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Expression, purification, refolding and *in vitro* recovery of active full length recombinant human gelatinase MMP-9 in *Escherichia coli*

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ABSTRACT

Human gelatinase (MMP-9) is a member of matrix metalloproteinases family (MMPs), which has been associated with malignant tumor progression and metastasis by matrix degradation. Herein, active full length recombinant human MMP-9 (amino acid residues 107–707) has been expressed in the form of inclusion bodies in *Escherichia coli* BL21, using pET21a vector. Solubilization of inclusion bodies was carried out in Tris-HCl buffer with 6 M urea, and refolding was performed using dilution and urea gradient methods. Tris-HCl buffer with 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ at pH 7.8 was used as a refolding buffer. Analysis of the structure by fluorescence and far-UV circular dichroism showed a well-formed structure by urea gradient method. Kinetic parameters in refolding conditions of rhMMP-9 were also analyzed, depicting increase in the enzyme's activity without any aggregation.

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases, commonly known for the ability to cleave components of the connective tissue both in the physiological and pathological conditions [1]. MMPs can be grouped into six main subclasses: collagenase, gelatinase, stromelysins, matrilysins, membrane-type MMPs, and others [2]. Gelatinase B/matrix metalloproteinase-9 (MMP-9) is secreted as a complex multi-domain proteolytic enzyme, consisting of a signal peptide (Met1-Ala19), a propeptide (Ala20- Arg106), a catalytic domain (Phe107-Gly223), a three fibronectin repeat domain (Asn224- Cys388), a Zn^{2+}- binding domain (Pro389- Pro447), an O-glycosylated domain (Glu448- Pro511) and a hemopexin domain (Val512- Asp707). MMP-9 is an interesting molecular target in acute inflammation, autoimmune diseases and invasive cancer [3]. Excess MMP-9 activation and expression has been implicated in the breakdown of connective tissue associated with pathological conditions such as arthritis, tumor invasion, metastasis, and multiple sclerosis [4,5]. Any disturbance of the generally well-balanced equilibrium between MMP-9 and its physiological inhibitors such as tissue inhibitor of matrix metalloproteinase-I (TIMP-I), can give rise to occurrence and development of pathological symptoms [6]. MMP-9 is one of the enzymes of neutrophil granules which are released after neutrophil activation [7]. Such sudden massive release of neutrophil MMP-9 within the circulation is not immediately counterbalanced by MMP inhibitors and may contribute to cancer processes [8]. Thus, it could be considered as an attractive target for therapeutic agents. There are several reports on cloning and expression of human MMP-9 [9,10]. Kroger and Tschesche reported cloning of the catalytic domain (AA residues 113–450) of human MMP-9 in pET-12b vector and its expression in *Escherichia coli* [11]. Kridel et al. cloned the catalytic domain of human MMP-9 into pCDNA3. The catalytic domain of MMP-9 was then purified by gelatin Sepharose chromatography [12].

In the present study, *active full length recombinant human* MMP-9 (amino acid residues 107–707) was cloned into pET21a, transformed and expressed in *E. coli* BL-21(DE3). Since the protein was expressed in the form of inclusion bodies, therefore, two different methods (dilution and urea gradient) were applied for the refolding process. Activity and structure of the enzyme in both methods were compared. Intrinsic fluorescence intensity and far-UV CD measurements were used to characterize conformational changes of the enzyme.

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2. Materials and methods

2.1. Materials

Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Restriction endonucleases and other modifying enzymes were obtained from Fermentas (Germany). *Escherichia coli* BL21 (DE3) cells were purchased from Novagen (USA). pET21a (+) vector was procured from GeneCust (Luxembourg). Other reagents (all of analytical grade) were provided from Merck (Darmstadt, Germany).

2.2. Construction of expression plasmid pET21a-active full length rhMMP-9

The optimized rhMMP-9 gene was synthesized and cloned by GeneCust into *NdeI* and *XhoI* restriction sites of pUC57. Then rhMMP9 gene was excised from the plasmid by digestion (*NdeI* and *XhoI* restriction endonucleases) and subcloned into pET21a.

2.3. Protein expression

To express MMP9, pET21a-MMP-9 was transformed into *E. coli* BL21 (DE3), using a routine procedure [13]. A single colony containing plasmid was then cultured in 10 mL Luria–Bertani (LB) medium in the presence of ampicillin (100 μg/mL), at 37 °C for overnight. 5 mL of this preculture medium was used to inoculate 500 mL of 2xYT medium broth (16 g/L of tryptone, 10 g/L of yeast extract, 5 g/L of NaCl, and pH 7.5, adjusted by 6 N NaOH), containing 100 μg/mL ampicillin. Cells were grown at different temperatures (18, 25 and 30 °C), while shaking until the optical density at 600 nm reached 0.8. The expression was induced by addition of IPTG (0.5 and 1 mM), using different times of induction (6 and 22 h). Cells were harvested by centrifugation (8000 × g, 15 min, 4 °C), lysed by sonication in Tris-HCl buffer, and centrifuged again (8000 × g, 20 min, 4 °C). Expressions were determined by SDS–PAGE (12.5% polyacrylamide). 25 μL of each sample was suspended in 5 μL Laemmli sample buffer (5X). 20 μL of this solution was added in each lane and the gel was stained with coomassie brilliant blue R-250 [14].

2.4. Separation of inclusion bodies

Bacterial pellets were resuspended in 20 mM Tris-HCl buffer, pH 7.8, containing 2 M urea, 0.5 M NaCl and 1% Triton X-100 (buffer A). The solutions were sonicated using pulse/rest (20 s). Soluble and insoluble protein fractions were separated by centrifugation (8000 × g, 20 min). Pellets were then washed with buffer B (same as buffer A, with 6 M urea and 5 mM imidazole, without 1% Triton X-100) for 2 h at room temperature. Finally, they were centrifuged (8000 × g, 30 min), and the supernatants were used for purification and refolding.

2.5. Purification and refolding of active full length rhMMP-9

The protein obtained in the previous stage (in denaturant condition, buffer B) was purified on column and refolded with the following methods.

2.5.1. Dilution method

The expressed rhMMP-9 in plasmid pET21a has 6 × His-tag at its c-terminal. Histidine tags have strong affinity for divalent metal ions (such as Ni^{2+} or Co^{2+}). Therefore, the unfolded protein was loaded on Ni–NTA agarose column (Amersham Biosciences), which was previously equilibrated with buffer B. Protein was allowed to bind to the resin for 2 h. The column was then washed by 20 vol of buffer B (containing 20 mM imidazole). Bound rhMMP-9 proteins were eluted with increasing imidazole concentration from 50 to 100 mM. Fractions of the enzyme (100 μL, 0.2 mg/mL) were added dropwise to 1 mL of the refolding buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 10% glycerol, 0.1 g/mL sucrose, pH 7.8), and incubated at 4 °C for 24 h.

2.5.2. Urea gradient method

Similar to dilution method, protein was loaded on Ni-NTA column for 2 h and washed with buffer B. The refolding buffer (20 mM Tris-HCl, 500 mM NaCl, and 5 mM imidazole) was used sequentially with different concentration of urea (6, 4, 2, 0 M), at a rate of 0.7 mL/min on column. Protein was then eluted with 20 mM Tris buffer, containing 500 mM NaCl, 50 and 100 mM imidazole.

To optimize the refolding process, different refolding and elution buffers were prepared by varying concentrations of $CaCl_2$ and $ZnCl_2$. The optimal pH was also determined, ranging from 4.5 to 9.5. Each individual parameter was changed in this set of experiment, while keeping other experimental conditions constant. Specific activity of the refolded rhMMP-9 was also considered to optimize the refolding process on column. Purity of the protein in both methods was ensured by SDS-PAGE according to the Laemmli method [14]. Protein concentration was determined by Bradford method [15].

2.6. Protease activity assay

The hydrolyzing activity of active full length rhMMP-9 was determined using denatured natural substrates such as gelatin. The enzyme (0.1 mg/L in a total volume of 0.44 mL Tris–HCl 20 mM, pH 7.8) was incubated at 37 °C for 1 min. Gelatin solution (1% w/v) was added to the enzyme, to the final volume of 0.5 mL. The reaction mixture was then incubated for 10 min at 37 °C. Reaction was stopped by addition of 0.1 mL TCA (100%), and kept at room temperature for 10 min. Soluble peptides were separated by centrifugation at 8000 × g for 10 min. Absorbance of the soluble peptides in the supernatant was measured at 280 nm. A control assay was performed in the absence of enzyme for all spectrophotometric measurements. One unit of enzyme activity is defined as the amount of enzyme, under given assay conditions that give rise to 1 unit of absorbance at 280 nm per minute of digestion. The number of units of activity per milligram of protein was taken as the specific activity of the enzyme [16].

2.7. Gelatin zymography

For zymography experiments, samples were diluted in sample buffer and prepared for electrophoresis (without reducing agent and boiling step) on a 12.5% SDS-PAGE co-polymerized with gelatin (0.01 g/mL) as the substrate [17]. SDS-PAGE gels were stained with coomassie brilliant blue R-250. Prior to staining, zymograms were washed in two baths of 2% Triton X-100 solution for 1 h at room temperature, followed by incubation in Tris–HCl 20 mM, 5 mM $CaCl_2$ (pH 7.8) at 37 °C for 16 h. Gelatinolytic activities were detected as unstained bands against the background of coomassie blue-stained gelatin.

2.8. Spectroscopic studies

2.8.1. Circular dichroism

Circular dichroism (CD) measurements were conducted using a JASCO (Tokyo, Japan) J-715 spectropolarimeter. For far-UV CD analysis (200–250 nm), the protein concentration was 0.25 mg/mL in 20 mM Tris buffer (pH 7.8). Results were expressed as molar