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Matrix metalloproteinase 2 fused to GFP, expressed in *E. coli*, successfully tracked MMP-2 distribution *in vivo*

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ABSTRACT

Matrix Metalloproteinases (MMPs) participate in many physiological and pathological processes. One major limitation to a better understanding of the role MMPs play in these processes is the lack of well-characterized chimeric proteins and characterization of their fluorescence. The specialized literature has reported on few constructs bearing MMPs fused to the sequence of the green fluorescent protein (GFP), but none of the described constructs have been intended for expression in bacteria or for purification and use *in vivo*. This work has tested a recombinant reporter protein containing the MMP-2 catalytic domain fused to GFP in terms of purification efficiency, degradation of substrates in solution and in zymograms, kinetic activity, GFP fluorescence, and GFP fluorescence in whole animals after injection of the purified and lyophilized fluorescent protein. This work has also characterized rhMMP-2 (recombinant human MMP-2) and inactive clones and used them as negative controls in experiments employing catMMP-2/GFP and rhMMP-2. To our knowledge, this is the first study that has fully characterized a chimeric protein with the MMP-2 catalytic domain fused to GFP, that has efficiently purified such protein from bacteria in a single-step, and that has obtained an adequate chimeric protein for injection in animals and tracking of MMP-2 fate and activity *in vivo*.

1. Introduction

Matrix Metalloproteinases (MMPs) are an important class of proteinases. They act on a broad range of substrates and participate in a myriad of physiological processes such as embryonic development, morphogenesis, angiogenesis, ovulation, cervical dilation, bone tissue remodeling, wound healing, and apoptosis [1,2] and pathological processes, particularly in vertebrates, including arthritis, fibrosis, tumor growth and metastasis, nephritis, neurological and periodontal diseases, hypertension, and atherosclerosis [1,3–5].

Excessive amounts of MMPs emerge in plasma during the course of many different diseases [3,6], from inflammatory [7–9] to neoplastic conditions [10–12], including an array of immunological disorders [13]. MMPs have a significant part in changes to structural proteins and cell-to-cell and cell-to-matrix messages (and *vice-versa*) [14]. MMP inhibitors can significantly reduce damage to a number of organs in certain diseases [15–18]. Nonetheless, the scarcity of good models to mimic increased MMP levels in plasma has prevented clarification of the role played by specific MMPs. Despite the publication of papers on molecular tools such as reporter proteins containing the catalytic domain of MMPs, MMP characterization regarding kinetic parameters (which are essential to understand the catalytic function and observe the effect of catalytically active MMPs) is still lacking. In other words, the paucity of thoroughly characterized chimeric proteins with catalytic activity appears to hinder insight into MMP involvement in physiological and pathological processes.

MMPs share a common domain structure that consists of a signal peptide, a propeptide, a catalytic domain, a hinge region, and, in the majority of the cases, a C-terminal domain [2]. In the early 1990s, researchers classified MMP-2 as gelatinase A. Enzymes of this type bear an insert of three fibronectin type II repeats in their catalytic domain, which generates a collagen-binding domain that links to and degrades type IV collagen or denatured collagens (gelatins)

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[19,20]. Thus, gelatinases can be purified in affinity columns containing gelatin bound to sepharose beads.

To pinpoint the particular changes due to excessive MMP-2 in different conditions, it is crucial to develop models that involve MMP-2 addition without major interferences with physiological cell responses, as it is the case with transgenes that express higher than normal levels of MMP-2 or animals in which MMP-2 has been knocked out. For instance, the validity of experiments conducted on MMP knockouts has been seriously challenged because of an increase of several other MMPs to compensate for the MMP knocked out [21–23]. Hence, availability of a well-characterized catalytically active fluorescent MMP-2 expressed in and purified from *Escherichia coli* seems to be critical to continue our investigations into the effect of MMP-2 on the cardiovascular system. Indeed, an injectable recombinant purified protein is easy to use and offers advantages such as the absence of co-purified inhibitors and proteinases in the final protein preparation.

In this context, this study aimed to obtain a recombinant reporter clone of the MMP-2 catalytic domain inserted in a framework bearing the GFP sequence and to characterize it in detail both *in vitro* and *in vivo*. To this end, this work has dealt with the detailed characterization of the chimera alongside the characterization of rhMMP-2 in terms of purification efficiency, degradation of substrates in solution and in zymograms, kinetic activity parameters, GFP fluorescence, and GFP fluorescence in whole animals after injection of the purified and lyophilized fluorescent protein. Characterization of inactive clones enabled their use as negative controls in experiments employing catMMP-2/GFP and rhMMP-2.

2. Materials and methods

2.1. Steps to construct the recombinant reporter plasmids

The human MMP-2 catalytic domain was amplified by polymerase chain reaction (PCR); the primer sequences were as follows: forward - TACAACTTCTTCCCTCGCAAGC; reverse - GCCCCATAGAGCTCCTGAAT. These nucleotides encoded the amino acids at position 110-446 (cDNA sequence: genbank accession n. BC002576.2) (**Y**NFFPRKPKWDKN. . .RLSQDDIKGIQELY**G** – first and last amino acids). Fig. S1 in the Supplementary material in the online version at DOI:10.1016/j.ijbiomac.2016.05.013 depicts the full human MMP-2 catalytic domain sequence.

The PCR products were inserted into the pGEM T-easy vector (Promega, Madison, WI, USA) with the aid of T4DNALigase (New England Biolabs, Ipswich, Massachusetts, USA). The recombinant plasmid was inserted in *E. coli* (DH5*alpha*) host cells by heat shock [24], and DNA was extracted from colonies by employing the Qiagen plasmid kit (Qiagen, Hilden, Germany).

The DNA of this recombinant plasmid was digested with the restriction endonucleases *Sph*I and *Sal*I. The resulting fragment containing the human MMP-2 catalytic domain was inserted into the pGFP vector (Clontech, Mountain View, CA, USA) 3′MCS, which had been opened *via* double digestion in the presence of *Sph*I and *Sal*I. The sequence encoding the rhMMP-2 catalytic domain was inserted in frame with the GFP reporter gene, to generate catMMP-2/GFP clones (Fig. S2 in the Supplementary material at DOI:10.1016/j.ijbiomac.2016.05.013). This recombinant plasmid was also used to transform *E. coli* by heat shock [24]. The DNA of these clones was extracted, and the positive clones were confirmed by PCR and digestion with *Sph*I/*Sal*I and *Eco*RI endonucleases. The plasmid was transformed in *E. coli* BL21(DE3) competent cells (Stratagene, La Jolla, CA, USA). The cultures were placed in ampicillin 100 μg/mL, and the correct sequence of several clones was initially checked by PCR reaction and digestion with restriction endonucleases and further DNA sequencing (ABI 3500XL, Life Technologies, Carlsbad, CA, USA).

2.2. Generation of catalytically inactive mutant clones

The catalytically inactive mutants, McatMMP-2/GFP and MrhMMP-2, were generated as follows. The mutation was performed using mutated primer pairs, which have three nucleotide mutations at position E^{375} (Glutamic acid residue). This mutation results in the substitution of the glutamic acid for a glutamine residue (Q^{375}) in same position of the sequence (E^{375} to Q^{375}). This position is within the catalytic domain. The mutated sequences of oligonucleotides are shown in bold: *Forward*: 5′ - C GTG GCA GCC CAC **GAC** TTT GGC CAC GCC -3′ e *Reverse*: 5′- GGC GTG GCC AAA **GTC** GTG GGC TGC CAC G.

Crabbe et al. described that the replacement of glutamic acid in the position E^{375} by glutamine (Q^{375}) entails a catalytic site completely inactive and that MMP-2 with this mutation has no catalytic activity [25]. The fibronectin domain sequence remains intact, still providing affinity for gelatin, which is a characteristic used for purification.

The procedure was carried out with the aid of the GeneArt® Site-Directed Mutagenesis PLUS Kit (Life Technologies, Carlsbad, CA, USA). The mutant clones were incubated with gelatin-sepharose (Gelatin Sepharose™ 4B, GE, Uppsala, Sweden) and further submitted to three washings with TNC buffer (Tris-HCl 50 mM, sodium chloride 150 mM, calcium chloride 10 mM, 0.005% Brij 35, pH 7.4) containing the protease inhibitors 1,10 *ortho*-phenanthroline (Phe), phenylmethanesulfonylfluoride (PMSF), and *N*-ethylmaleimide (NEM), all at 2 mM. Three clones from each mutant were evaluated by SDS-PAGE, gelatin zimography, and fluorimetric activity assay.

2.3. Protein expression and purification

The catMMP-2/GFP clone was plated in LB medium with agar (yeast extract 5.0 g/L, NaCl 10.0 g/L, tryptone 10.0 g/L, and agar 15 g/L) containing ampicillin 100 μg/mL, followed by incubation at 37 °C for 12–16 h. One colony was added to 20 mL of LB medium containing ampicillin 100 μg/mL at 37 °C, under shaking at 200 rpm, for 12–16 h (starter culture). Subsequently, 10 mL of the starter culture was added to 1 L of LB medium containing ampicillin 100 μg/mL, which was followed by incubation at 37 °C for approximately 3 h, under shaking at 200 rpm, until an OD_{600} of 0.5–0.7 was reached. Then, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the growth medium, to activate the gene expression under the control of the Lac Z promoter. To induce and express this protein, several concentrations of IPTG (5, 50, and 500 μM) and different times of bacteria collection (6, 8, 10, 12, and 18 h) were tested after IPTG addition at 18 °C, under shaking.

To purify the protein, the cells were harvested by centrifugation at 4000*g*, for 15 min. The bacterial pellet was re-suspended in 20 mL of TNC buffer containing protease inhibitors (Phe, PMSF, and NEM, all at 2 mM) and lysozyme 10 μg/mL, followed by incubation for 30 min on ice. Subsequently, the samples were submitted to 10 cycles of 10 s sonication, with an interval of 10 s between the cycles. The bacterial debris was removed by centrifugation at 18,000*g* for 30 min. The supernatants were applied to a gelatin-sepharose column (Gelatin Sepharose™ 4B, GE, Uppsala, Sweden) and run on an Akta Prime Plus Chromatography system (GE, Uppsala, Sweden) set up inside a refrigerator. The column was washed once with TNC buffer, followed by one washing with Triton X-114 (0.1%, v/v), to remove LPS from the bacterial cell walls [26]. The column was further washed with TNC, and the protein was finally eluted with DMSO (5%, v/v). Samples from all the fractions were collected and evaluated against MMP-2 and GFP on zymograms, silver-stained SDS-PAGE gels, and western blots. The protein was