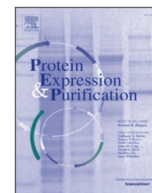
Protein Expression and Purification 126 (2016) 104–108

Contents lists available at ScienceDirect

Protein Expression and Purification

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



A facile method to prepare large quantities of active caspase-3 overexpressed by auto-induction in the C41(DE3) strain

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A R T I C L E I N F O

Article history:
Received 29 March 2016
Received in revised form
17 May 2016
Accepted 13 June 2016
Available online 15 June 2016

Keywords:
Caspase-3
Auto-induction
C41(DE3)
Soluble expression
Enzyme kinetics

A B S T R A C T

Since human Caspase-3, a member of the cysteine protease family, plays important roles not only in the apoptosis pathway as an executioner protein, but also in neurological disorders as a critical factor, biomedical researchers have been interested in the development of modulators of caspase-3 activity. Such studies require large quantities of purified active caspase-3. So far, purification of soluble caspase-3 from full-length human caspase-3 in *Escherichia coli* (*E. coli*) yields only several mg from a liter of culture media. Therefore, a number of alternative strategies to purify active caspase-3 have been described in the literature, including refolding and protein engineering. In this study, we systematically study the effects of host *E. coli* strains and growth conditions on purifications of active caspase-3 from full-length human caspase-3. Using a combination of conditions that include use of the C41(DE3) strain, low-temperature expression, and auto-induction that induces caspase-3 expression depending on metabolic state of the individual host cell, we are able to obtain 14–17 mg caspase-3 per liter of culture, an amount that is about 7 times larger than published results. This optimized expression and purification method for caspase-3 can be easily scaled up to facilitate the demand for active enzyme.

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1. Introduction

Caspase-3 (also designated CED-3, murine ICE, and a protease like protein ICE/CPP32 in humans) belongs to a cysteine protease family and plays essential roles in normal brain development, embryonic and hematopoietic stem cell differentiation, chromatin condensation, and apoptosis as an "executioner" protein. Caspase-3 is expressed as procaspase-3, a zymogen form of active caspase-3 which is cleaved into two subunits (large 17 kDa subunit and small 12 kDa subunit) by cellular apoptosis signals. Cleaved zymogen forms a heterodimer composed of two subunits which is the active protease [1]. Besides the apoptosis pathway, activated caspase-3 has been identified as a critical factor in neurological disorders such as Alzhehimer's, Huntington's, and Parkinson's diseases. In these diseases, abnormal cleavages of caspase-3's substrates provoke toxic effects on the neuronal cells and accelerate progression of disease [2,3]. Moreover, in cancer, activity of caspase-3 contributes re-progression of chemo- or radiotherapy resistant tumors by inducing cytokines through the paracrine signaling pathway [4–6]. Besides the importance of modulator developments of caspase-3 activity in the medicinal research field, purified active caspase-3 is also required in large quantities for various biochemical studies. Thus far, purification of active human caspase-3 from the full-length procaspase-3 *in Escherichia coli* (*E. coli*) has been limited by low yields, probably due to toxicity to the host. To obtain larger quantities of caspase-3, more complicated purification strategies have been developed. One commonly used method is refolding two subunits of caspase-3, which are expressed separately in *E. coli* as inclusion bodies [7]. Another method is to express engineered procaspase-3, which contains thrombin cleavable amino acid sequences in place of self-cleavage sites and requires thrombin cleavage of the engineered procaspase-3 in order

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http://dx.doi.org/10.1016/j.pep.2016.06.004
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to obtain fully activated caspase-3 [8]. Since reported expression conditions for active caspase-3 from full-length procaspase-3 were of such low yield (expressed full-length caspase-3 in BL21(DE3) or BL21(DE3)pLysS or Rosetta(DE3) at 30 or 18 °C in 2xYT or LB medium [9–11]), we decided to explore expression conditions for full-length caspase-3 in order to provide a facile method for large scale purification of active caspase-3. In this study, we systematically studied the effects of *E. coli* host strain and growth conditions on the yields of purified active caspase-3. Employing auto-induction method [12] in combination with the C41(DE3) host strain and low temperature growth, we are able to obtain 14–17 mg of active caspase-3 from 1 L culture. Our expression and purification method presented here can be easily scaled up to fulfill the high demand for active caspase-3 in the biomedical research community.

2. Materials and methods

2.1. Materials

The expression vector harboring human caspase-3 with 6x histidine tag on its C-terminus (pET23b-Casp3-His [13]) was purchased from addgene (Plasmid #11821). Chemically competent BL21-Gold (DE3)pLysS and Rosetta™2(DE3) cells were purchased from Agilent technologies and Novagen, respectively. C41(DE3) [14] and Ac-DEVD-*p*NA, were purchased from Sigma-Aldrich or Santa Cruz Biotechnology. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and imidazole were purchased from Bio basic (Canada). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from UBPBio (USA). tris(2-*carboxyethyl*)phosphine (TCEP) was purchased from Hampton research (USA). Precision Plus Protein™ standards and dithiothreitol (DTT) were purchased from Bio-Rad (USA). Other chemicals were purchased from Sigma-Aldrich.

2.2. Expression of human caspase-3 in 2xYT medium

BL21-Gold(DE3)pLysS, Rosetta™2(DE3), and C41(DE3) [15] harboring pET23b-Casp3-His [13] were streaked on LB plates which contained appropriate antibiotics. The single colony from each plate was used to inoculate 10 mL of 2xYT media (16 g/L tryptone, 10 g/L yeast extract, and 5 g/L NaCl) containing appropriate antibiotics and cultured during overnight at 37 °C. 1 L of 2xYT medium containing appropriate antibiotics in baffled flask was inoculated with 1:100 dilution of the overnight culture, and cultured at 37 °C. When OD_{600} reached at about 0.6, caspase-3 was induced with 0.2 mM IPTG at 30 °C. After 3 h of induction at 30 °C, cells were harvested.

2.3. Expression of human caspase-3 in auto-induction medium

A single colony of C41(DE3) [15] containing pET23b-Casp3-His [13] was inoculated into 1 mL of PG medium (50 mM Na_2HPO_4, 50 mM KH_2PO_4, 25 mM NH_4Cl, 25 mM Na_2SO_4, 2 mM Mg_2SO_4, 0.5% glucose, and 0.2x trace metals) [12] containing 50 μg/mL of ampicillin and the culture was grown at 37 °C overnight. This culture was inoculated with a 1:1000 dilution ratio into two 0.5 L aliquots of pre-warmed auto-induction medium [12] (ZYM-5052: 40 mM Na_2HPO_4, 10 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM Mg_2SO_4, 0.5% glycerol, 0.05% glucose, 0.2% lactose, and 0.2x trace metals) in 2 L baffled flasks at 37 °C with a shaking speed 170 rpm. When OD_{600} of the culture reached to 0.2 at 37 °C, the temperature of the culture was lowered to 18 °C with a shaking speed 170 rpm. After 22–24 h growth at 18 °C, the cultures reached at the maximum OD_{600} [9] and were harvested. Protein concentrations were determined by Bradford protein assay kit II (Bio-Rad, USA) with bovine serum albumin as the standard.

2.4. Purifications of human caspase-3 from 2xYT media

Each of three cell pellets from 1 L 2xYT medium was suspended with 40 mL of a buffer containing 20 mM HEPES (pH 7.5), 300 mM NaCl, 20 mM imidazole, and 10 mM beta-mercaptoethanol (BME). Cells were lysed by sonication and the lysate was centrifuged (20,000 × g for 30 min at 4 °C). The supernatant was mixed and incubated for 1 h at 4 °C with 1–2 mL of pre-equilibrated Ni-NTA resin (GE Healthcare Life Sciences) with buffer A (20 mM HEPES (pH 7.5), 300 mM NaCl, 20 mM imidazole). The resin was transferred into a poly-prep column (Bio-Rad, USA) and washed with 10 bed volumes of buffer A and 10 bed volumes of a buffer containing 20 mM HEPES (pH 7.5), 300 mM NaCl, 50 mM imidazole, and eluted with 5 bed volumes of buffer B (20 mM HEPES (pH 7.5), 300 mM NaCl, 250 mM imidazole) by gravity. Purities of caspase-3 from different hosts were analyzed with a 4–20% gradient Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Protein concentrations were determined by Bradford protein assay kit II (Bio-Rad, USA) with bovine serum albumin as the standard.

2.5. Purification of human caspase-3 grown in auto-induction medium

A cell pellet from 1 L auto-induction medium was suspended with the same buffer described above. Cells were lysed by sonication and lysate was centrifuged (20,000 g for 30 min at 4 °C). The supernatant was loaded twice on a prepacked HisTrap HP (GE Healthcare Life Sciences) pre-equilibrated with 5 column volumes of buffer A by peristaltic pump at 4 °C. The HisTrap HP column was washed with 10 column volumes of 43 mM imidazole and eluted by a linear gradient of imidazole concentrations from 43 mM (10% of buffer B) to 250 mM (100% of buffer B) over 10 column volume using AKTApurifier UPC 100 system (GE Healthcare Life Sciences). Then, 5 mM of TCEP (pH ~ 7.5) was immediately added into the fractions containing caspase-3. Fractions containing pure caspase-3 as determined by SDS-PAGE were pooled and concentrated to about 8 mL. A portion of purified protein was stored with 10% glycerol at −80 °C for SDS-PAGE analysis and determination of kinetic parameters. Concentrated protein was loaded onto a HiLoad 26/600 Superdex™ 200 pg column (GE Health-care Life Sciences). Caspase-3 was purified by size exclusion chromatography with buffer C (20 mM HEPES (pH 7.5), 500 mM NaCl, 3 mM TCEP, 1 mM ethylenediaminetetraacetic acid (EDTA) and 10% glycerol) using AKTApurifier UPC 100 system. Purity of caspase-3 was analyzed with 4–20% SDS-PAGE gels.

2.6. Determination of caspase-3 activity and kinetic constants

The activity of purified caspase-3 was measured by colorimetric assay using a substrate, Ac-DEVD-*p*NA (Sigma-Aldrich). The purified caspase-3 (2–5 nM) was assayed in reaction buffer (50 mM HEPES, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, and 5 mM DTT) containing different concentrations of the substrate (0, 12.5, 50, 100, 150, 180 and 200 μM). Release of *p*-nitroaniline (*p*NA) was monitored at 405 nm using a SpectraMax 340 (Molecular Devices, USA). A standard curve for *p*-nitroaniline (*p*NA) was made in the reaction buffer. All measurements were at least triplicated and carried out at room temperature. K_M and k_{cat} values were obtained using OriginPro 8.6. Standard deviations of k_{cat}/K_M were calculated using the propagation of errors method [16].

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