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Sardine procalcitonin amino-terminal cleavage peptide has a different action from calcitonin and promotes osteoblastic activity in the scales of goldfish

Yoichi Kase¹, Takahiro Ikari¹, Toshio Sekiguchi¹, Masayuki Sato¹, Shouzo Ogiso¹, Tsuyoshi Kawada², Shin Matsubara², Honoo Satake², Yuichi Sasayama¹, Masato Endo³, Kei-ichiro Kitamura⁴, Atsuhiko Hattori⁵, Takushi X. Watanabe⁶, Yusuke Maruyama⁵, Yoshinari Watanabe⁷, Hisayuki Funahashi⁸, Akira Kambegawa⁹ and Nobuo Suzuki^{1*}

¹Noto Marine Laboratory, Institute of Nature and Environmental Technology, Kanazawa University, Noto-cho, Ishikawa 927-0553, Japan

²Bioorganic Research Institute, Suntory Foundation for Life Sciences, 8-1-1, Seikadai, Seika-cho, Soraku-gun, Kyoto 619-0284, Japan

³Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo 108-8477, Japan

⁴Department of Clinical Laboratory Science, Graduate School of Medical Science, Kanazawa University, Kanazawa, Ishikawa 920-0942, Japan

⁵Department of Biology, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Ichikawa, Chiba 272-0827, Japan

⁶Peptide Institute Inc., Osaka 567-0085, Japan

⁷Organization of Frontier Science and Innovation, Kanazawa University, Kakuma, Ishikawa 920-1192, Japan

⁸Department of Anatomy, Showa University School of Medicine, Shinagawa-ku, Tokyo 142-8555, Japan

⁹Kambegawa Laboratory, Tokyo 201-0013, Japan

*Correspondence to: Dr. Nobuo. Suzuki,
Noto Marine Laboratory, Institute of Nature and Environmental Technology,
Kanazawa University, Ogi, Noto-cho, Ishikawa 927-0553, Japan.
Tel.: 81-768-74-1151; Fax 81-768-74-1644;
e-mail: nobuos@staff.kanazawa-u.ac.jp

ABSTRACT

The nucleotide sequence of a sardine preprocalcitonin precursor has been determined from their ultimobranchial glands in the present study. From our analysis of this sequence, we found that sardine procalcitonin was composed of procalcitonin amino-terminal cleavage peptide (N-proCT) (53 amino acids), CT (32 amino acids), and procalcitonin carboxyl-terminal cleavage peptide (C-proCT) (18 amino acids). As compared with C-proCT, N-proCT has been highly conserved among teleosts, reptiles, and birds, which suggests that N-proCT has some bioactivities. Therefore, both sardine N-proCT and sardine CT were synthesized, and their bioactivities for osteoblasts and osteoclasts were examined using our assay system with goldfish scales that consisted of osteoblasts and osteoclasts. As a result, sardine N-proCT (10^{-7} M) activated osteoblastic marker enzyme activity, while sardine CT did not change. On the other hand, sardine CT (10^{-9} to 10^{-7} M) suppressed osteoclastic marker enzyme activity, although sardine N-proCT did not influence enzyme activity. Furthermore, the mRNA expressions of osteoblastic markers such as type 1 collagen and osteocalcin were also promoted by sardine N-proCT (10^{-7} M) treatment; however, sardine CT did not influence their expressions. The osteoblastic effects of N-proCT lack agreement. In the present study, we can evaluate exactly the action for osteoblasts because our scale assay system is very sensitive and it is a co-culture system for osteoblasts and osteoclasts with calcified bone matrix. Both CT and N-proCT seem to influence osteoblasts and osteoclasts and promote bone formation by different actions in teleosts.

Keywords: Procalcitonin amino-terminal cleavage peptide; Calcitonin; Osteoblasts; Osteoclasts; Japanese sardine; Scales

Abbreviations: alkaline phosphatase, ALP; Calcitonin, CT; procalcitonin carboxyl-terminal cleavage peptide, C-proCT; procalcitonin amino-terminal cleavage peptide, N-proCT; para-nitrophenol, pNP; tartrate-resistant acid phosphatase, TRAP; rapid amplification of cDNA ends, RACE; reverse phase high performance liquid chromatography, RP-HPLC; ultimobranchial gland, UBG

1. INTRODUCTION

Calcitonin (CT) is composed of 32 amino acid residues and functions as a hypocalcemic hormone resulting from the suppression of osteoclastic activity in mammals (Azria, 1989). Furthermore, in teleosts as well as mammals, it has been demonstrated that CT suppresses osteoclastic activity in both freshwater and seawater teleosts (Suzuki et al., 2000; Sekiguchi et al., 2009). CT is mainly synthesized in the C-cell of the thyroid gland in mammals and the ultimobranchial gland (UBG) in non-mammalian vertebrates (Sasayama, 1999). In the process of CT production, this hormone is first generated in the form of a preprocalcitonin precursor, which is subsequently cleaved into 3 peptides: procalcitonin amino-terminal cleavage peptide (N-proCT), CT, and procalcitonin carboxyl-terminal cleavage peptide (C-proCT) in salmon, chickens, rats, and humans (Burns et al., 1989a; 1989b). In neoplastic C cell cultures, it has been reported that N-proCT and CT concentrations are nearly equimolar in both cellular extracts and basal media (Burns et al., 1989a). These facts strongly suggest that N-proCT has some bioactivities.

Until now, there has not been agreement in the literature regarding whether N-proCT has osteoblastic effects. Namely, human N-proCT had no mitogenic activity on osteoblasts or preosteoblasts in rats (Guenther and Fleisch, 1991) and has no accelerating effects on the mitosis and cell differentiation of human osteoblasts (Hassager et al., 1991). In contrast, Burns et al. (1989b) found that nanomolar concentrations of N-proCT (treatment duration 20–22 hr) were mitogenic for a human osteosarcoma cell line.

On the other hand, we previously determined the peptide sequence of the CT from UBGs of Japanese sardines (*Sardinops melanostictus*) using reversed phase high-performance liquid

chromatography (RP-HPLC) (Suzuki et al., 1994). The hypocalcemic activity of synthetic sardine CT was examined by comparing it with that of synthetic salmon CT using a rat bioassay. We found that hypocalcemic activity in sardine CT was more than double that in salmon CT (Suzuki et al., 1994). Therefore, sardine N-proCT may have high activity in osteoblasts as sardine CT has high activity in calcium metabolism. However, in general, sardine is difficult to keep long term in aquarium, although this fish was easily collected in Japan. Therefore, this species is not suitable for physiological experiments. To analyze the bioactivity of both CT and N-proCT, we have developed an original *in vitro* assay system with goldfish and have previously shown that goldfish CT suppressed osteoclastic activity in the scales of goldfish (*Carassius auratus*) (Suzuki et al., 2000; Suzuki et al., 2008; Sekiguchi et al., 2009). Furthermore, the osteoclast-activating hormone, parathyroid hormone, acted on osteoblasts and osteoclasts and then induced active-type osteoclasts (multinucleated osteoclasts) in goldfish (Suzuki et al., 2011). Therefore, our scale *in vitro* bioassay with goldfish is suitable for the elucidation of hormonal function.

In the present study, we determined the full-length sequence of the sardine preprocalcitonin precursor from their UBGs using 5' and 3' rapid amplification of cDNA ends (RACE) methods. Furthermore, both sardine N-proCT and sardine CT were synthesized, and their bioactivities for osteoblasts and osteoclasts were examined using our original assay system with goldfish scales.

2. MATERIALS AND METHODS

2.1. Animals

Japanese sardines (*Sardinops melanostictus*) were purchased from Choshi's Fishermen's Cooperative Association. The fish were used to determine a preprocalcitonin precursor.

Goldfish (*Carassius auratus*), purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan), were artificially fertilized from a female and a male goldfish (20–30 g) at the Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology. Fish were fed a commercial pellet diet every morning and were kept in tap water at 26°C. Growing fish of approximately 20 g of body weight were moved to Noto Marine Laboratory at Kanazawa University and used for the *in vitro* experiments.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Kanazawa University.

2.2. Sequencing of a preprocalcitonin precursor from sardine UBGs

UBGs were removed from Japanese sardines (*Sardinops melanostictus*) and kept in RNAlater® (Sigma-Aldrich, St. Louis, MO, USA). Total RNAs were extracted from the UBGs using a total RNA isolation kit (NucleoSpin RNA II, TaKaRa Bio Inc., Otsu, Japan). The full-length sequence of sardine preprocalcitonin was determined by RACE using the GeneRacer® Kit (Life Technologies, Tokyo, Japan). The first-strand cDNA was reverse transcribed from 1 µg total RNA using an oligo-dT-adaptor primer. The 3' RACE gene-specific primers (5'-GACTCTGAAAGACTGCGCCTTTTCC-3') were designed according to the sequence of a previous partial cloning (Suzuki et al., 1999). PCR fragments were obtained using a 3' RACE primer and adaptor primer provided from a kit, and were

directionally sequenced by the ABI PRISM™ 3130 Genetic Analyzer using a 3' RACE primer. Next, 5' RACE was performed using a 5' RACE primer (5'-GACTCTGAAAGACTGCGCCTTTTCC-3') and an adapter primer. The PCR product was subcloned using the pGEM-T Easy (Promega, Madison, Wisconsin, USA) and was sequenced with the ABI PRISM™ 3130 Genetic Analyzer using plasmid universal primers.

2.3. Molecular phylogenetic analysis

Molecular phylogenetic analysis was performed with modification of the previous study (Sekiguchi et al., 2016). In brief, the amino acid sequences were aligned using the ClustalW program's MEGA 6.06 software (Tamura et al., 2013). After manually removing gaps, alignments were calculated to construct unrooted phylogenetic trees using the neighbor-joining method with MEGA 6.06 software. Moreover, the bootstrap value was estimated by 1000 pseudoreplications using MEGA 6.06 software. The sequences of the CT precursors used are as follows: Japanese medaka, NP_001098365; fugu, CAC81278; Atlantic salmon, NP_001135058; green sea turtle, EMP38282; American alligator, XP_006275102; rock pigeon, EMC88082; chicken, ABY65359; African ostrich, XP_009683015; turkey, XP_003206284; platypus, ENSOANP00000017830; Tasmanian devil, XP_003773616; nine-banded armadillo, XP_004472573; cattle, NP_001137209; domestic cat, BAO79719; horse, AAF72526; human, AAA58403; rhesus monkey, EHH23079; house mouse, P70160; and Norway rat, AAA40849.

2.4. Peptide synthesis

Sardine CT was a gift from the Peptide Institute Inc. (Osaka, Japan). Sardine N-proCT was synthesized by a solid-phase peptide synthesizer (CS36X: CSBio Co. Inc., Menlo Park, CA, USA) using the Fmoc method, in accordance with the manufacturer's instructions. In

brief, 0.25 mmol Fmoc-NH-SAL-PEG resin and 1 mmol of each Fmoc amino acid were used in the reaction. Peptides collected from the reacted resin were separated using a RP-HPLC with a C18 column (Shimadzu LabSolutions: Shimadzu Co., Kyoto, Japan), and the fraction corresponding to the main peak was collected. Peptides in the fraction were measured using mass spectrometry (Ultraflex III: Bruker Daltonics Inc., Billerica, MA, USA), showing a single peak corresponding to the appropriate sardine N-proCT molecular weight.

2.5. Effects of sardine CT and sardine N-proCT on scale osteoblastic and osteoclastic activities using the cultured scales of goldfish

Scales were collected from goldfish under anesthesia in 0.03% ethyl 3-aminobenzoate methanesulfonic acid salt (Sigma-Aldrich, Inc., St. Louis, MO, USA); aeration was provided with an electric motor. Using these scales, we examined the influences of both sardine CT and sardine N-proCT on osteoblasts and osteoclasts with alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) as markers (Suzuki et al., 2000; Suzuki and Hattori, 2002). These scales were incubated for 6 h in Leibovitz's L-15 Medium (Thermo Fisher Scientific Inc., Grand Island, NY, USA) containing a 1% penicillin-streptomycin mixture (Thermo Fisher Scientific Inc.) supplemented with each peptide (10^{-9} , 10^{-8} , and 10^{-7} M).

The methods for measuring ALP and TRAP activities were as follows. After incubation, the medium of each well was removed. Then, each well was washed with phosphate buffered saline. Thereafter, an aliquot of 100 μ l of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$) or an acid buffer (0.1 M sodium acetate, including 20 mM tartrate, pH 5.3) was added to each well. This microplate was immediately frozen at $-80^{\circ}C$ and then kept at $-20^{\circ}C$ until analysis. The next, an aliquot of 100 μ l of 20 mM para-nitrophenyl phosphate in an alkaline or acid buffer was then added to each microplate well with a melted alkaline or acid buffer

solution. This plate was incubated at 23°C for 60 min while being shaken. After incubation, the reaction was stopped by adding 50 µl of 3 N NaOH. The 150 µl of a reacted solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced para-nitrophenol (pNP) using a standard curve for pNP.

After measuring both ALP and TRAP activities, the surface area of the scales was calculated using Image J. Thereafter, ALP and TRAP activities were normalized to the surface area (mm²) of each scale (Suzuki et al., 2009).

2.6. Effects of sardine CT and sardine N-proCT on scale osteoblastic marker mRNA expressions using cultured goldfish scales

Scales were collected from goldfish under anesthesia with 0.03% ethyl 3-aminobenzoate methanesulfonic acid salt (Sigma-Aldrich). To examine changes in genes expressed in osteoblasts that responded to sardine CT and sardine N-proCT (each 10⁻⁷ M), these scales were incubated for 6 h in Leibovitz's L-15 medium (Thermo Fisher Scientific Inc.) containing a 1% penicillin-streptomycin mixture (Thermo Fisher Scientific Inc.). After incubation, the scales were frozen at -80°C for mRNA analysis.

Total RNAs were prepared from goldfish scales using a total RNA isolation kit for fibrous tissue (QIAGEN GmbH, Hilden, Germany). Complementary DNA synthesis was performed using a kit (QIAGEN GmbH). To examine the osteoblastic activation of scales by sardine CT and sardine N-proCT treatment, the mRNA expression of osteoblastic markers (type 1 collagen and osteocalcin) were examined. Gene-specific primers for type 1 collagen (sense: 5'-TGCAACCAGGATGCCATCAA-3'; antisense: 5'-ATGAGGCGCAGGAAGGTGAG-3') and for osteocalcin (sense: 5'-

ATGCCTGAGCGCAGGTCTTC-3'; antisense: 5'-CACAGGCCAGGTTTGCTTCA-3') (Thamamongood et al., 2012) were used. The amplification of EF-1 α cDNA using a primer set (sense: 5'-ATTGTTGCTGGTGGTGGTGG-3'; antisense: 5'-GGCACTGACTTCCTTGGTGA-3') (AB979720) was performed. The PCR amplification was analyzed using real-time PCR apparatus (Mx3000p, Agilent Technologies, Santa Clara, CA, USA) (Suzuki et al. 2011; Thamamongood et al., 2012). The annealing temperature of type 1 collagen, osteocalcin, and EF-1 α was 60°C. Type 1 collagen and osteocalcin mRNA levels were normalized to the EF-1 α mRNA level.

2.7. Statistical analysis

All results are expressed as the means \pm SE. The statistical significance between the control and the experimental group was assessed by Student's *t*-test (*in vitro* cell activity experiment) or paired *t*-test (osteoblastic marker mRNA expression). In all cases, the selected significance level was $p < 0.05$.

3. RESULTS

3.1. Sequencing of the preprocalcitonin precursor from sardine UBGs

The sequence of the sardine preprocalcitonin precursor was obtained from the RNA of their UBGs using RACE methods. The sardine preprocalcitonin precursor was composed of 134 amino acid residues that included signal peptides (25 amino acids) (Fig. 1). We predicted the signal peptide sequence by using the program, SignalP 4 (Petersen et al., 2011). Sardine

procalcitonin was composed of N-proCT (53 amino acids), CT (32 amino acids), and C-proCT (18 amino acids) (Fig. 1).

These vertebrate N-proCTs have some common features (Fig. 2). These features are summarized as follows: 1) The first amino acid residue is mostly Ala (Thr or Val in some cases); 2) there is no Cys residue in the sequence of N-proCT; 3) eight residues (Lys⁴, Leu¹⁷, Leu²⁵, Leu²⁶, Ala²⁸, Gln³⁵, Ser⁵⁹, and Asp⁶¹) are well conserved in all N-proCTs; 4) insertion/deletion points are present in the sequence of N-proCT from the 9th to the 11th, from the 46th to the 50th, and from the 54th to the 58th positions.

As with CT, N-proCT has been highly conserved among vertebrates, as compared with C-proCT (Table 1). Namely, the amino acid sequence of sardine CT was more like that of teleosts, reptiles, and birds (93.8–96.9%) than that of Marsupialia (platypus: 93.8%; Tasmanian devil: 87.5%), and it is different from that of Placentalia (50.0–75.0%) (Table 1). Also, the predicted amino acid sequence of sardine N-proCT was more conserved among teleosts, reptiles, and birds (78.2–85.5%) than in Marsupialia (platypus 66.7%; Tasmanian devil: 69.6%) and Placentalia (48.3–63.2%) (Table 1).

3.2. Phylogenetic analysis of sardine CT and sardine N-proCT

The phylogenetic tree was constructed from known CTs and N-proCTs, together with sardine CT and sardine N-proCT. The form of the molecular phylogenetic tree of CTs was similar to that of N-proCTs (Fig. 3). Both sardine CT and sardine N-proCT are grouped into the clade that includes non-mammalian vertebrates and Marsupialia with the high support of bootstrap values. Sardine N-proCT was classified into the same group with Atlantic salmon, Japanese medaka, and fugu.

3.3. Effects of sardine CT and sardine N-proCT on scale osteoblastic and osteoclastic activities using cultured goldfish scales

The bioactivities of both sardine CT and sardine N-proCT were examined using our assay system with goldfish scales. The results are indicated in Figure 4.

Sardine N-proCT (10^{-7} M) activated ALP (osteoblastic marker enzyme) activity in the scales of goldfish, although sardine CT (10^{-9} to 10^{-7} M) did not influence ALP activity. In both peptides (10^{-9} and 10^{-8} M), ALP activity did not change significantly.

In the case of TRAP activity, sardine CT (10^{-9} to 10^{-7} M) responded to osteoclasts and suppressed TRAP activity in a dose-dependent manner; however, sardine N-proCT did not change their activities (Fig. 5).

3.4. Effects of sardine CT and sardine N-proCT on scale osteoblastic marker mRNA expressions using cultured goldfish scales

To confirm the promotive action of sardine N-proCT in osteoblasts, we examined osteoblastic marker mRNA expression in cultured scales.

The results are indicated in Figure 6. The mRNA expressions of osteoblastic markers such as type 1 collagen and osteocalcin were promoted by sardine N-proCT (10^{-7} M) treatment; however, sardine CT did not influence their expressions.

4. DISCUSSION

Using RACE methods, we determined the full length of the sardine preprocalcitonin precursor (Fig. 1). Our results indicate that sardine procalcitonin was composed of N-proCT

(53 amino acids), CT (32 amino acids), and C-proCT (18 amino acids) (Fig. 1). The amino acid sequences of 19 N-proCTs, including sardine N-proCT, were calculated by the neighbor-joining method using MEGA 6.06 software (Tamura et al., 2013). The molecular phylogenetic tree of CTs and N-proCTs revealed that these peptides are discriminated between mammalian and non-mammalian with high bootstrap values (Fig. 3). Interestingly, the CTs and N-proCTs of Marsupialia, which are primitive mammals, are located in the same clade formed by those of non-mammalian vertebrates (Fig. 3). The similarity of sardine N-proCT to that of Marsupialia (platypus: 66.7%; Tasmanian devil: 69.6%) was different from that to Placentalia (48.3–63.2%) (Table 1). Similar results were obtained for CTs. Sardine CT was more similar to that of Marsupialia (platypus: 93.8%; Tasmanian devil: 87.5%) than to that of Placentalia (50.0–75.0%) (Table 1). The sequence similarity was also supported by phylogenetic analysis.

Next, the bioactivity of sardine N-proCTs was examined with a goldfish scale *in vitro* assay system and compared with that of sardine CT. As a result, we are the first to demonstrate that sardine N-proCT (10^{-7} M) activated osteoblastic marker enzyme activity; however, sardine CT did not change (Fig. 4). On the other hand, sardine CT (10^{-7} to 10^{-9} M) suppressed osteoclastic marker enzyme activity, although sardine N-proCT did not influence enzyme activity (Fig. 5). In addition, osteoblastic marker (type 1 collagen and osteocalcin) mRNA expression was promoted by sardine N-proCT (10^{-7} M) treatment; however, sardine CT did not influence their expressions (Fig. 6). Our obtained bioactivity of sardine N-proCT was supported by a previous report that human N-proCT has mitogenic action in a human osteosarcoma cell line (Burns et al., 1989b). Furthermore, Burns et al. (1989a) reported that in rats, thyroidal levels of CT and N-proCT increase in parallel *in vivo* when C-cell hyperplasia is induced by a high-fat diet. In neoplastic C-cell cultures, also, N-proCT and CT

concentrations are nearly equimolar in both cellular extracts and basal medium; dexamethasone increases both the cellular and secreted concentration of these peptides (Burns et al., 1989a). Judging from the report of Burns et al. (1989a, 1989b), mammalian N-proCT appears to be present in equimolar amounts and coordinately regulated with CT both *in vivo* and *in vitro*. In vertebrates, including rats, humans, and teleosts, N-proCT might function in osteoblasts and regulate bone metabolism.

In the reproductive period, the plasma calcium level in female teleosts increases remarkably (Watts et al., 1975; Yamauchi et al., 1978; Norberg et al., 1989; Suzuki et al., 2004; Guerreiro et al., 2007). The candidate for this hypercalcemic hormone is estrogen. In female teleosts, estrogen promotes the synthesis of vitellogenin, which is a major component of egg protein and a calcium-binding protein (Kwon et al., 1993; Tinsley, 1985). At the same time, estrogen promotes calcium resorption from scales by activating osteoclasts (Persson et al., 1995; Suzuki et al., 2000; Suzuki and Hattori, 2003). In estrogen-injected goldfish, scale TRAP and ALP activities (Suzuki and Hattori, 2003) and plasma calcium levels (Suzuki et al., 2003) increased. In estrogen-injected goldfish, we demonstrated that estrogen promoted CT secretion and that estrogen directly acts on the UBG since the estrogen receptor was detected in the UBG (Suzuki et al., 2004). In teleost reproduction, CT suppresses the excess degradation of calcified tissues (Suzuki et al., 2004). In this period, N-proCT might be effective in bone formation. Namely, both CT and N-proCT cleaved from a preprocalcitonin precursor seem to influence osteoblasts and osteoclasts and protect from excess bone resorption by different actions.

We can detect bioactivities of N-proCT because our scale assay system in the present study is very sensitive. In addition, parathyroid hormone and prostaglandin E₂ function in

osteoblasts and osteoclasts of scales (Suzuki et al., 2011; Omori et al., 2012). The sensitivity of our assay system may be caused by the coexistence of osteoblasts, osteoclasts, and bone matrix (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Redruello et al., 2005; Suzuki et al., 2007; Ohira et al., 2007; Azuma et al., 2007; Suzuki et al., 2008). The mechanism of N-proCT for osteoblasts has not yet been elucidated, although we discovered a new function of N-proCT on osteoblasts in teleosts. We believe that N-proCT binds to a novel receptor in osteoblasts. Therefore, using our scale *in vitro* assay system with goldfish, we are planning to investigate the detailed mechanism of N-proCT on osteoblasts.

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Figure legends

Fig. 1. Nucleotide and deduced amino-acid sequences of sardine preprocalcitonin

The deduced amino acid sequence of preprocalcitonin is depicted under the preprocalcitonin cDNA sequence. The signal peptide, N-proCT, CT, and C-proCT are shown by the dotted underline, box, double underline, and underline, respectively. The stop codon TGA is indicated by an asterisk. This data is available under GenBank accession number LC191399.

Fig. 2. Multiple alignments of the amino-acid sequences of N-proCTs

Identical amino acids are shown in black boxes. DDBJ/EMBL/GenBank accession numbers are as follows: Japanese medaka, NP_001098365; fugu, CAC81278; Atlantic salmon, NP_001135058; green sea turtle, EMP38282; American alligator, XP_006275102; rock pigeon, EMC88082; chicken , ABY65359; African ostrich, XP_009683015; turkey, XP_003206284; platypus, ENSOANP00000017830; Tasmanian devil, XP_003773616; nine-banded armadillo, XP_004472573; cattle, NP_001137209; domestic cat, BAO79719; horse, AAF72526; human, AAA58403; rhesus monkey, EHH23079; house mouse, P70160; and Norway rat, AAA40849.

Fig. 3. Phylogenetic tree of CTs and N-proCTs

Phylogenetic trees of CTs (A) and N-proCTs (B) were calculated by the neighbor-joining method. The bootstrap value is shown besides each branch as the percentage of times. The scale bars show an evolutionary distance of 0.1 and 0.05 amino acid substitutions per protein.

Fig. 4. Effects of sardine CT (A) and sardine N-proCT (B) on ALP activities in cultured scales incubated for 6 h

** indicates a statistically significant difference at $p < 0.01$ from the values of control scales (n = 8).

Fig. 5. Effects of sardine CT (A) and sardine N-proCT (B) on TRAP activities in cultured scales incubated for 6 h

*, **, and *** indicate statistically significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, from the values of control scales (n = 8).

Fig. 6. Effects of sardine CT and sardine N-proCT on type 1 collagen (A: CT; B: N-proCT) and osteocalcin (C: CT; D: N-proCT) mRNA expressions in cultured scales incubated for 6 h

* and ** indicate statistically significant differences at $p < 0.05$ and $p < 0.01$, respectively.

n = 9 samples; one sample from one fish.

Table legend

Table 1. Similarity of sardine CT, sardine N-proCT, and sardine C-proCT to those of other vertebrates

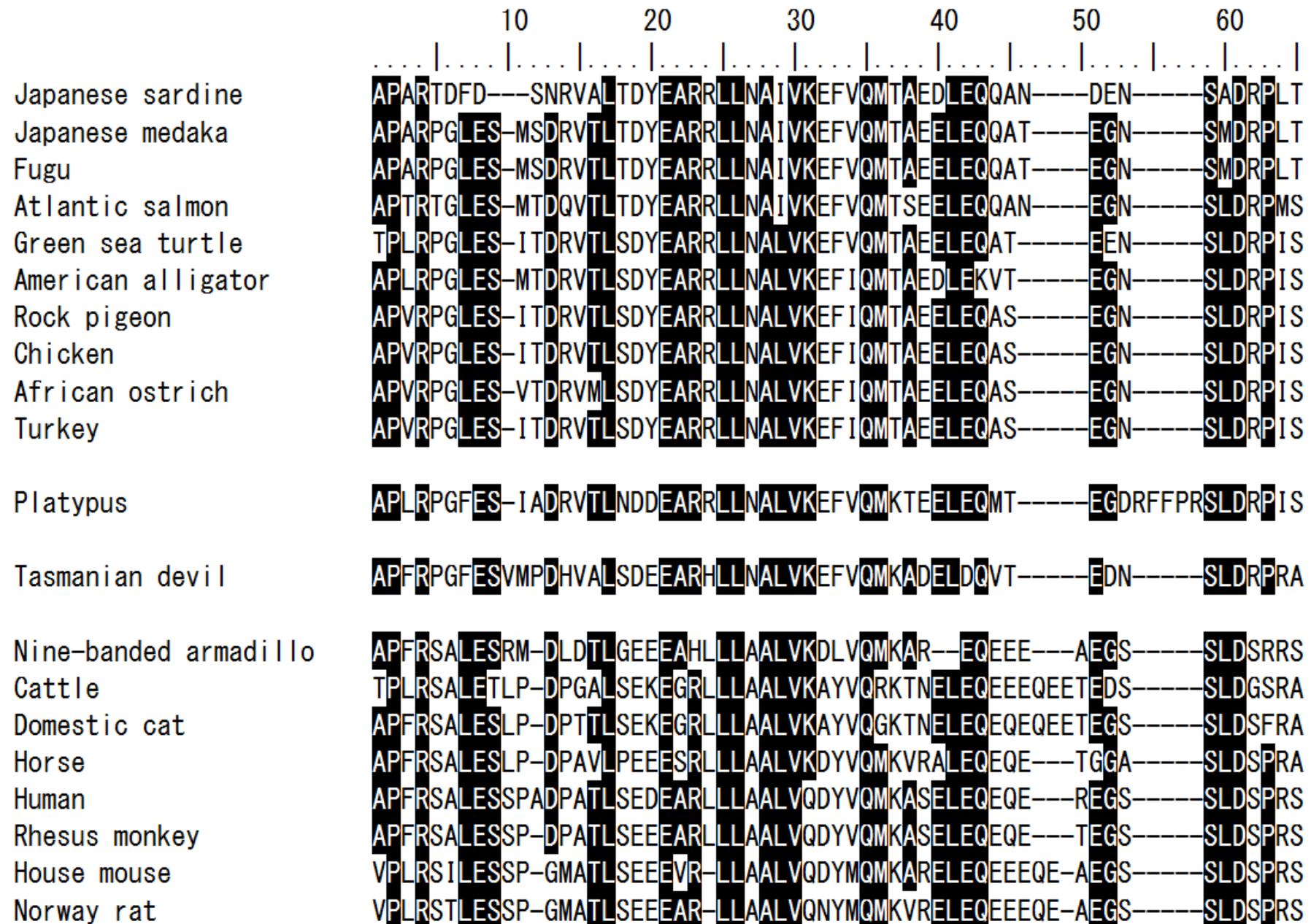
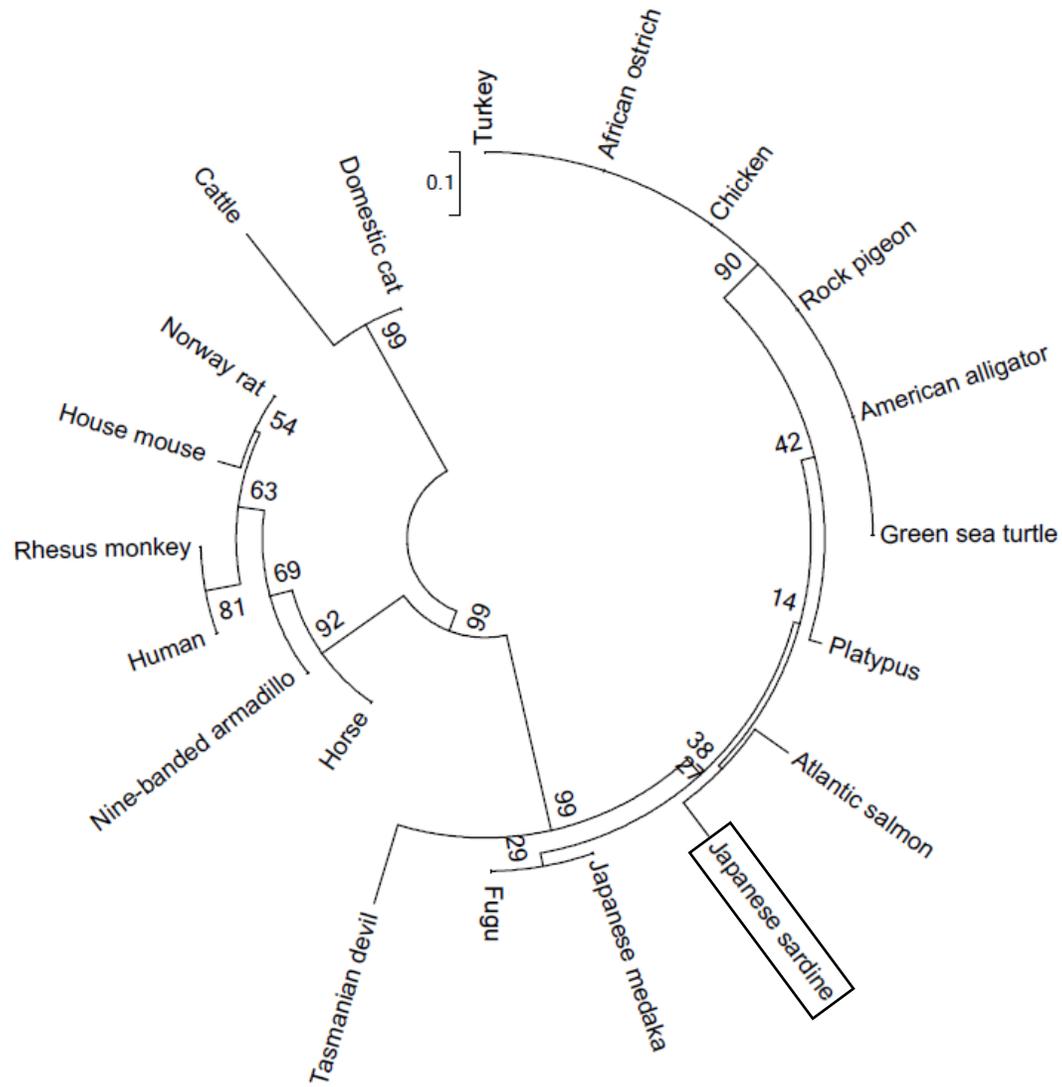


Figure 2 Kase et al.

(A) Phylogenetic tree of CTs



(B) Phylogenetic tree of N-proCTs

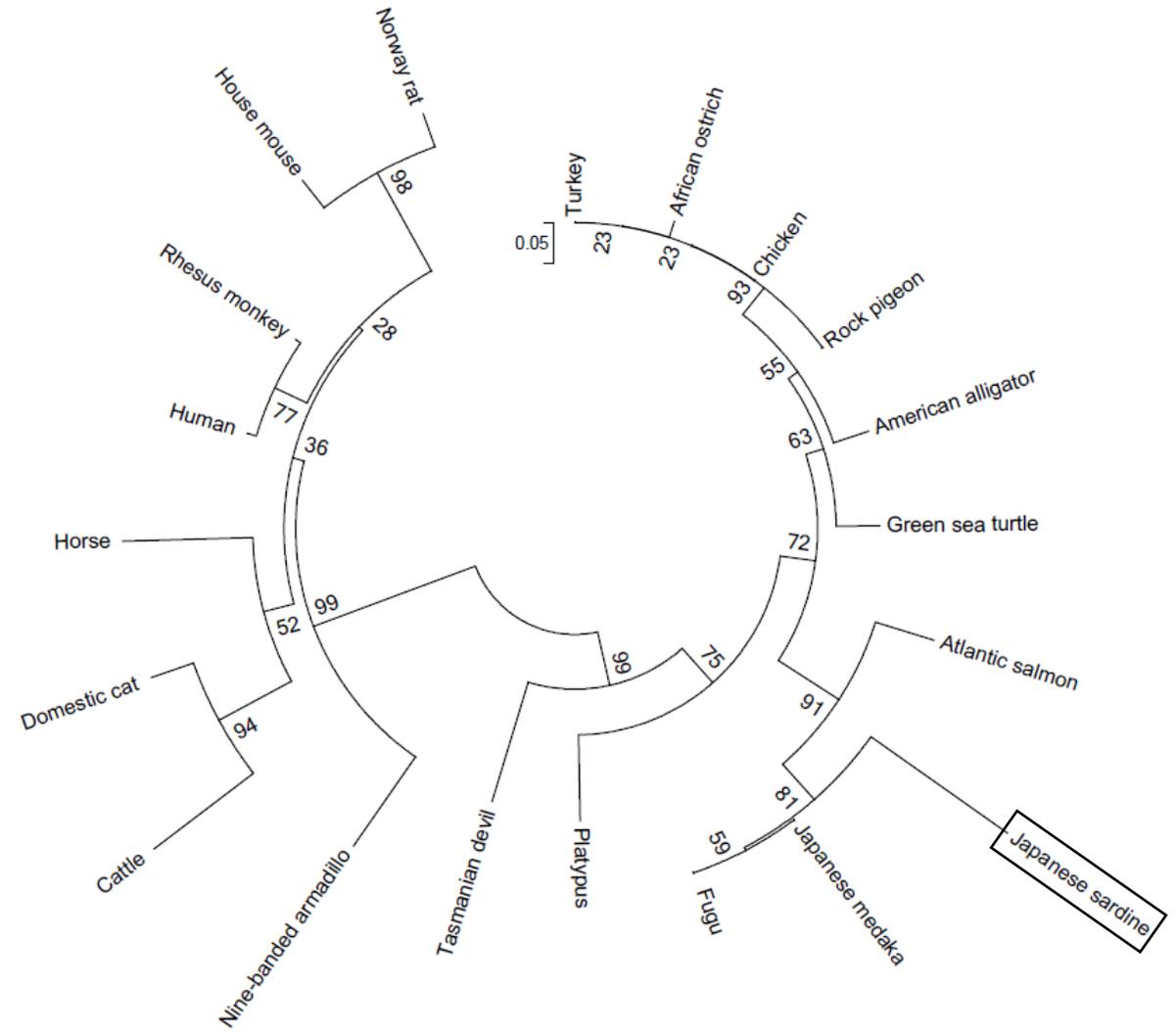
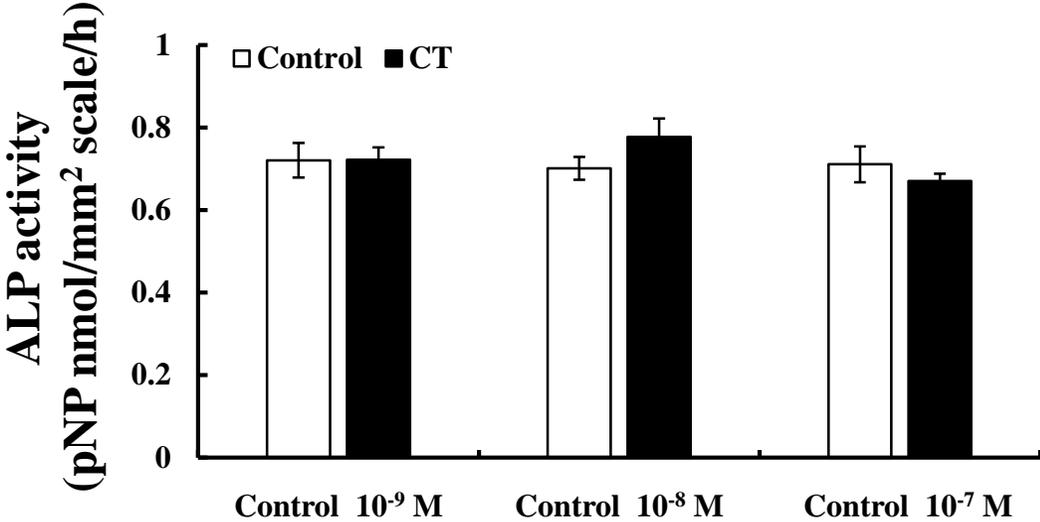


Figure 3 Kase et al.

(A) CT



(B) N-proCT

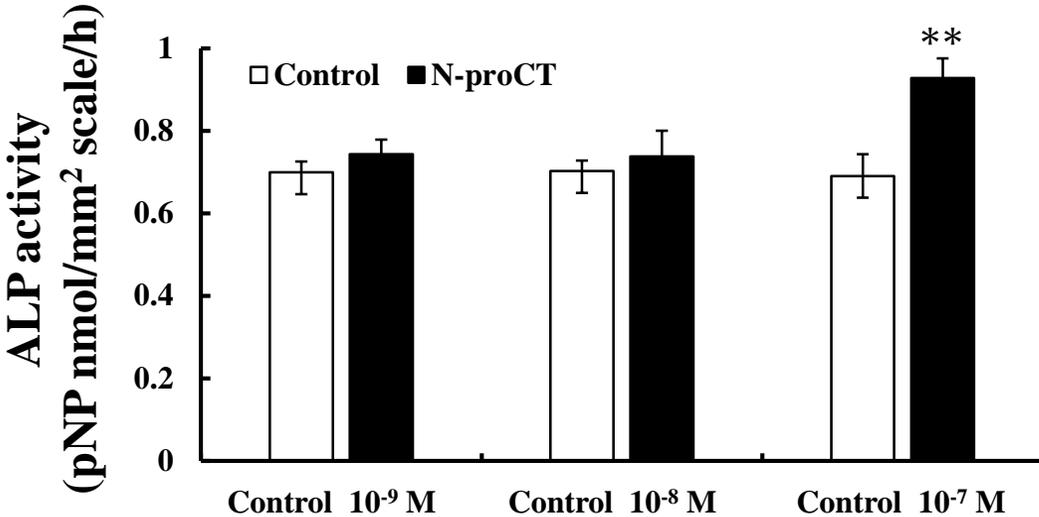
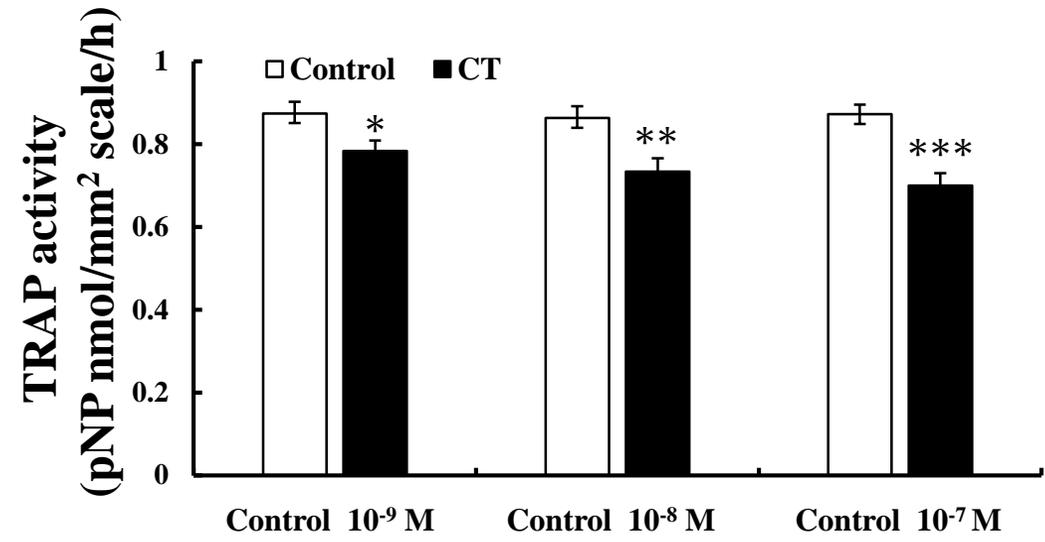


Figure 4 Kase et al.

(A) CT



(B) N-proCT

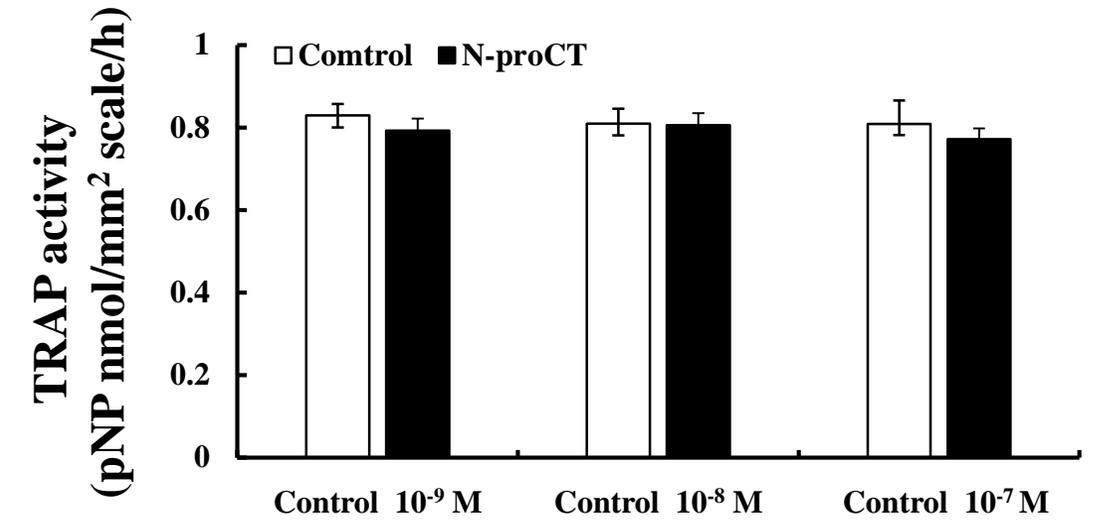


Figure 5 Kase et al.

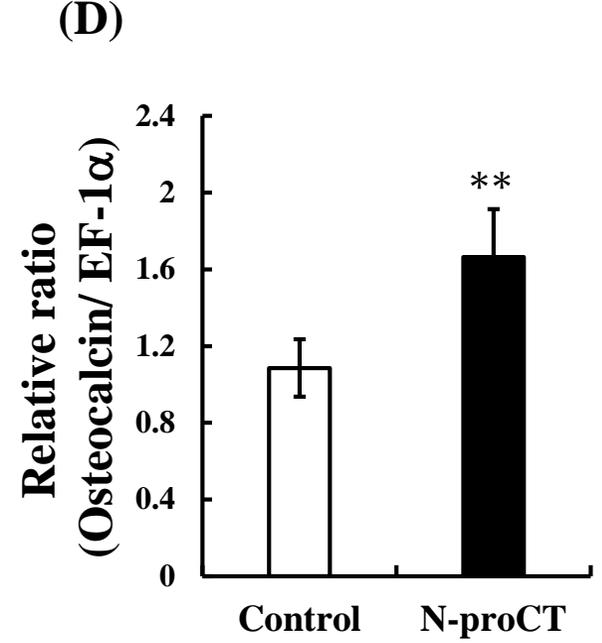
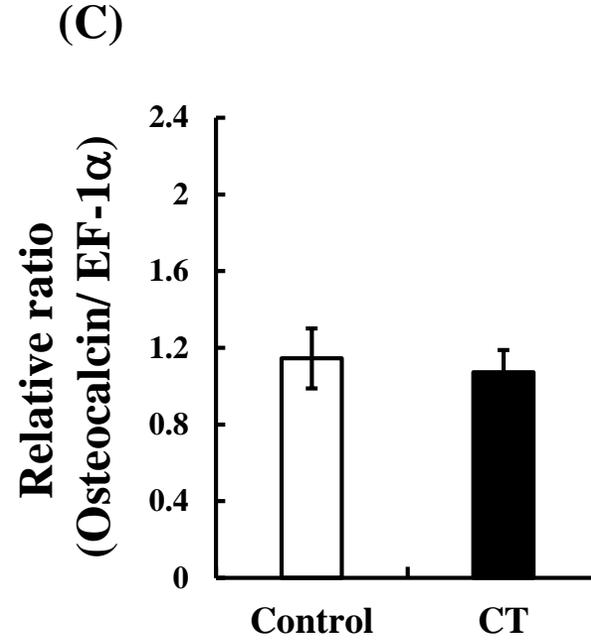
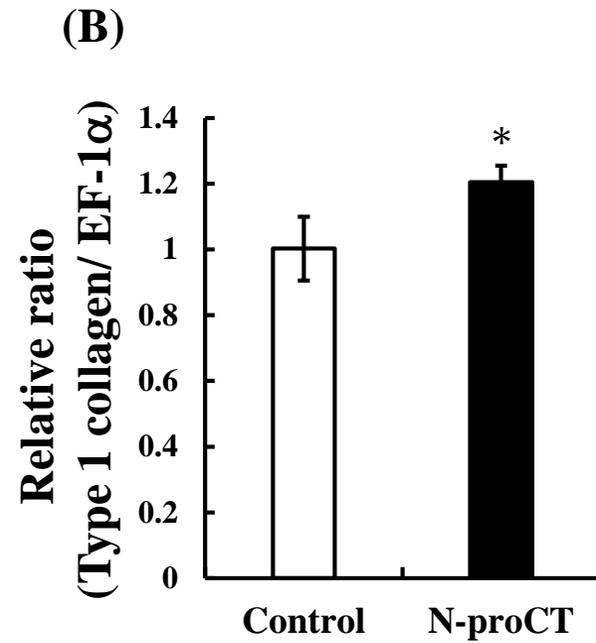
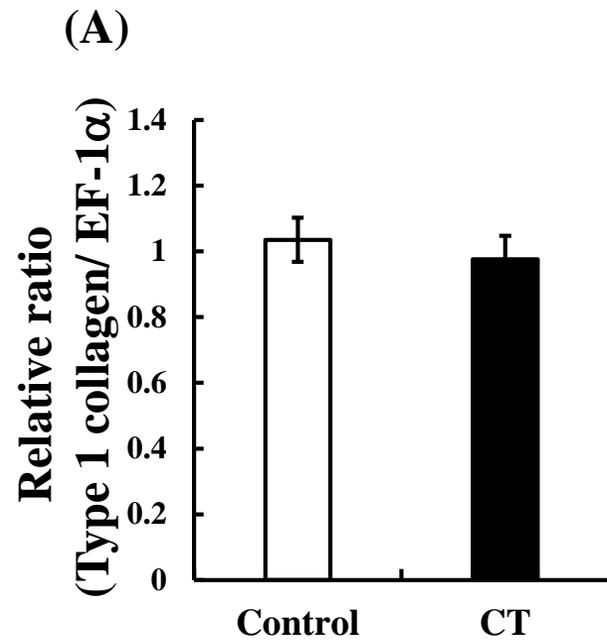


Figure 6 Kase et al.

Table 1. Similarity of sardine CT, sardine N-proCT, and sardine C-proCT to those of other vertebrates.

Animal	Scientific name	Similarity of CT(%)	Similarity of N-proCT (%)	Similarity of C-proCT (%)	Accession
Japanese sardine	<i>Sardinops melanostictus</i>	100.0%	100.0%	100.0%	LC191399
Japanese medaka	<i>Oryzias latipes</i>	96.9%	83.6%	66.7%	NP_001098365
Fugu	<i>Takifugu rubripes</i>	96.9%	83.6%	72.2%	CAC81278
Atlantic salmon	<i>Salmo salar</i>	93.8%	85.5%	66.7%	NP_001135058
Green sea turtle	<i>Chelonia mydas</i>	96.9%	78.2%	57.1%	EMP38282
American alligator	<i>Alligator mississippiensis</i>	96.9%	78.2%	57.1%	XP_006275102
Rock pigeon	<i>Columba livia</i>	96.9%	80.0%	52.4%	EMC88082
Chicken	<i>Gallus gallus</i>	96.9%	80.0%	52.4%	ABY65359
African ostrich	<i>Struthio camelus</i>	96.9%	80.0%	47.6%	XP_009683015
Turkey	<i>Meleagris gallopavo</i>	96.9%	80.0%	52.4%	XP_003206284
Platypus	<i>Ornithorhynchus anatinus</i>	93.8%	66.7%	57.1%	ENSOANP00000017830
Tasmanian devil	<i>Sarcophilus harrisii</i>	87.5%	69.6%	42.9%	XP_003773616
Nine-banded armadillo	<i>Dasypus novemcinctus</i>	71.9%	50.9%	33.3%	XP_004472573
Cattle	<i>Bos taurus</i>	50.0%	50.0%	38.1%	NP_001137209
Domestic cat	<i>Felis catus</i>	62.5%	48.3%	14.3%	BAO79719
Horse	<i>Equus caballus</i>	75.0%	50.9%	28.6%	AAF72526
Human	<i>Homo sapiens</i>	68.8%	63.2%	19.0%	AAA58403
Rhesus monkey	<i>Macaca mulatta</i>	68.8%	61.4%	23.8%	EHH23079
House mouse	<i>Mus musculus</i>	71.9%	54.2%	33.3%	P70160
Norway rat	<i>Rattus norvegicus</i>	75.0%	52.5%	28.6%	AAA40849