Published in final edited form as:

Methods Mol Biol. 2011 ; 705: 175–194. doi:10.1007/978-1-61737-967-3_10.

Expression of Recombinant Proteins with Uniform N-Termini

Orsolya Király¹, Lan Guan², and Miklós Sahin-Tóth¹

¹ Department of Molecular and Cell Biology, Henry M. Goldman School of Dental Medicine, Boston University, Boston, MA, 02118

² Department of Cell Physiology & Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, TX 79430

Abstract

Heterologously expressed proteins in *Escherichia coli* may undergo unwanted N-terminal processing by methionine and proline aminopeptidases. To overcome this problem, we present a system where the gene of interest is cloned as a fusion to a self-splicing mini-intein. Furthermore, this fusion construct is expressed in an engineered *Escherichia coli* strain from which the *pepP* gene coding for aminopeptidase P has been deleted. We describe a protocol using human cationic trypsinogen as an example to demonstrate that recombinant proteins produced in this expression system contain homogeneous, unprocessed N-termini.

Keywords

Escherichia coli; intein; human cationic trypsinogen; *in vitro* refolding; ecotin affinity chromatography; aminopeptidase P

1. Introduction

The expression system presented here was developed as part of an effort to elucidate the functional effect of the p.A16V mutation in human cationic trypsinogen, which has been reported to be associated with chronic pancreatitis by several studies (1–4). This variant alters the N-terminal amino acid residue of the mature, secreted trypsinogen protein. The amino-acid numbering starts with Met¹ of the pre-trypsinogen protein and the first 15 residues comprise the secretory signal peptide. It had previously been determined that other pancreatitis-associated mutations in human cationic trypsinogen increase the propensity of trypsinogen for autoactivation (5,6). We speculated that the p.A16V mutation might have a similar effect; however, functional characterization of the recombinantly expressed mutant trypsinogen required preparations with uniform, authentic N-termini.

For high-yield heterologous expression of human cationic trypsinogen in *Escherichia coli*, an expression plasmid was constructed in which the secretory signal peptide of trypsinogen was deleted and the initiator methionine was placed immediately upstream of the mature protein. Trypsinogen expressed from this construct accumulated in the cytoplasm as inclusion bodies. The native N-terminal sequence of trypsinogen isolated from pancreatic juice is Ala¹⁶-Pro¹⁷-Phe¹⁸. The expected N-terminal sequence of trypsinogen expressed in *E. coli* is Met- Ala¹⁶-Pro¹⁷-Phe¹⁸. However, we found that the N-terminal sequence of recombinant trypsinogen in the inclusion bodies was heterogeneous, consisting of ~30%

Pro¹⁷-Phe¹⁸ and approximately ~70% Met-Ala¹⁶-Pro¹⁷-Phe¹⁸. Apparently, part of the expressed trypsinogen was processed by methionine aminopeptidase and then by proline aminopeptidase (aminopeptidase P). Removal of the initiator methionine by methionine aminopeptidase is a well documented phenomenon in *E. coli* (7) which occurs if the second amino acid residue has a small, uncharged side chain. With high-level expression of heterologous proteins, the enzyme gets saturated and only a fraction of the proteins is processed. After cleavage of the initiator methionine, proteins can be subject to cleavage by aminopeptidase P, which cleaves the N-terminal amino acid of a protein if proline is in the penultimate position (8).

To address the problem of unwanted N-terminal processing by aminopeptidases, we developed a novel expression system (9). First, the gene of interest (in this case human cationic trypsinogen) was cloned in a C-terminal fusion with the *Synechocystis* DnaB mini-intein (10). In this fusion construct (see Fig. 1.), translation was initiated by the start codon of the intein gene and the intein moiety was subsequently removed through intein self-cleavage (11). To eliminate cleavage by aminopeptidase P, the fusion construct was expressed in the aminopeptidase P deficient *E. coli* LG-3 strain. This strain was engineered by deleting the *pepP* gene coding for aminopeptidase P from the *E. coli* chromosome (12, 13), using the recombination-based method described by Datsenko and Wanner (14). The intein-trypsinogen fusion was expressed in LG-3 cells as inclusion bodies, solubilized with guanidine and re-natured *in vitro* followed by affinity purification on immobilized ecotin (15). Finally, we used MonoS cation-exchange chromatography to remove the small fraction of uncleaved intein fusion proteins and to obtain a pure trypsinogen preparation with uniform, authentic N-termini. The expression system described here can be useful for the heterologous expression of proteins whose N-terminal integrity is compromised by aminopeptidase activity.

2. Materials

2.1. Expression Plasmid Construction

1. Expression plasmid pTrapT7-*PRSSI* harboring the human cationic trypsinogen gene under the control of the T7 promoter (5,6).
2. Plasmid pTWIN2 (New England Biolabs, Ipswich, MA).
3. Oligonucleotide primers (Sigma Genosys, The Woodlands, TX).

Primer A: 5'-CGG GAG TCC ATG GCT ATC TCT GGC GAT AGT CTG ATC AGC-3'

Primer B: 5'-GAA AGG AGC GTT GTG TAC AAT GAT GTC ATT CGC-3'

Primer C: 5'-ATT GTA CAC AAC GCT CCT TTC GAT GAT GAT GAC AAG-3'

Primer D: 5'-CTG CTC ATT GCC CTC AAG GAC-3'

4. Deep Vent[®] proofreading DNA polymerase with appropriate reaction buffer, 10 mM dNTP mix (New England Biolabs), diluted to 2 mM and stored in aliquots at -20 °C.
5. Agarose gel electrophoresis equipment (e.g. Mini-Sub Cell GT system, Bio-Rad, Hercules, CA).
6. QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).
7. Restriction enzymes *Nco*I and *Eco*RI, T4 DNA ligase (New England Biolabs).

8. α -Select Gold Competent *E. coli* cells (Bioline, Taunton, MA).
9. Luria-Bertani (LB) medium. Dissolve 25 g granulated LB broth, Miller (BP1426-500, Fisher Scientific) in 1 L distilled water, add 10 mL 1 M Tris-HCl, pH 7.5; sterilize by autoclaving.
10. LB agar plates containing 100 μ g/mL ampicillin, stored at 4 °C.
11. QIAprep Spin Miniprep kit (Qiagen).
12. DNA sequencing was performed with automated sequencing at a core facility.

2.2. Deletion of the *pepP* gene from *E. coli*

1. Plasmids pKD4 and pCP20 (available from Dr. Barry L. Wanner, Department of Biological Sciences, Purdue University, West Lafayette, IN, *see ref.* 14).
2. Oligonucleotide primers (Sigma Genosys).
P1-s: 5'-ACT CTA CAC TAA AAA CAA AAA ACG TAA GGA GAG TGT TAT GAG TGG TGT AGG CTG GAG CTG CTT C-3'
P2-as: 5'-AGC GCC AGC GTC GCG CCC GCC ATG CCG CCA CCG ACG ATG ATT ACG CAT ATG AAT ATC CTC CTT A-3'
T1: 5'-CCG CAA CCG ACC GCG CCA GAA G-3' k1: 5'-CAG TCA TAG CCG AAT AGC CT-3'
T2: 5'-CAA ATG TAC CGG CAG CGC CC-3' k2: 5'-CGG TGC CCT GAA TGA ACT GC-3'
3. Restriction enzyme *DpnI* (New England Biolabs).
4. Agarose gel electrophoresis equipment (e.g. Mini-Sub Cell GT system, Bio-Rad).
5. *E. coli* strain BW25113 harboring the helper plasmid pKD46 (14) (available from Dr. Barry L. Wanner).
6. Luria-Bertani (LB) medium. Dissolve 25 g granulated LB broth, Miller (BP1426-500, Fisher Scientific) in 1 L distilled water, add 10 mL 1 M Tris-HCl, pH 7.5; sterilize by autoclaving.
7. SOB medium: 20 g/L bacto-tryptone, 5 g/L yeast extract, 0.584 g/L NaCl, 0.186 g/L KCl, pH 7.0. Sterilize by autoclaving, store at 4 °C. For complete medium, add 1 mL of 2 M Mg^{2+} solution to 99 mL SOB medium.
8. 2 M Mg^{2+} solution: 1 M $MgCl_2 \cdot 6 H_2O$, 1 M $MgSO_4 \cdot 7 H_2O$. Dissolve 20.33 g $MgCl_2 \cdot 6 H_2O$ and 24.65 g $MgSO_4 \cdot 7 H_2O$ in 100 mL distilled water, filter sterilize.
9. 2 M glucose solution: dissolve 36.04 g glucose (Sigma, St. Louis, MO) in 100 mL distilled water, filter sterilize, store at 4 °C.
10. SOC medium: SOB medium with 20 mM glucose. Add 1 mL of 2 M Mg^{2+} solution and 1 mL 2 M glucose solution to 98 mL SOB medium.
11. Electroporator with 0.2 cm cuvettes (e.g. MicroPulser, Bio-Rad).
12. Kanamycin: Kanamycin sulfate (Sigma) is dissolved in sterile water at 16 mg/mL and stored at -20°C in aliquots.
13. Ampicillin: Ampicillin (Sigma) is dissolved in sterile water at 100 mg/mL and stored at -20°C in aliquots.

14. *L*-arabinose: *L*-arabinose (Sigma) is dissolved in sterile water at 1 *M* and stored at –20°C in aliquots.
15. Plasmid pGP1-2 harboring the T7 RNA polymerase under the control of a temperature- inducible λ promoter (16).
16. DNA sequencing was performed with automated sequencing at a core facility.

2.3. Expression and purification of trypsinogens

2.3.1. Trypsin Affinity Chromatography Column

1. Actigel ALD resin (#2701; 50 mL; Sterogene Bioseparations, Carlsbad, CA, *see* Note 1).
2. Coupling solution (#9704; 10 mL; 1 *M* sodium cyanoborohydride; Sterogene Bioseparations).
3. Bovine trypsin, TPCK treated (# 3744, Worthington Biochemical, Lakewood, NJ).
4. 60 mL Buchner funnel inserted into a side-arm flask through a rubber stopper.
5. Adams Nutator mixer or shaking table.
6. Empty chromatography column, ~8 mL volume (10×100 mm, e.g. HR 10/10, Amersham-Pharmacia, Piscataway, NJ).

2.3.2. Ecotin Affinity Chromatography Column

1. Plasmid pT7-7-ecotin harboring the recombinant ecotin gene (17).
2. Competent *E. coli* BL21 (DE3) cells (Novagen, Madison, WI).
3. Luria-Bertani (LB) medium. Dissolve 25 g granulated LB broth, Miller (BP1426-500, Fisher Scientific) in 1 L distilled water, add 10 mL 1 *M* Tris-HCl, pH 7.5; sterilize by autoclaving.
4. Ampicillin: Ampicillin (Sigma) is dissolved in sterile water at 100 mg/mL and stored at –20°C in aliquots.
5. IPTG: Isopropyl-1-thio- β , D-galactopyranoside (Sigma) is dissolved to 1 *M* in sterile distilled water and stored in aliquots at –20°C.
6. Osmotic shock buffer: 30% sucrose, 20 *mM* Tris-HCl, pH 8.0, 5 *mM* Na-EDTA. Prepare fresh and store at 4°C until use.
7. 1 *M* Tris-HCl (pH 8.0). Dissolve 121.14 g Tris base in 1 L distilled water and adjust pH to 8.0 with HCl under a pH meter.
8. 5 *M* NaCl. Dissolve 292.2 g NaCl in 1 L distilled water.
9. Lyophilizer (e.g. Freezone 4.5 Freeze Dry System, Labconco Corp., Kansas City, MO).
10. Fast Protein Liquid Chromatography (FPLC) system (e.g. LCC-501 Plus, Amersham-Pharmacia) with trypsin affinity column (*see* **step 5 of Subheading 3.3.1**).

¹There are several newer generation forms of Actigel ALD available (e.g. Ultraflow, Superflow) which claim to exhibit better flow rates. In our application we experienced no benefit from using these resins and we obtained the best results with the least expensive, regular Actigel ALD resin.

11. Spectrophotometer with quartz cuvette to measure ultraviolet (UV) absorbance at 280 nm.
12. Actigel ALD resin (#2701; 50 mL; Sterogene Bioseparations).
13. Coupling Solution (#9704; 10 mL; 1 M sodium cyanoborohydride, Sterogene Bioseparations).
14. Empty chromatography column with ~2 mL volume (5×100 mm; e.g. HR 5/10 or Tricorn 5/100, Amersham-Pharmacia).

2.3.3. Trypsinogen Expression and Purification using *E. coli* Strain LG-3

1. *E. coli* strain LG-3: described in **step 21** of **Subheading 3.2**.
2. Luria-Bertani (LB) medium. Dissolve 25 g granulated LB broth, Miller (BP1426-500, Fisher Scientific) in 1 L distilled water, add 10 mL 1 M Tris-HCl, pH 7.5; sterilize by autoclaving.
3. IPTG: Isopropyl-1-thio- β , D-galactopyranoside (Sigma) is dissolved to 1 M in sterile distilled water and stored in aliquots at -20°C .
4. Resuspension/wash buffer for inclusion bodies: 0.1 M Tris-HCl, pH 8.0, 5 mM K-EDTA.
5. Sonicator (e.g. Cell Disruptor Model W-200R with a microtip probe, Heat Systems Ultrasonics, Farmingdale, NY).
6. DTT: Dithiothreitol (Sigma) is dissolved in sterile distilled water to 1 M and stored in aliquots at -20°C .
7. Denaturing buffer for inclusion bodies: 4 M guanidine-HCl, 0.1 M Tris-HCl, pH 8.0, 2 mM K-EDTA, 30 mM dithiothreitol (*see* Note 2).
8. Refolding buffer: 0.9 M guanidine-HCl, 0.1 M Tris-HCl, pH 8.0, 2 mM K-EDTA, 1 mM L-cysteine, 1 mM L-cystine.
9. Argon gas (Grade 5).
10. Fast Protein Liquid Chromatography (FPLC) system (e.g. LCC-501 Plus) with Mono S HR 5/5 cation-exchange column (Amersham-Pharmacia).
11. 2 mL ecotin affinity column (*see* **step 18** of **Subheading 3.3.2**).
12. Wash buffer for ecotin affinity chromatography: 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl.
13. Elution solution for ecotin affinity chromatography: 50 mM HCl.
14. Buffer A for cation exchange chromatography: 20 mM Na-acetate, pH 5.0.
15. Buffer B for cation exchange chromatography: 20 mM Na-acetate, pH 5.0, 0.5 M NaCl.
16. Spectrophotometer with quartz cuvette to measure ultraviolet (UV) absorbance at 280 nm.

2.3.4. SDS-PAGE

1. Running gel buffer: 1.5 M Tris-HCl, pH 8.8, 0.4 % SDS.

²Add dithiothreitol immediately before use from a 1 M solution kept at -20°C in aliquots.

2. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8, 0.4 % SDS.
3. Acrylamide solution: 30% acrylamide with a acrylamide:bis-acrylamide ratio of 37.5:1.
4. Ammonium persulfate solution: 10 % ammonium persulfate (APS, Sigma) solution. Prepared fresh in distilled water.
5. N,N,N',N'-Tetramethylenediamine (TEMED, Sigma, electrophoresis grade).
6. 2× Laemmli sample buffer: 120 mM Tris-HCl, pH 6.8, 20% glycerol, 2 % SDS, 0.003% bromophenol blue. To make 10 mL of 2× sample buffer, mix 34 mL distilled water with 10 mL glycerol, 6 mL 1 M Tris-HCl, pH 6.8, 1 g SDS and 1.5 mg bromophenol blue (Sigma). Store the sample buffer at room temperature and add dithiothreitol to 100 mM just before use.
7. DTT: Dithiothreitol (Sigma) is dissolved in sterile distilled water to 1 M and stored in aliquots at −20 °C.
8. Protein markers: MultiMark multi-colored standards (Invitrogen, Carlsbad, CA).
9. Gel staining solution: 40% methanol, 10% acetic acid, 0.25% Coomassie Brilliant Blue R-250 (*see* Note 3). To prepare 1 L staining solution, mix 500 mL distilled water with 400 mL methanol, 100 mL glacial acetic acid and 2.5 g Coomassie Brilliant Blue R-250. Stir on magnetic stirrer until the dye is completely dissolved, then filter the solution.
10. Destaining solution: 30% methanol, 10% acetic acid (*see* Note 3).
11. DryEase® Mini-Gel Drying System (Invitrogen, *see* Note 4).

3. Methods

3.1. Construction of the expression plasmid harboring the intein-trypsinogen fusion

1. Amplify the ~460 nt DnaB mini-intein gene from the plasmid pTWIN2 using primers A and B. Reactions contain 1×reaction buffer, 0.2 mM dNTP, 1 μM of each primer and 0.02 units/μL of Deep Vent DNA polymerase. Cycling conditions are as follows: 35 cycles of 94°C for 20 sec, 50°C for 30 s, 72°C for 1 min (RapidCycler, Idaho Technology, Salt Lake City, UT).
2. A ~230 nt portion of the recombinant human cationic trypsinogen gene is amplified from plasmid pTrapT7-*PRSSI* using primers C and D. Reaction and cycling conditions are the same as in **step 1**.
3. Run PCR reactions from Steps 1 and 2 on an agarose gel and excise the PCR product bands from the gel using a razor blade.
4. Purify the PCR products from the gel with a gel extraction kit and elute in 10 μL elution buffer (10 mM Tris-HCl, pH 8.0).
5. Set up a new PCR reaction containing 1 μL of each product from **steps 1 and 2**, and primers A and D. Reaction and cycling conditions are the same as in step 1 (*see* Note 5).

³Solutions containing methanol are hazardous and should be disposed of accordingly.

⁴Gel-Dry™ solution contains ethanol, polyethylene glycol, methanol and isopropanol and should be disposed of as hazardous waste.

⁵The underlined sequences in primers B and C overlap and thus allow the hybridization of PCR products obtained in the first round of amplifications, (*see* ref 20).

6. The reaction product is run on an agarose gel, excised, purified and digested with *NcoI* and *EcoRI*.
7. Digest the expression plasmid pTrapT7-*PRSSI* (5,6) with *NcoI* and *EcoRI* and ligate the PCR product between these sites using T4 DNA ligase.
8. 1 μ L of the ligation reaction is transformed into chemically competent *E. coli* and the cells spread on LB agar plates containing 100 μ g/mL ampicillin. Incubate the plates overnight at 37°C.
9. Pick two colonies from the plate and grow in 2 mL LB medium with 100 μ g/mL ampicillin overnight with shaking.
10. Prepare plasmid DNA (miniprep) from the transformants and digest ~1 μ g DNA with *NcoI* and *EcoRI*. Verify the presence of the correct insert by separating and visualizing the digestion reaction on an agarose gel.
11. Choose a positive clone and sequence the entire *NcoI-EcoRI* insert.

3.2. Deletion of the *pepP* gene from *E. coli*

To remove the *pepP* gene that encodes an active proline aminopeptidase from the *E. coli* genome, the Red recombinase based gene deletion system described by Datsenko and Wanner (14) is used. First, a recombination substrate is produced by amplifying the kanamycin resistance gene flanked by two FRT sites using primers that contain 44–45 nucleotide extensions homologous to sequences flanking the *pepP* gene on the *E. coli* chromosome. This PCR product is introduced into *E. coli* cells harboring a helper plasmid expressing the λ Red recombinase that catalyzes homologous recombination between the recombination substrate and the *E. coli* chromosome. The target gene is thus exchanged for the recombination substrate containing the kanamycin resistance gene and recombinants can be selected for by kanamycin resistance. The kanamycin resistance gene is eliminated by site-specific recombination between the two FRT sites catalyzed by the FLP recombinase introduced on a second helper plasmid. Both helper plasmids are temperature sensitive and can be eliminated by growing at the non-permissive temperature. At the end of the procedure, a single FRT site remains at the locus of the *pepP* gene. This remaining FRT sequence is sometimes termed a “scar sequence”.

1. Amplify the kanamycin resistance gene from plasmid pKD4 using primers P1-s and P2-as with the following cycling program: 95°C for 8 min followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 2 min, and a final extension of 72 °C for 8 min.
2. The PCR reaction is run on an agarose gel, gel-purified and treated with *DpnI* to eliminate template DNA. *DpnI* cleaves only the methylated template plasmid DNA. 1 μ L *DpnI* and 5 μ L 10 $\times$ NEBuffer 4 are added to a 45 μ L PCR reaction and the digestion reaction is incubated at 37°C for 1 hour. The digested product is electrophoresed and gel-purified.
3. The *E. coli* strain BW25113 containing the helper plasmid pKD46 (harboring the Red recombinase system genes) is spread on a fresh LB agar plate containing 100 μ g/mL ampicillin.
4. After incubating the plate at 30°C overnight, 12 mL LB medium containing 100 μ g/mL ampicillin is inoculated with a colony and grown overnight at 30°C.
5. To make electrocompetent cells, 1 mL overnight culture is mixed with 20 mL SOB, 100 μ L 2 M $MgSO_4$ and 20 μ L 1 M *L*-arabinose and grown at 30°C for 5 hours.

6. Cells are harvested by centrifugation (1,000 ×g, 15 minutes at 4 °C), washed twice with 40 mL ice cold water and once with 40 mL ice cold 10% glycerol, and resuspended in 0.2 mL 10% glycerol.
7. 3 µL *DpnI*-treated PCR reaction (from **step 2**) is added to 100 µL of electrocompetent cells and pulsed in a 0.2 cm electroporation cuvette with an electroporator setting of 2.5 kV (Ec2 setting on MicroPulser).
8. 0.5 mL SOC medium is added and the cells are incubated at 37°C for 1 hour.
9. Cells are spread on LB plates containing 50 µg/mL kanamycin and incubated at 37°C overnight.
10. Seven colonies are picked from the plate and each is suspended in 50 µL water.
11. Using 5 µL of this suspension as template, colonies are screened by PCR using two primer sets: primers T-1 and k-1, and primers T-2 and k-2 (*see* Note 6). Reactions contain 1× Taq reaction buffer, 2.5 mM MgSO₄, 0.2 mM dNTP, 1 µM of each primer and 2 units of Taq polymerase in 50 µL final volume. Cycling conditions are 96°C for 4 min, followed by 25 cycles of 96°C for 45 s, 62°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 4 min.
12. PCR reactions are run on an agarose gel.
13. Colonies showing the expected size bands are streaked onto LB plates containing no antibiotics and incubated at 37°C overnight to cure the temperature sensitive pKD46 plasmid. All tested colonies are patched onto LB plates with 50 µg/mL kanamycin and LB plates with 100 µg/mL ampicillin. (*See* Note 7).
14. From colonies resistant to kanamycin but sensitive to ampicillin, one colony from the drug free LB plate is inoculated into 10 mL LB with 50 µg/mL kanamycin and grown overnight. The absence of the *pepP* gene is verified by amplifying the “scar sequence” using primers T-1 and T-2 and sequencing the PCR product. This kanamycin-resistant, aminopeptidase P-deleted strain is designated as LG-1. Electrocompetent LG-1 cells are prepared as described in **steps 5 and 6** (cells are grown at 37 °C).
15. Plasmid pCP20 harboring the FLP recombinase gene is purified by miniprep after growing the appropriate cells at 30°C overnight in LB medium with 100 µg/mL ampicillin.
16. Plasmid pCP20 is introduced into competent cells from **step 14** by electroporation and then 0.5 mL SOC medium is added and the cells are incubated at 30°C for 1 hour. Cells are spread on LB plates containing 100 µg/mL ampicillin and incubated at 30°C overnight.
17. 10 colonies are picked and streaked onto drug-free LB plates, then incubate at 43°C overnight.
18. Streaks from **step 17** are patched on drug-free LB plates, LB plates with 50 µg/mL kanamycin and LB plates containing 100 µg/mL ampicillin and incubated at 37 °C overnight.

⁶In both primer sets, one primer anneals within the kanamycin resistance gene and the other anneals to a nearby sequence in the *E. coli* chromosome. Amplification of PCR products indicates that recombination between the *pepP* gene and the recombination substrate has occurred.

⁷Colonies resistant to kanamycin have undergone homologous recombination and have lost the *pepP* gene, while resistance to ampicillin indicates that the helper plasmid has not been lost.

19. One of the colonies growing in the absence of antibiotics but sensitive to both kanamycin and ampicillin, indicating excision of the kanamycin resistance gene and loss of the helper plasmid pCP20, is picked and grown overnight in 10 mL LB. This strain is designated LG-2.
20. Electrocompetent LG-2 cells are prepared as described in **steps 5 and 6** (growing at 37°C).
21. Plasmid pGP1-2 is purified by a miniprep protocol and introduced into electrocompetent LG-2 cells by electroporation. Transformants are spread onto LB plates with 50 µg/mL kanamycin and the plates are incubated at 30°C. The new strain is designated LG-3 and is used for the expression of trypsinogens.

3.3. Expression and purification of trypsinogens

3.3.1. Preparation of the trypsin affinity chromatography column

1. The Actigel ALD resin (supplied in 20% ethanol) is resuspended by turning the bottle upside-down a few times. A 60 mL Buchner funnel is inserted into a side-arm flask through a rubber stopper and the side arm is attached to the house vacuum through a hose. Approximately 20 mL resin is poured into the funnel and washed several times with 50 mM Na-phosphate, pH 7.5 under suction to remove the ethanol and to equilibrate with the coupling buffer. (*See Note 8*).
2. The vacuum is disconnected and the resin in the funnel is resuspended in ~5 mL phosphate buffer. The resuspended resin is transferred from the funnel to a 50 mL Falcon tube and allowed to settle.
3. The supernatant is removed with a pipette and to the remaining ~16 mL wet resin 14 mL 50 mM Na-phosphate, pH 7.5 and 80 mg crystalline bovine trypsin are added. This corresponds to about 5 mg protein per mL resin immobilization ratio. Finally, 3 mL 1 M coupling solution is added to 0.1 M final concentration.
4. Seal the Falcon tube's cap with Parafilm and incubate the tube at room temperature for 1–3 hours. After the room temperature incubation, move the reaction to the 4°C cold room and leave overnight with gentle rocking on an Adams Nutator.
5. Pour the resin into a ~8 mL volume empty chromatography column and wash several times, alternating between 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl buffer and 50 mM HCl solution. Store the column equilibrated in 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl in the refrigerator.

3.3.2. Preparation of the ecotin affinity chromatography column—Ecotin is a pan-serine-protease inhibitor from *E. coli* (18) that forms a relatively tight complex with trypsinogen and thus can be used for affinity purification of the zymogen (15). Recombinant ecotin (17) is overexpressed in the periplasmic space of *E. coli* and isolated using osmotic shock (19) followed by trypsin affinity chromatography. Purified ecotin is immobilized on aldehyde activated resin by reductive amination using cyanoborohydride (15) and loaded into a chromatography column.

1. *E. coli* BL21 (DE3) cells are transformed with plasmid pT7-7-ecotin (17) and spread on an LB agar plate containing 100 µg/mL ampicillin to select for transformants. The plates are incubated overnight at 37°C.

⁸The coupling buffer must be free of amines, therefore, phosphate-based buffers are recommended. Do not use Tris buffer or other amine-containing buffers.

2. Two flasks containing 50 mL LB medium with 100 µg/mL ampicillin (or 50 µg/mL carbenicillin; Sigma) are each inoculated with a streak of the pT7-7 transformed *E. coli* colonies and incubated overnight at 37 °C with shaking.
3. Two flasks of 1,200 mL LB medium containing 100 µg/mL ampicillin are each inoculated with a 50 mL overnight culture and grown at 37 °C until OD₆₀₀ reaches 0.5 (*see* Note 9).
4. Expression of ecotin is induced by adding 1 mM IPTG to the cultures. Cultures are grown for an additional 4 hours at 37°C.
5. Harvest the cells by centrifugation at 15,000×g for 10 minutes at 4°C and resuspend in 500 mL ice cold osmotic shock buffer. Keep on ice for 10 minutes.
6. Centrifuge the suspension for 30 min at 15,000×g at 4 °C. Discard the supernatant.
7. Resuspend the pellet in 250 mL ice cold water and incubate on ice for 15 minutes.
8. Centrifuge the suspension for 15 minutes at 15,000×g at 4 °C. The supernatant, containing the periplasmic fraction, is removed and saved (*see* Note 10).
9. 5 mL 1 M Tris-HCl, pH 8.0 and 10 mL 5 M NaCl is added to the supernatant (*see* Note 11).
10. The supernatant, which is approximately 250 mL, is centrifuged for 15 minutes at 27,000×g at 4 °C and loaded on the trypsin affinity column in five runs of 50 mL each.
11. Collect the flow-through from all runs and pool. Apply the pooled flow-through back onto the column.
12. Wash the column with 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl. Elute the ecotin with 50 mM HCl. Pool the eluates from all the runs. Determine the concentration and yield of ecotin by reading the absorbance at 280 nm using a calculated molar extinction coefficient of 23,045 M⁻¹cm⁻¹ and the monomeric molecular mass of 16,099.5 Da (<http://ca.expasy.org/tools/protparam.html>). Typical yields vary between 20 and 40 mg of purified ecotin.
13. Dialyze the eluate against two changes of 3.5 L 1 mM HCl overnight. Determine its purity by subjecting samples of 1 µL, 5 µL and 10 µL to SDS-PAGE (*see Subheading 3.3.4*).
14. Lyophilize the dialyzed ecotin overnight (*see* Note 12).
15. Store the Lyophilized ecotin at -20 °C until use. When needed dissolve in water to obtain ~40 mg/mL concentration.
16. Wash the Actigel ALD resin as described in **step 1 of Subheading 3.3.1**.
17. Mix 1 mL ecotin solution (~40 mg protein) with 8 mL wet resin (~5 mg protein per wet resin immobilization ratio) and 1 mL 1 M sodium cyanoborohydride. Incubate

⁹Growing the bacterial culture followed by the isolation of the periplasmic fraction is a full-day procedure, so it is practical to start the culture as early as possible.

¹⁰At this step, the supernatant has to be clear. Centrifuge again if necessary before proceeding to **step 9**.

¹¹The procedure can be paused here and the supernatant stored on ice overnight.

¹²Distribute the dialyzed sample to 50 mL Falcon tubes (about 15 mL in each). Using adequate cold protection, hold the tubes tilted in a Dewar flask with liquid nitrogen and slowly rotate the tubes so that ecotin freezes onto the tube wall as a layer. Cover the tubes with Parafilm and punch holes in the Parafilm to let water evaporate. Place tubes in a larger freeze-dry bottle or flask and lyophilize overnight.

the mixture at room temperature for 1–3 hours on a shaking table followed by overnight incubation in the cold room.

18. Pack the resin into a 2 mL chromatography column and wash several times by alternating 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl buffer and 50 mM HCl. Store the column equilibrated with 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl in the refrigerator.

3.3.3. Expression and purification using *E. coli* strain LG-3—Trypsinogen variants cloned into the intein fusion constructs are expressed as inclusion bodies in *E. coli* LG-3. Analysis of solubilized inclusion bodies by SDS-PAGE (*see Subheading 3.3.4*) reveal that the intein moiety has been cleaved off approximately 70–80% of the fusion proteins in *E. coli* (Fig. 2A), therefore a separate *in vitro* intein cleavage step is not included. After *in vitro* refolding, trypsinogens are purified using ecotin affinity chromatography (15). SDS-PAGE analysis of purified samples show that eluted trypsinogens contain a small fraction of uncleaved fusion proteins (Fig. 2B) and further purification is necessary. The eluate is thus subjected to ion-exchange chromatography (Fig. 2D) which yields a homogenous preparation of recombinant trypsinogens with authentic N-termini (Fig. 2C).

1. Electroporate the plasmid containing the intein fusion construct (from **step 11** in **Subheading 3.1**) into the aminopeptidase P deficient *E. coli* strain LG-3 (from **step 21** in **Subheading 3.2**). Spread transformants onto LB agar plates containing 100 µg/mL ampicillin and 50 µg/mL kanamycin. Incubate the plates at 30°C overnight.
2. Establish starter cultures by inoculating 10 mL LB medium with 100 µg/mL ampicillin and 50 µg/mL kanamycin with a streak of colonies from each plate and incubating at 30 °C overnight with shaking.
3. Inoculate 200 mL LB medium containing 100 µg/mL ampicillin and 50 µg/mL kanamycin with 10 mL starter culture and grow until culture density reaches an OD₆₀₀ of 0.5. This will take approximately 2.5 hours.
4. Transfer cultures to a 42 °C incubator for 30 minutes and add 200 µL 1 M IPTG (*see Note 13*).
5. Grow cultures at 30 °C for an additional 5 hours (*see Note 14*).
6. Cultures are divided into four 50 mL Falcon tubes and cells are harvested by centrifugation at 2000×g for 10 minutes. Store pellets at –80 °C until use.
7. One pellet, corresponding to 50 mL of the original culture, is used per round of purification. Thaw the pellet on ice and resuspend in 4 mL resuspension buffer. Keep samples on ice from this step until **step 13**.
8. Divide the resulting suspension into 6 aliquots of approximately 700 µL in Eppendorf tubes for more efficient sonication.
9. Sonicate the aliquots three times for 20 seconds using continuous mode at power setting 4.
10. Centrifuge the sonicated samples in a microcentrifuge at 13,200 rpm for 5 minutes at 4 °C.

¹³Addition of IPTG is necessary because the region upstream of the intein-trypsinogen fusion contains the lac operator (5,6).

¹⁴It is useful to check expression levels before starting the purification procedure. After approximately 4 hours of culturing, a 1 mL sample is removed from each culture and centrifuged for 5 min at 12,300 rpm. Discard the supernatant and resuspend the pellet in 1 mL resuspension buffer and sonicate three times for 20 s (continuous mode, power setting 5). Microfuge the samples for 5 min at 12,300 rpm and discard the supernatants. Resuspend the pellets in 30 µL reducing Laemmli sample buffer, vortex briefly and incubate at 90°C for 5 minutes. Apply these samples to a SDS polyacrylamide gel (*see Subheading 3.3.4*). Purified trypsinogen should be loaded as positive control and samples with a strong band at the same position are processed further.

11. Discard the sample supernatants and resuspend the pellets in 1 mL wash buffer by pipetting. Centrifuge the resuspended samples in a microfuge at 13,200 rpm for 5 minutes at 4 °C. The supernatants are discarded and the pellets are resuspended and centrifuged again (*see* Note 15).
12. Washed pellets, corresponding to inclusion bodies, are resuspended in 500 µL denaturing buffer containing 30 mM dithiothreitol and incubated at 37°C for 30 min to reduce the disulfide bonds in trypsinogen.
13. During **step 12**, *L*-cystine and *L*-cysteine are added to 50 mL refolding buffer and stirred for 30 min on a magnetic stirrer under argon (*see* Note 16).
14. Microfuge samples at 13,200 rpm for 5 minutes at 4 °C to remove any insoluble material and then dilute in 50 mL refolding buffer (containing *L*-cystine and *L*-cysteine) under argon and stir for 5 minutes (*see* Note 17).
15. Refolding reactions are incubated overnight at 4°C.
16. Dilute the refolding reactions with 50 mL 0.4 M NaCl and centrifuge at 27,000×g for 15 minutes (*see* Note 18).
17. Refolded trypsinogen (100 mL) is loaded onto a 2 mL ecotin affinity column (from **step 18** of **Subheading 3.3.2**) at flow rate of 2 mL/min.
18. The column is washed with wash buffer at a flow rate of 2 mL/min until the UV signal recedes to baseline.
19. Trypsinogen is eluted from the column with 50 mM HCl at a flow rate of 2 mL/min and the protein concentration is determined by reading the absorbance at 280 nm and using a calculated molar extinction coefficient of 37,525 M⁻¹cm⁻¹ (<http://ca.expasy.org/cgi-bin/protparam>). Samples are then subjected to SDS-PAGE (described under **Subheading 3.3.4**) for assessment of purity (Fig. 2B).
20. The ecotin eluate is directly loaded onto a Mono S HR 5/5 cation exchange column equilibrated with 20 mM Na-acetate, pH 5.0.
21. The column is developed with a gradient of 0–0.5 M NaCl at a flow rate of 1 mL/min and 1 mL fractions are collected, *see* Fig. 2D.
22. Fractions showing the highest absorbance at 280 nm are run on an SDS-PAGE gel for verification of purity (Fig. 2C).

3.3.4. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

1. The Mini-PROTEAN Gel System (Bio-Rad) is described. Clean glass plates are rubbed with 95% ethanol to remove any grease and 13% running gels are prepared by mixing (for 4 gels) 9.4 mL water, 7.5 mL running gel buffer, 13 mL 30% acrylamide solution, 100 µL APS solution and 50 µL TEMED. The solution is

¹⁵Pellets can be combined to produce a single pellet at the end of the washing procedure.

¹⁶First, add *L*-cystine in powder form to 50 mL refolding buffer and dissolve by vigorous stirring at room temperature on a magnetic stirrer for about 30 minutes. There is no need for argon at this step. *L*-cystine is poorly soluble in water and undissolved crystals may still be visible after 30 min. This has no impact on the refolding procedure. Add *L*-cysteine after the refolding solution has been equilibrated with argon for a few minutes. *L*-cysteine should dissolve readily.

¹⁷A 200 µL pipet tip is attached to the tubing from the gas cylinder and punched through the Parafilm covering the flask with the refolding buffer. The flask is placed on a magnetic stirrer and after a few minutes of argon flow, the denatured trypsinogen sample is added dropwise to the buffer under moderate stirring. The solution is then stirred for another 5 min under argon.

¹⁸This step is included to dilute the guanidine content of the sample. Centrifugation is necessary to remove any precipitated proteins, which can clog up the chromatography system and the column.

gently mixed and gels are cast, overlayed with water-saturated 1-butanol, and allowed to set for at least 30 min.

2. Stacking gels (4 gels) are prepared by mixing 11.6 mL water, 5 mL stacking gel buffer, 3.33 mL 30% acrylamide solution, 60 μ L APS solution and 50 μ L TEMED.
3. Pour off the 1-butanol from the running gels and then cast the stacking gels and insert the combs. Stacking gels are left to polymerize for at least 30 min, then the combs are removed and the wells rinsed with distilled water (*see* Note 19).
4. The concentration of trypsinogen samples is determined as described in **step 19** under **Subheading 3.3.3**. Approximately 200 pmol trypsinogen (~5 μ g) is loaded per lane.
5. Trypsinogens are precipitated by adding trichloroacetic acid at 10% final concentration and incubating on ice for 10 minutes.
6. Centrifuge the samples at 13,200 rpm for 10 minutes in the microfuge at 4 °C and discard the supernatants.
7. Resuspend the pellets in 30 μ L reducing Laemmli sample buffer, briefly vortex and heat-denature by incubating at 90°C for 5 minutes.
8. Load the samples onto a gel using a 20 μ L pipet tip or Hamilton syringe. One lane on each gel should be loaded with protein markers.
9. Run the gel by applying a 30 mA current until the blue dye front exits the gel.
10. After electrophoresis, remove the gel from the unit and stain in Coomassie Blue staining solution for 20–30 minutes on a rocking table (*see* Note 20).
11. Excess staining is removed by incubating the gel in several changes of destaining solution on a rocking table until the gel background was clear (*see* Note 20).
12. Gels are preserved by soaking in Gel-Dry™ solution for 20 minutes and then stretching on a drying frame between two cellophane sheets and air-drying overnight (*see* Note 21).

Acknowledgments

We would like to thank Barry L. Wanner (Department of Biological Sciences, Purdue University, West Lafayette, IN) for sharing the plasmids and bacterial strains of the Red recombinase based gene deletion system. The valuable support and contributions of Ronald Kaback, Edit Szepessy, Zoltán Kukor and Miklós Tóth are gratefully acknowledged. This work was supported by NIH Grant DK058088 to M.S.-T.

References

1. Witt H, Luck W, Becker M. A signal peptide cleavage site mutation in the cationic trypsinogen gene is strongly associated with chronic pancreatitis. *Gastroenterology*. 1999; 117:7–10. [PubMed: 10381903]
2. Pfützer RH, Whitcomb DC. Trypsinogen mutations in chronic pancreatitis. *Gastroenterology*. 1999; 117:1507–8. [PubMed: 10610342]

¹⁹Gels can be stored for two weeks at 4°C wrapped in wet paper towels in airtight plastic bags.

²⁰To minimize exposure to methanol vapor, cover the dishes tightly with aluminum foil during these steps. Solutions containing methanol are hazardous and should be disposed of accordingly. To reduce the amount of hazardous waste, slightly stained destaining solutions can be re-used at the beginning of a destaining procedure. Speed and efficiency of destaining can be increased by placing a small sheet of Kimwipe in the dish which adsorbs colloidal stain particles.

²¹Care should be taken to avoid air bubbles between the cellophane sheets as these can result in white spots on the dried gels. The Gel-Dry™ solution contains ethanol, polyethylene glycol, methanol and isopropanol and should be covered during soaking of gels and disposed of as hazardous waste.

3. Chen JM, Ragueneas O, Ferec C, Deprez PH, Verellen-Dumoulin C, Andriulli A. The A16V signal peptide cleavage site mutation in the cationic trypsinogen gene and chronic pancreatitis. *Gastroenterology*. 1999; 117:1508–9. [PubMed: 10610343]
4. Teich N, Bauer N, Mössner J, Keim V. Mutational screening of patients with nonalcoholic chronic pancreatitis: identification of further trypsinogen variants. *Am J Gastroenterol*. 2002; 97:341–6. [PubMed: 11866271]
5. Sahin-Tóth M. Human cationic trypsinogen. Role of Asn-21 in zymogen activation and implications in hereditary pancreatitis. *J Biol Chem*. 2000; 275:22750–5. [PubMed: 10801865]
6. Sahin-Tóth M, Tóth M. Gain-of-function mutations associated with hereditary pancreatitis enhance autoactivation of human cationic trypsinogen. *Biochem Biophys Res Commun*. 2000; 278:286–9. [PubMed: 11097832]
7. Ben-Bassat A, Bauer K, Chang SY, Myambo K, Boosman A, Chang S. Processing of the initiation methionine from proteins: properties of the *Escherichia coli* methionine aminopeptidase and its gene structure. *J Bacteriol*. 1987; 169:751–7. [PubMed: 3027045]
8. Yaron A, Mlynar D. Aminopeptidase-P. *Biochem Biophys Res Commun*. 1968; 32:658–63. [PubMed: 4878817]
9. Király O, Guan L, Szepessy E, Tóth M, Kukor Z, Sahin-Tóth M. Expression of human cationic trypsinogen with an authentic N terminus using intein-mediated splicing in aminopeptidase P deficient *Escherichia coli*. *Protein Expr Purif*. 2006; 48:104–11. [PubMed: 16542853]
10. Wu H, Xu MQ, Liu XQ. Protein trans-splicing and functional mini-inteins of a cyanobacterial dnaB intein. *Biochim Biophys Acta*. 1998; 1387:422–32. [PubMed: 9748659]
11. Evans TC Jr, Benner J, Xu MQ. The cyclization and polymerization of bacterially expressed proteins using modified self-splicing inteins. *J Biol Chem*. 1999; 274:18359–18363. [PubMed: 10373440]
12. Yoshimoto T, Murayama N, Honda T, Tone H, Tsuru D. Cloning and expression of aminopeptidase P gene from *Escherichia coli* HB101 and characterization of expressed enzyme. *J Biochem*. 1988; 104:93–7. [PubMed: 2851590]
13. Yoshimoto T, Tone H, Honda T, Osatomi K, Kobayashi R, Tsuru D. Sequencing and high expression of aminopeptidase P gene from *Escherichia coli* HB101. *J Biochem*. 1989; 105:412–6. [PubMed: 2659585]
14. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A*. 2000; 97:6640–5. [PubMed: 10829079]
15. Lengyel Z, Pál G, Sahin-Tóth M. Affinity purification of recombinant trypsinogen using immobilized ecotin. *Protein Expr Purif*. 1998; 12:291–4. [PubMed: 9518472]
16. Tabor S, Richardson CC. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci U S A*. 1985; 82:1074–8. [PubMed: 3156376]
17. Pál G, Sprengel G, Patthy A, Gráf L. Alteration of the specificity of ecotin, an *E. coli* serine proteinase inhibitor, by site directed mutagenesis. *FEBS Lett*. 1994; 342:57–60. [PubMed: 8143850]
18. Chung CH, Ives HE, Almeda S, Goldberg AL. Purification from *Escherichia coli* of a periplasmic protein that is a potent inhibitor of pancreatic proteases. *J Biol Chem*. 1983; 258:11032–8. [PubMed: 6411724]
19. Neu HC, Heppel LA. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J Biol Chem*. 1965; 240:3685–92. [PubMed: 4284300]
20. Aiyyar A, Xiang Y, Leis J. Site-directed mutagenesis using overlap extension PCR. *Methods Mol Biol*. 1996; 57:177–91. [PubMed: 8850005]

Figure 1.

Primary structure of the intein–trypsinogen fusion protein. An initiator methionine was placed upstream of the 154 amino-acid long mini-intein, which was then fused in-frame with the 232 amino-acid long human cationic trypsinogen. At the fusion junction, the C-terminal Asn of the splice region is joined to Ala¹⁶ of the trypsinogen propeptide. The N-terminal cysteine of the mini-intein has been mutated to Ala to disable splicing at the N-terminus. The sequence of the fusion construct has been deposited with GenBank under accession number DQ371396. Reproduced from **ref. 9** with permission from Elsevier Science.

Figure 2.

Expression and purification of the intein–trypsinogen fusions. In the experiment shown here, wild type cationic trypsinogen and pancreatitis-associated mutants p.A16V and p.N29I were expressed and purified. SDS–PAGE analysis of **(A)** inclusion bodies from LG-3 cells expressing the intein–trypsinogen fusions; **(B)** trypsinogens eluted from the ecotin-affinity column; **(C)** trypsinogen fractions after Mono S chromatography. Inclusion body fraction prepared from 1.8 mL LG-3 culture ($OD_{600} = 1.6$) or $\sim 5 \mu\text{g}$ purified protein was loaded per lane. **(D)** Mono S chromatography of cationic trypsinogen eluted from the ecotin-affinity column. The column was developed with a 0–0.5M gradient of NaCl at a flow rate of 1mL/min. Under these conditions only pure trypsinogen is eluted, while unprocessed fusion proteins are not recovered. Reproduced from **ref. 9** with permission from Elsevier Science.