Journal of Biotechnology 177 (2014) 45–52

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Expression and characterization of common carp (*Cyprinus carpio*) matrix metalloproteinase-2 and its activity against type I collagen

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ARTICLE INFO

Article history:
Received 13 December 2013
Received in revised form 19 February 2014
Accepted 26 February 2014
Available online 6 March 2014

Keywords:
Matrix metalloproteinase-2
Common carp
Cloning
Catalytic domain
Gelatin zymography
Collagen

ABSTRACT

Matrix metalloproteinases (MMPs) play essential roles in the metabolism of animal collagen while few reports are available for MMPs in aquatic animals. In this study, we report the complete sequence of matrix metalloproteinase-2 (MMP-2) gene from common carp (*Cyprinus carpio*) skeletal muscle. The full-length cDNA of MMP-2 was 2792 bp which contains an open reading frame of 1974 bp, corresponding to a protein of 657 amino acid residues. Based on the structural feature of MMP-2, the gene of the catalytic domain containing 351 amino acid residues was cloned and expressed in *Escherichia coli*. SDS-PAGE showed that the truncated recombinant MMP-2 (trMMP-2) with molecular mass of approximately 38 kDa was in the form of inclusion body. The trMMP-2 was further purified by immobilized metal ion affinity chromatography. After renaturation, similar to native MMP-2, the trMMP-2 exhibited high hydrolyzing activity toward gelatin as appeared on gelatin zymography and optimal activity was at pH 8.0 and 40 °C. The activity of the trMMP-2 was completely suppressed by metalloproteinase inhibitors, including EDTA, EGTA and 1,10-phenanthroline while other proteinase inhibitors did not show any inhibitory effect. Divalent metal ion Ca^{2+} was necessary for the gelatinolytic activity, suggesting it is a calcium-dependent metalloproteinase. Moreover, the trMMP-2 effectively hydrolyzed native type I collagen at 37 °C and even at 4 °C, implying its potential application value as a collagenase for preparation of biologically active oligopeptides.

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1. Introduction

Matrix metalloproteinases (MMPs) are a family of endopeptidases commonly known for their abilities to cleave components of the connective tissue in physiological and pathological processes (Visse and Nagase, 2003). Based on their substrate specificities and sequence characteristics, they can be grouped into six main subclasses: collagenase, gelatinase, stromelysins, matrilysins, membrane-type MMPs, and others (Nagase et al., 2006). Similar to other MMPs, MMP-2 is produced in a latent form (proMMP) requiring activation and is inhibited by TIMPs (Fedarko et al., 2004). MMP-2 as gelatinase A, is secreted as a zymogen of 72 kDa and activated after removal of the inhibitory propeptide (Ra and Parks, 2007). A unique characteristic of MMP-2 is its ability to degrade type I, II, III and IV collagens, as well as troponin I, myosin light chain and poly (ADP-ribose) polymerase, which may cause cardiac dysfunction (Nagase et al., 2006).

Though MMP was first identified on the basis of its critical role in amphibian metamorphosis, most researches on MMPs are focused on mammalians and their physiological roles have been reviewed (Page-McCaw et al., 2007). For MMP study in fisheries, the cDNA of MMPs have been cloned from zebrafish (*Danio rerio*) (Zhang et al., 2003), Japanese flounder (*Paralichthys olivaceus*) (Kinoshita et al., 2002), grass carp (*Ctenopharyngodon idella*) (Xu et al., 2012), Atlantic salmon (*Salmo salar*) (Leong et al., 2010) and rainbow trout (*Oncorhynchus mykiss*) (Saito et al., 2000a, 2000b). We have purified such proteinases both from common carp (*Cyprinus carpio*) dark muscle (Wu et al., 2008) and red sea bream (*Pagrus major*) skeletal muscle (Wu et al., 2010), which could hydrolyze native type I collagen effectively.

In general, MMPs contain a signal peptide, a propeptide, a catalytic domain and C-terminal hemopexin-like domain (Nagase, 1999). From the X-ray three dimensional structure of human pro-matrix metalloproteinase-2, the catalytic domain connects with fibronectin-like domain and hemopexin-like domain through some peptide loops (Morgunova et al., 1999). The N-terminal propeptide

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http://dx.doi.org/10.1016/j.jbiotec.2014.02.019
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of MMPs is completely removed during their activation and the hemopexin-like domain involves in binding to TIMP-2 (Fridman et al., 1992). Several reports have indicated that the fibronectin-like domain plays a crucial role for properly orienting the native substrate relative to the active site of gelatinase (Steffensen et al., 1995; Ye et al., 1995), suggesting that the catalytic domain could function as an active enzyme independent of other domains. Steffensen et al. (1995) expressed a 21 kDa fragment of MMP-2 consisting of three repeated fibronectin type II-like modules in *Escherichia coli* and purified it from inclusion bodies. Cheng et al. (2003) expressed and purified the catalytic domain of gelatinase A with and without fibronectin insert domain. The absence of the fibronectin-like domain in MMP-2 resulted in decreased digestion rates apparently as tested using fluorogenic substrates. More recently, the full-length human MMP-2 was successfully expressed in the soluble fraction and was functional in gelatinolytic fluorometric assay, suggesting its correct folding (Gonçalves et al., 2012).

Rapid degradation of fish muscle could be observed during post-mortem storage due to endogenous proteolytic enzymes acting on muscular cells and connective tissues. These proteinases include gelatinases (Kubota et al., 2000), cathepsins and myofibril-bound serine proteinases (Zhong et al., 2012). MMPs have been proposed to participate in the metabolism of collagen and in the post-mortem degradation of fish muscle causing texture profile decreasing (Lødemel et al., 2004; Saito et al., 2000a,b). Collagens play important roles in meat texture formation and contribute a fixed amount of background toughness to meat (Alderton et al., 2004; Kubota et al., 2001). The gelatinase activation has been completely performed when muscle turned to be soft while the collagens reveal less stable and more disorderly structures (Martinez et al., 2011; Moreno et al., 2012), which then can be easily degraded to small peptides by gelatinases such as MMP-2 and MMP-9.

In order to elucidate the interaction between collagen and MMPs, especially their roles in physiological processing of fish muscular proteins, it is necessary to obtain a highly purified and active MMP(s). Because of its extremely low content in fish muscle together with a complicated purification process, it is quite difficult to obtain such proteinase in native form. Also, some other molecules such as fibronectin may be co-purified with MMP on gelatin columns (Steffensen et al., 2011). Due to these limitations, we tried to express the catalytic domain of MMP-2 with three fibronectins containing 351 amino acids in *E. coli*, and characterize its biochemical properties after refolding. The action of truncated recombinant MMP-2 on collagen was also investigated.

2. Materials and methods

2.1. Bacterial strains and materials

E. coli Top10 (saved in our laboratory) was used for cloning. BL21 (DE3) was used for expression. Vector pMD-18T, rTaq polymerase and DNA marker were purchased from Takara Biotechnology (Dalian, China Co. Ltd). The expression plasmid pET28a(+) and IPTG were obtained from Invitrogen (Carlsbad, CA, USA). Ni-NTA resin and protein marker were purchased from Tiangen (Beijing, China).

2.2. Cloning of MMP-2 cDNA

Total RNA was extracted by modified Trizol extraction procedure from common carp skeletal muscle. The first strand cDNA was synthesized with TIANScript RT Kit (Tiangen, Beijing, China) according to the manufacture's instruction. Based on the sequence of grass carp MMP-2 (GenBank: HQ153830.1), some primers (MMP2-F1, MMP2-R1, MMP2-R2) were designed (Table 1). Using these primers and cDNA synthesized, a fragment of the MMP-2 gene of approximately 1400 bp was amplified by PCR. The PCR product was purified and cloned into pMD-18T vector for DNA sequence analysis.

According to sequencing result of the above PCR product, specific oligonucleotide primers were designed for 5′-RACE (MMP2-5F1, MMP2-5F2) and 3′-RACE (MMP2-3R1, MMP2-3R2, P2-R1). All the primers were shown in Table 1. RACE and RACE-PCR were conducted using the SMART RACE Amplification Kit (Clontech, CA, USA). Nested-PCR was adopted to improve the specificity of SMARTer RACE amplification and the program used for 5′-RACE and 3′-RACE was performed as following: 10 min at 94 °C followed by 30 cycles of 50 s at 94 °C, 55 s at 50 °C, 60 s at 72 °C, and a final extension of 7 min at 72 °C. The PCR products, 5′-RACE (estimated 1400 bp) and 3′-RACE (estimated 430 bp) were then purified, cloned and sequenced. DNA sequencings were carried out by a DNA sequencer ABI PRISM 3730 (CA, USA). Protein sequence analysis was performed using DNAMAN software for multiple sequence alignments with proteins that are available from GenBank using the BLAST program (NCBI).

2.3. Construction of expression plasmids

To make an expression plasmid encoding the catalytic domain of MMP-2, the corresponding DNA fragment was amplified by PCR using cDNA as template. A pair of specific primers MMP2-F 5′-CATATGAATTTCTTTCACAGGAAG-3′ (containing the *Nde* I site) MMP2-R 5′-GGATCCTTATGTGACCGTTGGGAGTTC-3′ (containing the *Bam*H I site) were designed. Stop codon "TTA" was added into the reverse primer. The PCR product was cloned into pMD-18T vector for sequencing. Both the positive plasmid and the plasmid pET28a(+) were digested by *Nde* I and *Bam*H I. Then the truncated MMP-2 gene fragment and the linearized plasmid pET28a(+) purified by Tiangen universal DNA purification kit were ligated at 16 °C overnight. The ligated product was transformed into the competent *E. coli* TOP10 cells and transformants were screened for kanamycin resistance. Plasmid DNA from resistant colonies was confirmed by restriction enzyme digestion and sequencing. The truncated recombinant MMP-2 (trMMP-2) plasmid containing the catalytic domain gene was named as pET28a-trMMP-2.

To examine the enzymatic properties of MMP-2 containing different domains, we established other two expression constructs of fibronectin-like domain (trMMP-2-Fnt) and the catalytic domain with deletion of fibronectin-like domain (trMMP-2-ΔFnt). The coding region of trMMP-2-Fnt was amplified by PCR (forward primer, 5′-CATATGCAAGTGGTAAAGGTGA-3′; reverse primer, 5′-GAATTCTTATAGGAATAGACTGTAGC-3′;) from the plasmid pET28a-trMMP-2. To obtain the trMMP-2-ΔFnt, we amplified the N-terminal and C-terminal sequences from the plasmid pET28a-trMMP-2 with primers (MMP2-F, ΔFnt-R 5′-CCAGGGCATGTGTCCAGAGC-3′) and primers (MMP2-R, ΔFnt-F 5′-CTCTGGACACATGCCCTGG-3′) respectively. Then, full-length trMMP-2-ΔFnt sequence was amplified by splicing the fragments of N-terminal and C-terminal sequence with primers (MMP2-F, MMP2-R).

2.4. Expression and purification of truncated recombinant proteins

Recombinant plasmids were transformed into *E. coli* BL21(DE3) to yield expression strains BL21. One positive clone of *E. coli* BL21 (DE3) carrying the recombinant plasmids were inoculated into 2 mL Luria broth (LB) medium containing 100 μg/mL kanamycin and incubated at 37 °C overnight, respectively. Then the cells were diluted 100-fold in 200 mL LB medium containing 100 μg/mL kanamycin. Cells were grown at 37 °C while shaking until the optical density at 600 nm reached 0.6 followed by addition of IPTG

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