An Expression System for Trypsin

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The eukaryotic serine protease, rat anionic trypsin, and various mutants created by site-directed mutagenesis have been heterologously expressed in *Escherichia coli*. The bacterial alkaline phosphatase (*phoA*) promoter was used to control the expression of the enzymes in an induced or constitutive fashion. The DNA coding for the eukaryotic signal peptide of pretrypsinogen was replaced with DNA coding for the *phoA* signal peptide. The *phoA* signal peptide successfully directs the secretion of the mammalian trypsinogen to the periplasmic space of *E. coli*. Active trypsin was expressed in the periplasm of *E. coli* by deleting the DNA coding for the activation hexapeptide of the zymogen. The activity of trypsin in the periplasm suggests that the enzyme is correctly activated and has folded such that the 12 cysteine residues involved in the six disulfide bonds of rat anionic trypsin have paired correctly.

A transcription terminator increased the level of expression by a factor of two. However, increasing the copy number of the plasmid *decreased* the levels of expression. Localization of the active enzyme in the periplasm allows rapid screening of modified trypsin activities and facilitates the purification of protein to homogeneity and subsequently to crystallinity.

Key words: serine protease, heterologous expression, alkaline phosphatase promoter

The analysis of protein structure and function using in vitro mutagenesis requires practical and efficient expression systems. Enzymological and structural studies routinely require 1–100 mg of protein. Biophysical characterizations that employ spectroscopy, calorimetry, or hydrodynamics often demand even more. To facilitate the analysis of several mutants, an expression system should produce protein rapidly, easily, and inexpensively. Moreover, the protein of interest must be free from artifacts of the expression system.

The flexibility of bacterial plasmids and phages make them ideal vectors for the rapid, inexpensive generation and expression of mutant proteins [1]. The well-characterized biology of *Escherichia coli* makes it an excellent host for both molecular genetics studies and the subsequent overproduction of specific mutants. A bacterial expression system also meets the practical requirements for simplicity and cost. However, the successful application of a genetic screen based on the activity of the

protein requires that any post-translational processing events that are required for activity are complete. Bacteria are often unable to produce eukaryotic proteins in an active state. Often the initiator methionine is inefficiently removed from overexpressed, heterologous proteins in *E. coli*. Moreover, large amounts of heterologous protein in the cytoplasm of *E. coli* generally precipitate as inclusion bodies [2,3].

The protein isolated from inclusion bodies may contain both intermolecular and intramolecular disulfide bonds [4,5]. In some cases, the protein from inclusion bodies can be dissolved with a denaturant and refolded [3,5,6]. However, refolding proteins generally results in low yields of properly folded material, restricts the ability to screen a large number of mutants, and may cause artifacts due to chemical modifications of the protein by the denaturant. The formation of inclusion bodies can sometimes be avoided by modulating the level of expression, but such results are unique to each protein and contrary to the goal of obtaining high level expression.

Trypsin is an exceptionally good subject for a molecular genetics analysis of protein structure and function. The genes and cDNAs encoding cationic and anionic trypsinogens from various species have been isolated and characterized [7,8]. Several high resolution structures have been determined including bovine cationic trypsin [9,10], trypsin-pancreatic trypsin inhibitor complex [11], trypsinogen [12,13], trypsinogen-pancreatic trypsin inhibitor complex [14], and rat anionic Asn-102 trypsin [15] (chymotrypsinogen numbering system [16]). Detailed mechanisms for the catalytic hydrolysis of peptide and ester substrates by serine proteases have been established [17,18], and trypsin is a highly representative member of this diverse and important class of enzymes. The details of how particular residues mediate catalysis and substrate recognition by trypsin or trypsin mutants, or how particular residues confer biological and chemical stability to trypsin should generalize to other serine proteases and enzymes.

The structural biology of trypsin suggests several factors to be considered during the design of a heterologous expression system. In the native state, trypsin is synthesized, stored, and secreted as the inactive precursor trypsinogen [19]. Trypsinogen is converted to active trypsin upon the removal of a hexapeptide from the amino terminus by trypsin [20] or by enterokinase [19,21–23]. The nascent amino-terminal isoleucine (Ile-16) side chain fits snugly into a hydrophobic pocket in the protein, thus directing the amino terminus to form a critical salt bridge to Asp-194 [9,10,24]. A heterologous expression system for native trypsin must faithfully liberate the amino terminus of Ile-16 in the active enzyme.

Native trypsin forms six disulfide bonds, two of which can be selectively reduced with chemical reagents [25,26]. The more accessible disulfide bond, 191–220, stabilizes the structural integrity of the specificity pocket; selective reduction of this disulfide bond results in a loss of enzymatic activity due to an increase in the K_m for arginine or lysine containing substrates [27]. The proper formation of disulfide bonds in trypsin is essential for complete activity.

Previous efforts to express trypsin entailed the use of a mammalian tissue culture system [28,29]. While mammalian cells provide a more native environment for rat anionic trypsin, tissue culture is slow and expensive compared to culturing microorganisms. In order to develop a more practical expression system for our studies, we chose to develop a prokaryotic expression system for trypsin.

MATERIALS AND METHODS

Strains

HB-101 [F^-, hsdS20 (r_B^-,m_B^-), recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44, λ^-] was used in the initial development of the expression system. *E. coli* strain SM-138 (derived from MC4100) [F^-, araD149, Δ(lac)169, re1A, rpsL, phoR] was obtained from Dr. S. Michaelis. *E. coli* strain MH-1 (derived from MC1061) [araD139,ΔlacX74, galU, galK, hsr$^-$, hsm$^+$, rpsL] was obtained from Dr. M. Hall. Strain LE101 [araD139,ΔlacX74, galU, galK, hsr$^-$, hsm$^+$, rpsL, phoR, proC::Tn5] was prepared by P1 transduction of a phoR mutation linked to *proC*::Tn5 into MH-1 to create a phoR strain which has a transformation efficiency of 10^5–10^6 using standard protocols. The resulting strain, LE-101, is resistant to kanamycin.

Plasmids

The plasmids used in this expression system are depicted in Figure 1. Plasmids pTg (formerly pTRAP) [22], pTn, and pBSTn2 were prepared as described [1]. pTn2 was prepared by ligating the 0.72-kb EcoRI/SalI trypsin fragment of pBSTn2 to the 2.7-kb EcoRI/SalI pTn vector fragment. All of the constructions have been verified by sequence analysis of the DNA. Plasmids designated "Tg" direct the synthesis of trypsinogen while plasmids designated "Tn" direct the synthesis of trypsin (the zymogen peptide is deleted). Plasmids designated 2 contain the *trp a* transcription terminator [30] 12 bases after the trypsin stop codon. Plasmids pTg, pTn, and pTn2 are derived from pBR322. Plasmid pBSTn2 is derived from Bluescript, a higher-copy-number plasmid [31]. Plasmids expressing mutant trypsins are named for the mutant protein [32]. For example, "pTn2D102N" is a pTn2 construction which expresses a trypsin in which the aspartic acid at position 102 has been mutated to an asparagine.

Plasmid pFOG-402 was the gift of Dr. D. Shortle. Plasmid pFOG-402 directs the expression of staphylococcal nuclease under the control of the *phoA* promoter and signal peptide. The DNA construction is identical with pTg in Figure 1 except that DNA sequences encoding staphylococcal nuclease replace the trypsin coding sequences.

Induced and Constitutive Expression

The alkaline phosphatase promoter was derepressed by growing bacteria in low-phosphate (0.1 mM KH_2PO_4) media using the following protocol. A single colony was inoculated into 2ml of LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 20 μg/ml ampicillin and grown overnight to saturation at 37°C with shaking. Then, 5 ml of MOPS minimal media [33] containing 0.1 mM KH_2PO_4 and 20 μg/ml ampicillin was inoculated with 100 μl of the saturated culture. The low-phosphate culture was then grown for 2–12 h at 37°C with shaking. The induced protein appears to be stable to degradation in prolonged incubations. There is no discernible difference in the amount of expressed trypsin between 8 and 24 h after inoculation.

E. coli strains which constitutively express alkaline phosphatase, phoR strains, have been isolated [34,35]. Strains modified in their regulation of alkaline phosphatase were transformed with pTg and analyzed for constitutive expression of alkaline
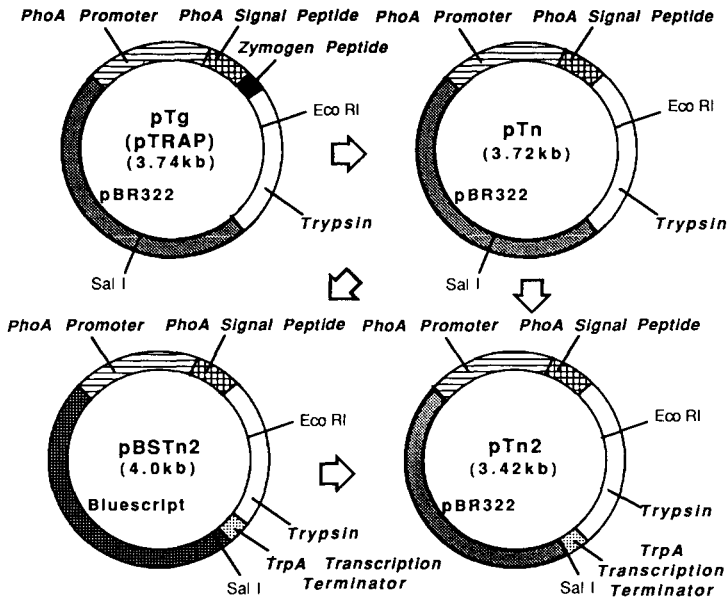
Fig. 1. A series of expression plasmids for rat anionic trypsin and trypsinogen. Arrows indicate the derivation of the plasmids (see Discussion).

phosphatase activity and trypsinogen protein. Strains that produced the highest levels of alkaline phosphatase produced the lowest levels of trypsinogen (data not shown). Of the strains which were tested, SM-138 was chosen for constitutive expression since it produced the highest levels of trypsinogen. Since SM-138 has a relatively low transformation efficiency of 10^3–10^4 using standard protocols, the phoR strain LE-101 was constructed which has a transformation efficiency of 10^5–10^6. For constitutive expression, *phoR* bacteria harboring the appropriate plasmid were grown for 12–17 h with shaking at 37°C in LB medium containing 50 μg/ml ampicillin.

Subcellular Fractionation

To prepare total cell extracts for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [36], pelleted cells from 1.5 ml of culture were resuspended in 100 μl of 2× Laemmli sample buffer and diluted with 100 μl of water. The samples were boiled for 10 min, vortexed vigorously for 5 min, and forced through a 200-μl pipet tip to shear the chromosomal DNA.

Periplasmic fractions were prepared by the lysozyme spheroplast procedure [37] with the following modifications. The cells from 1.5 ml of saturated culture were collected by centrifugation in a microfuge for 1 min at 12,000 rpm. The supernatant was carefully aspirated and the pellet resuspended in 20 μl of 25% sucrose, 10 mM Tris HCl, pH 8.0. The cell walls were digested with 6 μl of lysozyme (50 mg/ml) in 20 mM EDTA. The preparations were incubated at room temperature for 45 min, then centrifuged for 5 min at 12,000 rpm. A 20-μl aliquot of the supernatant was mixed with 20 μl of either 2× Laemmli sample buffer for Western analysis [38] or 2× Laemmli sample buffer without β-mercaptoethanol for activity analysis (see below). This method is scaled up for large-scale preparations of up to 200 L of culture [39]. Osmotic shock, an alternative method for the preparation of periplasmic proteins

from *E. coli* [40], has also proven to be a useful method for isolating trypsin from the periplasm (data not shown).

Immunological Analysis

The Western blot in Figure 2 was prepared as described [41]. The Western blot in Figure 3 was prepared similarly except that second antibody was a 1:500 dilution of goat antirabbit IgG conjugated to horseradish peroxidase (Boehringer Mannheim Biochemicals) in 50 ml of 3%BSA, 0.1% NP-40 in 0.154 M NaCl, 10 mM Tris, pH 7.4 (TBS). The color development was carried out in 5 min with 100 ml of 4 mM 4-chloro-1-napthol, 0.01% hydrogen peroxide in 20% methanol in TBS [42].

Activity Gel Analysis

Analysis of periplasmic fractions for trypsin activity was carried out as described [1]. Briefly, the periplasmic fraction of a 1.5-ml culture was fractionated by SDS-PAGE in the absence of β-mercaptoethanol. This gel was soaked for 40 min in 300 ml of 2.5% Triton X-100 to remove SDS, then in 300 mls of 5 mM $CaCl_2$ for 5 min to replace Ca^{++} [43,44]. Finally, the gel was overlaid on a polyacrylamide gel containing a trypsin ester substrate (in this case tosyl-arginine methyl ester) and phenol red pH indicator at pH 9. Trypsin activity was indicated by yellow (acidic) spots on the red (basic) field in 0.5 to 3 h at 37°C.

HPLC Analysis

Trypsin D102N,S195C protein was expressed as the zymogen and purified as described [39]. The affinity-purified protein was further purified by strong anion exchange HPLC on a Dupont Bio Series Zorbax® SAX resin (6.2 × 80 mm) packed in a Dupont Reliance Cartridge. A 20-μl sample of 14 μg of the protein was loaded and eluted at 1.5 ml/min with a 0–50% linear gradient of 1.0 M NaCl, 20 mM Tris, pH 7.0 (solvent B), in 20 mM Tris, pH7.0 (solvent A). The column effluent was monitored at 280 nm.

RESULTS

Expression of Trypsinogen and Trypsin in *E. coli*

We have established a bacterial expression system for rat anionic trypsin. Expression is controlled by the alkaline phosphatase (*phoA*) promoter (Fig. 1), which is induced in low-phosphate media [45]. Figure 2A shows the total cell extracts from HB 101 transformed with pTg. The *phoA* promoter was induced in two separate cultures by growth in low-phosphate media as described in Materials and Methods. A band of the approximate molecular weight of trypsinogen was induced (Fig. 2A, Lanes 3,4) and was shown to be immunologically cross-reactive with antitrypsinogen antibodies (Fig. 2B). The periplasmic extract analyzed in lane 6 was prepared from HB-101 transformed with the identical plasmid, pFOG-402, encoding staphylococcal nuclease A in place of trypsin. The periplasmic extract analyzed in lane 10, Figure 2B, was prepared from a strain in which the alkaline phosphatase promoter was cloned in the reverse orientation. Greater than 90% of the detectable trypsinogen can be recovered in the periplasm, although some protein occasionally adheres to the spheroplasts.
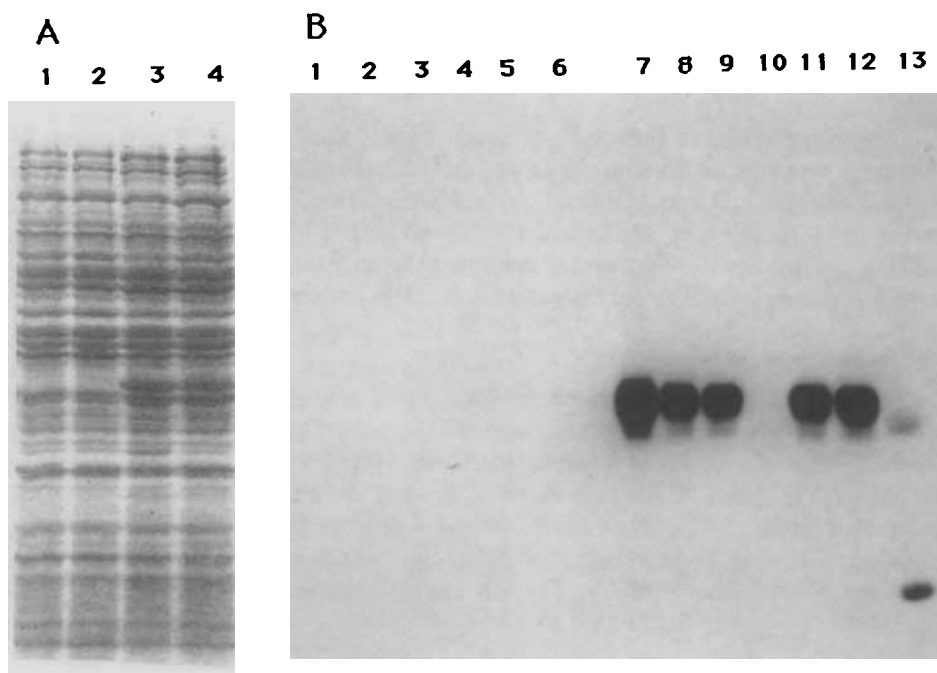
Fig. 2.   Induced production of trypsinogen in *E. coli* **A:** Two clones of HB-101 transformed with pTg were grown in high-phosphate MOPS (1 mM KH₂PO₄) to repress the phoA promoter (uninduced) or low-phosphate MOPS (0.1 mM KH₂PO₄) to induce the phoA promoter. Total cell extracts were prepared of each clone, fractionated by 12.5% SDS-PAGE, and stained with Coomassie Brilliant Blue. **Lanes 1,2:** Uninduced. **Lanes 3,4:** Induced. **B:** Total cell extracts of six clones of HB-101 transformed with pTg and one clone transformed with pFOG-402 were prepared as in A. The gels were blotted to nitrocellulose, probed with rabbit antitrypsinogen antibodies, then radioactive goat antirabbit IgG antibodies. Film was exposed to the radioactive Western blot for 2 h, then developed. **Lanes 1–5:** Uninduced. **Lane 6:** Induced pFOG-402 (makes staphylococcal nuclease). **Lanes 7–12:** Induced. **Lane 13:** High molecular weight standards (only α-chymotrypsinogen [25,700] and β-lactoglobulin [18,400] are visible). The clone in lane 10 was transformed with a pTg construction in which the promoter region was in the reverse orientation.

In order to develop a genetic screen for modified trypsin activities as well as to facilitate large-scale preparations of the protein, it was desirable to engineer the expression system to constitutively secrete active trypsin [1]. Strains containing a *phoR* mutation constitutively express genes which are under the transcriptional control of the *phoA* promoter. Constitutive expression of alkaline phosphatase [34,35] and trypsinogen [22] has been reported. The secretion of active trypsin into the periplasm of *E. coli* was initially achieved with pTn [1]. Figure 3 shows the Western analysis of the periplasmic fractions of phoR strains transformed with pTn and pTgD102N. The difference in size between the zymogen and the activated trypsin is readily apparent

and is consistent with the migration of the native forms [22]. In general, more protein is obtained from plasmids which direct the synthesis of the zymogen; however, the zymogen requires activation by enterokinase resulting in a loss of time and protein. Furthermore, it is difficult to obtain enterokinase which is not contaminated with trypsin [22].
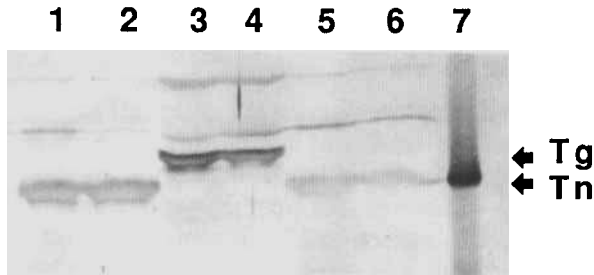
Fig. 3. Production of trypsin in *E. coli* and the effect of a transcription terminator on protein production. Periplasmic extracts were prepared of pairs of three strains which are constitutive for phoA. **Lanes 1,2:** pTn2 in SM-138. **Lanes 3,4:** pTgD102N in JF-210. **Lanes 5,6:** pTn in SM-138. **Lane 7:** Recombinant rat anionic trypsin standard. Tg = trypsinogen; Tn = trypsin.
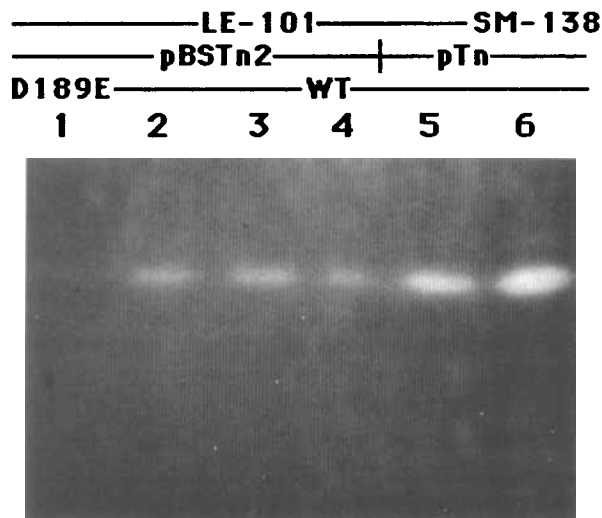
Fig. 4. Activity analysis of periplasmic extracts of *E. coli* transformed with trypsin-producing plasmids. **Lane 1:** pBSTn2D189E in LE-101. **Lanes 2–4:** pBSTn2 in LE-101. **Lane 5:** pTn in LE-101. **Lane 6:** pTn in SM-138.

The trypsin encoded by pTn is active. Figure 4, Lanes 5,6, shows the activity gel analysis of periplasmic fractions from, respectively, LE-101 and SM-138 transformed with pTn. SM-138 makes approximately twice as much active trypsin as LE-101. Periplasmic extracts that do not contain active trypsin but which instead contain trypsinogen or staphylococcal nuclease show no signal on the activity gel. SDS-PAGE in the absence of β-mercaptoethanol apparently separates endogenous inhibitors from heterologously expressed rat anionic trypsin while suppressing the activity of endogenous proteases [46–48]. The expression of trypsin apparently causes no deleterious effects on the growth of E. coli.

Optimizing Expression Levels

In an attempt to optimize the expression system, efforts were made to increase the number of copies and the stability of the trypsin mRNA. A pUC-derived plasmid with a transcription terminator at the end of the trypsin coding sequence (pBSTn2) was constructed. Figure 4 shows results from the activity gel analysis of periplasmic fractions: Lanes 2–4 contain extracts of LE-101 transformed with pBSTn2 while lane 5 is LE-101 transformed with pTn. Strains transformed with pBSTn2 make ~50% less trypsin than strains transformed with pTn.

This decrease in recoverable trypsin activity may be due to the increased amount of plasmid-encoded mRNA. Alternatively, the decrease in recovered trypsin activity may be due to a genetic aberration of the E. coli strains SM-138 and LE-101 which have been shown to have a PhoR *variable* phenotype (*variably* constitutive versus stably *constitutive*) [35]. The plasmid pBSTn2 apparently induces the phoR *variable* phenotype as assayed by streaking the strain on rich media plates and monitoring alkaline phosphatase activity with 5-bromo-4-chloro-3-indoyl-phosphate-*p*-toluidine (XP). The predicted variability in expression of trypsin occurs with pBSTn2 and much less frequently with the lower copy number plasmids.

Because of the lower levels of expression obtained with pBSTn2 compared to pTn, the transcription terminator was introduced into the lower-copy-number plasmid (pTn2), resulting in at least a twofold increase in the amount of recoverable protein (Fig. 3, lanes 1,2,5,6). The enhanced expression due to the transcription terminator has also been detected for several mutant trypsins (data not shown). A likely cause for the increase in trypsin protein synthesis is the secondary structure of the terminator. Hairpins form an important structural unit of rho-independent transcription terminators, stabilize E. coli mRNA, and consequently increase protein synthesis from a particular, stabilized mRNA [49–51].

Analysis of Mutant Trypsins

The periplasm preparation described in Materials and Methods quickly yields a crude, concentrated sample of trypsin. The activity gel assay has allowed the determination of the electrophoretic mobility and activity of several trypsin mutants with surface-loop deletions (data not shown) as well as substrate binding pocket mutants. Lane 1 of Figure 4 contains periplasmic extract of LE-101 transformed with pBSTn2D189E. The low level of TAME hydrolysis activity observed for pBSTn2D189E is due to the D189E mutation. Aspartic acid-189 is located at the bottom of the substrate binding pocket and forms a salt bridge with the lysine or arginine of bound substrate. The presence of glutamic acid at the bottom of the substrate binding pocket reduces, but does not abolish, trypsin activity.

Purification of Mutant Trypsins

E. coli strain SM-138 transformed with a pTn2 construction and grown in LB medium containing 50 µg/ml ampicillin in shaken flasks, without special aeration or other fermentor techniques, generates approximately 5-10 mg of unpurified protein per liter of culture and 1 mg of purified protein per liter of culture. These are average values, since the expression level varies for different trypsin mutants.

The trypsin mutant D102N,S195C was prepared as described from 200 L of SM-138 transformed with pTnD102N,S195C [39]. Low-pressure anion exchange, cation exchange, and affinity chromatography are used to prepare protein to greater than 95% purity. However, strong anion exchange HPLC is necessary to obtain protein of sufficient purity to yield high-quality crystals (unpublished results). Figure 5 is an HPLC chromatogram of a sample of D102N,S195C trypsin. Coomassie-stained SDS-PAGE gels and Western analysis reveal that both peak 2 and peak 3 contain trypsin, while peak 3 also contains an endogenous *E. coli* inhibitor [52]. A trypsin-inhibitor complex might inhibit crystal growth because the incorporation of the complex in a lattice of trypsin molecules could disrupt the addition of more trypsin molecules to the lattice.

DISCUSSION

The bacterial alkaline phosphatase (*phoA*) promoter and signal peptide [53] has been used to secrete human epidermal growth factor [54], staphylococcal nuclease [55], rat anionic trypsinogen [22], and α-lytic protease [56] into the periplasmic space of *E. coli*. We now report that the *phoA* signal peptide directs active rat anionic trypsin to the periplasm of *E. coli*. Expression can be induced in low phosphate media (0.1 mM inorganic phosphate) or can be maintained constitutively in phoR strains. The enzymatic activity of the product indicates that the protein is soluble and that all six disulfide bonds form correctly. Because the signal peptide is removed from the *phoA* signal peptide-trypsin fusion protein during secretion, the problem of post-translational processing of the N-terminal methionine is alleviated. Furthermore, the bacterial *phoA* signal peptide is a functional and sufficient activation peptide for the mammalian trypsin.
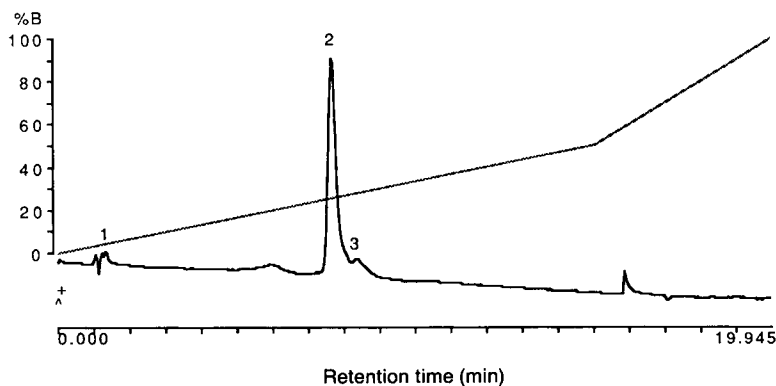

Fig. 5.   Strong anion exchange HPLC chromatogram of affinity-purified trypsin D102N,S195C. Peaks 2 and 3 contain rat anionic trypsin. Detection is absorbance at 280 nm.

The free amino terminus of trypsin is essential for activity [57,58]. In trypsin, the side chains of the two amino-terminal residues, Ile-16 and Val-17, fit snugly into hydrophobic pockets, thus directing the amino terminus toward the formation of a critical salt bridge to the γ-carboxylate of Asp-194 [12,13]. It has been shown that the positive charge which forms the salt bridge to Asp-194 *must* be the Ile-16 amino terminus in order for trypsin to be active [14,59]. Therefore, the alkaline phosphatase signal peptide must be cleaved precisely before the Ile-16 of the trypsin sequence. However, the signal peptide of pro-alkaline phosphatase is cleaved to leave an arginine at the amino terminus of alkaline phosphatase [53] and isozymes of alkaline phosphatase vary by the presence or absence of this arginine at the amino terminus [60]. The product of the *iap* gene removes this arginine residue [61]. Because of this variability in *phoA* signal peptide cleavage, arginyl-trypsin might be formed. If this product does exist, we have not detected it. Arginyl-trypsin might be purified from native trypsin either by diethylaminoethyl (DEAE) cellulose chromatography because of the difference in charge between the two proteins, or by affinity chromatography on bovine pancreatic trypsin inhibitor (BPTI) because of the decreased affinity of trypsinogen for BPTI. Notably, *E. coli* also generates the correct amino terminus of staphylococcal nuclease from the *phoA* signal peptide-staphylococcal nuclease fusion protein [55].

The *phoA* signal peptide is a sufficient activation peptide for rat anionic trypsin. For both of the bacterial proteases subtilisin E and α-lytic protease, the propeptide of the naturally occurring protease has been shown to be essential for the recovery of active protease. Replacement of the propeptide of subtilisin E with the *omp* A signal peptide resulted in the secretion of mature-length, but inactive subtilisin E [62]. Similar results have been obtained with α-lytic protease using the *phoA* signal peptide [56]. Both results have been explained as a requirement for the propeptide to obtain proper folding of the protease. Rat anionic trypsin and by structural similarity the other eukaryotic serine proteases, evidently do not require more than the proper liberation of the amino terminus for activation.

The availability of the various plasmids enhances the versatility of the expression system. While pTn2 plasmids allow optimized expression, the pBSTn2 construction allows the rapid generation and limited expression of mutants including libraries of changes at any given position [1]. The Bluescript plasmids also facilitate the rapid preparation of large amounts of either single-stranded or double-stranded DNA for sequencing and subcloning. Having constructions with and without the zymogen sequence makes switching between the two expression products simple. This flexibility is important. Assuming that the deleterious effect of pUC-based expression (pBSTn2) is due to trypsin activity, it may be advantageous to express certain trypsin mutants as zymogens from pUC derived plasmids (pBSTg2).

The ability to switch between constitutive and induced expression is also desirable. Induction may be the reason that certain expression systems yield enhanced levels of expression [56,63]. In fact, bacterial alkaline phosphatase is expressed at very high levels when induced [64,65], but at levels similar to trypsin when constitutive [66,67]. Constitutive expression is easier to maintain than induced expression for high-biomass fermenter cultures of *E. coli*. Constitutive expression also permits the use of genetic screening techniques [68], which may be confounded under conditions for induction.

For analyzing a collection of mutants obtained by in vitro mutagenesis, periplasmic localization permits the rapid, facile preparation of a concentrated extract

free of cytoplasmic macromolecules. This fraction can be analyzed by Western analysis for the levels of trypsin protein synthesis, the isoelectric point of the protein, and the relative size of the protein. A relative assay of the activity of the enzyme can then be determined by activity gel analysis.

We believe this expression system to be general for eukaryotic serine proteases. It has been used to express rat anionic trypsins with surface loop deletions as well as bovine cationic trypsin and an elastase-like protease secreted by cercaria of *Schistosoma mansoni* (J. McKerrow and C.S. Craik, unpublished results).This expression system has provided sufficient protein to permit the analysis of substrate binding pocket mutants [22] and active site mutants [39] of rat anionic trypsin.

In light of the potential problems with the heterologous expression of a eukaryotic protease in bacteria, the current levels of expression are reasonable. Attempts to maximize the levels of purified protein that can be obtained from this system must now address aspects such as 1) more efficient purification schemes, 2) regulation of promoter activity and strength, and 3) optimization of codon usage. By attempting to streamline the production of active and inactive proteases in *E. coli*, the rate-determining steps controlling heterologous expression will be elucidated. This in turn will provide insights into cellular biology as well as an efficient means to biosynthesize proteases and their mutants.

ACKNOWLEDGMENTS

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