Protein Expression and Purification 121 (2016) 66–72

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Highly efficient recombinant production and purification of streptococcal cysteine protease streptopain with increased enzymatic activity

Michael D. Lane [a, b], Burckhard Seelig [a, b, *]

[a] Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN, USA
[b] BioTechnology Institute, University of Minnesota, St. Paul, MN, USA

ARTICLE INFO

Article history:
Received 25 November 2015
Received in revised form
30 December 2015
Accepted 4 January 2016
Available online 7 January 2016

Keywords:
Enzyme purification
Protease
Zymogen
Maturation
Autoinduction
Streptococcal pyrogenic exotoxin B

ABSTRACT

Streptococcus pyogenes produces the cysteine protease streptopain (SpeB) as a critical virulence factor for pathogenesis. Despite having first been described seventy years ago, this protease still holds mysteries which are being investigated today. Streptopain can cleave a wide range of human proteins, including immunoglobulins, the complement activation system, chemokines, and structural proteins. Due to the broad activity of streptopain, it has been challenging to elucidate the functional results of its action and precise mechanisms for its contribution to *S. pyogenes* pathogenesis. To better study streptopain, several expression and purification schemes have been developed. These methods originally involved isolation from *S. pyogenes* culture but were more recently expanded to include recombinant *Escherichia coli* expression systems. While substantially easier to implement, the latter recombinant approach can prove challenging to reproduce, often resulting in mostly insoluble protein and poor purification yields. After extensive optimization of a wide range of expression and purification conditions, we applied the auto-induction method of protein expression and developed a two-step column purification scheme that reliably produces large amounts of purified soluble and highly active streptopain. This method reproducibly yielded 3 mg of streptopain from 50 mL of expression culture at >95% purity, with an activity of 5306 ± 315 U/mg, and no remaining affinity tags or artifacts from recombinant expression. This improved method therefore enables the facile production of the important virulence factor streptopain at higher yields, with no purification scars that might bias functional studies, and with an 8.1-fold increased enzymatic activity compared to previously described procedures.

1. Introduction

Streptococcus pyogenes is a human-specific pathogen responsible for over 500,000 deaths per year globally [1]. This ubiquitous bacterium commonly causes mild infections of the upper respiratory tract and skin. However, severe infections of the skin, blood stream, and soft tissues are possible and are frequently life-threatening. Additionally, recurrent infections can lead to a variety of autoimmune diseases including acute rheumatic fever, rheumatic heart disease, acute poststreptococcal glomerulonephritis, and possibly pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) [2]. *S. pyogenes* produces several virulence factors responsible for its infectivity, including secreted toxic superantigens and proteases [3].

Streptopain is a cysteine protease secreted by *S. pyogenes* that is critical for full host infectivity due to its ability to cleave host proteins (plasminogen, fibrinogen), antimicrobial peptides, and antibodies [2]. This protease is also known as SpeB (streptococcal pyrogenic exotoxin B) because it was initially believed to have superantigenic activity. However, this originally detected activity was found to be caused by contamination or co-purification with superantigens and therefore it was concluded that streptopain does not have superantigenic activity [4]. Despite first being isolated and characterized in the 1940's [5], the detailed mechanism of streptopain's proven role in bacterial pathogenesis is still poorly understood [6]. Streptopain frequently produces enigmatic results based on the proteins it is known to cleave. For example, its activity

* Corresponding author. Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN, USA.
E-mail address: seelig@umn.edu (B. Seelig).

http://dx.doi.org/10.1016/j.pep.2016.01.002
1046-5928/

seems to both inhibit and activate systems such as inflammation, complement, immunoglobulin defense, as well as cleave numerous proteins produced by *S. pyogenes* [6]. It is these seemingly contradictory activities that continue to make streptopain a relevant and challenging research target today.

Classically, streptopain was isolated from *S. pyogenes* culture supernatant by a variety of chromatography techniques [7–11]. These yielded purified protein because the bacteria secrete streptopain to act on the extracellular matrix. Recombinant production of streptopain variants in *Escherichia coli* was pursued for convenient exploration of point mutations [12]. These protein variants were purified by combinations of ion exchange chromatography [13–17], dye-ligand chromatography [13,15], size-exclusion chromatography [14,15,17], or Ni^{2+}-chelating chromatography [12,16,18–20].

In *S. pyogenes*, streptopain is initially expressed as a 40 kDa zymogen. Maturation is caused by cleavage of the 138 N-terminal amino acids, resulting in a 28 kDa active protease [21]. This cleavage can be performed by mature streptopain or by exogenous proteases [22]. Most previously published recombinant purifications yielded the zymogen, which was subsequently activated by incubation with mature streptopain [12,18–20], although some cases of streptopain self-activation during expression and purification were also reported [17,18].

Our efforts at replicating recombinant streptopain expression and purification methods in *E. coli* repeatedly met challenges and did not achieve high yields, purity, or activity. Specifically, we frequently were able to express large quantities of streptopain, but the protein remained in the insoluble fraction. Accordingly, we trialed a variety of expression and purification strategies to identify an improved method of purification. Here we report our most successful expression system and purification method whereby we obtained the highest reported yield (3 mg/50 mL) and activity (5306 ± 315 U/mg by azocasein assay) of a highly purified (>95% by SDS-PAGE) activated streptopain. Our approach has the added benefit of fully maturating the protease with no remaining affinity tags that might bias its activity or structure in subsequent experiments.

2. Materials & methods

2.1. Materials

The streptopain-containing plasmid pUMN701 was generously donated by Dr. Patrick Schlievert. All primers were synthesized by the University of Minnesota Genomics Center. The restriction enzymes *Bam*HI-HF and *Xho*I, the T4 DNA ligase, and BSA were purchased from New England Biolabs. BL21(DE3) cells, kanamycin, acetonitrile, and standard phosphate buffered saline were purchased from VWR. LB medium, tryptone, yeast extract, glycerol, and glucose were purchased from Fisher Scientific. The SP Sepharose FF resin and SP HP HiTrap column were products from GE Healthcare Life Sciences. All other reagents were purchased from Sigma–Aldrich.

2.2. Overexpression of streptopain by autoinduction in E. coli

The coding sequence of the streptopain zymogen was PCR amplified from the pUMN701 plasmid with PCR primers "SpeB_BamHI_FW" and "SpeB_XhoI_RV" (Supplementary Table S1), which added an N-terminal His6 tag. The PCR product was cloned into the pET24a plasmid using restriction digestion with *Bam*HI-HF/*Xho*I and ligation with T4 DNA Ligase. The resulting pET24a construct was sequenced to confirm insertion of the correctly oriented full coding sequence of streptopain (amino acids 1–398) with an N-terminal His6 tag. The plasmid was transformed into BL21(DE3) *E. coli* cells. A culture of 150 mL of standard LB medium and 36 μg/mL Kanamycin was inoculated with a single colony and grown overnight at 37 °C. 400 μL of the overnight culture was used to inoculate 200 mL of auto-induction media (50 mM Na_2HPO_4, 50 mM KH_2PO_4, 2 mM $MgSO_4$, 36 μg/mL Kanamycin, 2% tryptone, 0.5% yeast extract, 0.5% NaCl, 60% glycerol, 10% glucose, 8% lactose, w/v). Cultures were grown at 37 °C for ~5–6 h until OD_{600} ~0.3–0.4 and then transferred to 25 °C for an additional 24 h. Cells were divided into 50 mL aliquots, collected by centrifugation, and frozen at −80 °C.

2.3. Purification of streptopain by affinity chromatography

A frozen cell pellet from 50 mL of culture medium was thawed at 4 °C and resuspended in 5 mL of lysis buffer (20 mM sodium acetate, 50 mM NaCl, 1 mM $HgCl_2$, pH 5.0). Mercury (II) chloride was added to all steps to a final concentration of 1 mM to reversibly inhibit the activity of streptopain. $HgCl_2$ is a hazardous material, so standard precautions should be taken for all materials containing $HgCl_2$, and contaminated waste should be disposed of properly. The suspension was lysed by sonication and the lysate was centrifuged to separate the insoluble fraction. The soluble fraction was run over 4 mL of SP Sepharose FF resin equilibrated with lysis buffer. The column was washed with 5 × 4 mL of lysis buffer. Streptopain was eluted with 5 aliquots of 4 mL elution buffer (20 mM sodium acetate, 100 mM NaCl, 1 mM $HgCl_2$, pH 5.0) and contained about 5 mg of protein in fractions 2–5. These 4 fractions were combined, diluted with 20 mM sodium acetate, 25 mM NaCl, 1 mM $HgCl_2$, pH 5.0 buffer to reach a final NaCl concentration of 50 mM, and concentrated with an Amicon Ultra centrifugal filter 3 kDa MWCO to a final volume of 5.5 mL. This solution was applied to an SP HP HiTrap column equilibrated with 20 mM sodium acetate (pH 5.0), 50 mM NaCl, 1 mM $HgCl_2$ and eluted with a gradient of NaCl from 50 mM to 200 mM via FPLC. Fractions of 1.5 mL were collected and protein concentrations were determined by measuring absorbance at 280 nm using a Nanodrop spectrophotometer.

2.4. Confirmation of streptopain protein identity by mass spectrometry

Cleared supernatant from an *E. coli* autoinduction expression of streptopain was separated on a 4–12% SDS-PAGE. The overexpressed band at 28 kDa was cut out, solubilized, digested with trypsin, concentrated in a SpeedVac vacuum concentrator, desalted by the Stage Tip procedure [23], and dried in a SpeedVac. Tryptic peptides were rehydrated in water/acetonitrile (ACN)/formic acid (FA) 98:2:0.1 and loaded using a Paradigm AS1 autosampler system (Michrom Bioresources, Inc., Auburn, CA). Each sample was subjected to Paradigm Platinum Peptide Nanotrap (Michrom Bioresources, Inc.) pre-column (0.15 × 50 mm, 400 μL volume) followed by an analytical capillary column (100 μm × 12 cm) packed with C18 resin (5 μm, 200 Å MagicC18AG, Michrom Bioresources, Inc.) at a flow rate of 320 nL/min. Peptides were fractionated on a 60 min (5–35% ACN) gradient on a flow MS4 flow splitter (Michrom Bioresources, Inc.). Mass spectrometry (MS) was performed on an LTQ (Thermo Electron Corp., San Jose, CA). Ionized peptides eluting from the capillary column were subjected to an ionizing voltage (1.9 kV) and selected for MS/MS using a data-dependent procedure alternating between an MS scan followed by five MS/MS scans for the five most abundant precursor ions.

2.5. Proteolytic activity measured by azocasein assay

Purified streptopain was assayed with azocasein substrate to