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Author(s): Sanath Rajapakse, Katsueki Ogiwara and Takayki Takahashi
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Characterization and Expression of Trypsinogen and Trypsin in Medaka Testis

Sanath Rajapakse[1], Katsueki Ogiwara[2], and Takayki Takahashi[2]*

[1]*Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya, Peradeniya 20400, Sri Lanka*

[2]*Laboratory of Reproductive and Developmental Biology, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan*

Previously, we reported that the medaka testis abundantly expresses the mRNA for trypsinogen, which is a well-known pancreatic proenzyme that is secreted into and activated in the intestine. Currently, we report our characterization of the medaka trypsin using a recombinant enzyme and show that this protein is a serine protease that shares properties with trypsins from other species. Two polypeptides (28- and 26-kDa) were detected in the testis extracts by Western blot analysis using antibodies that are specific for medaka trypsinogen. The 28-kDa polypeptide was shown to be trypsinogen (inactive precursor), and the 26-kDa polypeptide was shown to be trypsin (active protease). We did not detect enteropeptidase, which is the specific activator of trypsinogen, in the testis extract. Immunohistochemical analyses using the same trypsinogen-specific antibody produced a strong signal in the spermatogonia and spermatozoa of the mature medaka testis. Substantial staining was found with spermatocytes, whereas extremely weak signals were observed with spermatids. In vitro incubation of testis fragments with the trypsinogen antibody strongly inhibited the release of sperm from the testis into the medium. Trypsin activity was detected in sperm extracts using gelatin zymographic analysis. Immunocytochemistry showed that trypsinogen and trypsin were localized to the cell membranes surrounding the sperm head. Collectively, these results suggest that trypsin plays an important role in the testis function of the medaka.

Key words: teleost, medaka, testis, spermiation, protease, trypsin

INTRODUCTION

Trypsin, which is a member of the large and diverse serine protease family, is generated from the precursor protein trypsinogen, which is synthesized in and secreted from the pancreas. Trypsin plays a central role in pancreatic exocrine physiology by triggering the activation of all other pancreatic digestive zymogens, including its own inactive precursor (Halfon et al., 2004). Trypsin and trypsinogen are among the most extensively studied enzyme models of protein structure and function as they are readily available in large quantities from the lumen of the vertebrate gut (Walsh and Wilcox, 1970). In particular, trypsinogen and trypsin have become prototypes for understanding the molecular mechanisms underlying the auto-activation of zymogens and the auto-degradation of the active proteases (Halfon et al., 2004; Varallyay et al., 1998; Szilagyi et al., 2001; Chen and Ferec, 2004). Studies concerning mutants and variants of trypsin have also provided a great deal of fundamental knowledge concerning the substrate-specific action of serine proteases (Hedstrom et al., 1992; Jelinek et al., 2004).

While mammalian trypsins are well characterized, there are only a few reports of the molecular and biochemical characterization of trypsin and trypsinogen from birds (Wang et al., 1995; Szenthe et al., 2005). However, trypsins from a variety of fish species have been isolated and characterized (Simpson and Haard, 1984; Genicot et al., 1996; Cao et al., 2000; Bezerra et al., 2001; Castillo-Yanez et al., 2005; Kurtovic et al., 2006; Klomklao et al., 2006). Fish trypsins are particularly interesting because these trypsins exhibit higher catalytic activity than that of their mammalian counterparts, making these proteins suitable for food processing and biotechnology applications (Simpson and Haard 1987; Simpson, 2000, Macouzet et al., 2005).

Recently, we reported the molecular and biochemical characterization of the enteropeptidase serine protease of the medaka *Oryzias latipes* (Ogiwara and Takahashi, 2007). In that study, we cloned a cDNA for medaka trypsinogen, which is the target zymogen of enteropeptidase, and deduced its amino acid sequence. Using Northern hybridization, we detected the trypsinogen mRNA in the medaka testis, spleen, and in the intestine (Ogiwara and Takahashi, 2007). More recently, Miura et al. (2009) provided evidence that testicular trypsin is an important factor in the control of meiosis, spermatogenesis, and of fertilization in the Japanese eel. Their results suggested that active trypsin, but not the zymogen trypsinogen, is involved in the control of meiosis, spermiogenesis, and fertilization; however, the precise role of this protease remains to be determined. These observa-

* Corresponding author. Tel. : +81-11-706-2748;
Fax : +81-11-706-4851;
E-mail: ttakaha@sci.hokudai.ac.jp
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tions prompted us to further investigate the possible role of trypsin in testicular physiology using the medaka, which is a good non-mammalian vertebrate model (Nagahama et al., 1994; Ozato and Wakamatsu, 1994; Ishikawa, 2000; Wittbrodt et al., 2002; Kasahara et al., 2007). During preliminary experiments using an in vitro medaka testis culture, we observed that the release of sperm from the mature testis into the medium was suppressed in the presence of soybean trypsin inhibitor (SBTI). Therefore, we speculated that trypsin might have a role in the spermiogenesis of the medaka.

The present study was performed to gain insights into the role of trypsin in the testis of adult medaka. We produced and characterized active recombinant medaka trypsin and determined the localization of trypsinogen and of trypsin in the testis by morphological analyses. We also examined the effect of a trypsinogen-specific antibody on the release of sperm from cultured testes. These results are reported in this study.

MATERIALS AND METHODS

Animals

Individuals of the adult orange-red variant of medaka (*Oryzias latipes*) were purchased from a local dealer and were kept in indoor tanks under artificial reproductive conditions (photoperiod, 10-h dark/14-h light; temperature, 27°C). The Committee of the Center for Experimental Plants and Animals at Hokkaido University approved all experiments conducted in this study.

Production and purification of recombinant medaka trypsinogen

Recombinant medaka trypsinogen was prepared as described previously (Ogiwara and Takahashi, 2007). Briefly, a 684-bp cDNA fragment (nucleotides 72–755, AB272106) containing the trypsinogen coding sequence, but without the putative signal sequence, was amplified by PCR. The product was digested with *Eco*RI and with *Hind*III, gel-purified and ligated into the pET30a expression vector (Novagen, Madison, WI). The recombinant medaka trypsinogen was expressed in *E. coli* and initially purified using a Ni^{2+}-Sepharose column, as described previously (Ogiwara et al., 2005). The purified recombinant protein was refolded by dialysis. The protein solution (20 ml) obtained from the above affinity chromatography was placed in dialysis tubing (dialysis membrane, size 36, Wako) and was extensively dialyzed against 50 mM Tris-HCl, pH 8.0 at 4°C. The dialyzed sample was then applied to a Resource Q column previously equilibrated with the same buffer. The resulting purified recombinant protein was a 279-residue fusion protein that contained a vector-derived 52-residue peptide at the N-terminus of the 227-residue medaka trypsinogen polypeptide. This recombinant trypsinogen contained two enteropeptidase cleavage sites: one from the vector sequence and the other from the trypsinogen sequence.

Activation of medaka trypsinogen by immobilized enteropeptidase

The active 32-kDa C-terminal serine protease domain of medaka enteropeptidase was prepared as described previously (Ogiwara and Takahashi, 2007) and immobilized on CNBr-activated Sepharose 4B (GE healthcare Biosciences, Piscataway, NJ) according to the manufacturer's protocol. Recombinant trypsinogen was incubated with the immobilized enteropeptidase for 1 h at room temperature. After removing the immobilized enteropeptidase by filtration, the filtrate was concentrated for further experiments.

Antibody preparation

The recombinant fusion protein of medaka trypsinogen (279-residues) prepared above was used as an antigen to raise anti-medaka trypsinogen antibodies in rats.

Anti-medaka enteropeptidase antibodies were as prepared previously (Ogiwara et al., 2007).

The recombinant medaka kallikrein-like protease was produced using an *E. coli* expression system. The coding region (nucleotides 70–828, AB242321) of the protein was amplified by PCR using medaka ovary cDNA. The product was digested with *Eco*RI and with *Xho*I, and the digested product was ligated into the pET30a vector. The kallikrein-like protease expressed in *E. coli* was purified using a Ni^{2+}-Sepharose column as described above. Specific antibodies were purified as previously described (Ogiwara et al., 2007).

Immunohistochemistry

Adult medaka testes were isolated and fixed with Bouin's solution (5% acetic acid, 9% formaldehyde, and 0.9% picric acid) for 2 h at room temperature. After fixing, specimens were dehydrated by passing the specimens through increasing concentrations of ethanol. The specimens were incubated in benzene for 30 min twice and then embedded into paraffin. Paraffin sections (5 μm thickness) were deparaffinized in xylene for 10 min, hydrated by passing the sections through decreasing concentrations of ethanol, and then placed in distilled water. After the sections were incubated in PBS including 3% H_2O_2 for 10 min at room temperature, the sections were incubated in Block Ace (Dainippon-Sumitomo Seiyaku, Osaka, Japan) for 60 min at room temperature. After blocking, the sections were reacted with an anti-trypsinogen antibody diluted with Immuno Shot immunostaining-fine (Cosmo Bio, Tokyo, Japan) for 60 min at room temperature. As a control, an anti-trypsinogen antibody previously treated with the recombinant medaka trypsinogen protein was used. After washing in PBS three times, the sections were reacted with an anti-rat IgG peroxidase-linked antibody (GE healthcare Biosciences) diluted with Immuno Shot immunostaining-fine for 60 min at room temperature. The sections were then washed with PBS three times and stained using a TSA fluorescence kit (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions. Signals were viewed using a fluorescent microscope.

Immunocytochemistry of medaka spermatozoa

Adult medaka testes were isolated and gently torn with tweezers. After the tissues were gently shaken in PBS, released spermatozoa were collected by centrifugation and used for immunocytochemical analysis. Spermatozoa were attached to the MAS-coated glass slides (Matsunami Glass Industries, Osaka, Japan) and fixed with 4% paraformaldehyde (Wako, Osaka, Japan) in 0.1 M phosphate buffer (pH 7.4) for 1 h. Then, slides were washed with PBS and were permeabilized in 1% Triton X-100 in PBS for 30 min. Next, the slides were incubated in Block Ace for 1 h at room temperature to block the non-specific binding sites, followed by incubation with a purified anti-medaka trypsinogen antibody for 1 h at room temperature and washed in PBS containing 0.1% Tween-20 (TPBS) three times. After washing, the slides were reacted with anti-rat IgG peroxidase-linked antibody for 1 h at room temperature. After three washes in TPBS, the sperm cells were stained using an AEC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Purified Rat IgG (1:1000 diluted) was used as the control.

Western blotting

Whole tissue samples of medaka testis, intestine, ovary, liver, spleen, brain, heart, and kidney were separately homogenized in PBS containing protease inhibitor cocktail IV (Wako) and 1 mM EDTA and centrifuged at 15,000 rpm for 10 min to obtain supernatant fractions. Protein concentrations of the extracts were determined using a BCA assay kit (Thermo Scientific, Yokohama,

Japan). Western blot analysis was performed with a trypsinogen antibody raised in rats.

Enzyme activity assay

Unless otherwise stated, the activity of recombinant medaka trypsin was determined at 37°C with a synthetic peptide substrate containing 4-methylcoumaryl-7-amide (MCA), butyloxycarbonyl (Boc)-Gln-Ala-Arg-MCA (Peptide Insitute, Osaka, Japan), according to the method of Barrett (1980) with the slight modifications reported in our previous study (Matsui and Takahashi, 2001). The release of the fluorophore 7-amino-4-methyl coumarin was measured by spectrofluorometry using an excitation wavelength of 370 nm and an emission wavelength of 460 nm.

The active trypsin concentration was determined using the active site titrant *p*-nitrophenyl-*p'*-guanidinobenzoate HCl.

Active medaka trypsin was preincubated with various protease inhibitors at 37°C in 500 μl of 0.1 M Tris-HCl buffer (pH 8.0). After incubation for 15 min, enzyme reactions were started by adding the substrate Boc-Gln-Ala-Arg-MCA.

Kinetic parameters

Kinetic parameters were determined for a variety of MCA-containing peptide substrates. Initial velocities were extrapolated from the plot of product versus time and transformed into double-reciprocal plots (Lineweaver and Burk, 1934). Maximum velocity (V_{max}), K_m, and k_{cat} values were obtained from the intercepts of these plots.

Digestion of protein substrates by recombinant medaka trypsin

Bovine plasma fibronectin (Sigma, St. Louis, MO), mouse laminin (Sigma), and medaka collagen type I (5 μg each) were incubated with recombinant medaka trypsin (0.1 μg for fibronectin and for laminin and 0.5 μg for collagen type I) in 20 μl of 50 mM Tris-HCl buffer (pH 8.0) for 16 h at 37°C. After incubation, 5 μl of SDS-PAGE sample buffer was added to each reaction, and the mixtures were boiled and subjected to SDS-PAGE under reducing conditions. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue. Medaka collagen type I was prepared as described previously (Horiguchi et al., 2008).

Medaka collagen type IV (1 μg) was incubated with 0.02 μg of recombinant medaka trypsin in 20 μl of 50 mM Tris-HCl buffer (pH 8.0) for 16 h at 37°C. The reactions were terminated by adding 5 μl of SDS-PAGE sample buffer. The mixtures were boiled and subjected to SDS-PAGE under reducing conditions, followed by Western blotting using the medaka collagen type IV antibody. Medaka collagen type IV and anti-medaka collagen type IV α1 chain antibodies were prepared as described previously (Kato et al., 2010).

Gelatin zymography

Gelatin zymography was performed as previously described (Ogiwara et al., 2012).

Reverse transcription and polymerase chain reaction (RT-PCR) of testicular enteropeptidase cDNA fragment

Total RNA was isolated from mature medaka testis using Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. The amount and purity of the fraction were determined by spectrophotometry. RT-PCR was performed with the RNA using a SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). The PCR amplification mixture consisted of the RT-PCR reaction, 1 × PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.2 μM of each primer, and 0.5 U Takara *Ex Taq* Hot start Version (Takara Bio, Ohtsu, Japan) (10 μl total volume). The following primers were used: EP-SS1, 5′-AGAACATCACAGGTGAACCGGTGA-3′ (sense, nucleotide no. 1-24, AB272104); EP-AS1, 5′-TAAGACATTAGAATGGACAGAGTC-3′ (antisense, no. 3497–3520). The amplification

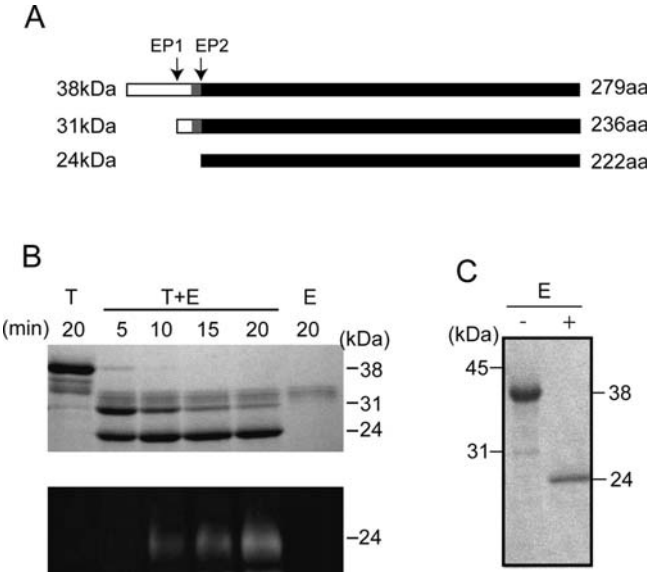

Fig. 1. Preparation of recombinant medaka trypsin. **(A)** Strategy for the preparation of active medaka trypsin. Our *E. coli* expression system using the pET30a expression vector was designed to produce inclusion bodies containing a 279-residue fusion protein with two enteropeptidase cleavage sites: one derived from the vector (EP1) and the other from the intrinsic medaka trypsinogen activation site (EP2). Enteropeptidase digestion thus generates two forms (236 and 222 residues) of the protein. The vector-derived 52-residue peptide (white), 5-residue medaka trypsinogen activation peptide (grey), and active trypsin (black) are indicated. The apparent molecular masses (38-, 31-, and 24-kDa) observed by SDS-PAGE analysis are shown for each polypeptide. **(B)** Proteolytic conversion of the 38-kDa trypsinogen fusion protein to smaller polypeptides (31- and 24-kDa) by treatment with enteropeptidase. T, a recombinant trypsinogen was incubated alone for 20 min; T+E, a recombinant trypsinogen was incubated with the medaka enteropeptidase serine protease domain at room temperature for 5 to 20 min; E, medaka enteropeptidase was incubated alone for 20 min. The incubated samples were subjected to SDS-PAGE under reducing conditions (upper panel). The same samples were electrophoresed under nonreducing conditions and analyzed by gelatin zymography (lower panel). The molecular masses of the polypeptides are shown on the right. **(C)** Recombinant trypsinogen fusion proteins treated with or without enteropeptidase **(E)** for 1 h at room temperature were analyzed by SDS-PAGE under reducing conditions. Untreated (–) and treated (+) samples are shown.

Table 1. Kinetic parameters of recombinant medaka trypsin as measured on MCA-containing substrates.

Substrates	V_{max}	K_m	k_{cat}	k_{cat}/K_m
	μmol/min/mg	mM	min^{-1}	mM^{-1}/min^{-1}
Boc-Gln-Ala-Arg MCA	1.80	0.10	42.9	429
Boc-Val-Pro-Arg-MCA	1.27	0.13	30.2	233
Boc-Leu-Lys-Arg-MCA	1.31	0.19	31.2	164
Boc-Glu-Lys-Lys-MCA	2.20	0.61	52.4	85.9
Z-Val-Val-Arg-MCA	0.57	0.16	13.6	84.8
Z-Leu-Arg-MCA	0.22	0.16	5.24	32.8
Suc-Leu-Leu-Val-Tyr-MCA	ND			
Ac-Ile-Glu-Thr-Asp-MCA	ND			
Suc-Ala-Ala-Pro-Phe-MCA	ND			

Boc, butyloxycarbonyl; Z, benzyloxycarbonyl; Suc, succinyl, Ac, acetyl; ND, not detected.

products were cloned into the pBluescript II KS(–) vector (Stratagene, La Jolla, CA) for sequencing.

Medaka testis culture

Adult medaka testes were removed, gently washed with L15 medium supplemented with 10% fetal bovine serum, 1 × penicillin-streptomycin-glutamine (Invitrogen) and with 10 mM HEPES, and incubated in the same medium. After incubating for 1.5 h at 26°C, the testes were removed carefully and washed gently with the medium. Then, the testes were individually placed in sterilized culture plate inserts (millicells, 0.4 μm pore size, Millipore Corporation, MA) with the same medium containing soybean trypsin inhibitor (SBTI, 0.4 mg/ml), purified rat anti-medaka trypsinogen antibodies (100 μg/ml), or rat normal IgG (100 μg/ml) for further incubation for 4.5 h. Twenty microliters of medium from each testis and from the control sample were removed, and the number of released spermatozoa were counted using a hemocytometer. The weight of each testis was measured, and the amount of released spermatozoa per milligram of testis was calculated.

Statistical analysis

All experiments were conducted at least three times to confirm the reproducibility of the results. The data are presented at the mean ± S.E.M.

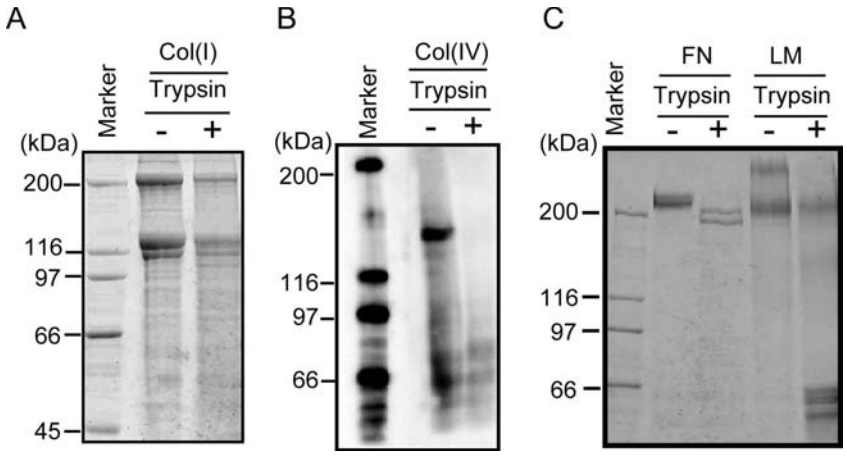

Fig. 2. Activity of recombinant medaka trypsin against protein substrates. **(A)** Digestion of medaka collagen type I by medaka trypsin. Medaka collagen type I was incubated in the absence (–) or presence (+) of medaka trypsin and analyzed by SDS-PAGE under reducing conditions. Col(I), collagen type I. **(B)** Digestion of medaka collagen type IV by medaka trypsin. Medaka collagen type IV was incubated in the absence (–) or presence (+) of medaka trypsin and analyzed by SDS-PAGE (under reducing conditions)/Western blotting using specific antibodies for medaka collagen type IV α1 chain. Col(IV), collagen type IV. **(C)** Digestion of fibronectin (FN) and laminin (LM) by medaka trypsin. Bovine plasma fibronectin and mouse laminin were separately incubated in the absence (–) or presence (+) of medaka trypsin and analyzed by SDS-PAGE under reducing conditions. Note that collagen type I, collagen type IV, fibronectin and laminin were all degraded by the medaka protease. Each experiment was performed in triplicate, with similar results, and one representative image is shown.

RESULTS

Characterization of recombinant medaka trypsin

Recombinant medaka trypsinogen was produced as a 279-amino acid (38-kDa) fusion protein in an *E. coli* expression system. The serine protease domain of recombinant medaka enteropeptidase was used to convert the fusion protein into a 24-kDa form via a 31-kDa intermediate (Fig. 1A). The 24-kDa product was capable of hydrolyzing gelatin (Fig. 1B), indicating that this product was active medaka trypsin. For further enzyme characterization, the pure 24-kDa enzyme sample generated by enteropeptidase digestion was used (Fig. 1C).

The activity of the recombinant medaka trypsin was tested against various synthetic MCA-containing peptide substrates (Table 1). The enzyme hydrolyzed substrates that contained arginine or lysine at the P_1 position; however, the enzyme did not hydrolyze substrates that contained tyrosine, phenylalanine, and asparagine at that location. The best substrate (highest *kcat*/*Km* value) was Boc-Gln-Ala-Arg-MCA, followed by Boc-Val-Pro-Arg-MCA. Both of these substrates had a small, hydrophobic residue at the P_2 position. Substrates with a bulky or basic residue at the P_2 position (Boc-Leu-Lys-Arg-MCA, Boc-Glu-Lys-Lys-MCA, Z-Val-Val-Arg-MCA, and Z-Leu-Arg-MCA) displayed smaller *kcat*/*Km* values.

The medaka trypsin also successfully degraded four extracellular matrix proteins, collagen type I, collagen type IV, fibronectin, and laminin (Fig. 2).

The effects of protease inhibitors on the activity of medaka trypsin were examined using the best peptide substrate, Boc-Gln-Ala-Arg-MCA (Table 2). The activity of the enzyme was strongly suppressed by well-known serine protease inhibitors. Interestingly, the enzyme was also substantially inhibited by chymostatin, which is a chymotrypsin-like protease inhibitor. Overall, these results strongly suggest that medaka trypsin is a serine protease that cleaves specifically at the carboxyl side of basic amino acid residues.

Presence of active trypsin in medaka testis

A Western blot analysis of medaka tissue extracts was conducted using antibodies specific for medaka trypsinogen. Clear signals were only detected in testis, intestine, and spleen tissues (Fig. 3A). The protein detected by the antibody appeared in two forms, a 28-kDa polypeptide that predominated in the testis and in the spleen and a 26-kDa polypeptide that was primarily visible in the intestine. The presence of both polypeptide species in the testis was confirmed using an excessive amount of the tissue extract (Fig. 3B). The signal intensity of the 26-kDa polypeptide was approximately 1/5 that of the 28-kDa species. The relation between the two polypeptides was examined by treating the testis extract with recombinant medaka enteropeptidase serine protease. The 28-kDa polypeptide was proteolytically converted to the 26-kDa protein by the treatment (Fig. 3C). A similar proteolytic conversion was observed when the intestinal extract was treated with the enteropeptidase; the 28-kDa band disappeared, and only the 26-kDa band was detected. These results indicate that the 28- and 26-kDa polypeptides are the precursor form (trypsinogen) and active trypsin, respectively.

The medaka testis extract exhibited enzymatic activity

Table 2. Effects of protease inhibitors on recombinant medaka trypsin activity.

Inhibitor	Concentration	inhibition (%)
Antipain	1 mM	100
Aprotinin	0.1 mg/ml	100
Leupeptin	1 mM	100
SBTI	0.2 mg/ml	100
Benzamidine	2 mM	90
DFP	1 mM	88
E-64	1 mM	25
Pepstatin	2 mM	0
TPCK	2 mM	0
TLCK	2 mM	100
Chymostatin	0.2 mM	50
EDTA	1 mM	25

Enzyme activities of the medaka recombinant trypsin were determined using Boc-Gln-Ala-Arg-MCA in the presence of inhibitors. Activities are expressed as the percentages of the respective controls. DFP, diisopropyl fluorophosphate; SBTI, soybean trypsin inhibitor; TPCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; EDTA, ethylenediamene-tetraacetic acid.

toward Boc-Gln-Ala-Arg-MCA. This activity was drastically reduced by pretreatment with SBTI and with the trypsinogen antibody (Fig. 4). Antibodies against the medaka kallikrein-like protein had no effect on this activity. These results indicate that active trypsin is present in the medaka testis.

Analyses of the expression of enteropeptidase in medaka testis

Medaka enteropeptidase cDNA was produced by RT-PCR using total RNA isolated from mature medaka testis. As expected, RT-PCR using the primer set EP-SS1/EP-AS1 produced a 3.5 kb cDNA (Data not shown). Parallel experiments using the intestinal total RNA as the template produced the same 3.5 kb cDNA. However, no immunoreactive material was detected in a Western blot analysis of the testis extract using antibodies specific for medaka enteropeptidase. These results indicate that although the medaka testis contains enteropeptidase mRNA that encodes the functional protein, it does not contain detectable levels of the translated product.

Immunohistochemical localization of trypsinogen and of trypsin in medaka testis

The mature medaka testis is a type of testis representing restricted distribution of spermatogonia in the germinal compartment (Grier, 1981; Schulz et al., 2010). The testis is composed of many cysts (Gresik et al., 1973; Grier et al., 1980; Shibata and Hamaguchi, 1986; Iwai et al., 2006). Spermatogonia are present in the cysts in the peripheral region of the testis, and the cysts migrate toward the center as the germ cells differentiate. Generally, cysts housing spermatogonia, spermatocytes, spermatids, and spermatozoa are distributed in that order from the periphery to the center of the testis (Fig. 5A). Strong signals detected using a trypsinogen-specific antibody were associated with spermatogonia and with spermatozoa (Fig. 5B and 5C). Signals were also observed, although to a lesser extent, with spermatocytes. Weak signals were detected with spermatids.

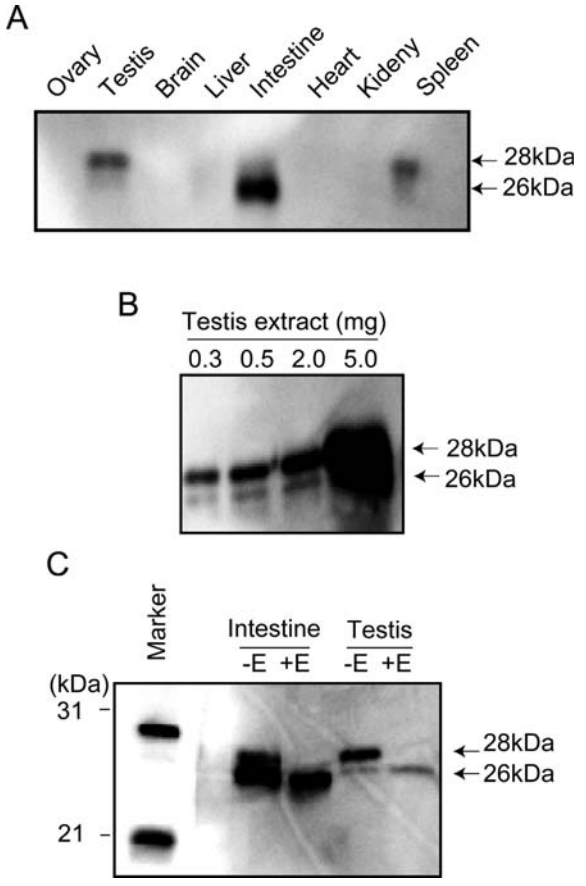

Fig. 3. Detection of active trypsin in the medaka testis. **(A)** Western blot analyses of medaka tissue extracts using specific anti-medaka trypsinogen antibodies. Various tissue extracts (0.5 μg protein) were electrophoresed in the presence of SDS under reducing conditions. **(B)** Occurrence of both the 26- and 28-kDa polypeptides in the medaka testis. Varying amounts (0.3, 0.5, 2.0, and 5.0 μg per lane) of the extract were analyzed by SDS-PAGE/Western blotting using specific anti-medaka trypsinogen antibodies. **(C)** Conversion of the 28-kDa polypeptide to the 26-kDa polypeptide by treatment with enteropeptidase (E). Extracts of medaka intestine (0.25 μg) and testis (2.5 μg) were separately incubated with active recombinant medaka enteropeptidase serine protease (0.05 μg) at 37°C for 1 h in 50 mM Tris-HCl (pH 8.0), and then analyzed by SDS-PAGE (reducing conditions)/Western blotting using specific anti-medaka trypsinogen antibodies. Note that enteropeptidase-treated intestine (intestine, +E) and testis extract (testis, +E) gave only a polypeptide band of 26-kDa. All experiments were performed in triplicate, and a representative image is shown for each.

Effect of trypsinogen antibodies on sperm release from medaka testes

Knowing that trypsinogen was detected in association with the spermatogonia and with the spermatozoa of the mature medaka testes, next, we conducted experiments examining the role of trypsin, specifically its possible involvement in the process of spermiation. In the in vitro culture of testis fragments, an inclusion of trypsinogen-specific antibodies dramatically reduced the release of sperm into the medium (Fig. 6A). The antibody caused a reduction in sperm release to an extent comparable to that caused by SBTI (Fig. 6B). Western blot analysis using the antibody with the sperm extracts showed the 28-KDa trypsinogen

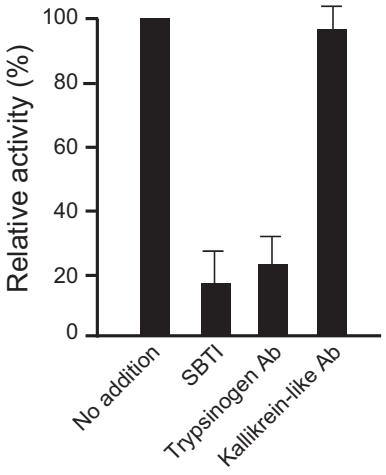

Fig. 4. Effect of soybean trypsin inhibitor (SBTI) and antibodies specific for medaka trypsinogen on ability of medaka testis extract to hydrolyze Boc-Gln-Ala-Arg-MCA. Testis extracts (25 μg) were preincubated with SBTI (0.2 mg/ml) or with purified anti-medaka trypsinogen antibodies (120 μg/ml) in 500 μl of 0.1 M Tris-HCl (pH 8.0) for 18 h at 4°C. The extract was also preincubated with purified anti-medaka kallikrein antibodies (120 μg/ml) as a control. After incubation, the amydolytic activity of each sample was determined using Boc-Gln-Ala-Arg-MCA as a substrate. Enzyme activities are expressed as the percentage of activity relative to the control (no addition). The data are presented as the mean values ± S.E.M. (n = 3).

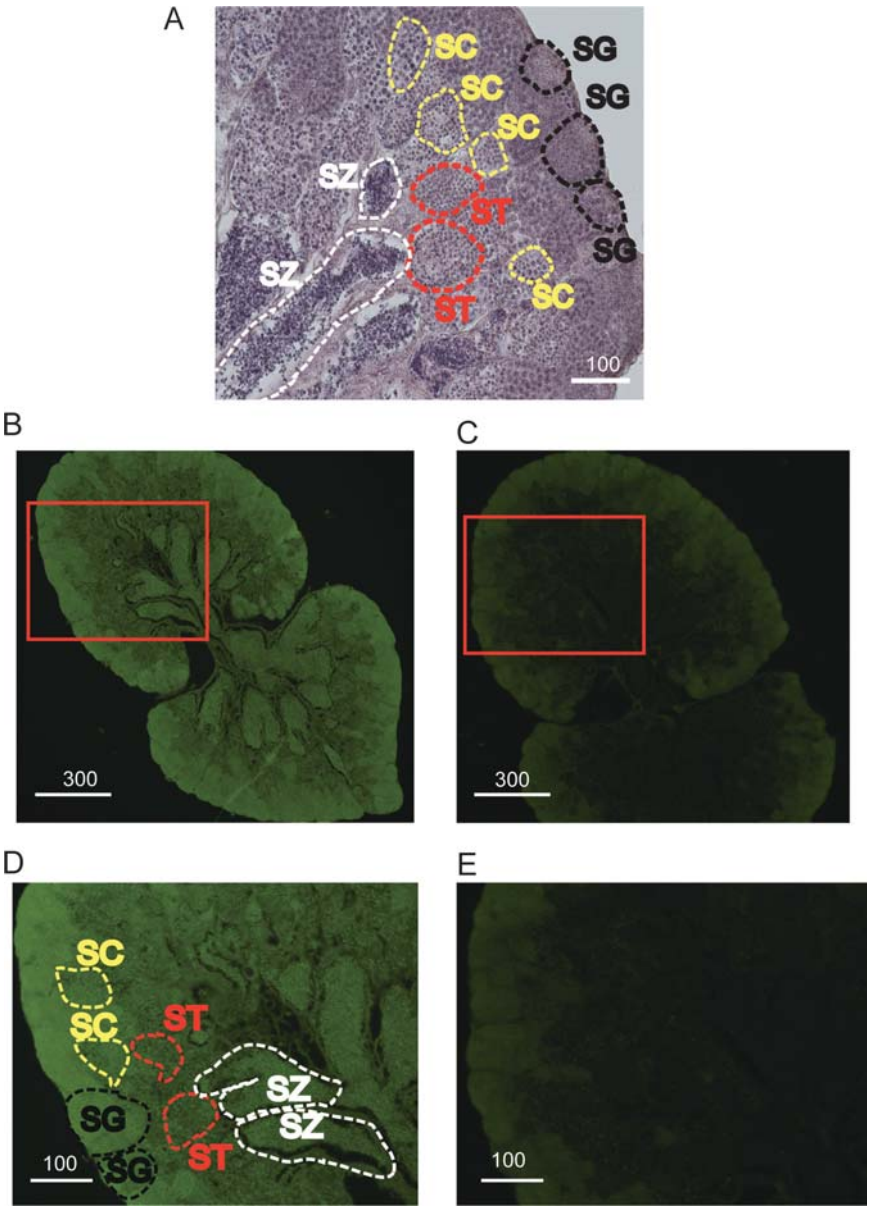

Fig. 5. Immunohistochemical detection of trypsinogen and trypsin in medaka testis. A testis section was stained with hematoxylin-eosin to identify individual cysts that contained spermatogonia (SG), spermatocytes (SC), spermatids (ST), or spermatozoa (SZ). Neighboring sections of the testis were incubated with a purified anti-medaka trypsinogen antibody (B and D) or with absorbed antibody (C and E). Boxed areas in (B) and (C) are shown at higher magnification in (D) and in (E), respectively. Positive signals were stained green. The scale bars are indicated in each panel. The reproducibility of the results was confirmed by repeating the experiments three times, and the results of a representative experiment are shown.

apparently with no 26-kDa active trypsin (Fig. 7A). However, the presence of the 26-kDa active enzyme in the sperm extracts was confirmed by gelatin zymography (Fig. 7B). An immunocytochemical analysis using anti-trypsinogen antibodies revealed that signals were associated with the cell membranes surrounding the sperm head (Fig. 7C). No significant staining was observed with the middle piece or with the tail of the sperm. These results indicate that active trypsin in the testes may have a role in the process of sperm release.

DISCUSSION

Trypsin is a pancreatic enzyme that is secreted into the intestine as an inactive precursor, trypsinogen. Previously, we found that mature medaka testes also abundantly express trypsinogen mRNA (Ogiwara and Takahashi, 2007). Intrigued, we pursued the biochemical and immunological analyses reported in this study. We found that medaka testes contain not only the inactive precursor protein trypsinogen but also the active enzyme trypsin. This strongly suggests a role for trypsin in testis function.

We prepared recombinant medaka trypsin and showed that this protein is enzymatically active and exhibited many features common to trypsins from other species. The medaka trypsin is a serine protease that hydrolyzes internal peptide bonds at the carboxyl terminal side of basic amino acids, such as arginine and lysine. Trypsins detected in the intestine and testis of the medaka were not distinguishable at the molecular level. The spleen also expressed trypsinogen to a considerable extent. A mechanism for the regulation of this tissue-specific expression of the trypsinogen

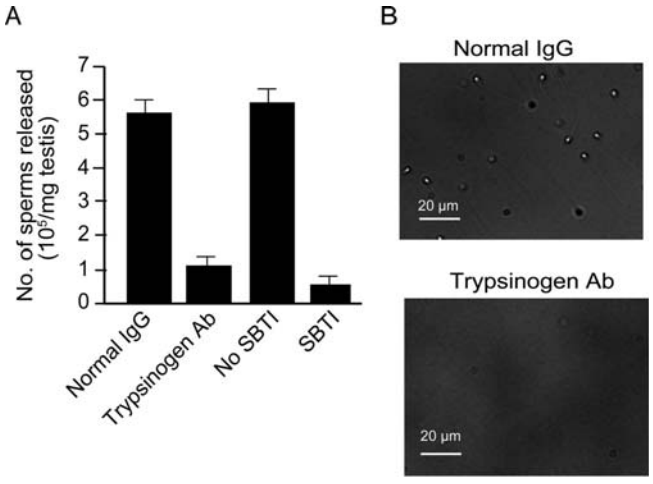

Fig. 6. Effects of specific anti-medaka trypsinogen antibodies and SBTI on sperm release from the testis in vitro. **(A)** Mature medaka testes were cultured at 26°C for 4.5 h in L15 medium with SBTI (0.4 mg/ml) or with purified rat anti-medaka trypsinogen antibodies (100 μg/ml), as described in the Materials and Methods. Controls were incubated without SBTI or with normal rat IgG (100 μg/ml). After incubation, the numbers of released sperm were counted. The data are presented as the mean values ± S.E.M. (n = 4). **(B)** Microphotographs of media from testes cultured in the presence of normal IgG (upper panel) or of anti-medaka trypsinogen antibodies (lower panel). The experiments were performed in triplicate, and the result of a representative experiment is shown. The scale bars (20 μm) are indicated in each panel.

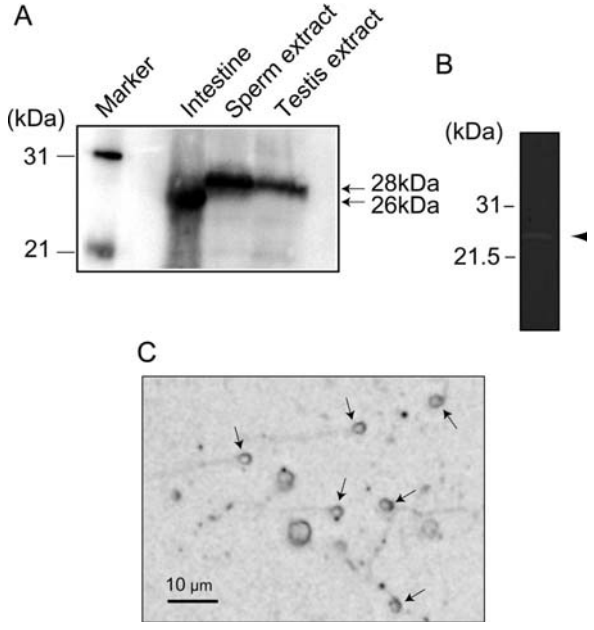

Fig. 7. Detection of trypsinogen and trypsin associated with medaka spermatozoa. **(A)** Adult medaka spermatozoa were collected, and the extract was prepared. The sperm extract (2.0 μg) was analyzed by Western blotting using an anti-trypsinogen antibody. Intestine (0.25 μg) and testis (1 μg) extracts were also analyzed for comparison. **(B)** The sperm extract (2.0 μg) was analyzed using gelatin zymography. The reproducibility of the result was confirmed by three independent experiments. An arrowhead indicates the position corresponding to 26-kDa. **(C)** Spermatozoa were collected from the testis of adult medaka and immune-stained with an anti-trypsinogen antibody. Sperm heads positively stained are indicated by an arrow. The scale bar (10 μm) is indicated. Experiments were conducted in triplicate, and the result of a representative experiment is shown.

gene in the medaka remains to be clarified. A recent observation by Miura et al. (2007) that trypsinogen is upregulated in the eel testis by 17,20-dihydroxy-4-pregnen-3-one (DHP), which is the steroid hormone that induces oocyte maturation in teleosts, is interesting in this context. DHP may also be a critical factor for testicular expression of the trypsinogen gene in the medaka.

As established by several studies using mammalian species, trypsinogen is activated by the specific processing enzyme enteropeptidase. Previously, we demonstrated that this activation is also true for medaka trypsinogen in the intestine (Ogiwara and Takahashi, 2007). Because the testis extract contained active trypsin in addition to the inactive precursor trypsinogen and because the precursor protein present in the extract could be readily converted in vitro to its active trypsin by recombinant medaka enteropeptidase, we suggest that the same activation mechanism involving enteropeptidase may operate in mature medaka testes. However, we did not detect enteropeptidase protein, although an mRNA transcript encoding the complete sequence of enteropeptidase was present in the testis. At present, we have no data concerning the mechanism of trypsinogen activation in the medaka testis; however, the following explanations may be possible: (a) the testis may produce active enteropeptidase in amounts that are sufficient for trypsinogen activation but that are too small to detect with our Western blot analysis, (b) an unknown protease may be involved in the activation process, and (c) trypsinogen may be autocatalytically activated by trypsin (Halfon and Craik, 2004). However, in the last case, the question of how trypsinogen is initially activated remains. Further studies are necessary to solve this problem.

Spermatogenesis in medaka is categorized as a cystic type (Gresik et al., 1973; Grier et al., 1980; Shibata and Hamaguchi, 1986; Iwai et al., 2006; Schulz et al., 2010). Spermatogenesis proceeds within a cyst that is delimited by somatic Sertoli cells. Each cyst is initially formed by a single primary spermatogonium and by several Sertoli cells in the peripheral regions of the testis near the tunica albuginea (Schulz et al., 2010). Mitotic divisions of the primary spermatogonium produce a cohort of secondary spermatogonia. As the cells enter meiosis, the cysts migrate toward the center of the testis. During spermatogenic progression, a clonal group of isogenic spermatocytes, spermatids, and spermatozoa is produced. Mature sperm are eventually released into the spermatic duct; this process is called spermiation (Pudney, 1995; Schulz et al., 2010). Our present observation that strong signals for trypsinogen and for trypsin were detected with spermatogonia and with spermatozoa most likely indicates roles of the proteins in the processes relevant to these cells. In this connection, the inhibition of sperm release from testis fragments by SBTI and by a trypsinogen-specific antibody in our in vitro experiment could be explained in several ways, given the involvement of active trypsin. During spermiation, spermatozoa should be freed from Sertoli cells, which play an indispensable role through-

out the course of spermatogenesis. The termination of Sertoli cell-germ cell contact may require proteolytic enzymes. We speculate that trypsin may be one such enzyme. The localization of trypsinogen and trypsin on the surface of the sperm head seems to favor this possibility. The reduction in the number of sperm released from the tissue into the culture medium by SBTI and by the antibody may reflect the deterioration in the process of Sertoli cell-germ cell detachment due to trypsin inhibition by the protease inhibitor or by the antibody. However, further studies demonstrating that trypsin would facilitate the release of spermatozoa from Sertoli cells in vivo are required in the future. Evidence for the critical role of proteolytic enzymes in the regulation of the Sertoli cell-germ cell interaction has been reported in mammals (Mruk et al., 1997; Longin et al., 2001; Siu and Chang, 2004). Notably, the inhibition of sperm release from the testis fragments by SBTI or by a trypsinogen antibody may have occurred through a trypsin-involving mechanism(s) different from the above-speculated mechanisms. For example, the protease might be involved in the process of passage from the spermatic duct to outside. Regardless, at present, the precise mechanism behind the release of sperm from the tissue remains unclear. However, our results strongly suggest the implication of trypsin in the process.

A previous study by Miura et al. (2007) reported that trypsin was detectable in the membranes of spermatozoa and found to be associated with fertilization in Japanese eel. Considering that this medaka counterpart is also localized to the membrane of spermatozoa, the medaka trypsin may play a role in fertilization, similar to the case of the eel. Interestingly, the expression of trypsin associated with spermatogonia is another common feature between the medaka and the Japanese eel (Miura et al., 2007). Such similarities in the expression and in the distribution of trypsin in the two fish tempt us to speculate concerning the biological importance of the protease in the male reproduction of teleost species.

Another interesting finding of the present study is that trypsinogen and trypsin were expressed in high abundance in the spermatogonia and in the spermatozoa compared with spermatocytes or with spermatids. This finding suggests a mechanism(s) regulating the differential expression of the trypsinogen gene during the development of germ cells. These cells express the trypsinogen gene during the early stage of spermatogenesis; however, its expression declines when the germ cells enter meiosis. However, germ cells resume trypsinogen gene expression in the extremely late stage of spermatogenesis. Considering that trypsinogen and trypsin proteins were detected in the lowest abundance in spermatids but in large abundance in spermatozoa, we speculate that the resumption of transcription and translation of the gene may occur in late spermatids, such that active trypsin expressed in the germ cells may be synchronized for their detachment from Sertoli cells. The validity of this assumption would be further strengthened by providing evidence that levels of trypsinogen mRNA expression is enhanced in late spermatids. Recent studies using mammalian species reveal that after meiosis, haploid germ cells initiate a gene expression program necessary for the differentiation of spermatids and of spermatozoa (Sassone-Corsi, 2002; Rousseaux et al., 2008; Gaucher et al., 2010, 2012; Montellier et al., 2013; Boussouar and Benahmed, 2004; Alves et al., 2013; Boussouar et al., 2014).

In summary, we have demonstrated the presence of trypsinogen and trypsin in the medaka testis. Our present results suggest that active trypsin may play a role in testis function in medaka.

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