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

determine proteolytic activity [24] by measuring absorbance at 366 nm over time. Undigested azocasein is precipitated in the protocol, while digested azocasein is freed to absorb at 366 nm. 20 μL of protease was added to 160 μL of a reaction mixture containing 2.7 mg/mL azocasein, 5 mM DTT, 5 mM EDTA in standard phosphate buffered saline. Time points between 5 s and 1 h were quenched with 40 μL of ice-cold 15% trichloroacetic acid. Absorbance at 366 nm was measured using a Nanodrop spectrophotometer. As previously described [18], one enzyme unit is defined as the amount of protease capable of releasing 1 μg of soluble azopeptides per minute using the reported specific absorption coefficient $A_{366}^{1\%} = 40 M^{-1} * cm^{-1}$.

2.6. Assay of streptopain cleavage specificity using peptides as substrates

A positive control peptide cleavage substrate (FAA**I**K↓AGARY) and a negative control peptide cleavage substrate (FAA**G**KAGARY) were synthesized by Chi Scientific to >90% purity. 1 nmol of each peptide was incubated with 10 pmol of purified active streptopain in 100 μL of 50 mM sodium phosphate (pH 7.0), 2 mM DTT, and 1 mM EDTA for 1 h at 37 °C. Streptopain was separated by filtering with a Nanosep 10 k MWCO filter. The flow-through was further purified by the Stage Tips procedure [23] using MCX extraction disks, a mixed-mode cation exchange sorbent. Disks were punctured and the small round piece of sorbent was placed in a 200 μL pipet tip and washed with 45 μL of 100% methanol. Peptide samples were acidified to pH 3 with formic acid, loaded into the tip, washed with 45 μL of 0.1% trifluoroacetic acid, washed with 45 μL of 100% methanol, and eluted with 50 μL of 5% ammonium hydroxide in methanol. The elution was dried in a SpeedVac vacuum concentrator and resuspended in 50 μL of 98% water, 2% acetonitrile, and 0.1% formic acid. Approximately 0.1 μg of peptide was loaded on the capillary LC column and analyzed by LC–MS/MS on the Orbitrap Velos system as described previously [25], except for the following modifications to the MS acquisition parameters: The MS1 scan was 250–1200 *m/z*; the MS2 first mass value was 116; dynamic exclusion time duration was 15 s; the lock mass value was 371.1012 *m/z*; the LC gradient was 0–38% B in 28 min, then increased to 80% B at 28.1, held at 80% B for 4 min, then the system was re-equilibrated at 2% B for 8 min. Peaks were manually inspected and peptide fragment ions were assigned.

3. Results

3.1. Testing multiple strategies to improve soluble protein expression

Our first efforts at recombinant expression of streptopain were to clone streptopain into a pET24a vector to render streptopain IPTG-inducible in an *E. coli* strain. These initial efforts at recombinant expression and purification of streptopain failed to produce significant quantities of soluble protein. Nearly all of the overexpressed protein was in the insoluble fraction after sonication. To optimize soluble protein expression, we tested 3 *E. coli* strains (BL21(DE3), BL21(DE3)pLysS, BL21(DE3)Rosetta), 2 chaperone systems (pTF16, pGRO7), 5 expression temperatures (16 °C, 25 °C, 28 °C, 30 °C, 37 °C), 4 induction concentrations of IPTG (0, 0.1, 0.5, 1.0 mM), 2 different culture media (LB and minimal media), 7 expression time points (0, 4, 8, 12, 16, 24, 48 h of culture post induction), 4 lysis methods (lysozyme, BugBuster®, French press, sonication), and 3 Ni-NTA purification methods (native, urea-denaturing, guanidine-denaturing). None of these efforts resulted in sufficient yields of soluble streptopain.

3.2. Overexpression of mature streptopain by autoinduction

We then performed an expression using the autoinduction method [26]. In this scheme, minimal media was supplemented with glycerol, glucose, and lactose, but IPTG was not used to induce expression. Over a longer period of time (24 h), protein was expressed to a lower intracellular concentration compared to an IPTG induced expression due to the weaker induction by lactose, but the cells grew to a higher density. Using this protocol, we obtained approximately 10 mg of soluble protease from a 50 mL culture (Fig. 1A). But the autoinduction approach introduced a new challenge: the ~40 kDa protease zymogen was cleaved into its ~28 kDa active form, thereby removing its N-terminal His6 tag that was to be used in the purification of the inactive zymogen. This cleavage occurred despite the attempt to inhibit streptopain from self-activation by the inclusion of 1 mM $HgCl_2$ in all lysis steps. In contrast, when we performed the purification in the absence of $HgCl_2$, we observed much more extensive proteolysis of all proteins present in the lysate, resulting in many products <15 kDa in the lysate, cleared lysate, flow-through and wash, and the resulting purified streptopain had much higher concentrations of streptopain fragments at ~12 and ~26 kDa, likely due to uninhibited activity of streptopain (Supplemental Fig. S1). We verified the identity of the 28 kDa protein product by isolating the respective band from an SDS-PAGE separation, digesting the protein with trypsin, and analyzing it by mass spectrometry. The ProteinPilot search engine was used to match proteins to the peptide spectra of trypsin digested streptopain. 259 unique streptopain peptides were identified (Supplementary Tables S2 and S3). These matched with >95% confidence to the predicted 253 amino acid sequence of the activated 28 kDa streptopain sequence according to the ProteinPilot Paragon scoring algorithm (Fig. 2). Peptides from the N-terminal cleaved region were also identified, and are likely present in low concentrations as residual cleavage fragments. These data confirmed that the principal soluble product was activated streptopain.

3.3. Purification of streptopain

Due to the loss of the N-terminal His6 tag through zymogen cleavage during the expression and lysis process, a method for purifying streptopain without the affinity tag was required. We tested a variety of column purifications, including multiple sequential orthogonal purifications. Overall, we tested weak anion exchange resin (DEAE), strong cation exchange resins (SP Sepharose FF by gravity flow, or HiTrap SP HP by FPLC), and size exclusion resins (G-25 by gravity flow, or G-75 by FPLC). We found that streptopain did not bind tightly to DEAE resin, but that a G-25 column to perform a crude cleared lysate clean-up followed with an SP Sepharose FF resin could purify the protein (data not shown). However, we achieved higher yields when we instead used SP Sepharose FF gravity flow resin to directly purify the cleared lysate without a prior G-25 column. We could reproducibly obtain ~90% purity streptopain from this single step purification (Fig. 1B), and the impurities were largely degradation products of streptopain itself (described below). This purity may be adequate for a variety of applications. We lost a significant quantity of streptopain in W1, which is simply a rinse with lysis buffer. This indicates that streptopain is only weakly binding to the column at 50 mM NaCl. In some attempts, we re-used this W1 on a fresh column and obtained additional purified streptopain (data not shown), indicating that it may be possible to maximize yield further at the potential cost of purity.

To further purify streptopain beyond 90% purity, we tried adding a subsequent G-75 size-exclusion column step, and found that we

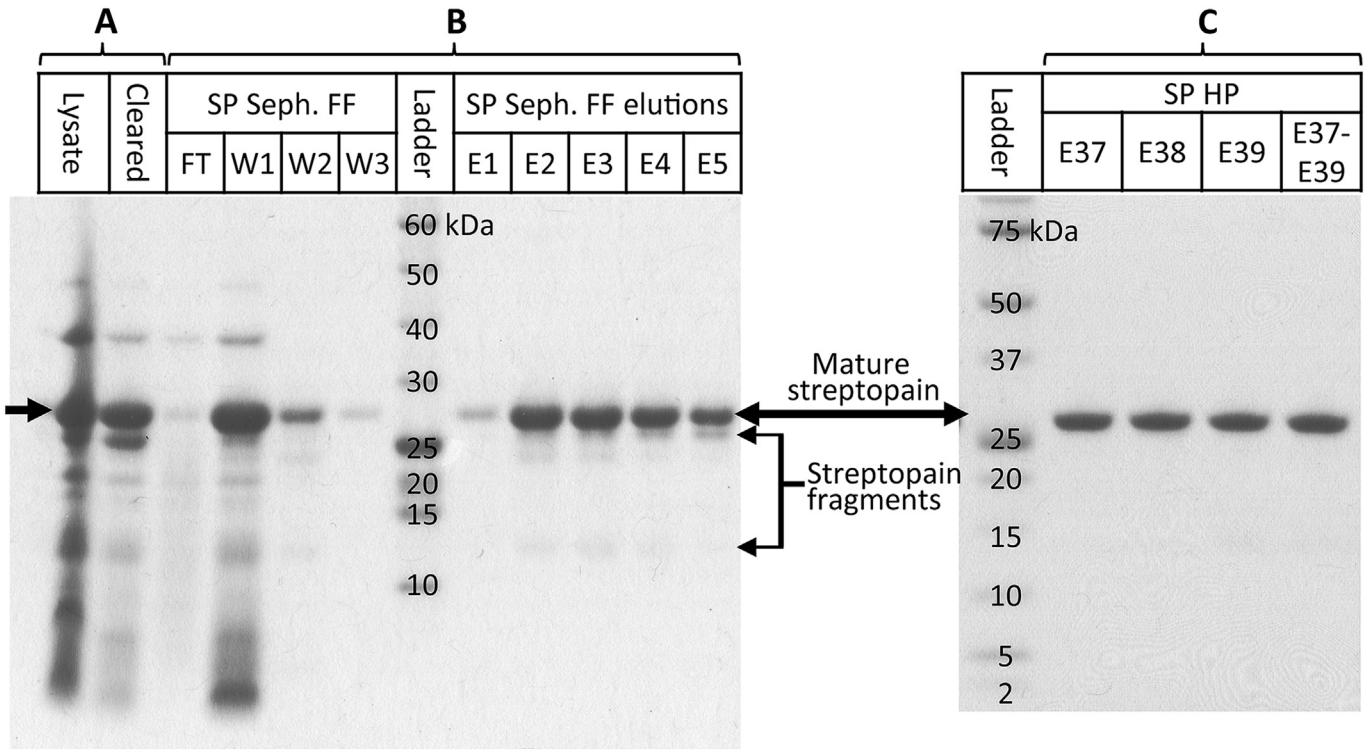

Fig. 1. SDS-PAGE gel of the expression and purification of mature streptopain. (A) Autoinduction lysate and cleared lysate. Arrow indicates mature streptopain migrating at ~28 kDa as expected. (B) SP Sepharose FF purification with flow-through (FT), washes 1–3 (W1–3), and elutions 1–5 (E1–5). Arrows indicate mature streptopain or streptopain degradation fragments. (C) SP HP HiTrap purification by FPLC with elutions 37, 38, and 39 and the combined elutions 37–39 more than one year after initial purification.

were unable to separate the 26 kDa impurity (shown in Fig. 1B, lane E5) from the 28 kDa desired product. In addition, the amount of streptopain degradation products (presumably caused by streptopain cleaving itself) would frequently increase during the size-exclusion column, even in the presence of 1 mM $HgCl_2$ throughout (data not shown). We found that a combination of two successive strong cation exchange resins gave the best results for soluble, pure enzyme with high activity. First, we used an SP Sepharose FF column (strong cation exchange) to isolate streptopain from the crude cleared lysate by gravity flow (Fig. 1B). This was followed with a HiTrap SP HP column (strong cation exchange) using an FPLC setup (Fig. 1C). Analyzing the protein by SDS-PAGE, we found our product to be >95% pure in fractions 37, 38, and 39 (Fig. 1C). The additional minor bands at ~26 kDa and ~12 kDa in the SP Sepharose FF were degradation products of streptopain as confirmed by mass spectrometry, performed as described above for the 28 kDa band (data not shown). The protease is stable in the elution buffer for more than a year at 4 °C (Fig. 1C). Our final yield after the second purification step was 3 mg of streptopain in these 3 fractions, from the original 50 mL culture. That was a 30% greater yield per volume of *E. coli* culture than the highest previously reported yield [18].

masmtggqqmgrgshhhhhhdqnfarnekeakdsaitfiqksaaikag
arsaedikldkvnlggelsgsn*myvynistggfvivs*gdkrspeilgystsg
sfdangkeniasf*mesyveqi*kenkkldttyagtaeik↓**QPVVKSLL**
DSKGIHYNQGNPYNLLTPVIEKVKPGEQSFVGQHAA
TGCVATATAQIMKYHNYPNKGLKDYTYTLSSNNPYF
NHPKNLFAAISTRQYNWNNILPTYSGRESNVQKMAI
SELMADVGISVDMDYGPSSGSAGSSRVQRALKENF
GYNQSVHQINRSDFSKQDWEAQIDKELSQNQPVYY
QGVGKVGGHAFVIDGADGRNFYHVNWGWGGVSD
GFFRLDALNPSALGTGGGAGGFNGYQSAVVGIKP

Fig. 2. Amino acid sequence of streptopain protein translated from pET24a vector. The first 20 amino acids indicated in light gray originate from the pET24a plasmid and are an N-terminal addition to the native streptopain zymogen. The ↓ indicates the beginning of the mature active streptopain sequence. Font styles indicate confidence of peptide matching from analyzing the 28 kDa gel band by mass spectrometry: bold underline uppercase is >95 confidence, lowercase is <50 confidence, italicized lowercase is no match.

3.4. Assays to confirm proteolytic activity and substrate specificity

We tested the activity of our purified streptopain protease by the standard azocasein protease activity assay. We found that the activity of our final purified protease in our pooled fractions was 5306 ± 315 U/mg (Fig. 3). This is 8.1-fold more active than the highest value reported to date [18]. To further confirm that our purified and active protease had a cleavage preference similar to previous reports for streptopain, we designed two test peptides based on published streptopain specificity experiments [27]. A positive control peptide (FAA**I**K↓AGARY) and a negative control peptide (FAA**G**KAGARY) were synthesized such that a cleavage should occur in the center of the positive control sequence as indicated by the arrow, but no cleavage should occur in the negative control peptide. Those two peptides only differed at a single amino acid position (I in place of G, shown in bold and underlined). In order to facilitate the peptide analysis by liquid chromatography–mass spectrometry (LC–MS) by improving separation on the C18 column, the large bulky amino acids F and Y were added to the N- and C-termini of the peptides, respectively. The two peptides were incubated with or without streptopain, purified, and then analyzed by MS (Fig. 4). When no protease was added to the incubation, the intact full length positive control peptide eluted at 25.2 min (Fig. 4A). The tandem mass spectrum of the doubly

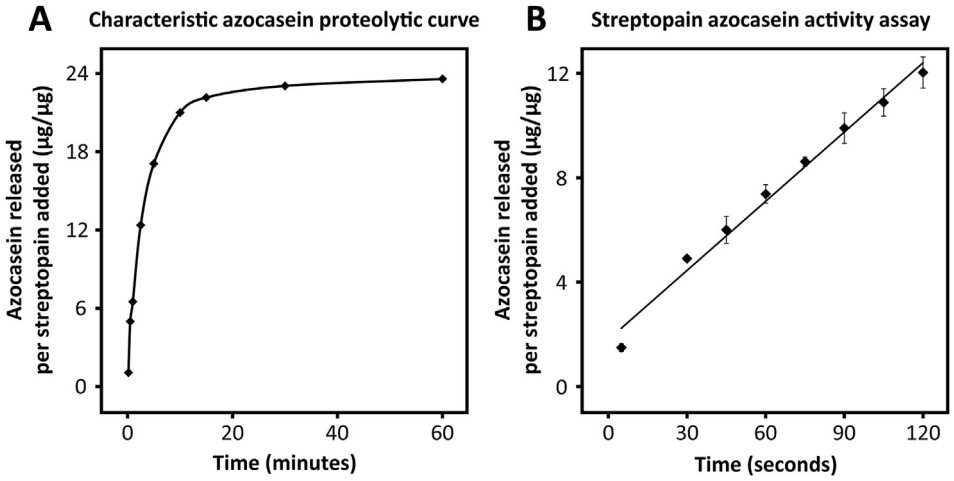

Fig. 3. Azocasein assays to determine protease activity of purified streptopain. (A) A characteristic azocasein proteolysis curve. (B) Azocasein assay of our purified streptopain (combined fractions E37–39) measured in the linear activity range at early time points to determine protease activity. The measurement was performed in triplicate and standard deviation was calculated between the three measurements.

charged precursor 534.3047 *m/z* (2.5 ppm mass error) labeled with all fragment ions can be found in the Supplementary Fig. S2. When streptopain was incubated with the positive control peptide, two products eluted at 21.9 and 25.1 min (Fig. 4C). The 21.9 min peak is the appropriate cleavage product FAAIK (doubly charged precursor) (<1 ppm mass error), while the 25.1 min peak is the methylated version of the same cleavage product (<1 ppm mass error). In contrast, only the uncleaved full length negative control peptide was detected both without and with streptopain at 21.9 and 21.3 min, respectively (Fig. 4 B and D). The doubly charged precursor in both of these samples was identified with <1 ppm mass error. The ion-labeled tandem mass spec of all samples are presented in Supplementary Figs. S2–6. In summary, the cleavage specificity of our streptopain preparation is identical to previous reports [27].

4. Discussion

At the outset of this study, we attempted to replicate published protocols for the recombinant expression of streptopain. However, we found that, in our hands, recombinant *E. coli* production of streptopain yielded largely insoluble protein. The reason for this is unclear. Induction of BL21(DE3) cells with IPTG produced large quantities of zymogen, but the protein was exclusively in the

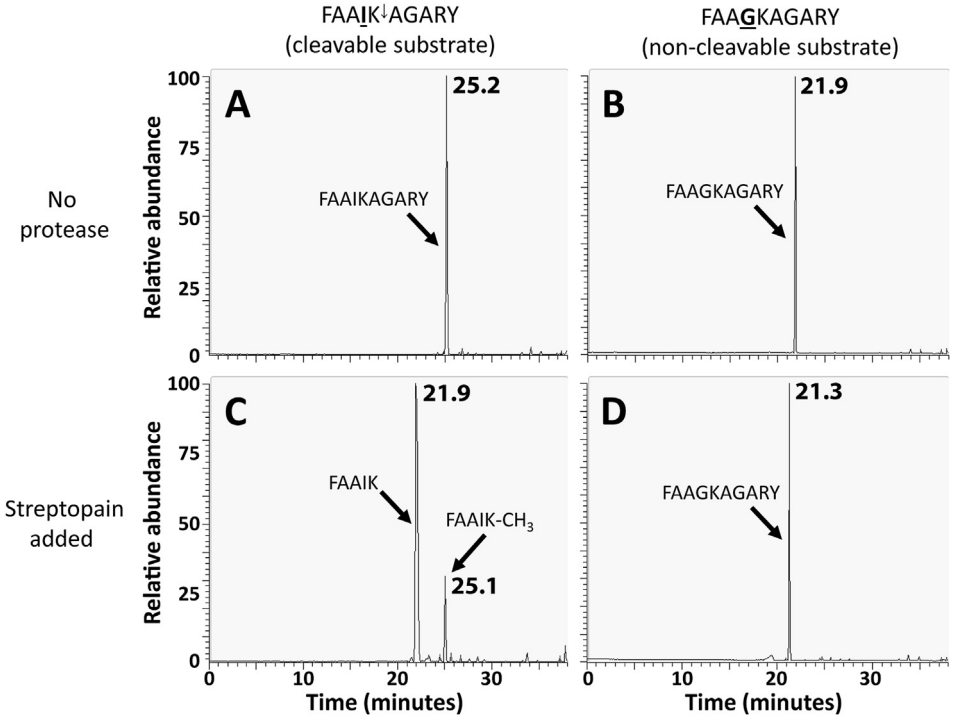

Fig. 4. LC–MS traces of two test peptide substrates without protease (A and B) and after an incubation with streptopain (C and D). Peptide incubation with streptopain resulted in digestion of the cleavable positive control peptide (FAA**I**K↓AGARY, C) but no digestion of the non-cleavable negative control peptide (FAA**G**KAGARY, D). Peaks are labeled with their sequences identified by MS analysis.

insoluble fraction. Varying IPTG concentrations made little difference, resulting in about the same overexpression from induction by 0.1 mM or 1.0 mM IPTG. We were concerned about poor protein folding, since varying IPTG induction concentration did not improve solubility. We therefore varied induction temperature between 16 °C and 37 °C, but observed no increase in soluble protease. BL21(DE3)pLysS or BL21(DE3) Rosetta strains did not yield soluble protein either. Further, we tried expressing in cells that also contained the pTF16 or pGRO7 plasmids, which produced the TF or GroES/GroEL chaperones, respectively, yet still did not see any improvement in soluble protein expression. We hypothesized that perhaps our lysis method was not adequately disrupting cells, yet cell lysis using French press, sonication, BugBuster reagent, and lysozyme all gave similar results.

Interestingly, we occasionally saw protease activity in our samples that were stored on ice in Laemmli buffer, including lysates and insoluble fractions. In these samples, the ~40 kDa streptopain zymogen activated to its mature 28 kDa protein, and also digested the other proteins present in that sample. This suggested that the protein we produced was capable of folding and activity, yet the expression conditions were still unsuited for soluble expression. Consequently, we attempted denaturing purifications of our successful insoluble expressions. In contrast to 4.5 M urea, 6 M guanidine hydrochloride successfully solubilized streptopain from the insoluble fraction, which was then immobilized on Ni-NTA agarose. The unfolded streptopain could be purified, but renaturation attempts were unsuccessful, including on-column renaturing, dialysis, and step-wise renaturation. These attempts consistently precipitated or degraded the protein, suggesting that fully denaturing streptopain was an irreversible process.

We were finally able to produce large quantities of soluble streptopain using the autoinduction method for *E. coli* recombinant protein expression. Our soluble streptopain was obtained as mature enzyme, indicating that at some point during autoinduction or cell lysis, the protease must have become active. If incubated long enough after lysis, the protease digested most proteins in the lysate (data not shown). Yet, the autoinduced bacteria continued to grow for at least 48 h. These data suggested that the protease did not become active until after lysis. As this approach eliminated the N-terminal His6 tag, we explored a variety of methods that had previously been used to purify streptopain directly from cell culture supernatants of *S. pyogenes* instead of *E. coli*. A combination of two successive strong cation exchange resins gave the best results for soluble, pure enzyme with the highest activity.

Cloning and expressing the protease with a C-terminal His6 tag using the autoinduction method, as an alternative to the cleaved N-terminal tag, was not explored. Although C-terminal His6 tags have been used with success to purify streptopain [17], we preferred to create a method to produce 100% native streptopain for subsequent downstream applications. A residual C-terminal His6 tag would not satisfy this goal. Furthermore, we did not attempt to directly clone and express the mature protein alone. The mature streptopain is capable of digesting the majority of proteins in the lysate, therefore it was assumed that overexpression of the active version of this broad specificity and robust protease would be incompatible with survival of the *E. coli* host.

Digesting our positive control peptide (FAA**I**K$^{\downarrow}$AGARY) with streptopain yielded the expected cleavage product FAAIK, and an additional C-terminally methylated version of that same FAAIK peptide. In contrast, streptopain digestion of our negative control peptide (FAA**G**KAGARY) yielded no detectable cleavage products. This result corroborates previous specificity analyses of streptopain [27]. The methylation product of FAAIK is a common adduct due to LC/MS sample preparation by purifying peptides with mixed-mode cation exchange sorbents and methanol (private communication with a Waters Corporation representative). We detected comparable methylation of other peptides in analogous experiments (data not shown).

Our optimized protocol improved streptopain activity by 8.1-fold and purification yield by 30%, compared to the best previously published protocols. The increase in protease yield is likely attributable to our use of the autoinduction method. This expression method has been employed previously for protease expression, where TEV protease was produced with high yields [28]. Autoinduction enables cell growth to a higher density, which results in higher protein yields, even though the expression per cell is lower. In our experience, IPTG induction of streptopain expression consistently led to insoluble protein. This IPTG-induced overexpression may have led to streptopain accumulation in inclusion bodies, which we found to be accessible only by a denaturing purification. Since autoinduction expresses continuously at a lower level, this may have prevented streptopain from aggregating in inclusion bodies. Furthermore, we can only speculate about the reason for the 8.1-fold increased activity of our purified streptopain over previous reports. Presumably, our autoinduction and dual cation exchange purification scheme yielded a higher fraction of properly folded and active streptopain. Unfortunately, of the few previously described streptopain purifications, even fewer reported any activity data, making it difficult to draw conclusions from our increased activity. Finally, a major strength of our approach is that we obtained fully mature streptopain with no residual affinity tags that could otherwise interfere with the activity or structure of the protease. Our purified protein is identical to wild type streptopain and functions with specificity appropriate for this protease.

5. Conclusion

Reliable production and purification methods are critical for continuing research on the challenging streptopain protease. We developed an expression protocol based on the autoinduction protein expression technique which produced large quantities of soluble, mature streptopain. Through applying classic column purification techniques, we found a purification scheme which resulted in highly pure (>95%) streptopain that also gave higher yield (3 mg/50 mL) of pure enzyme with higher activity (5306 ± 315 U/mg) than previously reported in the literature. Further, since the protease cleaves off its N-terminus during the purification steps, the final purified product has no remaining affinity tags, eliminating potential undesired effects on streptopain's natural activity due to modifications. This work combined techniques from modern and classic protein expression and purification systems to result in a scheme which is both more reliable and reproducible in our hands while obtaining protein of the highest activity and yield reported. This will be useful for further studies on streptopain, as this robust expression and purification method will enable the facile production of large quantities of active protease.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgments

We would like to thank Dr. Patrick Schlievert in the Department of Microbiology at the University of Iowa for the donation of the plasmid containing streptopain, Dr. Florian Seebeck for suggesting the autoinduction protocol, Dr. LeeAnn Higgins in the Center for Mass Spectrometry and Proteomics at the University of Minnesota for her assistance in the mass spectrometry studies and data analysis, Dr. Ebbing de Jong in the College of Biological Sciences at

the University of Minnesota for his assistance with STAGE TIP design and production, and Dr. Matilda Newton and Dr. Yari Cabezas for their comments on the manuscript. This work was funded by the US National Institutes of Health (NIH) (AI113406; MSTP grant T32 GM008244 to MDL), the Minnesota Medical Foundation, the American Heart Association (MDL), and by a grant from the Biocatalysis Initiative of the BioTechnology Institute at the University of Minnesota.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2016.01.002.

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