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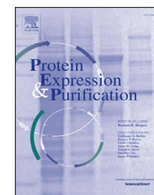

Efficient expression and purification of biologically active human cystatin proteins

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ABSTRACT

Cystatins are reversible cysteine protease inhibitor proteins. They are known to play important roles in controlling cathepsins, neurodegenerative disease, and in immune system regulation. Production of recombinant cystatin proteins is important for biochemical and function characterization. In this study, we cloned and expressed human stefin A, stefin B and cystatin C in *Escherichia coli*. Human stefin A, stefin B and cystatin C were purified from soluble fraction. For cystatin C, we used various chaperone plasmids to make cystatin C soluble, as it is reported to localize in inclusion bodies. Trigger factor, GroES-GroEL, DnaK-DnaJ-GrpE chaperones lead to the presence of cystatin C in the soluble fraction. Immobilized metal affinity chromatography, glutathione sepharose and anion exchange chromatography techniques were employed for efficient purification of these proteins. Their biological activities were tested by inhibition assays against cathepsin L and H3 protease.

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1. Introduction

Cystatins are protein inhibitors of cysteine proteases. They are reported to be reversible and competitive in nature. There are three families of cystatins 1) Stefins: Stefins are unglycosylated intracellular inhibitors having low molecular weight (11 kDa). They lack signal sequence and intramolecular disulfide bonds. Stefin A and stefin B are widely known members of this family. 2) Cystatins: They are extracellular low molecular weight inhibitors and some of them are glycosylated. In contrast to stefins they have signal sequence and intramolecular disulfide bonds. Some well known members of this family are cystatin C, D and SN. 3) Kininogens: They are high molecular weight glycoproteins (68–120 kDa). They are intravascular in nature. This family is represented by low and high molecular weight kininogens [1]. Stefin A is known to play an important role in maintaining normal physiology of skin [2]. Stefin B and cystatin C have broad range of biological roles. For example, stefin B is reported to be involved in protection of cancer cells by enhancing their resistance to oxidative stress and apoptosis [3]. Over expression results in delay of caspase activation in T98G cells and this function of stefin B was found to be independent of cathepsin inhibition [4]. Cystatin C has various functions; anti-inflammatory, anti-viral and anti-bacterial functions [5–7]. Production of soluble cystatins is of great importance as it will help in studying various biological applications of these proteins. There are various reports in which expression and purification of stefin A [8], stefin B [9] and cystatin C [10,11] have been discussed, however the yield is low and purification process involves way too many steps. In this work, we introduced chaperone plasmids as an approach to increase the yield of soluble cystatin protein and optimized a very convenient one step purification protocol. We managed to express high level of stefin A and stefin B and they can be efficiently purified by one step chromatography. All the purified cystatins are found to be very efficient in their biological activities.

2. Materials and methods

Escherichia coli BL21 (DE3) cells (Novagen) were maintained in our lab. HEK 293 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% bovine serum (Gibco) at 37 °C and 5% CO_2. Vectors pGEX-preScission protease and pGEX6P1 were gifted by Dr. Sunando Datta (IISER Bhopal, India). pET 28a vector and pcDNA 3.1-stefin B vector were kind gifts from Dr. Vikas Jain (IISER Bhopal, India) and Dr. Natasa Kopitar Jerala (Jozef Stefan Institute, Slovenia) respectively. The histone H3 construct was kindly provided by Dr. Hitoshi

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Kurumizaka (Waseda University, Japan) Chaperone plasmids (#3340) were purchased from TaKaRa. Restriction enzymes NdeI, BamHI, EcoRI and XhoI were from NEB. Ni-NTA agarose resin was purchased from Qiagen. Glutathione Sepharose resin and Hi Trap™ 5 ml DEAE chromatographic column were purchased from GE healthcare. Enzymes cathepsin L (C6854), thrombin (T4648) and TRI reagent (T9424) were purchased from Sigma. TOPO TA cloning kit (450641) and iScript™ cDNA synthesis kit (170-8891) were purchased from Invitrogen and Bio-Rad respectively. All the primers and plasmids used in this study are shown in Tables 1 and 2 respectively. Bradford's reagent was used for protein quantification.

2.1. Construction of plasmids

Stefin A cDNA was amplified from cDNA of HEK 293 cells. Firstly, RNA was isolated from HEK 293 cells via TRI reagent. iScript™ cDNA synthesis kit was used for synthesis of cDNA from isolated RNA. Stefin A cDNA was cloned into pCR 2.1 TOPO vector. pCR 2.1 TOPO-stefin A vector was digested with NdeI and BamHI restriction enzymes and obtained stefin A gene was further sub-cloned into pET 28a vector at NdeI and BamHI restriction sites. Stefin B was amplified from pcDNA 3.1-stefin B vector and subcloned into pGEX 6P1 vector at EcoRI and XhoI restriction sites. HEK cDNA was used for amplification of cystatin C which was then cloned into pCR2.1TOPO vector. Product of NdeI and BamHI restriction digestion of pCR 2.1 TOPO – cystatin C vector was further sub-cloned into pET 28a vector. Table 1 shows primers used for cloning. Plasmids used in this study shown in Table 2.

2.2. Preparation of histone proteins from chicken brain tissue

Histones were extracted from purified brain nuclei of chicken by hydroxyapatite chromatography as described earlier [12]. Nuclei isolation was done using a method previously described [13]. In short, brain tissue was homogenized using a motor driven Potter–Elvehjem homogenizer in Solution 1 (0.34 M sucrose, 15 mM Tris-Cl pH 7.5, 15 mM NaCl, 60 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.5 mM EGTA, 15 mM β-ME, and 0.2 mM PMSF) to prepare a 10% homogenate. After filtration of homogenate, Triton X-100 was added to the homogenate to a final concentration of 0.25% followed by centrifugation. Nuclei pellets were washed 3 times with Solution 1 to remove detergent. Nuclei were suspended in 10 mM Tris-Cl pH 7.5, 0.35 M NaCl, 0.2 mM PMSF, and 15 mM b-mercaptoethanol to remove non histone proteins. Washed nuclei were hand homogenized in a hypotonic solution (10 mM Tris-Cl, pH 7.5, 15 mM β-ME, and 2 mM PMSF) to prepare soluble chromatin. Soluble chromatin was mixed with hydroxyapatite resin equilibrated in 50 mM sodium phosphate buffer, pH 6.8. The volume was then increased to 10 times (10 ml/g resin) of the initial volume with the same buffer. NaCl was added to the final concentration of 0.6 M. The chromatin mixed hydroxyapatite was washed several times with a large volume of 50 mM phosphate buffer containing 0.6 M NaCl. Total histones were then eluted with phosphate buffer containing 2.0 M NaCl. Eluted histones were desalted by dialysis against 10 mM Tris-Cl, pH 7.5 and precipitated with 3.5 volumes of chilled acetone. The precipitated histones were collected by centrifugation at 10,000 rpm and washed 2–3 times with chilled acetone. The pellet was air dried and dissolved in 10 mM Tris-Cl (pH 7.5).

Table 1
Primers used for molecular cloning.

Primers	Sequence
Stefin A Forward	CATATGATACCTGGAGGCTTATCTGAGGCC
Stefin A Reverse	GGATCCCTAAAGCCCGTCAGCTCG
Stefin B Forward	CTGAATTCATGATGTGCGG
Stefin B Reverse	GTCTCGAGTCAGAAATAGGTCA
Cystatin C Forward	GCCATATGAGTCCCGGCAAGCCGCCG
Cystatin C Reverse	GGATCCCTAGGCGTCCTGACAGGTGGATTTC

2.3. Recombinant human histone H3 preparation

pHCE-H3 plasmid was transformed into BL21 (DE3) cells for recombinant human histone H3 expression and purification. Plasmid pHCE-H3 contains highly constitutive promoter for histone H3 protein expression so induction was not required. His tagged H3 was purified by Ni-NTA chromatography as described earlier with some modifications [14]. In brief, O/N grown culture of pHCE-H3 transformed BL21 (DE3) cells (500 ml LB medium having 100 μg/ml of ampicillin) were harvested by centrifugation (4 k rpm, 15 min, 4 °C) and lysed in 10 ml lysis buffer (50 mM Tris-Cl pH 8.0, 500 mM NaCl, 5% glycerol, 1 mM PMSF) by sonication (60% amplitude – 10 s on and 10 s off). After lysis, supernatant and pellet were separated by centrifugation at 4 °C for 15 min at 15 k rpm. Pellet (having histone H3) was further extracted with 10 ml of 7 M guanidine hydrochloride containing lysis buffer and centrifuged again (15 k rpm, 4 °C, 15 min). Supernatant obtained after centrifugation contained histone H3. Supernatant (10 ml) was incubated with 1 ml of Ni-NTA agarose resin, equilibrated with 7 M guanidine hydrochloride lysis buffer, at 4 °C. After 1 h of incubation on rotospin, resin was poured into gravity flow column and washed with 20 ml of washing buffer (50 mM Tris-Cl, pH-8.0, 500 mM NaCl, 5% glycerol, 6 M urea, 5 mM imidazole and 1 mM PMSF). Elution was performed in batch wise manner. Protein was eluted from resin with buffer (50 mM Tris-Cl, pH-8.0, 500 mM NaCl, 5% glycerol, 6 M urea, and 1 mM PMSF) having 50, 100, 200 and 500 mM imidazole sequentially. Volume of each elution fraction was 2 ml. Eluted fractions were dialyzed against buffer (25 mM Tris-Cl pH-7.5, 100 mM NaCl, 1 mM β-ME, 10% glycerol) overnight at 4 °C. Next day, dialyzed protein was centrifuged at 15 k rpm for 15 min at 4 °C to remove precipitates and aliquots were made.

2.4. Histone H3 protease preparation

Protease preparation was done as described earlier [15] with some modifications. Chicken liver microsomal extract was prepared and partially purified by 30% ammonium sulfate precipitation. After overnight dialysis at 4 °C against buffer (25 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM β-ME, 0.2 mM EDTA, 10% glycerol), Superose™6 10/300 GL chromatography was performed at 4 °C in buffer (25 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM β-ME, 0.2 mM EDTA, 10% glycerol) at a flow rate of 0.3 ml/min to further purify H3 specific protease activity.

2.5. In vitro activity assay and activity inhibition assay with H3 protease

Activity assays and activity inhibition assays were performed as described earlier [15,16]. Chicken brain core histones and recombinant human histone H3 were used as substrate for H3 protease activity. For activity assay 0.3 μg of partially purified H3 protease was mixed and incubated with 4 μg of brain core histones or 4 μg of recombinant histone H3 in 20 μl reaction for 1 h at 37 °C. Reaction buffer contains 10 mM HEPES, pH 5.5, 100 mM NaCl, 1 mM β-ME, 0.2 mM EDTA and 10% glycerol. Reaction was stopped by boiling the reaction in SDS-PAGE loading dye. Activity inhibition assay was performed in the similar manner as in vitro H3 clipping assay except increasing concentration of inhibitors were added to the reaction before adding enzyme to the reaction.

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