisakidae) is one of the most frequently encountered parasites in humans through the ingestion of marine products. A. simplex has a complicated life cycle that involves sea mammals, crustaceans, cephalopods, and fish. The human anisakiasis occurs when people eat raw or undercooked fish or squid. A. simplex can cause direct tissue damage following larval invasion in the gut wall, development of an eosinophilic granuloma, or perforation of the gut [1,2]. It has also been reported that an A. simplex larval infection can cause an allergic response due to anti-Anisakis IgE antibodies [3-5]. The allergic responses caused by anisakiasis include anaphylaxis, urticaria, angioedema, rheumatic manifestations, and nephrotic syndrome [6-11]. These allergic responses have been reported to be associated with A. simplex larvae excretory-secretory (ES) products. Immunoblot analysis of A. simplex ES products from the 3rd-stage larvae (L3) revealed several allergenic bands ranging from 10 to 186 kDa [12]. Moreover, the ES products from A. simplex larvae elicited proinflammatory cytokine

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and chemokine secretion from a mouse lung epithelial cell line and primary lung epithelial cells [13]. Although infected larvae are unable to complete their life cycle in the human body, they can survive a few weeks and even develop to further stages [14]. The human immune system can be activated with ES products during the developmental process of the larvae. It is usually assumed that there would be a plenty of ES products that could provoke immune responses in an infected human.

We immunoscreened cDNA library of *A. simplex* larvae with polyclonal rabbit serum raised against major ES products (35-40 kDa) of larvae to identify an antigen that elicits the immune response after *A. simplex* larval infection. This approach led to the identification of α -methylacyl CoA racemase (Amacr), which is an essential enzyme involved in the β -oxidation of bile acid intermediates and branched-chain fatty acid degradation. We also analyzed its homology with other Amacr orthologs, its regulation by RT-PCR, and localization by immunohistochemistry.

Live A. *simplex* larvae were collected from the body cavity and the viscera of the chub mackerel, *Scomber japonicus*, which were purchased from a local fish market. To obtain ES products, live L3 were incubated in DMEM (Dulbecco's Modified Eagle Medium, gentamycin 150 mg/ml, vancomycin 10 mg/ml, and 0.5 ml/larva) at 37°C for 48 hr. The ES products were cen-

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*Corresponding author (sunnyock@kosin.ac.kr)

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trifuged and concentrated (Amicon stirred cells, Millipore Corp., Billeria, Massachusetts, USA). After the concentrated ES products were resolved by SDS-PAGE on a 12% gel, the strong protein bands between 35 and 40 kDa were cut out and minced with a razor blade (Fig. 1). The finely minced gel was mixed with Freund's adjuvant and PBS to make a homogeneous suspension. The suspension (1 ml) was injected intraperitoneally into 8 to 10 week-old ICR mice. After the last injection conducted at 1-week intervals, the same suspension was mixed with Freund's incomplete adjuvant and administered intraperitoneally. The sera were collected 2 weeks later.

An *A. simplex* L3 lambda Zap II library [15] was screened using 1:500 diluted polyclonal anti-major *Anisakis* ES (against 35-40 kDa) mouse sera (pre-absorbed with *Escherichia coli* lysates) and 1:1,000 diluted horseradish peroxidase-labeled goat anti-mouse IgG (Serotec, Oxford, UK). Nylon transfer membranes (Amersham Biosciences, Piscataway, New Jersey, USA) were developed using DAB substrate (Thermo Fisher Scientific Inc., Pittsburgh, Philadelphia, USA). Positive plaques were picked and purified by plating at successively lower densities. Immunopositive plaques from the final round of purification were subcloned using the lambda ZAP II in vivo excision method (ExAssist/SOLR system; Stratagene, La Jolla, California, USA). One clone was isolated, and then the purified plasmid DNA was sequenced (Macrogen, DNA Sequencing



Fig. 1. SDS-PAGE of excretory-secretory (ES) products from *Anisakis simplex* L3 at 37°C for 24 hr. ES products ranging from 35 to 40 kDa were injected into mice for the production of polyclonal antibodies. Lane 1, molecular marker; Lane 2, ES products.

Service, Seoul, Korea).

Nucleotide and amino acid sequences of the selected clone were compared via BLAST analysis at the National Center for Biotechnology Information. Twenty amino acid sequences, including the A. simplex Amacr, were chosen and analyzed. The significance and evolutionary conservation of Amacr was investigated by constructing a phylogram using the maximum Parsimony method [16] in MEGA4 [17]. Amino acid sequence accession numbers are as follows; A. simplex (ADW54022.1), Caenorhabditis elegans (NP 509517.2), Sus scrofa (XP 0031339 21.1), Danio rerio (NP 001018462.1), Gallus gallus (NP 00102 6619.1), Bos taurus (AAI18287.1), Anopheles gambiae (XP 3170 33.2), Xenopus laevis (NP 001167512.1), Ailuropoda melanoleuca (XP 002919481.1), Mus musculus (NP 032563.2), Branchiostoma floridae (XP_002595975.1), Homo sapiens (BAD96551.1), Canis familiaris (XP 855429.1), Taeniopygia gutata (XP 002191 692.1), Saccoglossus kowalevskii (XP_002730894.1), Oryctolagus cuniculus (XP 002714167.1), Equus caballus (XP 001500301.1), Drosophila melanogaster (NP_610054.1), Rattus norvegicus (NP_ 036948.1), and Pan troglodytes (XP_001152632.1).

The recombinant protein was expressed as a glutathione Stransferase (GST) fusion protein. The full-length sequence of the selected clone was subcloned into the pGEX-4T-2 vector (Amersham BioSciences) and was used to transform *E. coli* strain BL21. Bacteria were cultured in 1.0 mM IPTG from 35°C to 37°C. The recombinant protein was purified using a MicroSpin GST Purification Module (Amersham Biosciences).

Western blotting was performed to check the reactivity of the recombinant protein obtained. The recombinant protein was separated by SDS-PAGE on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Transferred proteins were confirmed by Ponceau staining and blocked with PBS containing 5% skim milk at room temperature for 1 hr. After washing 3 times with PBST for 10 min, membranes were incubated overnight with 1:500 rabbit polyclonal Amacr antibodies to AMCR (Abcam, Cambridge, UK) at 4°C. The membranes were then washed with TBST and incubated with 1:1,000 diluted horseradish peroxidase-labeled goat polyclonal antibodies to rabbit IgG (Abcam). The ECL plus Western blotting system (Amersham Biosciences) was used for detection.

To study the effects of incubation time and temperature on mRNA expression of the selected clone, 100 *A. simplex* larvae were incubated in DMEM at 10°C, 15°C, 25°C, 37°C, and 40°C for 24 or 48 hr. After the total RNA was extracted (TRIzol, Invi-

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Anicakie	PLAPCKT	TRUVNLER	KONKHUPNN	CLESD	TTDPYPPC	TER	CL.NP	TELLKEN	FRITVAR	TTCECOT	GELSOR	PELNYU	ALSCIMPT	206
Caeporhabditis	PMNPCKS	WEFFEL PK	SEDIKKUPDI	CRTCD	ULL DPYPPC	TER	CT.DP	LSLWNDN	CLITCP	TSCYCOT	CRMCOR	CHDINYV	AMSCHLDT	136
Sile	PLARCKR	SL.VVNL KP	POGPAVMPRI	CAT.2 D	TVI.EDEPDC	TNEK	OLCP	ETLIPEN	PRITYAR	LSCECOS	CREEOV	CHDTNYL.	ALSCVI.SP	139
Danio	TOPRCKO	VALNIKS	PKGVAVLKRI	CLOSD	VIEPYRKG	TNEK	GL GP	EDITKEN	PRITYAR	LTGYGOS	GSYAKA	CHDTNYL.	AMSGLUSM	135
Gallug	VOCRGKR	SLALDLER	PPGAAALPRI	CSG2 D	VI.TEPERHG	TNEK	GLOP	EVILIHEN	PRITYAR	LTGEGOT	CKYAKS	CHDINYV	ALSCVISK	136
Bos	RLARGKR	SLVVDLKO	PRGAAVLERI	CARAD	MIEPERPG	TNEK	OLGP	ELLOKEN	PRITYAR	LSGEGOS	GRESKM	CHDTNYL.	ALSOVISE	134
Anonheles	VLC GGKR	SLALNLKO	PKATNVVRSI	CRSCD	TTEPERPO	TNEK	CL.CP	TVLLSDN	PRIVYAR	LTGEGOT	GTHAAR	CHDLNVV	ALSOVI.SM	134
Xenopus	TLARGER	TAVNLKS	PEGISLIKKI	CKKSD	VITEPERHG	MEN	GLGP	DIMLOEN	POLTYAR	LTGEGOS	GKYAKA	CHDINYV	STSGLLSK	135
Ailuropoda	VLARAKR	SLAVDLER	POGAAVLERI	CARTD	VIEPERPG	THEK	OLGP	ELLOKEN	PKLIYAR	LSGEGOS	GRESRV	GHDINYL	ALSGVISK	135
Mus	FLARGER	SLALDLKR	SOGVTVLERM	CARAD	VI.LEPERCO	MEK	OLGP	ETLLODN	PKLTYAR	LSGEGOS	GIESKV	GHDTNYL.	ALSOVISK	134
Branchiostoma	MLARGKR	SVALNLKO	OEGVDVLERM	COKSD	LIEPFRAG	MER	GLGP	DTLMKDN	PGLIYAR	MTGFGOS	GSESOM	GHDINYI	GLSGMLSL	135
Homo	RLCRGKR	SLVLDLKO	PRGAAVLERI	CKROD	VI.LEPERRG	MEK	OLGP	ELLOREN	PRLIVAR	LSGEGOS	GSECRL	GHDINYL	ALSGVISK	135
Canis	ILA RGKR	SLALDLOR	POGAAVLEPI	CARAD	VLDPYRHG	MEK	OLGP	EILOREN	PRLIYAR	LSGEGOS	GRESKT	GHDINFL	ALSGVLSK	135
Taeniopygia	VOARGKR	SLALDLKO	POGAAVLERI	CGAAD	VLIEPFRHG	/MER	GLGP	EVLLOEN	PRLIYAR	LTGFGOT	GKYAKS	GHDINYL	ALSGVLSK	165
Saccoglossus	LLSRGKR	SIVLDLKS	SEGLKIAKKI	CLASD	VLIEPFRPG	/MEK	LGLGP	TVLLKHN	PGLIYAR	LTGFGOS	GKYSOM	GHDINYL	AMSGLLSR	137
Orvctolagus	RLSRGKR	SLVLDLOR	POGVSVLRRL	CARAD	VLLEPFRCG	/MEKI	LNLGP	EILOOEN	PRLIYAR	LSGFGOS	GRESRV	GHDINYL	ALSGILSK	135
Equus	YLA RGKR	SLALDLKR	PRGAAVLRRL	CTRAD	VVLDPFRAG	/MEKI	LOLGP	EILOREN	PSLIYAR	LSGFGOL	GRFSRL	GHDINYL	ALSGVLSK	135
Drosophila	VLCOGKR	FLCLDLKN	PKGOOAVORL	VKKCD	VLIEPFRPG	/MEKI	LNLGP	TDLCTAN	PRLIYAR	LTGFGOH	GRLAOR	GHDINYA	ALSGVLSM	134
Rattus	HLA RGKR	SLALDLKR	SPGAAVLRRM	CARAD	VLLEPFRCG	/MEKI	LQLGP	ETLRODN	PKLIYAR	LSGFGQS	GIFSKV	GHDINYV	ALSGVLSK	114
Pan	RLCRGKR	SLVLDLKQ	PRGAAVLRRL	CKRSD	VLLEPFRRG	/MEKI	LOLGP	EILOREN	PRLIYAR	LSGFGQS	GSFSWL	GHDINYL	ALSGVLSK	135
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Anisakis	FAGHSVK	.*	*::*.** NMLA <mark>GFAGG</mark> S	:	** :: * <mark>JILAAIHQR</mark>	.* IIN <mark>GO</mark>		:* * . IDVSMVD	GLAYLGS	FQ T IYKD	IDNVW-	NKPFSW	FS <mark>GDCPV</mark>	293
Anisakis Caenorhabditis	FAGHSVK	.* RFPYWTPA RPWPPV	*::*.** NMLAGFAGGS	LMAAF	** :: * JILAAIHOR JIVSAIHAR	.* IINGO	* : SKGSI SQGCV	:* * . IDVSMVD LDCSMTE	GLAYLGS GVAYLAS	FQ <mark>T</mark> IYKD FVQYYYE	IDNVW-	N <mark>KP</mark> FSW TDKYAA	FSGDCPV FTGECPI	293 221
Anisakis Caenorhabditis Sus	FAGHSVK FAGAEAS	.* RFPYWTPA RPWPPV PQPPM	*::*.**. NMLAGFAGGS NMLADFAGGG NLLADFGGGS	LMAAF LSAAF LMCAL	** :: * JILAAIHQR JIVSAIHAR JIMMALFER	IINGO THNGO TRSG-	* : SKGS I SQGC V -KGC I	:* *. IDVSMVD LDCSMTE IDASMVE	GLAYLGS GVAYLAS GTAYLSS	FQTIYKD FVQYYYE FLW <mark>KTQ</mark> L	IDNVW- QSHLF- T <mark>G</mark> LWD	N <mark>KP</mark> FSW TDKYAA - Q <mark>PRGQN</mark> M	FSGDCPV FTGECPI LDGGAPF	293 221 223
Anisakis Caenorhabditis Sus Danio	FAGHSVK FAGAEAS IGRSGEN LGRSSEK	.* RFPYWTPA RPWPPV PQPPM PYAPI	*::*.**. NMLAGFAGGS NMLADFAGGG NLLADFGGGS NLVADFAGGG	LMAAF LSAAF LSAAF LMCAL	** :: * JILAAIHQR JIVSAIHAR JIMMALFER JIVLALL <mark>E</mark> R	.* IINGO THNGO TRSG- SRSG-	* : 3KGS I 3QGC V -KGC I -QGC I	:* * . IDVSMVD LDCSMTE IDASMVE	GLAYLGS GVAYLAS GTAYLSS GAAYVGS	FQTIYKD FVQYYYE FLWKTQL FMW <mark>KS</mark> RS	IDNVW- QSHLF- TGLWD- IGLWN-	N <mark>KP</mark> FSW TDKYAA - Q <mark>PRGQNM</mark> - R <mark>PRGENL</mark>	FSGDCPV FTGECPI LDGGAPF LDSGAPF	293 221 223 219
Anisakis Caenorhabditis Sus Danio Gallus	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN	.* RFPYWTPA RPWPPV PQPPM PYAPI PYAPI	*::*.**. NMLAGFAGGS NMLADFAGGG NLLADFGGGS NLVADFAGGG NLLADFAGGG	LMAAFO LSAAFO LMCALO LMCAFO	** :: * Silaaihqr Sivsaihar Simmalfer Sivlaller Siilalyer	.* IINGO THNGO TRSG- SRSG- TISG-	* : SKGS I SQGC V - KGC I - QGC I - KGC V	:* * . IDVSMVD LDCSMTE IDASMVE IDASMVE VDASMVE	SLAYLGS GVAYLAS GTAYLSS GAAYVGS GIAYISS	FQTIYKD FVQYYYE FLWKTQL FMWKSRS FLWKSQN	IDNVW- QSHLF- TGLWD- IGLWN- LGLWN-	N <mark>KP</mark> FSW - TDKYAA -QPRGQNM -RPRGENL -RPRGENL	FSGDCPV FTGECPI LDGGAPF LDSGAPF LDSGAPF	293 221 223 219 220
Anisakis Caenorhabditis Sus Danio Gallus Bos	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN IGRSGEN	.* RFPYWTPA RPWPPV PQPPM PYAPI PYAPI PYAPI	*::*.** NMLAGFAGGG NLLADFGGGS NLLADFGGGG NLLADFAGGG NLLADFGGGG	LMAAFO LSAAFO LMCALO LMCALO VLCALO LMCAMO	** :: * SILAAIHQR SIVSAIHAR SIMMALFER SIVLALLER SIVLALLER SIIIALYER SIIMALFER	IINGO THNGO TRSG- SRSG- TISG- TRSG-	* : SKGS I SQGC V - KGC I - QGC I - KGC V - KGC V	:* * . IDVSMVD LDCSMTE IDASMVE IDASMVE VDASMVE IDASMVE	SLAYLGS SVAYLAS STAYLSS SAAYVGS SIAYISS STAYLSS	FQTIYKD FVQYYYE FLWKTQL FMWKSRS FLWKSQN FMWKTQE	IDNVW- QSHLF- IGLWD- IGLWN- LGLWN- TGLWE-	N <mark>KP</mark> FSW - TDKYAA -QPRGQNM -RPRGENL -RPRGENL -QPRGQNM	FSGDCPV FTGECPI LDGGAPF LDSGAPF LDSGAPF LDGGAPF	293 221 223 219 220 218
Anisakis Caenorhabditis Danio Gallus Bos Anopheles	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN IGRSGEN LGRKENK	.* RFPYWTPA RPWPPV PQPPN PYAPI PYAPI PYAPI PTAPI	*::*.** NMLAGFAGGS NMLADFAGGG NLLADFGGGS NLVADFAGGG NLLADFGGGG NLLADFGGGG	LMAAFO LSAAFO LMCALO LMCALO VLCALO LMCAMO LMCAFO	** :: * SILAAIHQR SIVSAIHAR SIMMALFER SIVLALLER SIIIALYER SIIMALFER SILAALVER	.* THNGG TRSG- SRSG- TISG- TRSG- HH <mark>SG</mark> -	* : SKGS I SQGC V KGC I - KGC V - KGC V - KGC V	:* * . IDVSMVD LDCSMTE IDASMVE IDASMVE VDASMVE IDASMVE VDHAMVE	GLAYLGS SVAYLAS STAYLSS GAAYVGS GIAYISS GTAYLSS GAAYVGS	FQTIYKD FVQYYYE FLWKTQL FMWKSRS FLWKSQN FMWKTQE WLYRSQS	IDNVW - QSHLF - TGLWD - IGLWN - LGLWN - TGLWE - LPVWG -	NKPFSW TDKYAA - QPRGQNM - RPRGENL - RPRGENL - QPRGQNM - KARGEN I	FSGDCPV FTGECPI LDGGAPF LDSGAPF LDSGAPF LDGGAPF LDGGAHF	293 221 223 219 220 218 218
Anisakis Caenorhabditis Sus Danio Gallus Bos Anopheles Xenopus	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN IGRSGEN LGRKENK LGDKN-S	.* RFPYWTP2 RP WPPV PQ PPM PY API PY API PT API PT PPI	*::*.** NMLAGFAGGS NLLADFGGGS NLLADFAGGG NLLADFAGGG NLLADFAGGG NLLADFAGGG	LMAAFO LSAAFO LMCALO LMCAFO VLCALO LMCAFO LMCAFO LMCAFO	** :: * SILAAIHQR SIVSAIHAR SIVLALFER SIVLALLER SIIIALYER SIIMALFER SILAALVER SIVMSLFER	.* THNGO TRSG- SRSG- TISG- TRSG- HHSG- TKSG-	* : SKGS I SQGC V -KGC I -KGC V -KGC V -KGC V	IDVSMVD LDCSMTE IDASMVE IDASMVE VDASMVE IDASMVE VDHAMVE IDCSMVE	GLAYLGS GVAYLAS GTAYLSS GAAYVGS GTAYLSS GAAYLGS GAAYLGS	FQTIYKD FVQYYYE FLWKTQL FMWKSRS FLWKSQN FMWKTQE WLYRSQS FVWKSQK	IDNVW - QSHLF - TGLWD - IGLWN - LGLWN - TGLWE - LPVWG - LGLWS -	NKPFSW TDKYAA -QPRGQNM -RPRGENL -RPRGENL -QPRGQNM -KARGENI -NSPGENM	FSGDCPV FTGECPI LDGGAPF LDSGAPF LDGGAPF LDGGAHF LDGGAHF	293 221 223 219 220 218 218 218
Anisakis Caenorhabditis Sus Danio Gallus Bos Anopheles Xenopus Ailuropoda	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN IGRSGEN LGRKENK LGDKN-S IGRNGEN	.* RFPYWTPP RP - WPPV PQ PPN PY API PY API PT API PT PPI PF PPI	*::*.* NMLAGFAGGG NLLADFGGGG NLLADFGGGG NLLADFAGGG NLLADFAGGG NLLADFAGGG NLLADFAGGG	LMAAFO LSAAFO LMCALO LMCAFO LMCAFO LMCAFO LTCALO LMCTLO	SILAAIHQR SIVSAIHAR SIVSAIHAR SIVLALLER SIVLALLER SIILALYER SILAALVER SILAALVER SILMALFER	.* THNGG TRSG- SRSG- TRSG- TRSG- TRSG- TRSG- TRSG-	* : SKGS I SQGC V - KGC I - KGC V - KGC V - KGC V - KGC V	IDVSMVD LDCSMTE IDASMVE IDASMVE VDASMVE IDASMVE IDASMVE IDCSMVE	SLAYLGS SVAYLAS STAYLSS SAAYVGS SIAYISS STAYLSS SAAYVGS SAAYLGS STAYLSS	FQTIYKD FVQYYYE FLWKTQL FMWKSRS FLWKSQN FMWKTQE WLYRSQS FVWKSQK FLWKTQQ	IDNVW- QSHLF- IGLWD- IGLWN- LGLWN- LGLWE- LPVWG- LGLWS- IGLWD-	NKPFSW - TDKYAA - QPRGQNM - RPRGENL - QPRGQNM - CARGENI - NSPGENM - QPQGQNL	FSGDCPV FTGECPI LDSGAPF LDSGAPF LDGGAPF LDGGAFF LDGGAFF LDGGAPF	293 221 223 219 220 218 218 218 218 219
Anisakis Caenorhabditis Danio Gallus Bos Anopheles Xenopus Ailuropoda Mus	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN IGRSGEN LGRKENK LGDKN-S IGRNGEN IGRSGEN	.* RFPYWTP2 RP WPPV PQ PPN PY API PY API PT API PT PPI PF PPI PY PPI	*::*** NMLAGFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFGGG NLLADFGGG	LMAAF LSAAF LMCAL LMCAL LMCAL LMCAL LMCAL LMCAL LMCAL LMCTL	** :: * ILAAIHQR IVSAIHAR IVSAIHAR IVALLER IVALLER IIIALYER IIAALVER IVSLFER IVSLFER IVLALFER	.* THNGG TRSG- SRSG- TISG- TRSG- TRSG- TRSG- TRSG- TRSG-	* : JKGS I JQGC V - KGC I - KGC V - KGC V - KGC V - KGC V - KGC V	IDVSMVD LDCSMTB IDASMVB IDASMVB VDASMVB IDASMVB UDHAMVB IDCSMVB IDANMVB IDSSMVB	SLAYLGS SVAYLAS STAYLSS SAAYVGS STAYLSS SAAYVGS SAAYLGS STAYLSS STAYLSS	FQTIYKD FVQYYYE FLWKTQL FMWKSRS FLWKSQN FMWKTQE MLYRSQS FLWKTQQ FLWKTQQ FLWKTQQ	IDNVW- QSHLF- IGLWD- IGLWN- LGLWN- LGLWS- LGLWS- IGLWD- MGLWK-	NKPFSW - TDKYAA - QPRGQNM - RPRGENL - QPRGQNM - QPRGQNL - NSPGENM - QPQGQNL - QPRGQNI	FSGDCPV FTGECPI LDSGAPF LDSGAPF LDSGAPF LDGGAFF LDGGAPF LDGGAPF LDGGAPF	293 221 223 219 220 218 218 218 218 219 218
Anisakis Caenorhabditis Sus Danio Gallus Bos Anopheles Xenopus Ailuropoda Mus Branchiostoma	FAGHSVK FAGAEAS IGRSGEN LGRKGEN IGRSGEN LGRKENK LGRKENK LGRKN-S IGRNGEN LGRKHEN	.* RFPYWTP2 RP - WPPV PQ - PPP PY - API PY - API PT - PPI PT - PPI PT - PPI PF - PPI PM - PPI	MILAGFAGGS NMLADFAGGG NLLADFGGGS NLLADFGGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG	LAAAF LSAAF LCAL LMCAL LMCAL LMCAF VLCAL LMCAF LTCAL LMCTL LMCTL LMCTL LMCTL LMCTL	SILAAIHQR SIVSAIHAR SIVSAIHAR SIVALLER SIVLALLER SIILAALVER SIVALFER SIVMALFER SIVMALFER SIVMALLER	.* IINGG THNGG SRSG- TISG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG-	* : JKGS I JQGC V - KGC I - KGC V - KGC V - KGC V - KGC V - KGC V - KGC V	IDVSMVD LDCSMTE IDASMVE UDASMVE UDASMVE UDASMVE UDCSMVE IDCSMVE IDSMVE IDSMVE	SLAYLGS SVAYLAS STAYLSS SIAYVGS SIAYLSS SAAYLGS STAYLGS STAYLSS STAYLSS SASYVGS	FQTIYKD FVQYYYE FLWKTQL FMWKSQN FLWKSQN FMWKTQE WLYRSQS FVWKSQK FLWKTQQ FLWKTQQ FLWKTQP	IDNVW- QSHLF- IGLWD- IGLWN- LGLWS- LGLWS- LGLWS- IGLWD- MGLWK- MF	NKPFSW - TDKYAA - QPRGQNM - RPRGENL - QPRGQNM - KARGENI - NSPGENM - QPQGQNL - QPRGQNI - HGRGENL	FSGDCPV FTGECPI LDSGAPF LDSGAPF LDSGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF	293 221 223 219 220 218 218 218 218 219 218 217
Anisakis Caenorhabditis Sus Danio Gallus Bos Anopheles Xenopus Ailuropoda Mus Branchiostoma Homo	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN IGRSGEN IGRNGEN IGRNGEN IGRSGEN IGRSGEN	.* RFPYWTPA RPPPN PYAPI PYAPI PTPPI PTPPI PTPPI PYPPI PYPPI PYPPI PYAPI	*::*.** NMLADFAGG NLLADFGGGS NLVADFAGG NLLADFAGGG NLLADFAGGG NLLADFAGGG NLLADFAGGG NLLADFGGGG NLLADFGGGG NLLADFAGGG	LACTLC LMCAPC	** :: * JILAAIHQR JIVSAIHAR JIVSAIHAR JIVIALLER JIITALYER JILALVER JILALVER JILALVER JILALVER JIVALFER JIVALFER JIVALFER JIVALFER JIMALFOR	.* IINGG THNGG SRSG- TISG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRTG-	* : GGC V - KGC I - KGC V - KGC V - KGC V - KGC V - KGC V - KGC V - KGC V	IDVSMVD LDCSMTE IDASMVE IDASMVE IDASMVE IDASMVE IDCSMVE IDANMVE IDSSMVE IDANMVE	SLAYLGS SVAYLAS STAYLSS SAAYVGS STAYLSS SAAYVGS STAYLSS STAYLSS SASYVGS STAYLSS SASYVGS	FQTIYKD FVQYYYE FLWKTQL FMWKSRS FLWKSQN FMWKTQE MLYRSQS FVWKSQK FLWKTQP FLWKTQP FLWKTQK	IDNVW QSHLF - TGLWD - IGLWD LGLWE LGLWS IGLWG IGLWD MGLWK LSLWE	NKPFSW - TDKYAA - QPRGQNM RPRGENL - QPRGQNM KARGENI - NSPGENM - QPQGQNL - QPRGQNI - APRGQNM	FSGDCPV FTGECPI LDGGAPF LDSGAPF LDSGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF	293 221 223 219 220 218 218 218 218 219 218 219 218 217 219
Anisakis Caenorhabditis Sus Danio Gallus Bos Anopheles Xenopus Ailuropoda Mus Branchiostoma Homo Canis	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN IGRSGEN LGRKENK IGRNGEN IGRSGEN IGRSGEN IGRSGEN	.* RFPYWTPA RPWPPV PQPPN PYAPI PYAPI PTPPI PTPPI PYPPI PYAPI PYAPI PYAPI	*::*.** MMLADFAGGG NLLADFAGGG NLLADFAGGG NLLADFAGGG NLLADFAGGG NLLADFAGGG NLLADFGGGG NLLADFGGGG NLLADFGGGG NLLADFGGGG NLLADFGGGG	LACTL LACAL LACAP LCAP LCAP LACAP LACAP LACAL LACAL LACAL LACAL LACAL	SILAAIHQR SIVSAIHAR SIVSAIHAR SIVNALFER SIVLALFER SILAALVER SILAALVER SIVALFER SIVLALFER SIVMALER SILMALFER SILMALFER	.* IINGG THNGG SRSG- TISG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG-	* : GGC V - KGC I - KGC V - KGC V	IDVSMVD LDCSMTE IDASMVE VDASMVE VDASMVE VDHAMVE IDCSMVE IDCSMVE IDSMVE IDANMVE IDANMVE	SLAYLGS SVAYLAS STAYLSS SAAYVGS STAYLSS SAAYLGS STAYLSS STAYLSS SASYVGS STAYLSS STAYLSS STAYLSS	FQTIYKD FVQYYE FLWKTQL FMWKSRS FLWKSQN FMWKTQE MLYRSQS FLWKTQQ FLWKTQP FLWKTQP FLWKTQP FLWKTQF	IDNVW - QSHLF - TGLWD - IGLWN - LGLWN - LGLWS - IGLWE - IGLWD - MGLWK - MGLWE - LSLWE - MGLWE -	- NKPFSW - TDKYAA QPRGQNM RPRGENL QPRGUN KARGENL - NSPGENM - QPQGQNL - QPGQNL HGRGENL - APRGQNL	FSGDCPI LDGGAPF LDSGAPF LDSGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF	293 221 223 219 220 218 218 218 219 219 219 219
Anisakis Caenorhabditis Sus Danio Gallus Bos Anopheles Xenopus Ailuropoda Mus Branchiostoma Homo Canis Taeniopygia	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN LGRKENK LGRKENK LGRKHEN LGRKHEN LGRSGEN LGRSGEN LGRKNEN	.* RFPYWTP2 PQ PPN PY API PY API PY API PT PPI PT PPI PY PPI PY API PY API PY API	*::*.** NMLAGFAGGS NLLADFGGGS NLLADFAGGG NLLADFAGGG NLLADFAGGG NLLADFGGGG NLLADFGGGG NLLADFGGGG NLLAGFAGGG NLLADFGGGA	LMAAP(LSAAF LMCAL LMCAL LMCAL LMCAL LMCAL LMCAL LMCAL LMCTL LMCTL LMCTL LTCAL LMCTL LTCAL LMCTL LMCTL LMCAL	SILAAIHQR SIVSAIHAR SIVSAIHAR SIVLALLER SIVLALLER SIVLALFER SILAALVER SILMALFER SIVMALFER SIVMALLER SIVMALLER SILMALFER SIVALFER	.* IINGG TRSG- SRSG- TISG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- ARSG-	* : SKGS I SQGC I - KGC I - KGC V - KGC V	IDVSMVD LDCSMTB LDASMVB IDASMVB VDASMVB IDASMVB IDCSMVB IDCSMVB IDCSMVB IDANMVB IDANMVB IDANMVB IDANMVB IDANMVB	SLAYLGS GVAYLAS STAYLSS SIAYVGS GAYLGS GAYLGS GTAYLSS GAYLGS STAYLSS GASYLGS STAYLSS STAYLSS STAYLSS STAYLSS	FQTIYKD FVQYYYE FLWKTQL FMWKRTQE WLYRSQS FLWKTQQ FLWKTQQ FLWKTQQ FLWKTQQ FLWKTQK FLWKTQK FLWKTQK FLWKSQN	IDNVW - OSHLF - TGLWD - IGLWD - LGLWN - TGLWE - LGLWS - MGLWK - MGLWK - MGLWE - LSLWE - LGLWS -	NKPFSW - TDKYAA - QPRGQNM - RPRGENL - QPRGQNM - KARGENI - NSPGENM - QPQGQNL - QPRGQNI - HGRGENL - APRGQNM - NPRGQNL - QPRGENL	FSGDCPV FTGECPI LDSGAPF LDSGAPF LDGGAPF LDGGAFF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF	293 221 223 219 220 218 218 218 218 218 219 218 217 219 219 219 249
Anisakis Caenorhabditis Sus Danio Gallus Bos Anopheles Xenopus Ailuropoda Mus Branchiostoma Homo Canis Taeniopygia Saccoglossus	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN IGRSGEN IGRSGEN IGRSGEN IGRSGEN IGRSGEN IGRSGEN IGRSGEN LGRKNEN LGRKNEN	* RFPYWTPP RP - WPPV PQ - PPN PY - API PY - API PT - PPI PF - PPI PY - PPI PY - API PY - API PY - API PY - API	MILAFAGG NMLAFAGG NLADFAGG NLLADFAGG NLLADFAGG NLLAFAGG NLLAFAGG NLLAFGGG NLLAFGGG NLLAFGGG NLLAFAGG NLLAFAGG NLLAFAGG NLLAFAGG	LMAAFC LSAAF LSAAF LCAL LMCAK VLCAL LMCAK LMCAK LMCAL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCL LICL LMCAL	SILAAIHQR SIVSAIHAR SIVSAIHAR SIVSAIHAR SIVALFER SIVIALFER SILAALVER SILAALVER SILAALFER SIVALFER SILMALFER SILMALFER SIVIALFER SIVIALFER	TRSG SRSG TRSG TRSG TRSG TRSG TRSG TRSG	* : SKGS I SQGC I - KGC I - KGC V - KGC V	IDVSMVD IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE	SLAYLGS SVAYLAS STAYLSS SAAYVGS STAYLSS SAAYLGS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS	PQTIYAD FVQYYYE FLWKTQL FMWKSRS FLWKSQN WLYRSQS FVWKSQK FLWKTQQ FLWKTQQ FLWKTQK FLWKSQK FLWKSQK WIFQGKN	IDNVW - QSHLF - TGLWD - IGLWD - LGLWS - LGLWS - IGLWS - MGLWK - MF LSLWE - MGLWE - MGLWE - TFAIWP	NKPFSW - TDKYAA - OPRGONM RPRGENL - OPRGONM - OPRGONM - OPRGONL - OPRGONL - OPRGONL - OPRGONL - OPRGONL - OPRGONL - OPRGONL - OPRGONL	FSGDCPV FTGECPI LDSGAPF LDSGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDSGAPF	293 221 223 219 220 218 218 218 218 219 218 217 219 219 219 229 223
Anisakis Caenorhabditis Sus Danio Gallus Bos Anopheles Xenopus Ailuropoda Mus Branchiostoma Homo Canis Taeniopygia Saccoglossus Oryctolagus	FAGHSVK FAGASASS IGRSGEN LGRSSEK LGRKGEN LGRKENK LGDKN-S IGRSGEN IGRSGEN IGRSGEN IGRSGEN LGRKNEN LGRKNEN LGRYEN LGRYEN		MILAGFAGGS NMLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFAGG NLLADFAGG NLLADFAGG	LMAAF LMAAF LSAAF LMCAF LMCAF LMCAF LMCAF LMCAL LMCAL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCLL LMCLL LMCLL	ILAAIHQR IVSAIHAR IVVSAIHAR IVIALLER IIVALLER IIVALFER IVALFER IVALFER IVALFER IVALFER IVIALFER IVIALFER IVIALFER IVIALFER	TRSG TRSG SRSG TISG TRSG TRSG TRSG TRSG TRSG TRTG TRSG TRS	* SKGS I SGG V - KGC V - KGC V - KGC V	IDVSMVD LDCSMT& LDCSMT& IDASMVB IDASMVB VDASMVB VDASMVB IDASMVB IDASMVB IDANNVB IDASMVB IDASMVB IDASMVB	GLAYLGS GVAYLAS GVAYLSS GAAYVGS GTAYLSS GTAYLSS GAAYVGS GTAYLSS GTAYLSS GTAYLSS GTAYLSS GVAYLSS GCAYIGS TAYLSS	FQTIYKD FVQYYYE FLWKTQL FMWKTQS FVWKSQK FVWKSQK FLWKSQK FLWKTQK FLWHSLQ FLWHSLQ FLWKSQN FLWKTQK	IDNVW - QSHLF - TGLWD - IGLWN - LGLWN - LGLWS - LGLWS - IGLWD - MGLWK - MGLWK - MGLWE - LSLWE - LGLWS - TFAIWP SGLWE -	- NKPFSW - TDKVAA - QPRGQNM RPRGENL - QPRGQNM KARGENI - NSPGENM - QPGQNL - QPGQNL - QPRGQNL - QPRGQNL	FSGDCPV FTGECPI LDSGAPF LDSGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDSGAPF LDSGAPF LDSGAPF	293 221 223 219 220 218 218 218 219 219 219 219 219 2219 249 223 219
Anisakis Sus Danio Gallus Bos Anopheles Xenopus Ailuropoda Mus Branchiostoma Homo Canis Taeniopygia Saccoglossus Oryctolagus Equus	FAGHSK FAGASAS IGRSGEN LGRSGEN LGRSGEN LGRKENK LGRKEN IGRSGEN IGRSGEN IGRSGEN LGRKNEN LGRKNEN LGRSGN		MILA GRAGG NULAD FAGG NULAD FAGG	LICAL LICAL LICAL LICAL LICAL LICAL LICAL LICAL LICAL LICAL LICAL LICAL LICAL LICAL LICAL LICAL LICAL	SILAAIHOR JUVALHAR JIVALLER JIVLALLER JIVLALLER JIVLALFER JIVALFER JIVALFER JIVALFER JIVALFER JIVALFER JIVALFER JIVALFER JIVALFER JIVALFER JIVALFER JIVALFER JIVALFER	.* THNGG TRSG- SRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG-	* SKGS I SQGC V KGC V K KGC V K KGC V K KGC V K KGC V K KGC V K KGC V K K K K K K K K K K K K K K K K K K K	IDVSMVD IDVSMVD IDASMVE IDASMVE VDASMVE VDASMVE IDASMVE IDASMVE IDASMVE IDANNVE IDANNVE IDANNVE IDANNVE IDANNVE	SLAYLGS SVAYLAS STAYLSS SAAYVGS SIAYISS SAAYUGS SAAYUGS STAYLSS STAYLSS STAYLSS STAYLSS SVAYLSS SCAYIGS STAYLSS STAYLSS STAYLSS	FQTIYKD FVQYYYE FLWKIGL FLWKSGS FLWKSGS VLYRSGS FVWKSGK FLWKIGQ FLWKIG FLWKIG FLWKIG FLWKSGN VIFGGKN FLWKSGN	IDNVW- QSHLF- TGLWD- IGLWN- LGLWN- LGLWS- IGLWS- IGLWS- IGLWS- LSLWE- MGLWE- LGLWS- TFAIWP SGLWE- MGLWE-	- NKPFSW - TDKYAA - TDKYAA - OPRGONM RPRGENL - PRGENL - OPRGONM - OPRGONM - OPRGONL - OPRGONL - OPRGONL - OPRGONL	FSGDCPV FTGECPI LDSGAPF LDSGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF	293 221 223 219 220 218 218 218 219 218 219 219 229 2219 221
Anisakis Caenorhabditis Sus Danio Gallus Bos Anopheles Xenopus Ailuropoda Mus Branchiostoma Homo Canis Taeniopygia Saccoglossus Oryctolagus Equus Drosophila	FAGHSYK FAGAEAS IGRSGEN LGRSSEK LGRSGEN LGRSGEN IGRGGEN IGRGGEN IGRGGEN IGRSGEN LGRNEN LGRYNEN LGRSCN LGRREK	* RPPWEP RP - MPPV PC PPN PY API PY API PT API PT API PY PPI PY PPI PY API PY API PY API TY API TY API TY API TY API TY API TY API TY API	MILAGFAGGS NMLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFAGGG NLLADFAGGG NLLADFGGG NLLADFGGG	LACAL LACAL LACAL LACAL LACAL LACAR LACAR LACAR LACAR LACAL	SILAAIHOR SIVALLES SIVALLES SIVALLES SIVALLES SIVALLES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES SIVALES	.* THNGG TRSG- SRSG- TISG- TRSG- TRSG- TRSG- TRSG- SRTG- TRSG- SRTG- TRSG- TRSG- TRSG- HRSG-	* SQGC V SQGC V KGC II KGC V KGC V K KGC V KGC V KGC V KGC V KGC V K KGC V KGC V K KGC V K KGC V K KGC V K KGC V K KGC V K K K K K K K K K K K K K K K K K K K	LIDVSMVD LDCSMTS IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDANVE IDANVE IDASMVE IDASMVE IDANVE IDANVE	SLAYLGS SVAYLAS STAYLSS SAYVGS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS STAYLSS	FQIIYKD FVQYYYE FLWKTQL FMWKSRS FLWKTQE VLYRSQS FLWKTQE FLWKTQK FLWKTQK FLWHTP FLWKSQN VIFQGKN VIFQGKN FVWKTQK FVWKTQK	IDNVW - QSHLF - TGLWD - IGLWN - LGLWN - LGLWS - LGLWS - LSLWD - MGLWK - MF LSLWE - MGLWE - SGLWE - SGLWE - MGLWE - LVIWG -	- NKPFSW - TDKVAA - TDKVAA - OPRGONM RPRGENL - OPRGONM - OPGQNL - OPRGONL - OPRG	FSGDCPV FTGECPI LDSGAPF LDSGAPF LDSGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF	293 221 223 219 220 218 218 218 219 218 219 219 229 229 229 229 229 229 229 229
Anisakis Caenorhabditis Sus Danio Gallus Bos Anopheles Xenopus Alluropoda Mus Branchiostoma Homo Canis Taeniopygia Saccoglossus Oryctolagus Equus Drosophila Rattus	FAGHSK FAGASAS IGRSGEN LGRSGEN LGRSGEN LGRSGEN LGRKEN LGRKEN LGRKEN LGRKEN LGRYEN LGRSGEN LGRSGEN LGRSGEN	* RPPWEP POPPN POPPN PYAPI PYAPI PYPPI PTPPI PYPPI PYPP	MILAGFAGG NULADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFGGG NLLADFGGG NLLAPFAGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG	LACAL LACAL	ILAAIHOK IVAALPE IVVALPE IVVALPE IIVAALPE IIVAALPE IIVAALPE IIVAALPE IVVALPE IIVAALPE IIVAALPE IIVAALPE IIVAALPE IIVAALPE IIVAALPE IIVAALPE IIVAALPE IIVAALPE IIVAALPE IIVAALPE IIVAALPE	.* IINGG THNGG SRSG- TISG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- HRSG- TRSG-	* SQGC V H V SQGC V H V SQGC V H V SQGC V H V SQGC V V SQGC V V SQGC V V SQGC V V SQGC	IDVSMVD LDCSMTS IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE IDASMVE	GLAYLGS GVAYLAS GAAYVGS GTAYLSS GAAYVGS GTAYLSS GTAYLSS GTAYLSS GVAYLSS GTAYLSS GTAYLSS GTAYLSS GTAYLSS GTAYLSS GTAYLST	FQIIYKD FVQYYYB FLWKTQL FMWKSRS FMWKTQB FWKTQS FVWKTQK FLWKTQA FLWKTQA FLWKTQA FLWKSQN WIFQGKN FLWKSQN WIFQGKN FLWGTQB FLWKTQA	IDNVW - QSHLF - TGLWD - IGLWN - LGLWN - LGLWS - IGLWE - LGLWS - MGLWE - LGLWS - MGLWE - LGLWS - MGLWE - LVIWG - MGLWA -	- NKP FSW - TDKYAA - TDKYAA OPRGONM - PPGENL OPRGONM OPGGNI - OPRGONM - OPRGONM	FSGDCPV FTGECPI LDSGAPF LDSGAPF LDSGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF VDGGSPF	293 221 223 219 220 218 218 218 219 219 219 219 223 219 223 219 219 218 198
Anisakis Sus Danio Gallus Bos Anopheles Xenopus Ailuropoda Mus Branchiostoma Homo Canis Taeniopygia Saccoglossus Oryctolagus Equus Drosophila Rattus Pan	FAGHSVK FAGAEAS IGRSGEN LGRSSEK LGRKGEN IGRSGEN IGRSGEN IGRSGEN LGRKHEN IGRSGEN LGRXFEN LGRYNEN LGRSVN LGRSVN LGRSVN IGRSGEN IGRSGEN	**************************************	NHLAFAGG NHLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFAGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG NLLADFGGG	LACAL LMCAL LMCAL LMCAF VLCAL LMCAF LTCAL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCTL LMCAL LMCAL LMCAL	GILAAIHOR IVSAIHAR IVSAIHAR IVMALER IIMALER IIMALER IIMALER IVMALER IVMALER IVMALER IVMALER IVMALER IVMALER IVMALER IVMALER IVMALER IVMALER IIMALER IIMALER IIMALER IIMALER	.* THNGG TRSG- SRSG- TISG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG- TRSG-	* SQGC V KGC V K KGC V K KGC V K KGC V K KGC V K K K K K K K K K K K K K K K K K K K	IDVSMVD LDCSMTB IDASMVB IDASMVB VDRAMVB IDASMVB IDASMVB IDASMVB IDASMVB IDANNVB IDASMVB IDASMVB IDASMVB IDASMVB IDANNVB	SLAYLGS GVAYLAS GTAYLSS GAAYLGS GTAYLSS GTAYLSS GTAYLSS GTAYLSS GTAYLSS GTAYLSS GCAYIGS GCAYIGS GCAYIGS GCAYIGS GCAYIGS GCAYIGS GTAYLSS GTAYLSS GTAYLSS GTAYLST	FQTIYKD FVQYYYE FLWKTQL FMWKSRS FMWKTQE WLYRSQS FLWKTQP FLWKTQP FLWKTQF FLWKTQK FLWSQGN FLWSQGN FLWGTQE FLWSQGN FLWQTQE FLWGTQE	IDNVW - QSHLF - TGLWD - IGLWD - LGLWS - LPVWG - LGLWS - IGLWD - MGLWE - LSLWE - MGLWE - TFAIWP SGLWE - LVIWG - MGLWE - SLWE -	- NKPFSW - TDKXAA OPRGQNM RPRGENL OPRGQNM KARGENL NSPGENM OPRGQNL OPRGQNL NPRGQNL OPRGQNL OPRGQNL OPRGQNL OPRGQNL APRGQNL	FSGDCEV FTGECPI LDSGAPF LDSGAPF LDSGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF LDGGAPF	293 221 223 219 220 218 218 218 218 219 219 219 229 2219 219 219 219 219 21



Fig. 2. Complete nucleotide and amino acid sequences (A) and phylogenetic analysis (B) of α-methylacyl CoA racemase (Amacr) from *Anisakis simplex* L3 (GenBank; HQ662605). The Amacr gene consists of 1,414 bp and 418 amino acids. The start codon (ATG), stop codon (TAA), and poly-adenylation signal (aataaa) are marked with an asterisk. The phylogenetic analyses were conducted in MEGA4 [23] using the maximum Parsimony method. The *A. simplex* Amacr was grouped with the nematode *Caenorhabditis elegans*.

trogen, Carlsbad, California, USA) and reverse transcribed using RT-PCR Master Mix (Takara, Tokyo, Japan), PCR was performed with Amacr primers (5'-AGC CCG AGG TAA AAC CAT TA and 3'-ACA ATC ACC CGA AAA CCA C). PCR cycles consisted of an initial 5 min denaturation at 94°C and 30 cycles of 94°C (30 sec), 56°C (30 sec), 72°C (30 sec), followed by a final 7 min extension at 72°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

Immunohistochemistry was performed to confirm the distribution of the Amacr protein. Paraffin-embedded larvae slides were deparaffinized and hydrated. For antigen retrieval, slides were immersed in citrate buffer (0.01 M, pH 6.0) and heated twice in a microwave (700 W) for 5 min. The slides were then quenched with endogenous peroxidase by incubation with a 3% hydrogen peroxide solution for 5 min and washed 3 times in PBS for 5 min. Slides were immunostained with rabbit polyconal Amacr antibody (1:500 dilution, Abcam) at 4°C overnight. After that slides were washed 3 times with PBS for 5 min and incubated with horseradish peroxidase-labeled goat polyclonal antibodies to rabbit IgG (1:2,000 dilution, Abcam) for 2 hr at room temperature. Slides were washed 4 times with PBS for 5 min each. The color reaction was developed with Dako's EnVision[™] System (DAKO, Carpinteria, California, USA). Slides were stained with DAB and counterstained with Meyer's hematoxylin (DAKO) for 20 sec, dehydrated, and mounted with Permount (Fisher Scientific) and observed by microscopy (Nikon, Eclipse 80i, Star Capture Basic V1.0, Tokyo, Japan).

We selected 1 clone from the final round of immunoscreening, designated as Asim37, which was the most consistently reactive with the polyclonal anti-Anisakis ES mouse sera during the screening process. The Amacr clone had an insert size of 1,412 bp and yielded 1 open reading frame (ORF) that encoded for a 418 amino acid protein with a predicted molecular weight of 46.7 kDa (GenBank; HQ662605). The complete sequence of the Amacr clone was identified as a α-methylacyl CoA racemase ortholog, a member of the CoA-transferase family III (Fig. 2A). Sequence analysis showed that the cDNA included 5' (32 bp) and 3' (188 bp) untranslated sequences. The cDNA construct contained an ATG start codon, a TAA stop codon, and a poly-A tail that comprised of 31 adenine residues. A typical polyadenylation signal was also present in 13 nucleotides upstream of the poly-A sequence. Interestingly, the A. simplex Amacr amino acid sequence was longer than that of other species at the N-terminus. Sequence homology analysis of the Anisakis Amacr gene displayed a homology of 36-50% and highly conserved amino acid sequences compared with 19 Amacr orthologs of different species (data not shown). The sequence analysis and phylogenetic analysis classified *A. simplex* Amacr as the most closely related to the *C. elegans* group (Fig. 2B).

The expression of the *Anisakis* Amacr was constitutively high under all conditions tested, suggesting that a large amount of Amacr gene expression in *A. simplex* L3 is required over a long period for the larval survival (Fig. 3). The expression of recombinant *A. simplex* Amacr protein was confirmed with SDS-PAGE. The GST-fusion protein revealed a 73 kDa band on the gel (Fig. 4). The recombinant Amacr protein showed a reactive band against Amacr antibody, a rabbit polyclonal to AMCR (Abcam) (Fig. 5). Immunohistochemical analysis showed that the Amacr protein was mainly localized at the ventriculus of *A. simplex* larvae, regardless of different time points (24 and 48 hr) (Fig. 6).

We have demonstrated the Amacr gene from A. *simplex* larvae. The Amacr gene contained a 1,412 bp cDNA transcript that encoded 418 amino acids. The expression of Amacr mRNA was constitutively high regardless of temperature (10-40°C) and time (24-48 hr). Amacr protein was expressed mainly in the ventriculus of *A. simplex* larva. The phylogenetic analysis showed that this gene was clustered to *C. elegans* which was the only nematode species that the Amacr gene has been known.

The Amacr gene encodes a racemase. The encoded enzyme racemase interconverts pristanoyl-CoA and C27-bile acyl CoAs between their (R)- and (S)-stereoisomers. The conversion to the (S)-stereoisomers is necessary for degradation of these substrates by peroxisomal β -oxidation. Encoded enzyme from this locus localizes at both mitochondria and peroxisomes. The function of this enzyme is presumed to be involved in energy production. In humans, an Amacr overexpression, deficiency, or mutant variants are known to be associated with a disease state [18,19]. Specifically, the Amacr overexpression is consid-



Fig. 3. RT-PCR anaylsis of the Amacr expression in *Anisakis* simplex L3 according to time and temperature. (A) α -actin. (B) RT-PCR of the Amacr mRNA. Lane 1-2, 10°C; Lane 3-4, 20°C; Lane 5-6, 25°C; Lane 7-8, 37°C; Lane 9-10, 40°C. Lanes 1, 3, 5, 7, and 9, 24 hr incubation; Lanes 2, 4, 6, 8, and 10, 48 hr incubation. The Amacr expression level was high and constitutive, regardless of time and temperature.

ered one of the most potent diagnostic markers of the prostate cancer [20].



Fig. 4. Expression of the *Anisakis simplex* L3 Amacr in *E. coli*. Lane 1, molecular marker; Lane 2, BL21(DE3); Lane 3, pGEX 4T-2; Lane 4, IPTG-induced; Lane 5, purified recombinant protein. The recombinant protein was constructed as a GST fusion protein and purified with a MicroSpin GST purification module. A GST fusion protein of the *A. simplex* Amacr was presented as 73 kDa band on the gel.



It is interesting to note that the function of the Amacr is related to the synthesis of the bile acid. The bile acid has long been known to play an important role in the host-parasite relationships, including penetration, immune suppression, and inflammation or modulation of homeostasis, enabling parasite invasion, and establishment [21]. The Amacr gene from *A*.



Fig. 5. Immunoblot analysis of the recombinant Amacr protein with rabbit polyclonal Amacr antibodies. Lane 1, IPTG-induced BL21(DE3); Lane 2, purified recombinant protein Amacr-GST fusion protein marked by an arrow (73 kDa). The recombinant Amacr protein showed a single reactive band against the Amacr antibody, rabbit polyclonal to AMCR (Abcam).

Fig. 6. Expression of the Amacr in *Anisakis simplex* larvae. The Amacr product was observed primarily in the ventriculus. (A) Control staining of larval ventriculus (arrows) with normal mouse serum (×40). (B) Specifically stained ventriculus (arrows). The L3 were incubated at 24°C for 24 hr (×40). (C) Larval Amacr immunostaining incubated for 48 hr also represents the same localization as 24 hr larvae. Ventriculus (arrows) (×100). Immuno-histochemical study showed that the Amacr product was found specifically in the ventriculus of the larvae.

simplex is thus presumably involved in the parasite-host relationships.

Generation of ATP from lipids occurs within mitochondria via the β -oxidation of fatty acids. However, the β -oxidation of fatty acids has not been reported in adult or larval helminths due to their anaerobic condition within the host even though the genes for the TCA cycle are present in several nematodes [22,23]. Recently, the oxidization of fatty acids and the activity of fatty acid oxidation enzymes were confirmed in infective larvae of *Angiostrongylus cantonensis* [24]. The uptake of arachidonic acid by *A. cantonensis* and the presence of oxidation enzymes strongly suggest that some nematodes can use the β -oxidation pathway.

The Amacr mRNA expression did not change depending on the incubation time (24 and 48 hr) and temperature ranging from 10°C to 40°C. These results imply that a large amount of the Amacr gene expression is required over a long period of time. The expression of high levels of the Amacr gene in *A. simplex* L3 can contribute to the generation of energy for further development. The immunohistochemical findings revealed that the Amacr was mainly localized in the ventriculus. The secretion from the ventriculus can play many roles, including invasion of the gastrointestinal mucosa of the hosts.

In conclusion, we have demonstrated the Amacr gene from *A. simplex* larval cDNA library by immunoscreening with ES antibody. Our data showed that *A. simplex* Amacr needs a large amount over an extended period of time to involve in energy production for the larval development.

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