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ORIGINAL ARTICLE

Characterization of Stearoyl-CoA Desaturases from a Psychrophilic Antarctic Copepod, *Tigriopus kingsejongensis*

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Abstract Stearoyl-CoA desaturase is a key regulator in fatty acid metabolism that catalyzes the desaturation of stearic acid to oleic acid and controls the intracellular levels of monounsaturated fatty acids (MUFAs). Two stearoyl-CoA desaturases (SCD, Δ9 desaturases) genes were identified in an Antarctic copepod, *Tigriopus kingsejongensis*, that was collected in a tidal pool near the King Sejong Station, King George Island, Antarctica. Full-length complementary DNA (cDNA) sequences of two *T. kingsejongensis SCD*s (*TkSCD*s) were obtained from next-generation sequencing and isolated by reverse transcription PCR. DNA sequence lengths of the open reading frames of *TkSCD-1* and *TkSCD-2* were determined to be 1110 and 681 bp, respectively. The molecular weights deduced from the corresponding genes were estimated to be 43.1 kDa (TkSCD-1) and 26.1 kDa (TkSCD-2). The amino acid sequences were compared with those of fatty acid desaturases and sterol desaturases from various organisms and used to analyze the relationships among TkSCDs. As assessed by heterologous expression of recombinant proteins in *Escherichia coli*, the enzymatic functions of both stearoyl-CoA desaturases revealed that the amount of C16:1 and C18:1 fatty acids increased by greater than 3-fold after induction with isopropyl β-D-thiogalactopyranoside. In particular, C18:1 fatty acid production increased greater than 10-fold in *E. coli* expressing TkSCD-1 and TkSCD-2. The results of this study suggest that both *SCD* genes from an Antarctic marine copepod encode a functional desaturase that is capable of increasing the amounts of palmitoleic acid and oleic acid in a prokaryotic expression system.

Keywords Antarctic copepod · *Tigriopus kingsejongensis* · Psychrophilic · Palmitoleic acid methyl ester · Oleic acid methyl ester · FAME (fatty acid methyl ester)

Introduction

In aquatic ecosystems, zooplanktons are important microorganisms supporting a higher trophic level of organisms in the food web. Among the zooplankton, *Tigriopus*, an intertidal copepod, is distributed worldwide on shallow supratidal regions near rock pools, and it undergoes dramatic fluctuations under flood, drought, and temperature conditions (Davenport et al. 1997; Raisuddin et al. 2007). Under various environmental stresses, this small organism has thrived and developed several survival strategies. Thus, *Tigriopus* has been used as a representative model in a wide range of studies on environmental biology and toxicology (Raisuddin et al. 2007). *Tigriopus* has a short life cycle, small body size, and unique life stages. Cellular functions and genetic information on *Tigriopus* have been elucidated from data obtained from mitochondrial genomic and complementary DNA (cDNA) sequences (Machida et al. 2002; Kim et al. 2004; Jung et al. 2006). Recently, cDNAs of *Tigriopus japonicus* were determined to have greater than 40,000 expressed sequence tags (ESTs) and approximately 5000 ESTs with significant hits among all ESTs (Raisuddin et al. 2007). From the cDNA data

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of *T. japonicus*, a large amount of genetic information related to DNA repair, mutations, detoxification, and stress-responsible genes was revealed.

Antarctic microorganisms have the ability to avoid or tolerate cold and freezing conditions. In particular, these microorganisms endure the conditions associated with semisolid ice matrices due to salts and other solutes and extremely cold seawater temperatures during the winter season. Most psychrophilic microorganisms that grow and thrive under cold conditions have developed various types of survival strategies against nutrient unavailability, reduced biochemical reaction rates (Wiebe et al. 1992; Karasova-Lipovova et al. 2003), membrane fluidity fluctuation (Nichols et al. 1993; White et al. 2000), protein misfolding, and ice formation at the intracellular compartment (Zhang et al. 2011). In addition, psychrophilic organisms tend to increase the amount of monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs) within their membranes to maintain fluidity under polar conditions. Many studies have reported that increased amounts of fatty acids enhance cold tolerance; furthermore, delta 9 desaturase forms a cold adaptation pathway by increasing unsaturated fatty acids to retain membrane fluidity at low temperatures (Bertin et al. 1998; Cruz et al. 2010; Svensk et al. 2013). In addition, copepod lipids provide new marine oil for commercial use; thus, genes encoding lipid-related enzymes were our primary targets to identify possible differences in enzymatic activity or efficiency as a cold adaptive strategy in the Antarctic species, *Tigriopus kingsejongensis*. *T. kingsejongensis* is a good candidate because (1) it can be cultured in the laboratory, (2) it is closely related to temperate species *T. japonicas* for comparison, and (3) it is an endemic species in Antarctica.

MUFAs are incorporated into membranous glycerolipids and can reduce the transition temperature from the solid to liquid-crystalline phase, which confers essential fluidity in membranes (Mouritsen and Jorgensen 1992). Stearoyl-CoA desaturases (SCDs) play a critical role in MUFA synthesis and metabolic regulation of fatty acid composition (Paton and Ntambi 2009). SCDs generate oleoyl-CoA and palmitoleoyl-CoA as crucial elements in triglycerides, membrane phospholipids, and other materials (Enoch et al. 1976; Los and Murata 1998; Tocher et al. 1998). Based on the enzymatic activities of SCDs, unsaturated fatty acids, including MUFAs, are capable of acting appropriately on the biological membranes of living organisms (Paton and Ntambi 2009).

In the present study, we isolated and investigated two *SCD* genes from *Tigriopus kingsejongensis*. This marine copepod is one of the dominant zooplankton species in the tidal pool regions of Antarctica. Two *SCD* genes were identified by comparison with other enzymes related to fatty acid metabolism from various living organisms. In addition, the structural properties of SCDs were predicted, and enzymatic activity was analyzed by the generation of recombinant proteins from a heterologous expression system in *Escherichia coli*. The expression of recombinant SCDs was detected by immunoblot analysis, and fatty acid components were analyzed by gas chromatography.

Materials and Methods

Collection of *Tigriopus kingsejongensis*

The Antarctic copepod *T. kingsejongensis* was harvested in intertidal zones at the penguin rookery near King Sejong Station, King George Island, Antarctica (62° 13′ S, 58° 47′ W) (Supplemental Fig. 1). *T. kingsejongensis* exhibited a body length of approximately 1 mm (excluding the tails) under microscopic observation (Leica M205C, Leica Microsystems, Mannheim, Germany) (Fig. 1). The strain was isolated by collection of a single adult using sterilized glass Pasteur pipettes. *T. kingsejongensis* was cultured in filtered Antarctic seawater and fed *Chlorella* sp. (obtained commercially from Danyang Chlorella Inc., Korea) without shaking. *T. kingsejongensis* was grown under 25 μmol photon m^{-2} s^{-1} light intensity and under a 16-h light/8-h dark cycle, and it was maintained at 3 °C until further study.

Isolation of *T. kingsejongensis* Stearoyl-CoA Desaturase Open Reading Frames

Stearoyl-CoA desaturase (SCDs, *TkSCD-1* and *TkSCD-2*) sequences were obtained from the *T. kingsejongensis* transcriptome during the Antarctic genome project at Korea Polar Research Institute (KOPRI) (paper in preparation). To isolate the open reading frames (ORFs) encoding *SCD* genes,

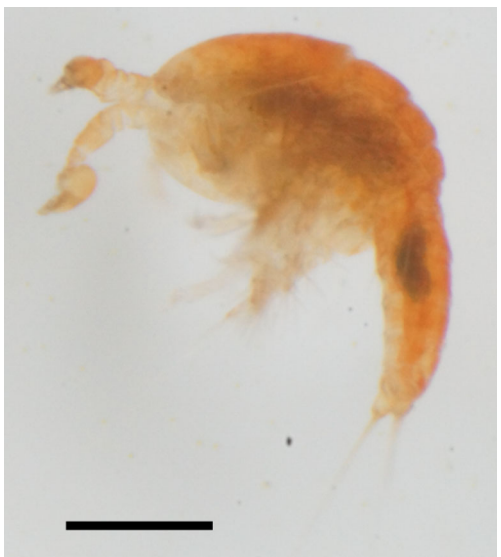


Fig. 1 Morphology of *T. kingsejongensis* observed by optical microscopy. *Scale bar* indicates 500 μm

total RNA was extracted using the RNeasy Mini Kit according to the manufacturer's protocols (Qiagen). After preparation of *T. kingsejongensis* RNA, complementary DNA (cDNA) was synthesized by reverse transcription polymerase chain reaction (RT-PCR) using SuperScript III Reverse Transcriptase (Invitrogen). Two pairs of primers specific to both *SCD*s were used to amplify the template cDNA (Table 1). Amplified PCR products were examined by agarose gel electrophoresis. Purified PCR products were ligated into the pTA-TOPO vector (DoctorProtein, Korea) and transformed into DH5α *E. coli* competent cells (Enzynomics, Korea). The plasmid DNAs extracted from *E. coli* transformants were analyzed by DNA sequencing (Macrogen, Korea). *TkSCD-1* and *TkSCD-2* were identified by comparison to the sequences in NCBI using tBlastx. The deduced amino acid sequences of *TkSCD-1* and *TkSCD-2* were investigated using SignalP (Petersen et al. 2011) to identify signal peptides and using TMpred (Hofmann and Stoffel 1993) and DAS (Cserzo et al. 1997) to predict the transmembrane topology of the two TkSCDs.

Amino Acid Sequence Alignment and Phylogenetic Analysis

TkSCD amino acid sequences were aligned with those from other organisms using the ClustalW algorithm (Thompson et al. 1994) in the BioEdit program (Hall 1999). Omega-3 fatty acid desaturases (FADs) of *Caligus rogercresseyi* (Cal_roger, ACO10720), *Pythium aphanidermatum* (Pyt_aphan, AGS55978), *Pythium splendens* (Pyt_splen, AGC59912), *Phytophthora infestans* (Phy_infes, XP_002902599), and *Saprolegnia diclina* (Sap_dicli, AAR20444) were used to align the amino acid sequences of TkSCD-1 (Fig. 2a). For TkSCD-2 amino acid sequence alignment, sterol desaturases (SDs) of *Arabidopsis thaliana* (Ara_thali, NP_186907), *Trypanosoma cruzi* (Try_cruzi, XP_804155), *Ashbya gossypii* (Ash_gossy, NP_985698), *Neurospora crassa* (Neu_crass, XP_962923), and *Mus musculus* (Mus_muscu, O88822) were selected (Fig. 2b). The amino acid sequences of polypeptides exhibiting high identity to TkSCD-1 and TkSCD-2 were searched in the NCBI database using the BlastP algorithm (*Microcoleus vaginatus*, WP_006634013; *Neosynechococcus sphagnicola*, WP_036533587; *Microcoleus* sp., WP_015184746; *Synechocystis* sp., WP_010872924, WP_038019042 and WP_041429662; *Lyngbya* sp. PCC8106, WP_009787974; *Planktothrix prolifica*, WP_026798760; *Pseudanabaena* sp. PCC6802, WP_026103003; *Leptolyngbya* sp., WP_023073403, WP_015134446 and WP_035993872; *Pseudanabaena biceps*, WP_040689762; *Crocosphaera watsonii*, WP_007305601; *Rivularia* sp., WP_015120383; *Calothrix parietina*, WP_015197146; *Scytonema tolypothrichoides*, KIJ85050; *Calothrix* sp., WP_015128827; *Nodularia spumigena*, WP_006199120; *Nostoc* sp., WP_015140910 and WP_010995766; *Anabaena variabilis*, WP_011320890; *Caligus rogercresseyi*, ACO10720; *Pythium aphanidermatum*, AGS55978; *Saprolegnia diclina*, AAR20444; *Pythium splendens*, AGC59912; *Phytophthora infestans*, XP_002902599; *Paramecium tetraurelia*, XP_001440490; *Arabidopsis thaliana*, NP_186907; *Trypanosoma cruzi*, XP_804155; *Mus musculus*, O88822; *Ashbya gossypii*, NP_985698; *Neurospora crassa*, XP_962923). The selected amino acid sequences were aligned by the same algorithm in an alignment analysis. Amino acid relationships were investigated by the distance method using 10,000 bootstrap repetitions. The unweighted pair group method with arithmetic mean (UPGMA) was adopted to construct a phylogenetic tree using MEGA6 software (Tamura et al. 2013).

Cloning of Recombinant TkSCDs in *E. coli*

The gene sequences used to express TkSCDs were amplified with forward and reverse primers that included *Nco*I and *Xho*I restriction endonuclease sites, respectively, and cloned into the pET32a (+) expression vector (Table 1) using T7 DNA Ligase (Elpisbio, Korea). Ligated plasmids were transformed into DH5α competent cells (Enzynomics, Korea). The expression plasmids extracted from *E. coli* transformants were confirmed by DNA sequencing (Macrogen, Korea). The verified plasmid was transformed into BL21 (DE3) competent cells (Enzynomics, Korea). The circular pET32a (+) plasmid was transformed as a negative control. Single colonies were transferred into LB broth containing ampicillin at a concentration of 100 μg/mL and incubated at 37 °C with continuous shaking until the OD_{600} reached 0.5. Isopropyl β-D-thiogalactopyranoside (1 mM final concentration) was added to the culture and

Table 1 Primers used in this experiment

Name of primers	Sequences of primers (5′ → 3′)
TkSCD-1_pET32_For	GCGGGAATTC*ATGAGCACTGAGACAGTCACTCAGGC*
TkSCD-1_pET32_Rev	GCGGCTCGAG*TTACAGCTCCTTCTTGGGTACATAGGT*
TkSCD-2_pET32_For	GCGGGAATTC*ATGGAATATCCGTCGGTGGAAAAGGT*
TkSCD-2_pET32_Rev	GCGGCTCGAG*TTAAGCCATGAGTCTCGACCAAGGGGC*

Italics indicate gene-specific nucleotides for TkSCD-1 and TkSCD-2. Underlines indicate restriction endonuclease sites for ligation to pET-32 expression vectors

incubated for 3 h at 37 °C with shaking. Transfected cells were analyzed by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, SDS-PAGE gels were stained with Coomassie staining solution to visualize the protein bands of transfected cells. To detect induced recombinant TkSCDs, Western blot analysis was performed. Protein samples were separated on SDS-PAGE gels and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA) using a semidry transfer instrument (Atto, Japan). A commercial anti-His antibody raised in mouse (Young-In Frontier, Korea) and anti-mouse IgG-horseradish peroxidase (Young-In Frontier, Korea) were used for immunoblot analysis for the two recombinant TkSCDs. Protein bands were detected using a colorimetric Western blot system (Opti-4CN Substrate Kit, Bio-Rad, USA). The expression and detection of recombinant TkSCDs were performed thrice independently.

Investigation of Fatty Acid Composition

The composition of fatty acids induced from each TkSCD was examined by fatty acid methyl ester (FAME) analysis using gas chromatography (YL-6100GC, Korea) equipped with a flame ionization detector and a capillary column (Agilent, USA). Freeze-dried samples (20 mg) were used to extract total lipids according to a previous study (Sasser 1990). The organic phases prepared were loaded into a glass vial, and fatty acid components were analyzed. FAME analysis was performed as follows: (1) 3 mL/min constant flow mode, (2) incubation at 100 °C for 5 min and temperature increase to 240 °C (4 °C/min) followed by holding the program for 20 min, and (3) a 250 °C detector temperature. Each chromatographic peak was designated and quantified using the Supelco 37 Component FAME Mix as a standard (Sigma, USA). Total fatty acid contents were evaluated by integration of chromatographic peaks from gas chromatography with the internal standard (1 mg of the C22:0 (Sigma, USA) in hexane). Production yields from *E. coli* cells are expressed as the unit (mg) FAMEs per gram dry weight. The gas chromatography analysis was performed thrice from each fatty acid samples extracted from recombinant *E. coli* cells.

Results

Isolation of TkSCD Genes and Phylogenetic Analysis of Gene Products

From the transcriptomic data of *T. kingsejongensis*, two stearoyl-CoA desaturase genes (*TkSCD-1* and *TkSCD-2*) were identified by BlastX and amplified using gene-specific primers. *TkSCD-1* and *TkSCD-2* are 1110 and 681 bp in length, respectively, including the start and stop codons. The

Fig. 2 Alignment of amino acid sequences of enzymes for fatty acid metabolism from various organisms related to those of stearoyl-CoA desaturases (SCDs) of *Tigriopus kingsejongensis*. Symbols of asterisks indicate typical histidine residues known as the characteristics of membrane-bound fatty acid desaturases. **a** Sequence alignment of amino acids of FADs related to those of TkSCD-1. *Tig_kings T. kingsejongensis*, *Cal_roger Caligus rogercresseyi*, *Pyt_splen Pythium splendens*, *Phy_infes Phytophthora infestans*, *Pyt_aphan Pythium aphanidermatum*, *Sap_dicli Saprolegnia diclina*. **b** Sequence alignment of amino acids of FADs related to those of TkSCD-2. *Tig_kings T. kingsejongensis*, *Ara_thali Arabidopsis thaliana*, *Try_cruzi Trypanosoma cruzi*, *Ash_gossy Ashbya gossypii*, *Neu_crass Neurospora crassa*, *Mus_muscu Mus musculus*

TkSCD-1 and TkSCD-2 sequences deduced by the corresponding ORFs comprised 369 and 226 amino acids, generating proteins of 43 and 26 kDa molecular weights, respectively. The deduced amino acid sequences of TkSCD-1 and TkSCD-2 were searched in the NCBI database, and the polypeptide sequences closely related to both TkSCDs were used for sequence alignment and phylogenic analyses. TkSCD-1 shared 52 % sequence identity with omega-3 fatty acid desaturase of *Caligus rogercresseyi* (GenBank ID, ACO10720), and TkSCD-2 shared 49 % amino acid identity with SD of *Sandaracinus amylolyticus* (GenBank ID, AKF03555). Each SCD amino acid sequence from *T. kingsejongensis* was compared with those of fatty acid enzymes originating from various types of organisms using the ClustalW algorithm (Thompson et al. 1994) in the BioEdit package (Hall 1999). Multiple alignment of amino acid sequences revealed three conserved regions composed of a high number of His residues associated with crucial motifs for catalytic reactions (Thiede et al. 1986; Los and Murata 1998). These results correlate with the membrane-spanning characteristics of TkSCDs.

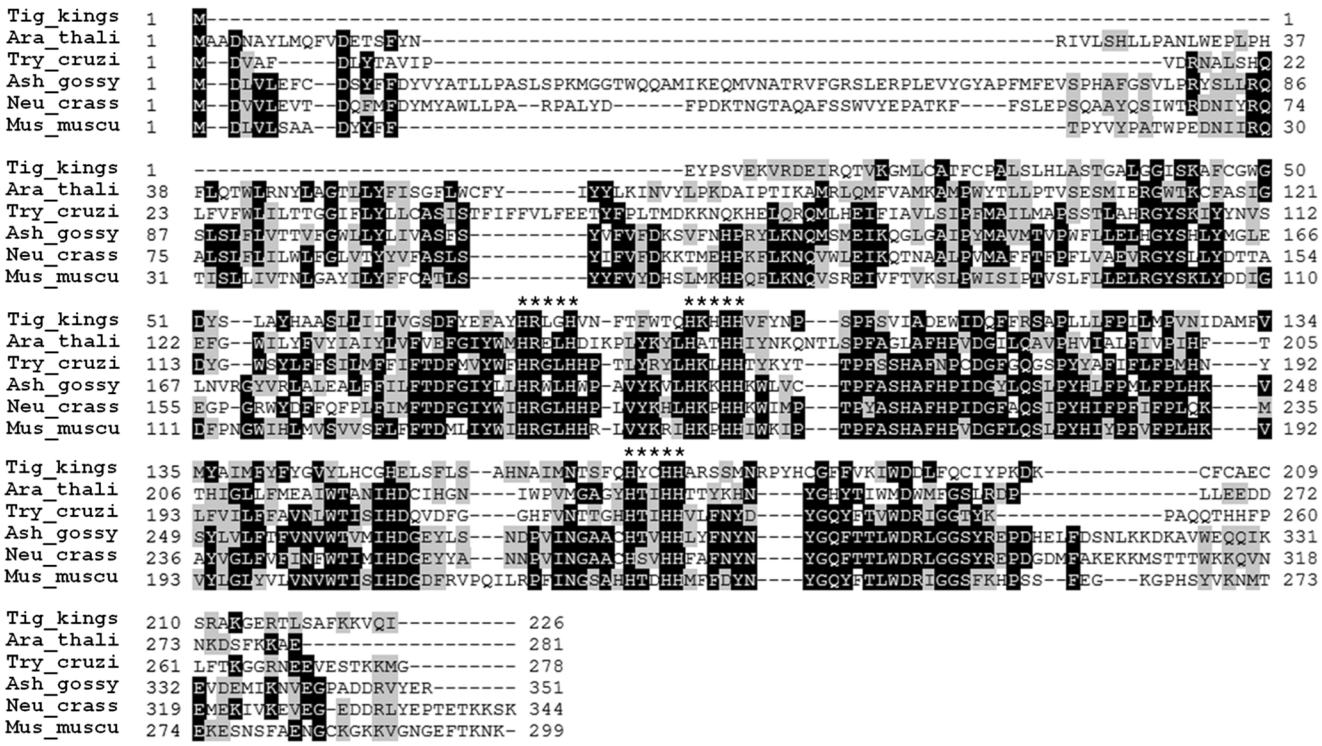
The amino acid sequences were compared to identify and differentiate TkSCDs from other fatty acid enzymes from various organisms. Upon comparison with other fatty acid enzymes, TkSCD-1 and TkSCD-2 belonged to two different groups of fatty acid enzymes related to fatty acid desaturases and SD (Fig. 3). This result suggests that these two TkSCDs might represent different types of fatty acid enzymes in *T. kingsejongensis*.

Characterization of TkSCD Amino Acid Sequences

The TkSCD amino acid sequences were characterized to investigate structural features of the TkSCD proteins. According to SignalP program analysis (Petersen et al. 2011), signal peptides were not detected in TkSCDs, suggesting that TkSCDs may be located intracellularly (Supplemental Fig. 1). Based on the finding that the conserved regions contained His residues, the topology of TkSCDs within the cellular membranes was analyzed. The results from TMpred (Hofmann and Stoffel 1993) revealed six regions of TkSCD-1 (62–83, 89–106,

(A)

(B)



119–139, 171–189, 215–233, and 243–262) that span the cell membrane, whereas the N- and C-termini were located in the outer membrane (Figs. 4a and 5b). TkSCD-2 was predicted to possess three membrane-spanning regions (29–59, 62–84, and 132–153), whereas both termini were placed to opposite sides of the membrane (Figs. 4b and 5d). In addition, the

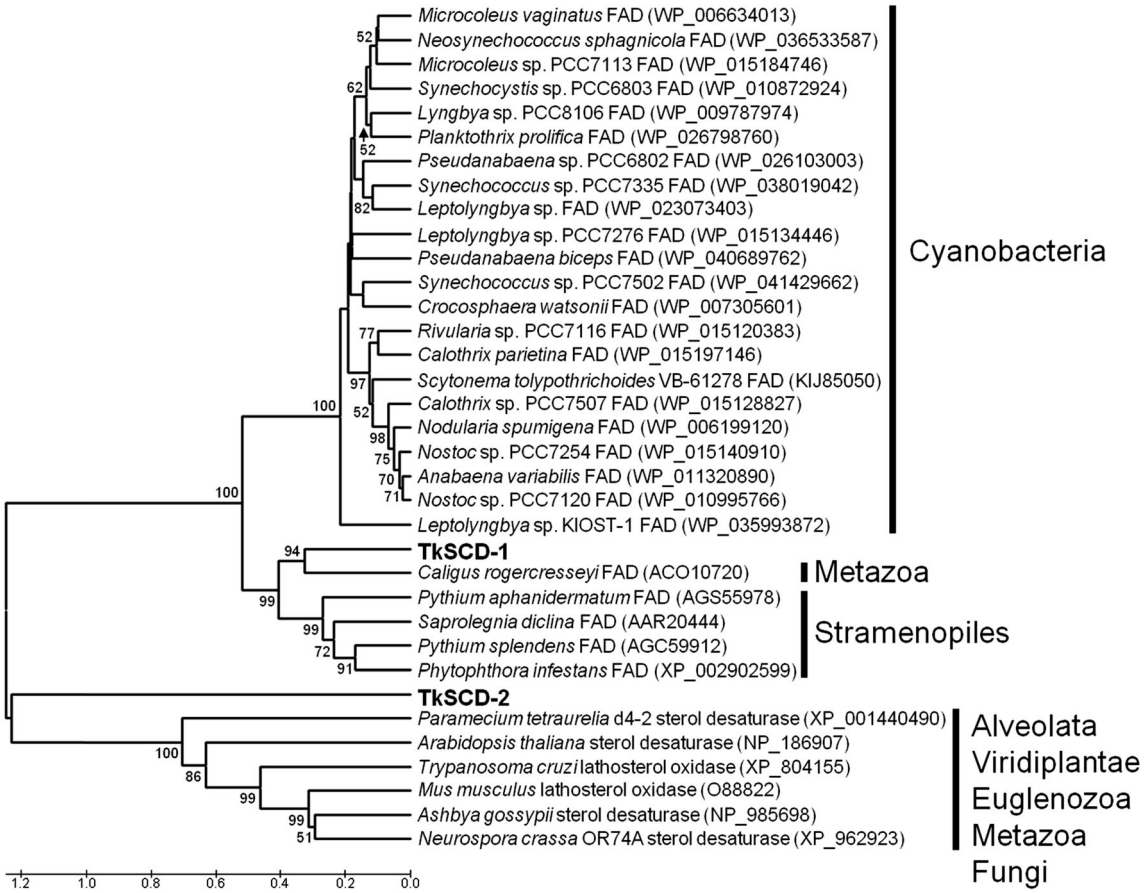


Fig. 3 Phylogenetic tree of FADs from organisms related to both SCDs of *T. kingsejongensis* generated by unweighted pair group method with arithmetic mean (UPGMA). Numbers on each branch show bootstrap values from 10,000 repetitions. Bootstrap values below 50 % supports were rejected from the phylogenetic tree

Fig. 4 Amino acid sequences of **a** TkSCD-1 and **b** TkSCD-2. *Italic*, *bold*, and *underlined regions* represent the integrated regions in the membranes

(A)

MSTETVTQAA NGAEKFSEVD FQKGVPQKVP SILEIKKALP AHCFQPDLST SFYYMFKDLA

L***VAGLYIVML LMEIQPFFWL QAA***YTPIY***WY VCGTLGASIF IVGHDC***GHES FSHNSLIN***DI***

VGNFMHTIIL VPYYPWKLSH RHHHKNTGNI DKDEVFYPVR ETHIGQDPGF ***MIPYFGLGVG***

WIFYLFKGYS PRTINHFNPF NHLFIKHSVN CLIS***MACMAA WISFVIVPYG SAF***GFTRLFV

HY***LMPVFSFM CWIVFITFLH HH***DENVPWYA DDKWDFVRGQ LSSVDRDYGW AHDVLHNIGT

HQIHHLFSKI PHYHLEEATQ VFREQFPELV RKSDERLIPA FVRLFHMFSE QVWIPSETKI

HTYVPKKEL

(B)

MEYPSVEKVR DEIRQTVKGM LCATFCPA***LS LHLASTGALG GISKAFCGWG DYSLAYHAA***S

L***LIILVGSDF YEFAYHRLGH VNFT***FWTQHK HHHVFYNPSP FSVIADEWID QFFRSAPLLL

FPILMPVNID A***MFVMYAIMF YFYGVYLHCG HEL***SFLSAHN AIMNTSFQHY CHHARSSMNR

PYHCGFFVKI WDDLFQCIYP KDKCFCAECS RAKGERTLSA FKKVQI

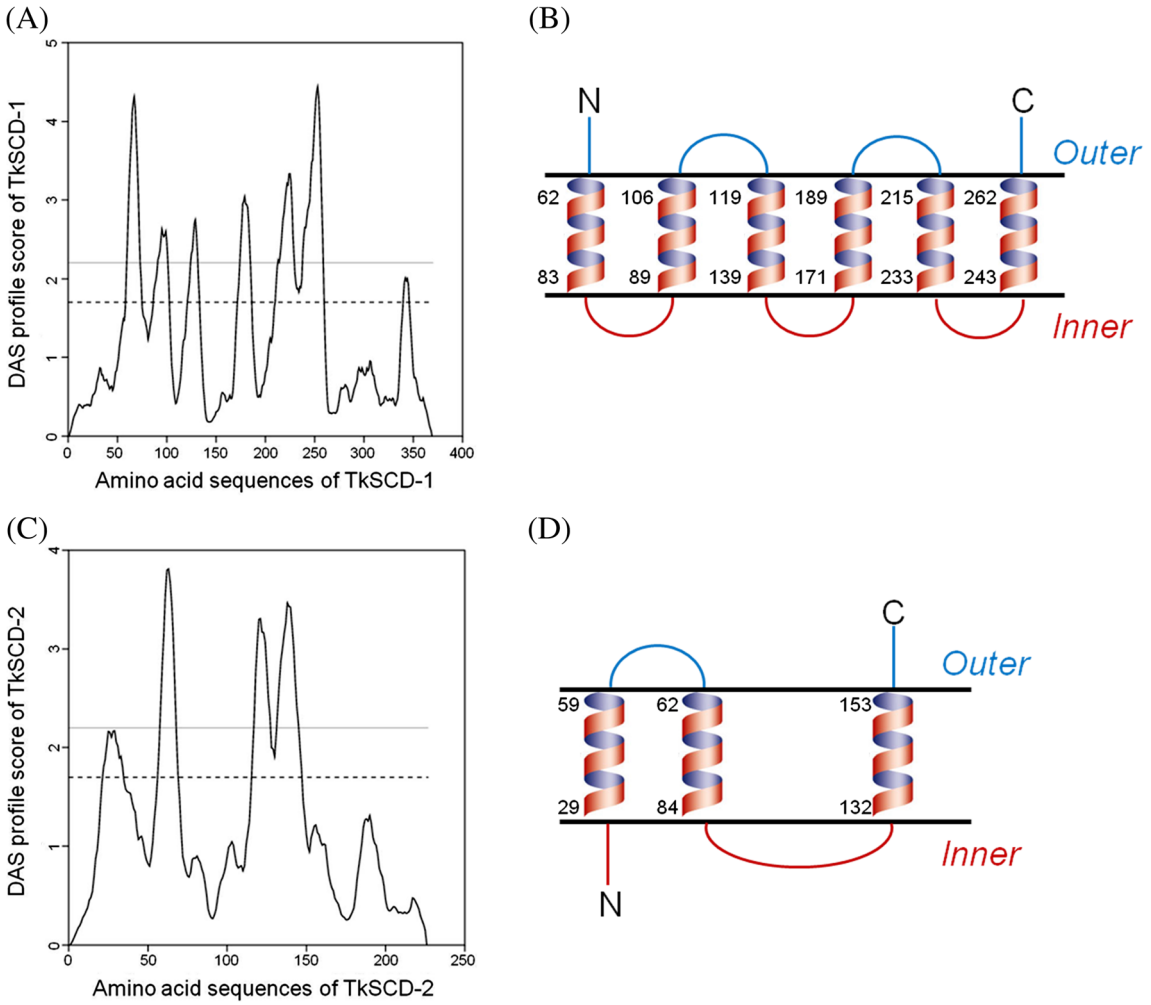


Fig. 5 Prediction and illustrations of transmembraneous parts of TkSCD-1 and TkSCD-2. **a**, **c** DAS profile for TkSCD-1 and TkSCD-2. Strict cutoffs are shown as the *lines* on DAS diagrams. **b**, **d** Illustration of transmembraneous patterns for TkSCD-1 and TkSCD-2. *Numbers* near the helices indicate the locations of amino acids of TkSCDs

transmembrane characteristics of TkSCDs were supported by the analytical results of the DAS program (Cserzo et al. 1997), which revealed six and three transmembrane regions in TkSCD-1 and TkSCD-2, respectively (Fig. 5a, c).

Expression of two TkSCDs in *E. coli*

The recombinant proteins induced in *E. coli* were investigated to characterize the activity of SCDs from *T. kingsejongensis* due to the limited amount of SCDs from this microorganism. For the expression of both recombinant TkSCDs, the pET32a (+) expression vector was used due to the high number of Cys residues in TkSCD-1 and TkSCD-2 (6 and 10 Cys residues, respectively). The recombinant fusion TkSCDs (Trx-TkSCDs), which contain thioredoxin to facilitate the formation of disulfide bonds and residual amino acid tags for detection, were expected to have molecular weights of 61 and 44 kDa for Trx-TkSCD-1 and Trx-TkSCD-2, respectively. However, the expressed proteins exhibiting the expected molecular weights for both Trx-TkSCDs were not detected in the crude samples by Coomassie staining (Fig. 6a). Therefore, immunoblot analysis was performed for sensitive detection. Both Trx-TkSCDs were detected at the expected sizes of the corresponding recombinant proteins (Fig. 6b).

Fatty Acid Composition Induced by Expression of Recombinant TkSCDs in *E. coli*

The compositional change of fatty acids in prokaryotic microorganisms induced by *TkSCD*s was examined by FAME analysis using gas chromatography. Total fatty acids were extracted from 20-mg dry weight of *E. coli* cells expressing the vector-only (negative control), Trx-TkSCD-1 and Trx-TkSCD-2. Total fatty acids were generated at concentrations of 34.33 ± 3.74, 47.96 ± 3.66, and 37.89 ± 2.54 mg/g DCW from the vector-only, Trx-TkSCD-1 and Trx-TkSCD-2

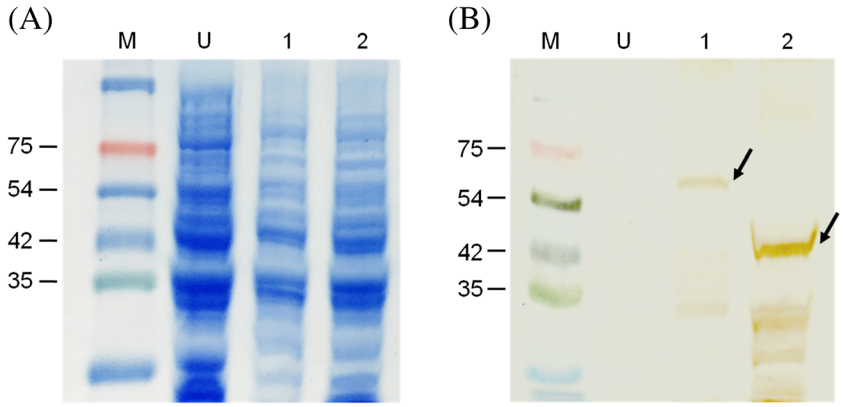


Fig. 6 Heterologous expression of recombinant TkSCDs from *E. coli* and their detection of expression by immunoblot analysis. **a** Coomassie blue staining and **b** Western blot analysis. *M* and *U* indicate the protein size marker and uninduced *E. coli* cells (pET32a(+) vector), respectively. *1* and *2* indicate the induced cells expressing TkSCD-1 and TkSCD-2, respectively. *Black arrows* in **b** represent TkSCDs detected by anti-His antibodies

samples, respectively (Table 2). The chromatograms demonstrated two fatty acid peaks corresponding to palmitoleic acid (C16:1, *n*-7) and oleic acid (C18:1, *n*-9), according to comparison with the FAME standard (Fig. 7). From the quantitative analysis of each chromatographic peak, the concentrations of palmitoleic acid (C16:1, *n*-7) and oleic acid (C18:1, *n*-9) increased following induction of both TkSCD genes. Compared with the palmitoleic acid concentrations of the negative control, TkSCD-1 and TkSCD-2 exhibited improvements of 2.30 and 0.20 mg/g DCW of palmitoleic acid, respectively (Table 2). The oleic acid concentrations increased to 8.87 mg/g DCW (17.7-fold) and 4.81 mg/g DCW (10.1-fold) with TkSCD-1 and TkSCD-2, respectively, compared with the negative control (Table 2). The conversion ratios from C16:0 to C16:1 of the negative control, TkSCD-1, and TkSCD-2 were calculated as 4.85, 12.45, and 5.95 %, respectively, and the C18:1 fatty acid from C18:0 was 19.13, 74.28, and 69.17 %, respectively.

Discussion

T. kingsejongensis is an endemic species living in marine and intertidal regions in Antarctica. Although it is important to understand the physiology involved in the adaptation of *Tigriopus* to various environmental stresses, minimal genetic information on this microorganism has been reported (Lenz et al. 2012). Due to the importance of spatial distribution, biological evolution and physiological characteristics in Antarctica, the overall genetic information of *T. kingsejongensis* has been investigated by next-generation sequencing (NGS) at the Korea Polar Research Institute (KOPRI). From the database of an Antarctic *Tigriopus*, two genes closely related to fatty acid biosynthesis were identified and designated as *TkSCD-1* and *TkSCD-2*, which are 1110 and 681 bp in length, encoding 369 and 226 amino acids, respectively (Fig. 4). Amino acid sequence analysis of the two TkSCDs suggests that they are different types of enzymes

Table 2 Major fatty acids in *E. coli* transformed with *TkSCD*s

	Microorganisms analyzed			
	E. coli			*C. reinhardtii* CC-125
	pET32a(+) (negative control)	Transformed cells from TkSCD-1	Transformed cells from TkSCD-2	
C16:0 (mg/g DCW)	13.99 ± 0.60	15.90 ± 0.41	12.68 ± 0.39	27.75 ± 0.38
Content (%)	40.74 ± 1.73	33.14 ± 0.87	33.45 ± 1.02	21.34 ± 0.29
C16:1 (mg/g DCW)	1.04 ± 0.08	3.34 ± 0.28	1.24 ± 0.22	1 ± 0.02
Content (%)	3.02 ± 0.22	6.95 ± 0.57	3.27 ± 0.58	0.77 ± 0.25
C18:0 (mg/g DCW)	0.33 ± 0.02	0.37 ± 0.03	0.28 ± 0.03	1.43 ± 0.13
Content (%)	0.96 ± 0.06	0.76 ± 0.05	0.73 ± 0.07	1.10 ± 0.10
C18:1 (mg/g DCW)	0.53 ± 0.04	9.40 ± 0.58	5.34 ± 0.31	1.40 ± 0.04
Content (%)	1.53 ± 0.10	19.59 ± 1.20	14.08 ± 0.80	1.08 ± 1.56
Total fatty acid (mg/g DCW)	34.33 ± 3.74	47.96 ± 3.66	37.89 ± 2.54	130 ± 1.56

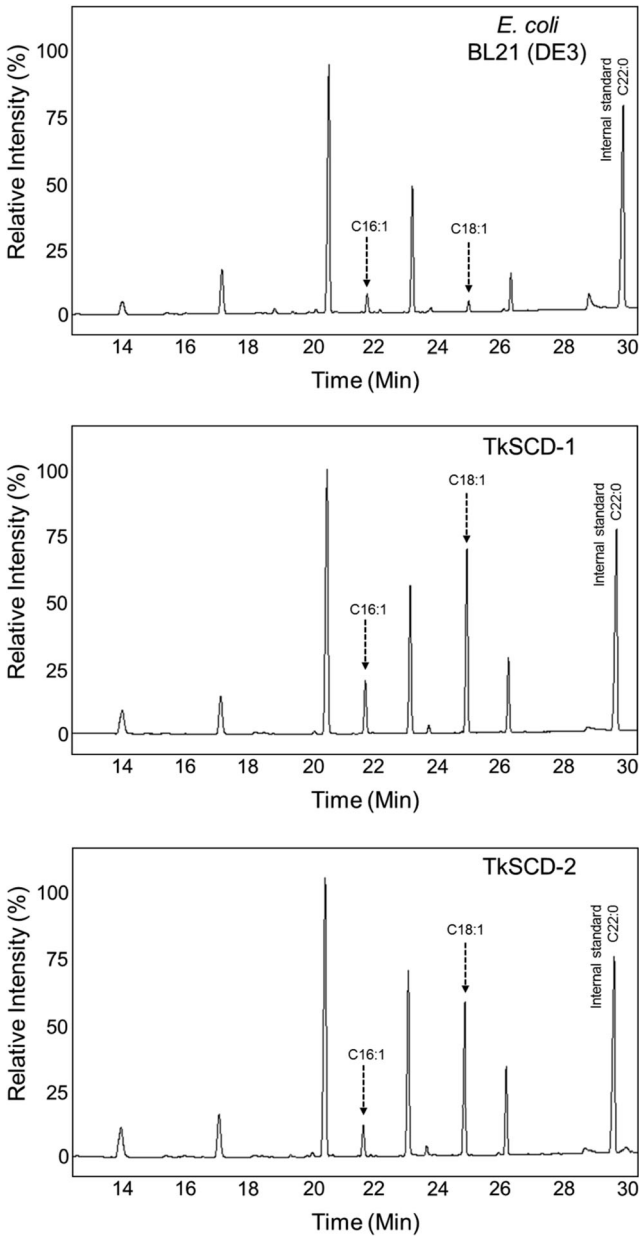


Fig. 7 The peak profiles of gas chromatography for total fatty acids from uninduced and two of induced *E. coli* cells. Uninduced *E. coli* cells (*top*), induced *E. coli* by TkSCD-1 gene (*middle*), and induced *E. coli* by TkSCD-2 gene (*bottom*)

due to their complete separation into two distinct groups (Fig. 3). However, each TkSCD was grouped together with several enzymes whose functions are not yet defined. Therefore, it is possible that the two TkSCDs could be the same or similar types of fatty acid enzymes.

The deduced amino acid sequences of TkSCDs were determined to have three histidine-rich motifs (HDCGH, HRHHH, and HQIHH for TkSCD-1; HRLGH, HKHHH, and HYCHH for TkSCD-2), as shown in Fig. 2. Based on the typical characteristics of these motifs, TkSCDs may be membrane-integrated desaturases. Three conserved histidine-rich motifs are a unique feature of SCDs and are believed to provide the catalytic sites for binding ferric iron as a ligand (Los and Murata 1998; Heinemann and Ozols 2003; Meesapyodsuk and Qiu 2014). The conserved histidine segments in Δ12 acyl-lipid desaturase and ferric iron have been predicted to form a catalytic center within the cytoplasmic region of the protein (Los and Murata 1998). Recently, structural determinants of acyl-CoA Δ9 desaturases were investigated from a marine copepod, *Calanus hyperboreus* (Meesapyodsuk and Qiu 2014). An experimental study on Δ9 desaturases from *C. hyperboreus* confirmed that two Δ9 desaturases of *C. hyperboreus* (ChDes9–1 and ChDes9–2) located on the endoplasmic reticulum are integral membrane proteins (IMPs), the N- and C-termini of which are oriented toward the cytosolic compartment. Specifically, a second transmembrane domain integrated in the membrane is critical for desaturase activity. In particular, the tyrosine residue in the second transmembrane domain plays an important role in determining the length of the fatty acid chains of the substrates (Meesapyodsuk and Qiu 2014). TkSCD-1 exhibited membranous structures similar to those of ChDes9–1 and ChDes9–2. Based on the predicted location of the integrated regions of TkSCD-1, the tyrosine residues are likely positioned within the second transmembrane fragment (Fig. 4). In addition, two histidine residues between the second and third transmembrane regions and one histidine residue near the C-terminus were predicted to be exposed to the cytoplasmic space. Based on the structural similarities to the desaturases described in previous studies, TkSCD-1 may express high desaturase activity intracellularly. In the case of TkSCD-2, this SCD may exhibit reduced desaturase activity compared with TkSCD-1 because the exposed space between the second and third transmembrane fragments was too narrow to form the histidine cluster with ferric iron. IMPs have been investigated for their use biologically and medically due to their associations with photosynthesis, respiration, molecular transport, and catalytic reactions (Carpenter et al. 2008). Despite the importance of IMPs in biological processes, structural studies on these proteins, especially eukaryotic proteins, have been challenged by insufficient quantities of well-expressed recombinant proteins with proper functions and the structural integrity of IMPs on cellular membranes (Carpenter et al. 2008; He et al. 2014). Recently, Wang et al. (2015) reported a human SCD structure (PDB ID, 4ZYO) with stearoyl-CoA as a substrate and presented four membrane-spanning regions composed of α-helix structures. We found that the amino acid sequences and length between 4ZYO and TkSCDs were considerably different and that the sequence identity was less than 11 % (data not shown). Therefore, TkSCDs provide a potential model for understanding the biological functions and crystal structure of SCDs across the organisms. To investigate the desaturase activity of TkSCDs, we generated recombinant proteins in an *E. coli* expression system. Despite the lack of crucial lipids and

posttranslational modifications in *E. coli*, this prokaryotic protein expression system has been adopted to characterize the function of proteins due to its advantages of rapid replication, economical cost, and time-saving processes (Kalscheuer et al. 2006; Feng and Cronan 2009; Gratraud et al. 2009; Beld et al. 2014; Cao et al. 2014). As a result of heterologous expression of TkSCDs, expressed recombinant protein bands were detected by immunoblotting using optimized 4-chloro-1-naphthol (4CN) reagents but not Coomassie blue staining (Fig. 6). Hence, this experimental result could allow the prediction of the enzymatic activity of SCDs using this *E. coli* system.

SCDs convert saturated fatty acids (SFAs) into monounsaturated fatty acids (MUFAs), which play key roles in cellular processes, such as regulation of membrane fluidity and synthesis (Los and Murata 1998; Heinemann and Ozols 2003; Meesapyodsuk and Qiu 2014). Gas chromatographic analysis was performed to investigate the resulting fatty acid composition of *E. coli* affected by the enzymatic activity of recombinant TkSCDs. In the gas chromatographic profiles, two distinctive peaks for palmitoleic acid (C16:1, Δ9, *n*-7) and oleic acid (C18:1, Δ9, *n*-9) were detected (Fig. 7). In particular, oleic acid influenced by the activity of TkSCD-1 increased dramatically. Quantitative analysis revealed that the total fatty acid (TFA) concentration in cells expressing TkSCD-1 and TkSCD-2 increased to 47.96 and 37.89 mg/g DCW, respectively, (Table 2) and was enhanced relative to the vector-only control (34.33 ± 3.74 mg/g DCW). The palmitoleic fatty acid concentrations were 3.34 ± 0.28 and 1.24 ± 0.22 mg/g DCW in cells expressing TkSCD-1 and TkSCD-2 cells, respectively. Particularly, oleic acid exhibiting remarkable increments on GC profiles increased to 9.40 ± 0.58 and 5.34 ± 0.31 mg/g DCW in cells expressing TkSCD-1 and TkSCD-2, respectively, demonstrating that 17.7-fold and 10.1-fold, respectively, improved oleic acid production compared with the negative control (Table 2). Gas chromatography results revealed that two Δ9-TkSCDs significantly affected the generation of double bonds to stearic acid (C18:0) rather than palmitic acid (C16:0). Recently, a metabolic engineering study was performed to increase free MUFAs in *E. coli* strains (Cao et al. 2014). In the study by Cao et al. (2014), heterologous expression of fatty acyl-ACP thioesterase and fatty acid desaturase originating from *Arabidopsis thaliana* increased to approximately 10.7 (~45.7 %) and 5.0 mg/L (~21.4 %) palmitoleic acid and oleic acid, respectively, among 23.4 mg/L TFAs. This pattern is in contrast to that observed in our study. The differential fatty acid increment patterns might result from the specific characteristics of double-bond accumulation by corresponding fatty acid desaturases. Therefore, we assume that the two types of SCDs from *T. kingsejongensis* have strong activity, particularly in the conversion of SFAs to MUFAs (especially oleic acid). For survival against environmental stresses, such as cold and freezing temperatures, these enzymatic activities would be advantageous to maintain cellular membrane fluidity, playing a key role in physiological adaptation to the Antarctic environment. Indeed, we determined that both TkSCDs are related to fatty acid desaturases from metazoa, stramenopiles, protozoa, fungi, plants, and animals. However, only a limited number of genes were characterized functionally. Novel SCDs from Antarctic copepods would provide new insights in lipid metabolism and the physiological adaptation mechanism of Antarctic organisms.

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