Enzyme and Microbial Technology 92 (2016) 9–17

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Trivalent metal ions based on inorganic compounds with in vitro inhibitory activity of matrix metalloproteinase 13

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ARTICLE INFO

Article history:
Received 3 February 2016
Received in revised form 7 June 2016
Accepted 10 June 2016
Available online 16 June 2016

Keyword:
Collagenase-3 catalytic domain
Metal ions
Protein refolding
Circular dichroism spectral
Isothermal titration calorimetry
Endothelial tube formation

ABSTRACT

Collagenase-3 (MMP-13) inhibitors have attracted considerable attention in recent years and have been developed as a therapeutic target for a variety of diseases, including cancer. Matrix metalloproteinases (MMPs) can be inhibited by a multitude of compounds, including hydroxamic acids. Studies have shown that materials and compounds containing trivalent metal ions, particularly potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$), exhibit cdMMP-13 inhibitory potential with a half maximal inhibitory concentration (IC_{50}) of 1.3 μM. The target protein was obtained by refolding the recombinant histidine-tagged cdMMP-13 using size exclusion chromatography (SEC). The secondary structures of the refolded cdMMP-13 with or without metal ions were further analyzed via circular dichroism and the results indicate that upon binding with metal ions, an altered structure with increased domain stability was obtained. Furthermore, isothermal titration calorimetry (ITC) experiments demonstrated that $K_3[Fe(CN)_6]$is able to bind to MMP-13 and endothelial cell tube formation tests provide further evidence for this interaction to exhibit anti-angiogenesis potential. To the best of our knowledge, no previous report of an inorganic compound featuring a MMP-13 inhibitory activity has ever been reported in the literature. Our results demonstrate that $K_3[Fe(CN)_6]$ is useful as a new effective and specific inhibitor for cdMMP-13 which may be of great potential for future drug screening applications.

1. Introduction

Matrix metalloproteinases (matrixins or MMPs) represent a family of zinc metallproteinases that is capable of degrading and remodeling structural proteins in the extracellular matrix such as gelatin, elastins, collagens, matrix glycoproteins and proteoglycan. MMPs play a major role in almost all aspects of cell behavior and belong to the metzincin superfamily of metalloproteinases. The latter is characterized by the presence of a divalent zinc atom (Zn^{2+}) in the active center of the enzyme. The typical structure of MMPs consists of a signal peptide (SP), a catalytic domain and a haemopexin-like domain [1]. The Zn^{2+} binding site in the catalytic core domain of all MMPs features a HEXXHXXGXXH amino acid, and the individual sequences bear high similarity in their 3D structures. The pocket in the core domain is hydrophobic in nature, but variable in depth depending on the MMPs, representing a determining factor of substrate specificity of MMPs. In fact, the S1′ pocket in the catalytic domains of MMPs has been reported to be particularly important. The depth and the length of the S1′ pocket and amino acids all represent critical foundations for the design and synthesis of matrix metalloproteinase inhibitors (MMPIs) [2–6].

MMPs mainly regulate the synthesis and secretion of cytokines, growth factors, hormone receptors, and cell adhesion molecules. Moreover, MMPs are also found to be critically involved in processes including tissue remodeling, angiogenesis, arthritis, cardiovascular disease, stroke, multiple sclerosis, neurodegenerative diseases, and allergies [7,8]. In tumor progression, MMPs play a key role not only in invasion processes, angiogenesis, and metastasis, but also in cancer cell transformation, growth, apoptosis, signal transduction and immune regulation [9,10]. It has been shown before that the secretion of MMPs by microvascular endothelial

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http://dx.doi.org/10.1016/j.enzmictec.2016.06.006
0141-0229/

cells represents a critical step in the formation of new blood vessels and most of the MMPs may initiate and promote angiogenesis [11,12]. In recent years, research groups around the globe have focussed on finding suitable inhibitors for MMPs to treat cancer or other related serious health conditions [13–18]. The activities of MMPs can be regulated by endogenous inhibitors, such as tissue inhibitors of metalloproteinase (MMPIs) [19–21]. In general, MMPIs can be classified as follows: natural MMPIs secreted by tissues, synthetic MMPIs, MMPIs screened from natural products and MMPIs screened from phage. Originally, the first generation of MMPIs, used as peptides or derivatives, has been designed to simulate the substrate of MMPs. Inhibition is achieved by competing chelation of the central zinc ion (Zn^{2+}) in MMP by ligands present in the inhibitors, e.g. hydroxylamine, carboxyl, thiols, etc [22]. In recent years, synthetic MMPIs have been used in clinical trials for MMP inhibition in the treatment of progressive lung cancer. However, due to a variety of side effects caused by the use of synthetic small molecule inhibitors in clinical applications, research has now focussed on finding MMPIs derived from natural products [23–25]. Anti-MMPs properties have been reported from the components of various natural products. For example, epigallocatechin gallate (EGCG) extracted from green tea exhibits a potent MMPI activity [26,27]. The Korean monkshood root (*Guanbaifu* in Chinese), is commonly used in the treatment of many diseases such as arthralgia, headache, convulsive epilepsy, coronary heart disease, ischemic arrhythmia, pyocutaneous disease and anemogenous phlegm. It has been demonstrated to exhibit a strong inhibitory effect on different MMPs, such as MMP-2 and MMP-9 [28–30]. The active compound featuring an inhibitory effect on MMPs extracted from the Korean monkshood root was identified as aluminum ammonium sulfate dodecahydrate ($NH_4Al(SO_4)_2{\cdot}12H_2O$). Further experiments have confirmed that the aluminum(III) ion (Al^{3+}) is responsible for the inhibitory activity. This was the first time that an inorganic substrate, i.e. aluminum ammonium sulfate, has been demonstrated to exhibit MMP inhibitory activity, potent enough to interfere with MMP related cellular processes [31].

Human matrix metallopeptidase 13 (MMP-13), also known as collagenase-3, represents a key member of the MMP family and has been identified as an important target for the treatment of osteoarthritis (OA) [32–35]. Until now, MMP-13 inhibitors have been identified mainly viahigh-throughput screening (HTS) in the synthetic compound library. Hydroxamic acid is a typical inhibitor for MMP-13 and the reaction mechanism for this activity has been suggested to be due to a functional group that is able to coordinate the central zinc(II) ion to two oxygen atoms via a bidentate ligand interaction [36–39]. In our previous work, Al^{3+} has been shown to exhibit strong inhibitory effects on both MMP-2 and MMP-9 using a HT1080 cell line [31]. We are now interested to determine whether Al^{3+} or other metal ions would exhibit similar effects on the catalytic domain in MMP-13 (cdMMP-13) in vitro. Therefore, we further characterized the cdMMP-13 inhibitory activity using different metal ions and analyzed the corresponding active components. In this work, we obtained the target protein by refolding the recombinant histidine-tagged catalytic domain of MMP-13 [40]. Circular dichroism (CD) analysis confirmed that cdMMP-13, desalted with the metal ions, increases the stability of the secondary structure of the protein. By comparison of the inhibitory activity of compounds containing different valence metal ions with a positive control inhibitor (i.e. CL-82198) on cdMMP-13, the half maximal inhibitory concentration value (IC_{50}) showed that trivalent metal compounds in particular exhibit a strong cdMMP-13 inhibition. Compared to other compounds, potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$) has been shown to exhibit the best inhibitory effect [36]. Furthermore, the binding event between $K_3[Fe(CN)_6]$ and cdMMP-13 has been confirmed by isothermal titration calorimetry (ITC) and an endothelial cell tube formation test provided evidence for the notion that this interaction may have a potential effect on anti-angiogenesis.

2. Materials and methods

2.1. Materials

Yeast extract and tryptone were purchased from OXOID (Basingstoke, UK). Isopropyl β-D-1-thiogalactopyranoside (IPTG), kanamycin sulfate, urea, imidazole, acrylamide, tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), N,N,-methylenebisacrylamide and glycine were purchased from Sangon Biotech (Shanghai, China). The markers for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Thermo Scientific (Waltham, MA, USA). Brij-35 was purchased from Amresco (Solon, OH, USA). CL-82198 was purchased from Biovision (San Francisco, CA, USA). DQ-gelatin was purchased from Molecular Probes (Eugene, OR, USA). The *Escherichia coli* (*E. coli*) strain BL21, to express the His-tagged cdMMP-13 catalytic domain, was provided from Xuexun Fang (Jilin University, China). Other reagents used in this series of experiments were of analytical grade and were purchased from Kermel (Tianjin, China). Purified water was prepared using a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.2. Cloning, expression and purification of cdMMP-13

The cdMMP-13 protein was recombinantly produced in the *E. coli* strain BL21 (DE3) with a N-terminal affinity tag using the plasmid pET22b. A detailed method for cloning, expression and purification of cdMMP-13 was carried out as previously reported. cdMMP-13 was cultured in an LB medium containing kanamycin at 37 °C. When OD600 reached 0.8, IPTG was added to the culture medium to reach a final concentration for induction of 1 mM. The induced culture was further incubated for 4 h at 37 °C. The cellswere harvested by centrifugation (8000*g* for 30 min at 4 °C) and resuspended in50 mL of lysis buffer (pH 7.5, 20 mM Tris). The inclusion bodies were recovered by centrifugation at 12,000*g*, rinsed with washing buffer I (20 mM Tris, 1 M urea, 100 mM NaCl, and 1% Trition-100) and washing buffer II (10 times diluted washing buffer I). Finally, the inclusion bodies were solubilized in the solubilization buffer consisting of8 M urea in 20 mM Tris (pH7.5) for 12 h at 4 °C and were clarified by centrifugation at 12,000*g* for 30 min at 4 °C. The clarified supernatant included the target protein.

The clarified supernatant was purified using a HisTrap FF 5 mL column. The column was equilibrated with binding buffer consisting of8 M urea in 20 mM Tris (pH7.5)and 75 mM imidazole [41]. The column was loaded with 1 mL of the clarified supernatant and was washed with the binding buffer to remove the contaminating proteins. The target protein was then eluted by the elution buffer consisting of 8 M urea in 20 mM Tris (pH 7.5)and 300 mM imidazole. All steps were performed at a flow rate of 1 mL/min. The fractions containing the target protein were pooled for renaturation. The overall purity of the purified protein was confirmed by 15% sodium dodecyl sulfate-polyacrylamidegel electrophoresis (SDS-PAGE) and the protein concentration wasdetermined by the Bradford method using bovine serum albumin as a protein standard.

2.3. Refolding of cdMMP-13 using SEC

Direct refolding was performed using a HisTrap desalted 5 mL column [42]. The column was equilibrated with the refolding buffer (pH 7.5, 20 mM Tris, 20 mM $CaCl_2$, 20 μmol $ZnCl_2$). The purified target protein was loaded, and a flow rate of 1.0 mL/min was maintained. The fractions containing cdMMP-13 were detected using a