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Tissue inhibitor of metalloproteinase-2 (TIMP-2) from red seabream (*Pagrus major*): Molecular cloning and biochemical characterization of highly expressed recombinant protein

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ABSTRACT

The tissue inhibitor of metalloproteinase-2 (TIMP-2) is originally characterized as an endogenous inhibitor of matrix metalloproteinases (MMPs) to response collagenolysis associated with immune challenge. In this study, the cDNA encoding TIMP-2a gene from red seabream (*Pagrus major*) muscle was cloned. It was 585 bp encoding a putative protein of 194 amino acids, which comprised all recognized functional domains and showed the high identity to TIMP-2as from other teleost fishes, revealing it belongs to TIMP-2a family. Soluble rTIMP-2a was efficiently expressed using a new constructed pPIC9K-rTIMP-2a vector with high inhibitory activity against to MMP-2 and MMP-9. The recombinant TIMP-2a tagged with 6 histidine residues showed the molecular mass of 23 kDa and isoelectric point of 6.50. Furthermore, the 6 disulfide bonds formed by 12 conserved cysteine residues were identified as functional motifs for its structural stability. In addition, rTIMP-2a possessed the high inhibitory activity against gelatinolytic hydrolysis and degradation of type I collagen which induced by endogenous MMPs in muscle. The results revealed the properties and inhibitory function of rTIMP-2a, which may be a pivotal role in regulation gelatinolytic MMPs metabolization during defense mechanism.

1. Introduction

In extracellular matrix (ECM), collagens are most abundant protein, while they are highly resistant to hydrolysis by almost endogenous proteinases except collagenolytic matrix metalloproteinases (MMPs) [1]. The MMPs, especially MMP-2 and MMP-9, were regarded to cause collagenolysis. These collagenolysis were generally occurred in various physiological and pathological processes, including muscle wound [2,3], major immune-mediated diseases [4,5] and muscle softening during postmortem storage [6,7]. Moreover, the growing number of evidences showed that MMP-2/MMP-9 participate in the activation of immune response in several teleost fishes muscle, playing the key role on immune imbalance [2,4,8].

Usually, the dynamic alteration of MMP/endogenous tissue inhibitors of metalloproteinase (TIMP) affect the activity level of MMP [9]. TIMP is tissue specific and primary endogenous inhibitor of MMP [9,10]. Generally, there are four members of TIMP family, including TIMP-1, TIMP-2, TIMP-3 and TIMP-4. To data, TIMP-2 has been reported from various vertebrate and invertebrate tissues due to its fine-tuned balance of TIMP-2/MMPs, such as zebrafish (*Danio rerio*) [11,12], grass carp (*Ctenopharyngodon idella*) [13], human (*Homo sapiens*) [14], acting the important role in several immune-related diseases. Compared to TIMP-2s found in mammal, which have only one type, teleost fishes have two distinct types of TIMP-2s (TIMP-2a and TIMP-2b), such as Fugu rubripes (*Takifugu rubripes*) [15], Japanese flounder (*Paralichthys olivaceus*) [16,17], gilthead seabream (*Sparus aurata*) [18]. Although the physiologic function distinction between TIMP-2a and TIMP-2b in teleost fish tissues are not clear, their amino acid sequences showed significant differences, which may link to their structures and properties.

Red seabream (*Pagrus major*) is a kind of important economic marine fish species. It has high nutritional value and is widely cultured in China and Japan. The production in China reached 234,500 tons in 2018 [19]. However, it is prone to have infectious diseases or immune disorder during farming, which usually result in fatal economic losses [20,21]. Thus, the researches about immune-related enzymes inhibitors and their functional properties are increasingly important, such as the inhibitors against MMP-2/MMP-9. In previous study, GST tagged TIMP-2a, as a endogenous inhibitor of MMP-9, was expressed in the form of inclusion using pGEX-6P-1 vector from red seabream embryo cell [22].

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The buffer containing 8 M urea was used to obtain soluble TIMP-2a. This is obviously problematic because of protein denaturation and inhibitory activity decline. Moreover, the conjugation of GST in N-terminal domain of TIMP-2 prevents its interaction with the catalytic domain of MMP. This may further result in its lack of inhibitory activity. Hence, in our study, a cDNA that encodes red seabream muscle TIMP-2a is identified and a new expression vector is constructed to produce soluble TIMP-2a with high inhibitory activity and yield. Additionally, it is investigated that the biochemical properties of TIMP-2a and its inhibitory effect on gelatinolytic MMPs activity to provide theoretical reference for collagens related immune response in teleost fish muscle.

2. Materials and methods

2.1. Experimental fish treatment

Cultured red seabream (*Pagrus major*) (body weight 600–700 g) were purchased alive from a local fish market, Xiamen, China. The skeletal muscle of sacrificed red seabream was immediately collected and frozen in liquid nitrogen for total RNA extraction. The muscles were also obtained for sarcoplasmic proteinase and type I collagen preparation.

2.2. Molecular cloning of rTIMP-2a cDNA sequence

Total RNA was extracted from skeletal muscle of red seabream using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the user's manual provided by the manufacturer. Approximately 1 μg of RNA was obtained from 1 g of skeletal muscle. Then, first-strand cDNA was reverse-transcribed from 2 μg total RNA by SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) using a universal oligo$(dT)_{15}$ primer according to the manufacture's instruction.

Based on the sequence of TIMP-2 mRNA from red seabream cultured embryos cells (GenBank accession: AB189640.1) [22], a fragment of TIMP-$2_{27\text{-}220}$ gene with the size of 585 bp was amplified by Polymerase Chain Reaction (PCR) in a GeneAmp 9700 thermal cycler (Applied Biosystems, USA). Two specific primers, sense primer F1 (5′-TGCAGC TGCGCCCCGGTCCACCC-3′) and antisense primer R1 (5′-AGGCTCCTC GCCGTCCAG-3′), were designed for cloning the whole protein without a signal peptide (rTIMP-2a). Then, these two primers and the cDNA synthesized were further used in the second round of PCR. Thermocycling conditions were 94 °C for 3 min, followed by 35 cycles of at 94 °C for 1 min, 53 °C for 40 s, 72 °C for 1 min, and final extension of 72 °C for 7 min. The PCR product was purified from agarose gel and cloned into pGEM-T Easy vector (Promega, Madison, USA). Its cDNA sequence was analyzed and confirmed by the Invitrogen Biotechnological Co. Ltd. (Shanghai, China) for three times. The deduced amino acid sequence identity and prediction of conserved domain in peptide were performed using DNAMAN software or BLAST program (GenBank, NCBI) [13].

2.3. Functional expression of rTIMP-2a

The sequence encoded rTIMP-2a was subcloned by high fidelity PCR with the Pfu polymerase (Invitrogen, Carlsbad, CA) employing two specific primers: sense primer F2 (5′ -TTGAATTCTGCAGCTGCGCCCC GGTCCACCC-3′) and antisense primer R2 (5′ -AGGGCGGCCGCTTA*GT GATGGTGATGGTGATG*AGGCTCCTCGCCGTCCAG-3′). The letters marked with underlined or italic sequences in these two specific primers is corresponding to the *EcoR* I and *Not* I restriction sites and coding 6 histidine residues, respectively. After gel-purified and digested with *EcoR* I and *Not* I of PCR product, it was cloned into *EcoR* I/*Not* I-digested pPIC9K vector (Invitrogen, Carlsbad, CA). The recombinant plasmid was transformed into *E. coli* DH5α and selected with ampicillin (100 μg/mL). The resulting rTIMP-2a expressing vector (pPIC9K-rTIMP-2a) was confirmed by DNA sequencing and the recombinant plasmid prepared was linearized using *Sac* I for easy homologous recombination with the genome of *Pichia pastoris* (*P. pastoris*) GS115 strain. Then, the vector was transformed into competent GS115 cells by electroporation according to the manual of the *P. pastoris* expression kit v. 3.0 (Invitrogen, Carlsbad, CA). For negative control, *Sac* I-linearized pPIC9K vector was also transformed into GS115 cells. Recombinant yeast clones were obtained from MD plates containing 1.34% yeast nitrogen base (YNB) (Difco), 4×10^{-5}% biotin, 1% dextrose, and 1.5% agar. Positive transformants were characterized by PCR using α-factor primer and 3′AOX1 primer (on the pPIC9K vector) as described in the manual.

2.4. Purification of rTIMP-2a

All procedures were performed at 4 °C. The crude yeast culture was centrifuged to remove cells and supernatant was collected. This supernatant was extensively dialyzed against buffer A (50 mM Na_2HPO_4 containing 5 mM imidazole and 0.3 M NaCl, pH 8.0), then it was subsequently applied to a Ni-NTA Sepharose (Pharmacia) column (1.5 × 3 cm) previously equilibrated with buffer A. Contaminating proteins were removed by washing the column with starting buffer until the absorbance at 280 nm reached baseline. Adsorbed proteins were eluted with a liner gradient of imidazole from 5 to 300 mM in buffer A in a total volume of 100 mL at a flow rate of 1.0 mL/min. Pooled fractions containing the pure rTIMP-2a were dialyzed against 20 mM PBS, pH 7.0 and used for further analysis. Protein concentration was determined by measuring the absorbance at 280 nm of the sample solution on column chromatography or with the method of Lowry with bovine serum albumin as standard [23].

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional electrophoresis (2D-PAGE) and western blotting analysis

2D-PAGE was performed on 7 cm ReadyStrip IPG strips (BioRad, CA, USA) with pH 3–10. Purified rTIMP-2a was resuspended in isoelectric focusing buffer containing 2 M thiourea, 7 M urea, 2% (w/v) Chaps, 0.2% (v/v) pH 3–10 ampholine, 1 M dithiothreitol (DTT) and bromophenol blue. IEF was performed in Ettan IPGphor apparatus according to the instructions of the manufacturer (GE Healthcare) at 20 °C as following: the strip was rehydrated in isoelectric focusing buffer at 50 V for 13 h, 30 min at 300 V, followed by 30 min at 1000 V, the voltage was then increased to 5000 until 6000 Vh was reached, and thereafter the voltage was decreased to 500 V and held at that voltage for 3 h. After IEF, the strip was equilibrated with 50 mM Tris-HCl (pH 8.8) containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 2% (w/v) DTT for 10 min followed by another 10 min using the same buffer with 2.5% iodoacetamide instead of DTT, then for SDS-PAGE. SDS-PAGE was performed according to the method of Laemmli using 12% gels [24]. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (CBB).

Western blotting was carried out as described in our previous study [25]. Briefly, rTIMP-2a was electrophoresed in SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 1 h and then incubated with the mouse anti-6 × histidine monoclonal antibody (Clontech) for 2 h as the first antibody. After extensive washing with Tris-buffered saline Tween 20 (TBST), blots were then revealed with corresponding horseradish-peroxidase-conjugated IgG (Pierce) as the second antibody using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as a chromogenic substrate.

2.6. Molecular mass of rTIMP-2a by Q-TOF mass spectrometry

An aliquot of purified rTIMP-2a was diluted to a concentration of 5 μM into 50% methanol (Burdick & Jackson)/0.2% formic acid (Pierce). An accurate whole mass was performed using a positive mode

ESI static nanospray on a Waters Q-TOF Global Ultima (Waters, Milford, MA, USA). The data was transformed using the MaxEnt 1 function in Waters MassLynx 4.0. The instrument was calibrated from 50 to 2000 *m/z* using a solution of sodium iodide.

2.7. Disulfide bond cleavage and formation of rTIMP-2a

To investigate the fabricated disulfide bonds of rTIMP-2a, the relative electrophoretic migration rates of rTIMP-2a were analyzed after adding disulfide reducing reagents dithiothreitol (DTT) and β-Mercaptoethanol (β-ME). The purified protein at concentration of 0.1 mg/mL was mixed with β-ME at different final concentrations from 0.015% to 0.5%. After 15 min of incubation at room temperature, the samples were prepared for SDS-PAGE analysis as described above.

2.8. Inhibition of rTIMP-2a on the activity of gelatinolytic MMP

Enzymatic activity of gelatinolytic MMP was analyzed by gelatin zymography, which was performed according to the method reported in our previous study [6]. Briefly, the sample was mixed with one-fourth of SDS sample loading buffer (200 mM Tris-HCl, pH 6.8, containing 8% SDS, 0.4% bromophenol blue, and 40% glycerol) and electrophoresed in 12% polyacrylamide gels containing 1 mg/mL bovine gelatin (Amresco) at 4 °C. After electrophoresis, the gels were washed with 2.5% (v/v) Triton X-100 for two 30 min periods followed by gentle shaking to remove SDS and rinsing with deionized water. The gels were incubated at 37 °C for about 15 h in buffer B (50 mM Tris-HCl and 5 mM $CaCl_2$, pH 8.0) and followed by CBB staining. The area of enzyme activity appeared as a clear band on CBB-stained dark blue background, and its clearness is in positive correspondence with enzymatic activity.

The minced red seabream muscle was homogenized in 3-fold of 25 mM sodium phosphate, buffer (pH 7.5) using a homogenizer (Kinematica, PT-2100, Switzerland) and centrifuged at 10,000 × g for 15 min in a centrifuge (Avanti J-25, Beckman Coulter, USA). Sarcoplasmic fraction was preincubated with purified rTIMP-2a (w/w = 100:1) at room temperature for 30 min. Samples were preincubated with or without 10 mM EDTA as positive or negative control, respectively. After gelatin zymography electrophoresis, the gels were washed and rinsed as described above and subsequently allowed to incubate in corresponding buffer (the test with buffer B containing 0.3 μM rTIMP-2a, the positive control with buffer B containing 10 mM EDTA, and the negative control only with buffer B) at 37 °C for 15 h, followed by CBB staining.

3. Results

3.1. Molecular cloning, expression and purification of rTIMP-2a

The partial cDNA sequence (585 bp) of red seabream TIMP-2 was obtained, which was predicted to encode 194 amino acid residues of mature TIMP-2 (GenBank accession: FJ460209.1) by analysis with DNAMAN software, as shown in Fig. 1A. BLASTp analysis suggested that the TIMP-2 from red seabream muscle shares 100% identity to that from embryos cultured cell of red seabream TIMP-2 (AB189640.1), 97.50% of gilthead seabream TIMP-2a (*Sparus aurata*, AM905937.1), 93.30% of fugu rubripes TIMP-2a (*Takifugu rubripes*, AB161713) and 91.24% of Japanese flounder TIMP-2a (*Paralichthys olivaceus*, AB106131); While only 70.33%, 64.25% and 63.21% similarity of TIMP-2b from gilthead seabream (*Sparus aurata*, AM905936.1), fugu rubripes (*Takifugu rubripes*, AB154540) and Japanese flounder (*Paralichthys olivaceus*, C82324, C82325) were observed, respectively (Fig. 1B). Moreover, it also showed 73.17% identity to TIMP-2 from human (*Homo sapiens*, S48568.1) and 28.65% to that from TIMP-2 Pacific oyster (*Crassostrea gigas*, AF321279). In addition, the sequence of rTIMP-2a was obviously divided into N-terminal domain with 127 amino acid residues and C-terminal domain with 67 amino acid

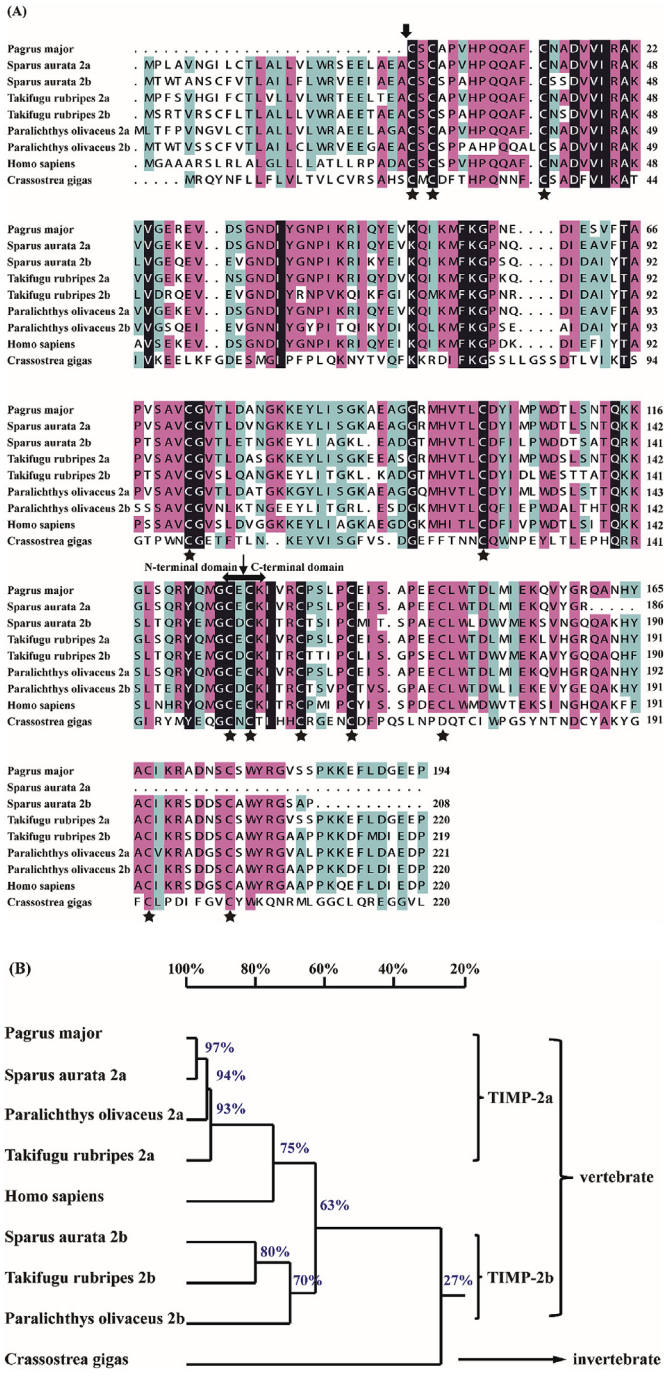

Fig. 1. Alignment (A) and homology analysis (B) of deduced amino acid sequence of rTIMP-2a from red seabream skeletal muscle with TIMP-2s from other teleost fishes, human and aquatic invertebrate orthologues. The same color show identical residues among those sequences shown. Asterisks indicate cysteine residues highly conserved in TIMP-2 that form intramolecular disulfide bonds. The longitudinal arrow indicates the putative cleavage site by signal peptides. The horizontal arrow indicates protein domain (N-terminal and C-terminal domain). The protein sequence used for homology analysis were as follows: TIMP-2a Fugu rubripes (*Takifugu rubripes*, AB161713), TIMP-2b Fugu rubripes (*Takifugu rubripes*, AB154540), TIMP-2a Japanese flounder (*Paralichthys olivaceus*, AB106131), TIMP-2b Japanese flounder (*Paralichthys olivaceus*, C82324, C82325), TIMP-2a Gilthead seabream (*Sparus aurata*, AM905937.1), TIMP-2b Gilthead seabream (*Sparus aurata*, AM905936.1), TIMP-2 human (*Homo sapiens*, S48568.1), TIMP-2 Pacific oyster (*Crassostrea gigas*, AF321279). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

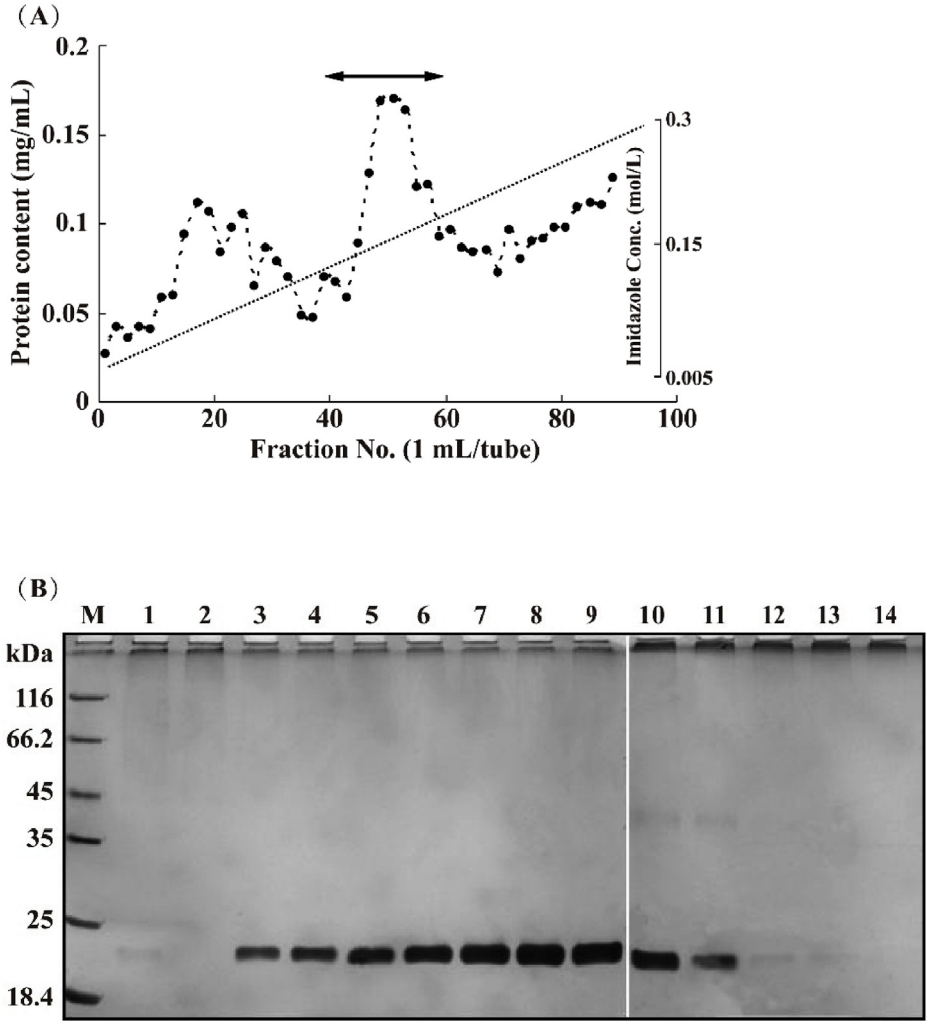

Fig. 2. The purification of rTIMP-2a from red seabream skeletal muscle. (A), Ni-NTA chromatography of rTIMP-2a (B), Analysis of Ni-NTA affinity column purified rTIMP-2a by SDS-PAGE followed silver staining. Protein content is represented by a dashed line. Fraction of rTIMP-2a pooled are represented by double arrow line. Lane M, protein marker; Lane 1, total dialyzed supernatant of GS115; Lane 2, unbounded proteins of Ni-NTA; Lanes 3–14, No. 17, 25, 43, 45, 48, 51, 55, 58, 61, 67, 70 and 74.

residues, as marked by horizontal arrow in Fig. 1A.

The DNA fragment encoding the mature rTIMP-2a and 6-His-Tagged in C-terminal was inserted into the expression vector pPIC9K and transformed into *E. coli* DH 5α. Then, the objective rTIMP-2a was collected in supernatant and purified using an affinity Ni-NTA Sepharose column. As shown in Fig. 2A, several fractions were eluted with buffer 20 mM Tris-HCl (pH 8.0) containing 30–180 mM imidazole, and the high protein peak was analyzed using SDS-PAGE, revealing only one single band with a molecular mass of about 23 kDa (Fig. 2B). The yield of rTIMP-2a expressed using *E. coli* DH 5α was about 40 mg/L yeast culture media after purification.

3.2. Molecular mass and isoelectric point analysis of rTIMP-2a

According to the deduced 208 amino acid sequence of rTIMP-2a, the calculated molecular mass and isoelectric point (pI) were 23,368 Da and 6.37, respectively (Fig. 1a). These were further investigated by MALDI-TOF-MS and 2D-PAGE. According to the results of MALDI-TOF-MS, the rTIMP-2a was exactly 23,397.03 Da with purity more than 95% (Fig. 3A), which was consistent with theoretical value (23,368 Da). The single spot about 23 kDa on 2D-PAGE also suggested rTIMP-2a was highly purified and its estimated pI value was approximately 6.50, which is similar with the theoretical pI value of 6.37 (Fig. 3B).

3.3. Disulfide bond cleavage and formation of rTIMP-2a

Using western blotting analysis, the purified rTIMP-2a showed a clear band corresponding to about 23 kDa in Fig. 4A. Furthermore, its dimer with about 46 kDa and a band with about 21 kDa were also found. Interestingly, the molecular mass of TIMP-2a obviously increased to about 25 kDa when it mixed with disulfide reducing reagents (DTT and β-ME) as shown in Fig. 4 B. Moreover, the similarly increased molecular mass bands were also investigated under reduced condition with different final concentration of β-ME from 0.015% to 0.5% using SDS-PAGE (Fig. 4C). With the increasing of molar ratio (β-ME/rTIMP-2), the molecular mass change of rTIMP-2a was more and more obvious after 15 min incubation at room temperature measured by gel shift analysis as shown in Fig. 4D. The rTIMP-2a presented molecular mass of 21.39 kDa in the absence of β-ME, while it showed molecular mass with 23.50 kDa when the concentration of β-ME was 0.03%. Moreover, the molecular mass of rTIMP-2a increased to 25.03 kDa after adding β-ME of 0.5%.

3.4. Inhibition of rTIMP-2a on gelatinolytic MMP activity

The inhibitory effects of rTIMP-2a on gelatinolytic proteinase of dark and white muscle from red seabream were investigated. As shown

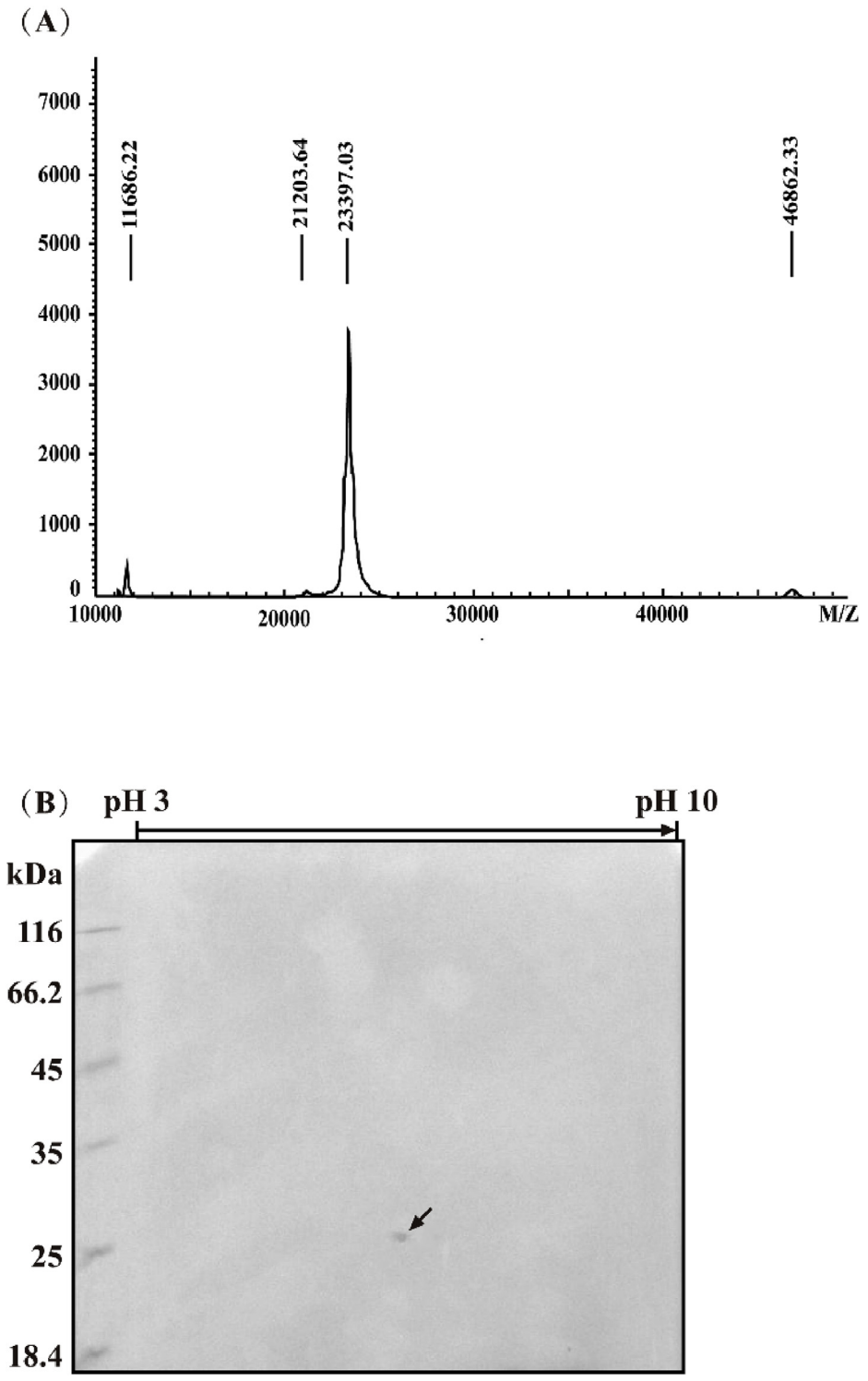

Fig. 3. Analysis of molecular mass (A) and isoelectric point (B)of rTIMP-2a by MALDI-TOF MS or 2D-PAGE.

in Fig. 5A, B and C, four types of gelatinolytic proteinases, marked as E1, E2, E3 and E4, were found in dark muscle using gelatin zymography. While only E1, E2 and E3 were observed from white muscle. The corresponding molecular mass of them were about 85 kDa, 65 kDa, 60 kDa and 55 kDa, respectively. After adding a type of MMP zymogen activator APMA, only the activities of E3 and E4 were changes, E3 showed lower and E4 was higher (Line2 and Line 4, Fig. 5A). In addition, the activities of E3 and E4 were completely disappeared after incubation with buffer containing 10 mmol/L EDTA, which was generally regarded as MMP inhibitor (Fig. 5C). However, the activities of E1 and E2 were not markedly changed under the corresponding conditions.

3.5. Inhibition of rTIMP-2a on type I collagen degradation

The effect of rTIMP-2a on the degradation of type I collagen produced by gelatinolytic proteinases from red seabream muscle were surveyed. The major bands of collagen I (α1, α2 and β chain) from red seabream muscle were presented and they suffer from obvious

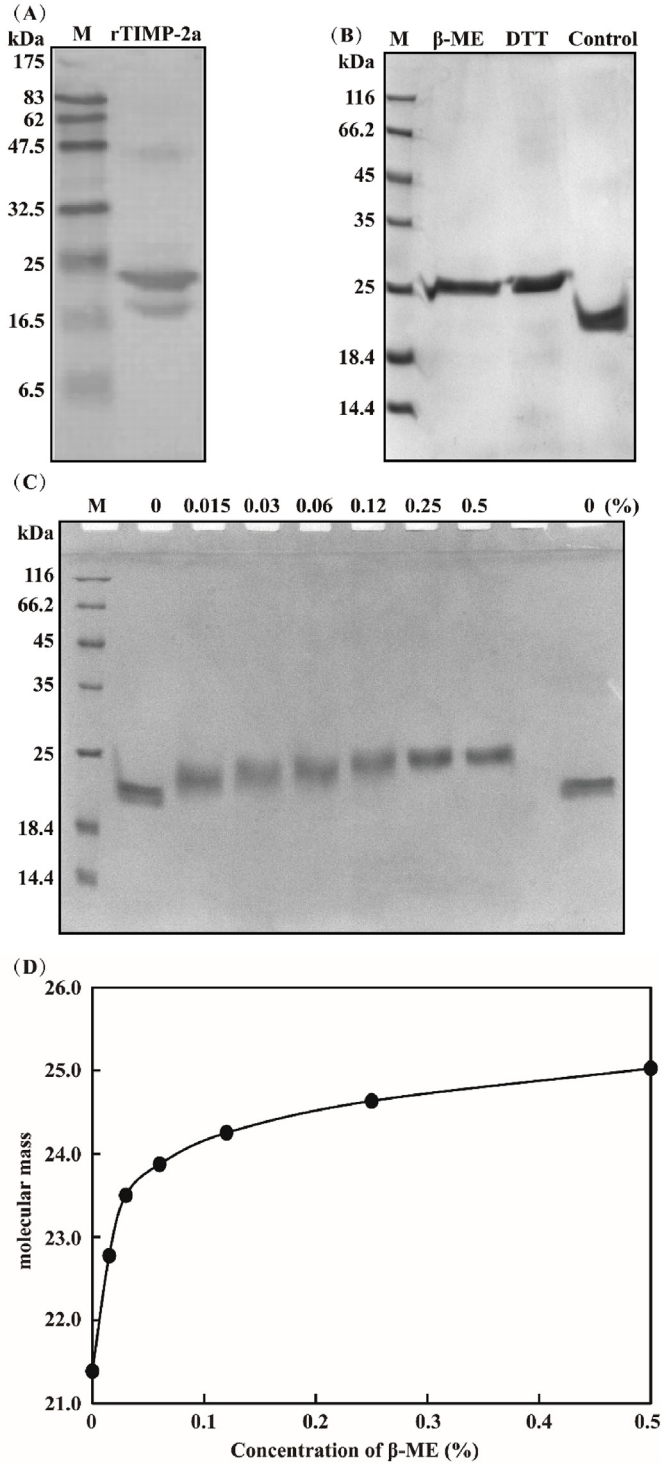

Fig. 4. The disulfide bond cleavage and formation of purified rTIMP-2a. (A) Western blotting analysis; (B) SDS-PAGE analysis of rTIMP-2a treated without or with disulfide reducing reagents; (C) SDS-PAGE analysis in the presence of β-ME with different concentration; (D) Correlation between molecular mass of rTIMP-2a on SDS-PAGE and concentration of β-ME. Molecular weight of rTIMP-2a incubated in different concentration of β-ME were calculated by the linear equation with log molecular mass vs migrated distance according to standard proteins. Line M: protein marker; Line β-ME: reduced sample with β-ME; Line DTT: reduced sample with DTT; Line Control: non-reduced sample; Lines 0.015, 0.03, 0.06, 0.12, 0.25, 0.5: reduced sample with β-ME of different concentrations (0.015, 0.03, 0.06, 0.12, 0.25, 0.5%).

proteolysis after gelatinolytic MMPs treatment (Fig. 6, line 1 and Line 2). Furthermore, as shown in Fig. 6 (Line 3), the degradation α1, α2 and β chain of type I collagen due to the function of gelatinolytic MMPs were strongly prevented after addition of rTIMP-2a.

4. Discussion

TIMP-2, as a multifunctional protein, is secreted to regulate proteinaceous ECM homeostasis and involves in a wide range of physiological processes, including in immune response during health and disease [4, 8, 26]. In the present study, the partial cDNA of TIMP-2 was obtained from red seabream muscle, which is composed of 585 bp nucleotides and codes 194 amino acids residues (Fig. 1A). The deduced amino acid sequence shares high similarity with TIMP-2as from other teleost fish (97.50%–91.24%) [16–18,27], while it shows relatively low homology to TIMP-2bs from the same species (70.33%–63.21%) [16–18,27], as shown in Fig. 1B. The homology analysis of TIMP-2s among species reveals that the cloned gene from red seabream muscle is TIMP-2a (rTIMP-2a). After the expression and purification of objective gene with 6-His-Tagged using expression vector pPIC9K and Ni-NTA Sepharose column, it was 23 kDa band in SDS-PAGE stained with CBB (Fig. 2B) or western blotting analysis by mouse anti-6 × histidine monoclonal antibody (Fig. 4A). The MALDI-TOF MS further presented its accurate molecular mass was 23,397.03 Da (Fig. 3A), which is in close agreement with the theoretical value (23,368 Da). A slight discrepancy between these two molecular masses might be attributed to the formation of disulfide bonds or mistranslation of an amino acid residue [28]. The molecular mass of r-TIMP2a is also similar as those of other TIMP-2s from teleost fish (21–23 kDa), such as Atlantic cod (21 kDa) [29], Pacific oyster (23 kDa) [30], zebrafish (21 kDa) [11]. All these results revealed that the purified protein was the objective rTIMP-2a. Actually, the similar gene has been cloned from red seabream embryo cell [22]. In previous study, the recombinant GST tagged TIMP-2 was expressed in the form of inclusion using expression vector pGEX-6P-1, which was treated with buffer containing 8 M urea to obtain the soluble protein. This extreme way also negative impact its inhibitory activity. Furthermore, TIMP-2 tagged with GST in N-terminal domain did not showed inhibitory activity only if the protein was cleaved with PreScission proteinase to remove its GST tag [25]. To overcome this shortcoming, the $(His)_6$-tagged rTIMP-2a was expressed using constructed expression vector pPIC9K-rTIMP-2a in our study. The advantage of this method is that rTIMP-2a is soluble and it tagged His in C-terminal domain instead of N-terminal domain. It is speculated that the N-terminal domain of TIMP-2s is crucial to its interaction with MMPs [14]. The similar soluble protein was also expressed for human TIMP-2-6XHis-tagged protein using HEK-293-F cell [31]. Thus, the new constructed expression system is more suitable for soluble rTIMP-2a from red seabream muscle to perform its inhibitory function. Furthermore, more information about the properties of rTIMP-2a have been revealed. The pI of rTIMP-2a (6.50) from red seabream (Fig. 3B) is similar to the theoretical pI of TIMP-2as from fugu rubripes (6.52) [27] and Japanese flounder (6.42) [16,17], but different from TIMP-2a of gilthead seabream (5.76) [18]. Moreover, it is also different from TIMP-2bs of fugu rubripes (7.42) [27], Japanese flounder (5.16) [16,17] and gilthead seabream (5.44) [18], which might be attributed to their amino acid sequences.

In addition, the conformation structure or structural motifs is more important factor to a mature TIMP-2a [13,30]. Formation accurate disulfide bonds is critical for TIMPs production to solve protein solubility because of improper or incomplete folding [32]. According to the crystal structure of human TIMP-2, the conserved 12 cysteine residues have able to form six disulfide bonds that fold the protein into two domains each containing three loops, including a N-terminal domain (loop 1–3) and a C-terminal domain (loop 4–6) [33]. The similar 12 conserved amino acid residues was also found in our rTIMP-2a sequence marked with asterisks in Fig. 1a, as same as TIMP-2s from other

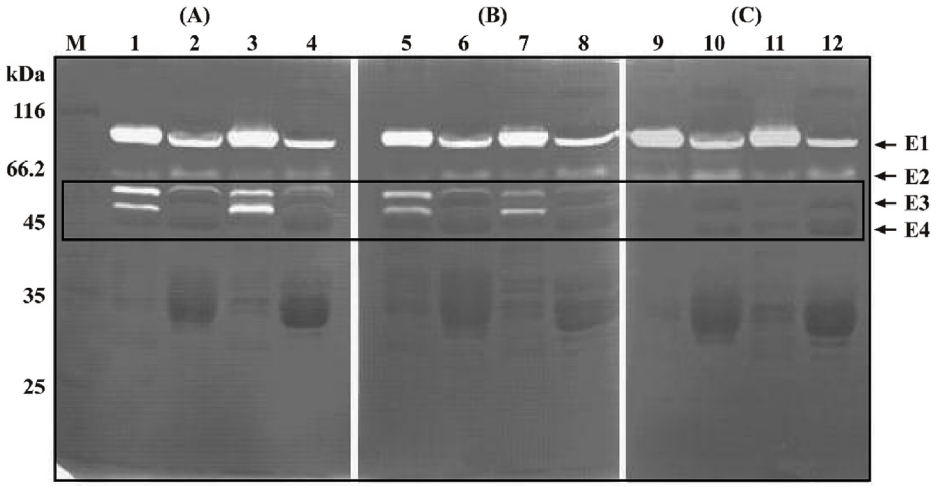

Fig. 5. Inhibitory effect of rTIMP-2a on activity of sarcoplasmic proteinases in red seabream skeletal muscle using gelatin zymography. (A) control (buffer C); (B) buffer C containing 0.3 μmol/L rTIMP-2a; (C) buffer C containing 10 mmol/L EDTA. Gelatin-incorporated gels could incubate with different reagents at 37 °C for 15 h, respectively, followed by CBB staining. Lane M, protein marker; Lanes 1 and 3, sarcoplasmic proteinases of dark muscle were treated with 0 and 2 mmol/L APMA at 37 °C for 3 h, respectively; Lanes 2 and 4, Sarcoplasmic proteinases of white muscle were treated with 0 and 2 mmol/L APMA for 3 h at 37 °C, respectively.

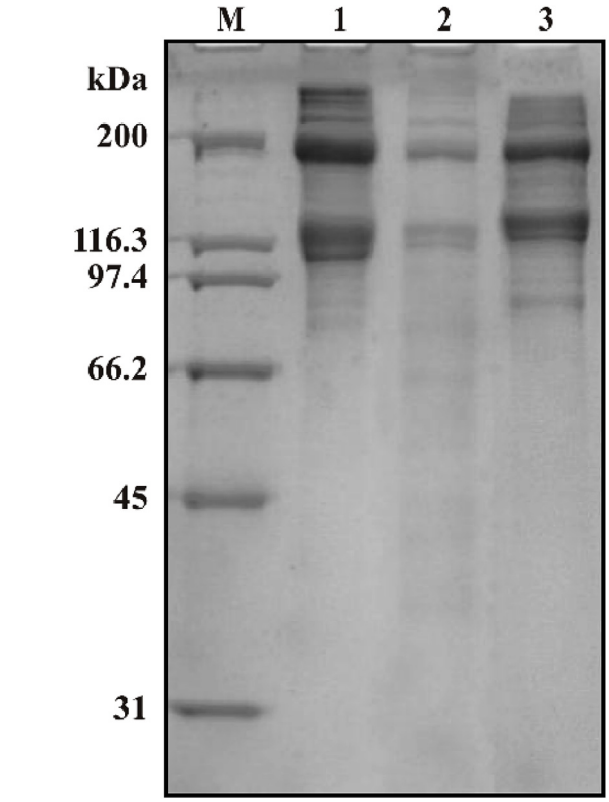

Fig. 6. Inhibitory effect of rTIMP-2a on type I collagen degradation of red seabream muscle produced by endogenous gelatinolytic proteinase. Lane M, broad protein marker; Lane 1, control, sample without incubation; Lane 2, sample incubated at 37 °C for 3 h; Lane 3, sample incubated in buffer C containing rTIMP-2a at 37 °C for 3 h.

teleost fishes [16,27]. As shown in Fig. 1a, rTIMP-2a have potential to form intramolecular disulfide bonds and six loops structures (loop 1–6), which are Cys1-Cys72, Cys3-Cys101, Cys13-Cys126, Cys128-Cys175, Cys133-Cys138 and Cys146-Cys167, respectively. However, the cleavage and formation of disulfide bonds of rTIMP-2a are not clear. Three bands of 21 kDa, 23 kDa and 46 kDa were observed for mature rTIMP-2a protein in Fig. 4A. The intermolecular disulfide bonds contributed to form the dimer of 46 kDa band. Furthermore, it seems that the intramolecular disulfide bonds of rTIMP-2a is more complicated. To investigate the cleavage and formation of disulfide bonds of rTIMP-2a, it was mixed with disulfide reducing reagents (DTT and β-ME). DTT and β-ME are the most commonly used reagents for the reduction of disulfide bonds [28,34,35]. The results displayed the change of electrophoretic migration rates on SDS-PAGE and revealed that the molecular mass of rTIMP-2a had significantly increased after the cleavage of disulfide bonds as shown in Fig. 4B, which was also presented when the concentration of β-ME increases from 0 to 0.05% in more detail (Fig. 4C and D). The molecular mass of rTIMP-2a treated with 0.03% of β-ME dramatically increased and presented bands from 21.39 kDa to 23.50 kDa. Moreover, it showed molecular mass of 25.03 kDa when the addition of β-ME was 0.5%. During the reduction process of β-ME, the intermediate mixed disulfide is produced, resulting in a molecular mass increase of the protein of 78 Da per disulfide bond [28]. It is reasonable to presume that the effect of β-ME on the molecular mass of rTIMP-2a is obviously insignificant. This behavior of molecular mass change is probably and mainly because that the breaking of disulfide bonds decrease of loop structure and increase structural flexibility of rTIMP-2a [14,34]. The first fast and then slow down change tendency of molecular mass of r-TIMP-2a was also found in Fig. 4C and D, which might be associated with the loop size formed by disulfide bonds, resulting in different degree of conformational structure change. According to the computational structure of human TIMP-2, the size of loops 1–3 (Cys1-Cys72, Cys3-Cys101, Cys13-Cys126) for r-TIMP2a in N-terminal domain is larger than those three loops in C-terminal domains [33].

The major physiological function of rTIMP-2 is considered as important factor to regulate the activity of gelatinolytic MMP and further involve in collagen metabolism of muscle [5,8]. Actually, various MMPs participate in the suppression or regulation type I collagen degradation or metabolization of teleost fish, such as grass carp (*Ctenopharyngodon idellus*) [7], common carp (*Cyprinus carpio*) [36] and red seabream (*Pagrus major*) [37]. The inhibition activity of red seabream recombinant TIMP-2a of gelatin hydrolysis with recombinant Japanese flounder MMP-9 has been revealed [22]. However, the inhibitory effect of recombinant TIMP-2a on other endogenous gelatinolytic MMPs, including MMP-2, is not clear. Thus, the endogenous gelatinolytic MMPs E3 of 60 kDa and E4 of 55 kDa from red seabream muscle, activated by APMA and inhibited by EDTA, respectively, were found in our study (Fig. 5). It is reported that they belong to MMP-2 and MMP-9, respectively, and could hydrolyzed effectively gelatin and type I collage in our previous studies [6, 38]. These gelatinolytic MMPs presented obvious type I collagen proteolysis and this proteolysis could be strongly inhibited by rTIMP-2a in Fig. 6, indicating that rTIMP-2a was successfully folded to the correct three-dimensional structure and showed relatively high inhibitory activity to suppress the degradation of type I collagen induced by these MMPs. All these results suggest that the rTIMP-2a is effective inhibitor to involve in collagen metabolism regulated by gelatinolytic MMPs.

5. Conclusion

In conclusion, a rTIMP-2a gene was successfully cloned from red seabream muscle. Using a new constructed expression vector pPIC9K-rTIMP-2a, the purified rTIMP-2a with molecular mass of 23 kDa showed high inhibitory activity to gelatinolytic MMPs during gelatin and type I collagen degradation. Furthermore, the formation of 6 disulfide bonds might contribute to conformational stability of mature rTIMP-2a and its active region. Our results strongly suggested that TIMP-2a might involve in immune response of ECM homeostasis for teleost fishes.

Declaration of competing interest

There are no conflicts to declare.

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