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Development of a rapid high-efficiency scalable process for acetylated *Sus scrofa* cationic trypsin production from *Escherichia coli* inclusion bodies

Mingzhi Zhao [a], Feilin Wu [a,c], Ping Xu [a,b,d,*]

[a] State Key Laboratory of Proteomics, National Engineering Research Center for Protein Drugs, Beijing Proteome Research Center, National Center for Protein Sciences, Beijing Institute of Radiation Medicine, Beijing 102206, PR China
[b] Key Laboratory of Combinatorial Biosynthesis and Drug Discovery (Wuhan University), Ministry of Education and Wuhan University School of Pharmaceutical Sciences, Wuhan 430071, PR China
[c] Life Science College, Southwest Forestry University, Kunming 650224, PR China
[d] Anhui Medical University, Hefei 230032, Anhui, PR China

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ABSTRACT

Trypsin is one of the most important enzymatic tools in proteomics and biopharmaceutical studies. Here, we describe the complete recombinant expression and purification from a trypsinogen expression vector construct. The *Sus scrofa* cationic trypsin gene with a propeptide sequence was optimized according to *Escherichia coli* codon-usage bias and chemically synthesized. The gene was inserted into pET-11c plasmid to yield an expression vector. Using high-density *E. coli* fed-batch fermentation, trypsinogen was expressed in inclusion bodies at 1.47 g/L. The inclusion body was refolded with a high yield of 36%. The purified trypsinogen was then activated to produce trypsin. To address stability problems, the trypsin thus produced was acetylated. The final product was generated upon gel filtration. The final yield of acetylated trypsin was 182 mg/L from a 5-L fermenter. Our acetylated trypsin product demonstrated higher BAEE activity (30,100 BAEE unit/mg) than a commercial product (9500 BAEE unit/mg, Promega). It also demonstrated resistance to autolysis. This is the first report of production of acetylated recombinant trypsin that is stable and suitable for scale-up.

1. Introduction

Trypsins (EC3.4.21.4) comprise a set of serine proteases. In human, these trypsins are expressed as trypsinogens that are encoded by *PRSS1* (cationic trypsinogen), *PRSS2* (anionic trypsinogen), and *PRSS3* (mesotrypsinogen) [1]. Cationic trypsinogen accounts for approximately 2/3 of the total amount of trypsinogens in human pancreatic juice. Anionic trypsinogen accounts for approximately 1/3 [2–4]. Mesotrypsinogen is a minor species and accounts for no more than 5% of trypsinogens [5]. Upon transfer from pancreas to small intestine, trypsinogens are activated into β-trypsins by enterokinase (EK) that removes the N-terminal propeptide sequences [6,7]. The activated β-trypsins can then activate additional trypsinogens. Trypsin can cleave peptides at the carboxyl end of lysine and arginine residues, but the digestion is blocked if the lysine or arginine is followed by a proline. However, β-trypsin is unstable in solution. For example, bovine β-trypsin can cleave itself further between Lys131 and Ser132 to produce α-trypsin. The activity of α-trypsin is much lower than that of β-trypsin. This autolysis property makes the purification and storage of trypsin difficult. Based on the structure analysis of *Sus scrofa* cationic trypsin (PDB ID: 2A31), when trypsinogen is activated to β-trypsin, the newly formed N-terminal residue Ile16 inserts into a cleft where its α-amino group forms an ion pair with Asp102 near the active site Ser195. This results in a conformational rearrangement of other residues. The amino group of Gly193 orients itself into the correct position, which completes the oxyanion hole of the active site to generate activated trypsin. The active site residues of trypsin include His57, Asp102, and Ser195 [8,9].

Crystallization has been widely used in routine purification of trypsin from pancreatic juice [10]. However, trypsin in pancreas is often co-crystallized with chymotrypsin which is difficult to remove later. Chymotrypsin can lead to non-specific cleavage activity of trypsin that is prepared from animal sources. To inhibit the activity of contaminated chymotrypsin, treatment of trypsin with tosyl phenylalanyl chloromethyl ketone (TPCK) is employed.

* Corresponding author at: Beijing Proteome Research Center, 33 Science Park Road, Changping District, Beijing 102206, PR China.
E-mail address: xuping_bprc@126.com (P. Xu).

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TPCK can alkylate the His57 moiety in the active site of chymotrypsin and chymotrypsin-like serine proteases. Alternately, chemical modifications such as acetylation and methylation can also be used to decrease the autolysis activity. The recombinant expressions of human anionic and cationic trypsinogen in *Escherichia coli* have been reported previously, but the expression levels were low and the purifications used expensive ecotin affinity chromatography [11,12].

In this paper, we build a prokaryotic expression vector containing the coding sequence for an optimized cationic trypsinogen gene with a propeptide sequence from *S. scrofa* and transformed it into the *E. coli* BL21 (DE3) strain. We then developed a complete production process that includes high-density fermentation, refolding, purification, activation, acetylation and further purification. The activity of our acetylated trypsin is higher than that of other commercially available product. It is also more stable than trypsin. The whole procedure is fast and scalable. The product can be safely used in proteomics research and biopharmaceutical production.

2. Materials and methods

All experiments were performed at room temperature (20 °C) unless otherwise noted.

2.1. Bacterial strains, plasmids and genetic manipulation

The *E. coli* BL21 (DE3) strain and the pET-11c vector were from Novagen (Germany). The strain was maintained on Luria–Bertani (LB) medium (1.0% tryptone, 0.5% yeast extract and 1.0% NaCl, pH 7.5) containing 100 μg/mL ampicillin at 37 °C. The open reading frame (ORF) of the *S. scrofa* cationic trypsinogen gene (Genbank accession: **XM_005657724.1**) was codon optimized, synthesized and inserted into the *Nde*I and *Bam*H I-digested pET-11c plasmid to yield a pET-11c-trypsinogen vector. The vector was transformed into the *E. coli* BL21 (DE3) strain.

2.2. Trypsinogen expression in a bioreactor

Shake-flask culture. The strain containing the pET-11c-trypsinogen vector was grown on a solid LB/Amp plate. A single colony was inoculated into a 250-mL flask containing liquid LB/Amp medium and cultured in a shaker at 37 °C to an optical density at 600 nm (OD_{600}) of 2 and saved as the seed culture.

The seed culture was inoculated into a 5-L fermenter (Applikon Biotechnology, the Netherlands) with a working volume of 2.5 L of fermentation complex medium (FCM) [26.7 g/L glycerol, 40.0 g/L yeast extract, 1.80 g/L KH_2PO_4, 1.80 g/L citric acid, 5.00 ml/L salt solution (250 g/L $MgCl_2{\cdot}6H_2O$, 100 g/L $CaCl_2{\cdot}2H_2O$, 100 g/L KCl, 2.00 M citric acid), 1.00 ml/L metal salt solution (6.80 g/L $ZnCl_2$, 54.0 g/L $FeCl_3{\cdot}6H_2O$, 16.2 g/L $MnCl_2{\cdot}4H_2O$, 2.20 g/L $CuSO_4{\cdot}5H_2O$, 4.80 g/L $CoCl_2{\cdot}6H_2O$, 0.024 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.20 g/L KI, 119 ml/L 37% hydrochloric acid, 1.00 ml/L Antifoam 402, pH 7.2]. The starting OD_{600} value was approximately 0.01, and air was sparged at 6.00 L/min. The temperature was set to 37 °C, and the pH value was maintained at 7.0 by acid or base addition through automatic feedback controls. Dissolved oxygen (DO) was maintained during the batch period at 30% air saturation. When the DO was lower than 15%, the stirring speed was controlled in a cascade mode depending on the dissolved oxygen. The maximum stir speed was 1200 rpm. The feed medium (275 mL/L glycerol, 225 g/L yeast extract) was added at 18.0 mL/h into the fermenter when the OD_{600} reached 25.0. The temperature was decreased to 32 °C. Protein expression was induced by the addition of 1 mM isopropyl thio-β-D-galactoside (IPTG) when OD_{600} reached 47.2. At 10 h post-induction, cells were recovered by centrifugation in a J6-MI centrifugal machine (Beckman, USA) at 4000 rpm for 30 min at 4 °C. The cell density measurement was performed by an Ultraspec 10 at 600 nm (GE Healthcare, USA). The culture was appropriately diluted with PBS until the OD_{600} was in the linear range of measurement (0.1–0.8).

2.3. Cell disruption and IB recovery

The *E. coli* cell pellet was re-suspended in PBS and lysed by high-pressure homogenization on an APV1000 (SPX, USA) for three times at 800 bar. To prevent heating, the suspension was cooled on ice. The IB was removed from the cell lysate by centrifugation at 4000 rpm on a J6-MI (Beckman, USA, rotor type: JS-4.2) centrifuge for 30 min at 4 °C. The pellet was washed with PBS containing 2 M urea and maintained at room temperature for 1 h with stirring. The suspension was then centrifuged at 4000 rpm at 4 °C for 30 min.

2.4. Optimization of trypsinogen refolding

The clean IB was suspended in a denaturing solution (20 mM Tris, 8 M urea, 20 mM DTT, pH 8.5) at a ratio of 1:10 (gram wet weight of IB: mL volume) and shaken gently for 4 h at room temperature. After centrifugation in an Avant J-26XP (Beckman, USA, rotor type: JA-25.50) at 12,000 rpm for 20 min, the clarified denatured inclusion body supernatant was recovered. Refolding optimization was designed by JMP software (SAS Institute, USA). The refolding volume of each sample was 20 mL, and the basic refolding buffer was composed of 20 mM Tris, 1 mM cystine and 3 mM cysteine. The pH, dilution ratio and urea concentration were set at 3 levels (Table 1).

The refolding yield was evaluated by C18-HPLC. The HPLC used buffer A [0.1% trifluoroacetic acid (TFA), 5% acetonitrile in water] and buffer B [0.1% TFA, 70% acetonitrile, and 20% isopropanol in water]. The flow rate was 0.8 mL/min. The column temperature was 50 °C. The experiment duration was 20 min. The initial concentration of buffer B was 10%. After sample injection on a C18 column (Kinetex 2.6 μm C18, 50 × 4.60 mm, Phenomenex), the buffer concentration was held at 10% buffer B for 5 min and then increased to 30% buffer B over 2 min. The buffer B was increased from 30% to 70% from 7 min to 18 min. The column was washed with 100% B and re-equilibrated for the next sample from 18 min

Table 1
Detailed comparison of refolding conditions and the relative efficiency.

Sample No.	pH	Dilute ratio	Urea (M)	Peak area (μAU*S)	IB (mL)	IB (peak area/mL)
1	9	40	1	372923	0.5	745846
2	9	20	2	499748	1	499748
3	7.5	40	1	44167	0.5	88334
4	8.25	10	0	90262	2	45131
5	9	10	1	615596	2	307798
6	7.5	40	0	17814	0.5	35628
7	9	40	0	229228	0.5	458456
8	7.5	40	2	415757	0.5	831514
9	7.5	10	0	16902	2	8451
10	9	20	1	462075	1	462075
11	7.5	10	1	145653	2	72827
12	9	40	2	353304	0.5	706608
13	7.5	20	1	63809	1	63809
14	7.5	20	0	7361	1	7361
15	9	10	0	378647	2	189324
16	9	10	2	492255	2	246128
17	9	20	0	343175	1	343175
18	7.5	10	2	413851	2	206926
19	7.5	20	2	492685	1	492685