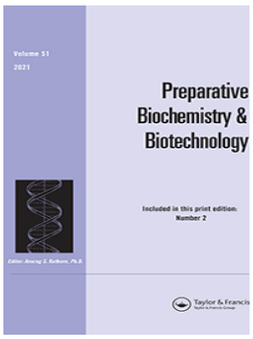

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Development of clone with novel TrpE fusion tag in *E. coli* for overexpression of trypsin in a bench-scale bioreactor

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ABSTRACT

Trypsin is a key enzyme under the serine proteases that is found in the pancreas which plays a key role in protein digestion. It cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine. This enzyme has received greater attention mainly due to its increased use in the removal of fusion tags during protein purification and its role in the processing of biosimilars like insulin. The present study was carried out to develop a clone with Novel TrpLE1413(TrpE) Fusion Tag for enhanced expression of trypsin which helps in cost reduction of biosimilar processing. In our experiment we have used a synthetic bovine trypsin gene containing a novel fusion tag TrpE at its N terminus, which was cloned into the pET41b (+) vector and expressed in *E. coli* BL21 (DE3) in a lab-scale bioreactor. Using the optimized fermentation process with TrpE Fusion Tag, 27.8 g/L inclusion bodies were produced at the end of fermentation, of which 209 mg/L of active trypsin was obtained after purification. In contrast, previous reports have claimed to produce a maximum of 60 mg/L of the enzyme without the fusion tag. Thus based on our findings, the small size (less than 2 kDa) of TrpE tag and its hydrophobicity may reduce the loss incurred during the purification process. Hence, it could be discerned that the use of the TrpE fusion tag along with a robust fermentation process led to 3– 4 fold higher yield making it a commercially viable process facilitating an improved recovery of the enzyme.

KEYWORDS

Trypsin; TrpE; fusion tag; recombinant enzymes; protein purification

Introduction

Trypsin (EC 3.4.21.4) is a highly valuable serine protease of molecular weight 23.3 kDa, which targets basic amino acids such as lysine and arginine at the C-terminus. The zymogen form of the enzyme called trypsinogen gets converted to trypsin by the addition of either trypsin or enterokinase. Trypsin plays a major role in metabolism, digestion and coagulation in mammals.[1] Besides, the enzyme is useful in leather bating, food processing, pharmaceuticals and clinical diagnosis.[2] The application of trypsin in cell culture mainly lies in the removal of adherent cells from the culture surface and in the resuspension of cells.[3] The optimal pH for trypsin activity is 7–9, hence the formulation buffer should be of acidic pH to prevent self-activation.[4] To date, trypsin used in the laboratory as well as on the commercial scale is obtained from bovine and swine pancreas. Nonetheless, if extracted from these sources, the risk of microbial load being carried over even after the purification step is high.[5] The use of recombinant enzymes can help to overcome this complication. The bovine trypsin gene has been widely expressed in both prokaryotes and eukaryotes. Even though proteins are expressed without any fusion tag, the use of such tags are desirable as the enzymes are highly prone to degradation even when expressed in prokaryotic hosts. Fusion tags increase enzyme stability. However, the size of the tag is an important factor as it determines the final yield. The tag-protein ratio when using glutathione S transferase, maltose binding protein and thioredoxin fusion tag with trypsin is about 1:1–1:3. Although the expression levels are high, the total product percentage is only around 33–50% of the inclusion bodies produced. Besides, some amount of the protein is present in the soluble fraction, resulting in the reduction of yield after the purification and refolding steps. In order to convert the soluble fraction of protein into insoluble fraction, the use of an insoluble fusion partner with a hydrophobic core is desirable. In this context, the use of fusion tags that are less than 2 kDa in size and a ratio of up to 1:8–1:10 (tag: protein) might reduce the cost of production by increasing the protein yield. This approach could result in 80–90% of the protein being pushed into the inclusion bodies fraction.

Both prokaryotes and eukaryotes have been largely exploited for producing recombinant trypsin. Trypsin and fused trypsin were expressed in the form of inclusion bodies by Greaney and Rosteck,[6] Peterson et al.,[7] Kopetzki et al. [8] and szilagyi et al.,[9] who claimed production in the range of 30–60 mg/L. Even though the fermentation process was optimized, Yee and Blanch, were not able to achieve a high yield (56 mg/L at 92 g/L dry cell weight).[10] Chen et al. attempted to express trypsin without any fusion tag, but regulatory issues

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Table 1. Nucleotide and amino acid sequence of the TrpE fusion tag.

Construct	Sequence
TrpLE1413 tag nucleotide sequence	AAAGCCATCTTTGTGCTGAAAGGTAGCCTGGATCGTGATCCGGAATTT
TrpE 1413 tag amino acid sequence	KAIFVLKGSLDRDPEF

*Start codon is not included in nucleotide and amino acid sequence.

linked with the removal and clearance of methionine were observed.[11] In another study, Hohenblum et al. expressed trypsin in E. coli and obtained 200 mg/g of dry cell mass.[12] Researchers have also tried expressing trypsin in hosts such as *Pichia pastoris* and other eukaryotes. Min Shu et al. were able to produce 201 mg/L of trypsin successfully using minimal media,[1] but the major disadvantage was the time consumption for the fermentation process. Considering all the above strategies, the usage of fusion tag seems to be the best option to achieve maximum yield. Further, *E. coli* seems to be a time-saving and cost-effective host for operating the bioreactors and was therefore selected for enhanced protein expression.

In this paper, we describe the use of novel fusion tag suitable for expressing enzymes that are difficult to produce in a bioreactor and achieve high product yield. The partial sequence of the TrpE fusion tag was derived from the tryptophan operon comprised of 16 amino acids. The synthetic bovine trypsin gene fused to the TrpE tag was cloned into the pET41b (+) vector, and the protein produced in the form of inclusion bodies were washed, solubilized and renatured to obtain its tertiary structure. When favorable conditions were provided, the fusion tag gets cleaved from the protein. The protein was selectively purified using cation exchange chromatography. Kinetic measurements were made with a spectrophotometric assay in which Nα-Benzoyl-L- arginine ethyl ester (BAEE) was used as a substrate to determine trypsin activity. This novel fusion tag displayed higher activity and increased titer when compared with the traditional tags used for the expression of enzymes.

Materials and methods

Strains, plasmids, reagents and media

E. coli DH5α procured from Thermofisher was used for the storage of plasmids, and *E. coli* BL21 (DE3) procured from New England Biolabs was employed as the expression host. The synthetic TrpE trypsin gene was procured from GeneArt. The pET41b (+) vector was purchased from Novagen. The plasmid isolation and gel elution kits were sourced from Qiagen. Standard trypsin was obtained from Roche and BBI solutions (2500 U/mg). The enzymes, ligase and polymerase were from New England Bio Labs. Pierce BCA protein estimation kit was bought from Thermo Fisher. Applikon and Sartorius bench-scale bioreactors were used for process development. Luria Bertani broth, kanamycin, isopropyl β–thio galactopyranoside (IPTG) and sodium dodecyl sulfate were acquired from Sigma. Nα- Benzoyl-L-arginine ethyl ester was from Bachem, and the ion exchange resin was from Biorad.

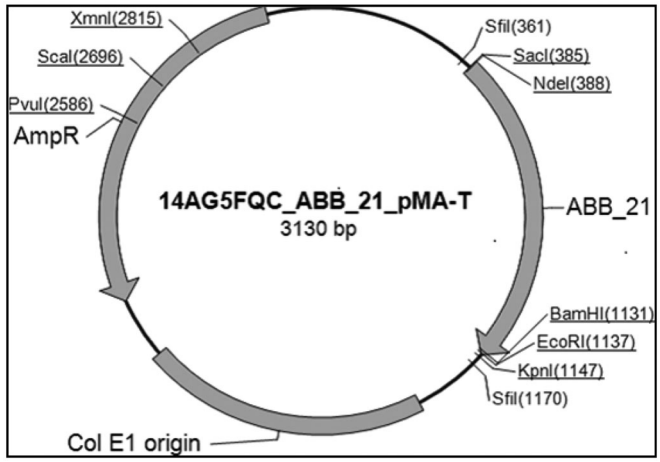

Figure 1. Vector map of the synthetic gene obtained from GeneArt (provided with the quality assurance document).

Construction of the expression vector and cloning of the gene

The nucleotide and amino acid sequence of TrpE fusion tag used for cloning with trypsin is shown in Table 1. In this study, 200 µL of reaction mixture was prepared for the restriction digestion of the insert and vector as follows: For digestion of insert, 5 µg of ABB 21/pMAT (Figure 1) was taken along with NdeI and BamHI enzymes, constituting a reaction volume of 200 µL including buffer and water. For digestion of vector, 5 µg of pET41b (+) was taken along with NdeI and BamHI enzymes, again comprising a reaction volume of 200 µL. The reaction mixture was incubated at 37 °C for 3 h. After the completion of the reaction, the samples were loaded onto 1.8% agarose gel. The digested bands were subjected to gel elution as per the protocol provided in the kit and used for ligation based on the insert and vector concentrations. The 1:3 ratio of vector and insert was set based on the NEBioCalculator software from New England Bio labs. Six colonies were grown after the ligation of gene with pET41b (+) and transformation into DH5α cells (Figure 2). The Ape file shown in Figure 2 was created in-house for our reference to clone the TrpE trypsin into the pET41b (+) vector. The colonies were subjected to colony PCR, and one clone found to be positive was screened by restriction digestion using the unique restriction sites in the vector and insert, with a reaction volume of 20 µL. After restriction digestion confirmation, the plasmid was sent for sequencing using T7 forward and reverse primers.

Small-scale expression and induction studies

Once the positive clones were screened by restriction digestion, the TrpE trypsin gene was transformed into *E. coli* BL21 (DE3), and the cells were cultivated at 37 °C (180 rpm)

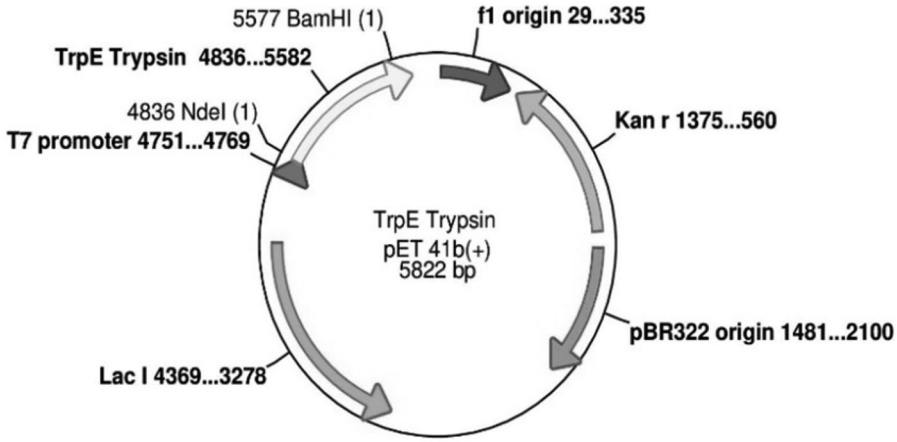

Figure 2. Schematic representation of TrpE trypsin cloned into pET41b (+) using ApE file software.

in 50 mL super broth containing 30 mg/L kanamycin for initial screening studies. The cells were allowed to reach an optical density (OD) of 0.6–0.8 and induced overnight with 1 mM IPTG at 37 °C. 0.5 OD cells were spun and the pellet was resuspended in 80 µL PBS and 20 µL of 5X sample solubilization buffer (SSB – 10% SDS, 250 mM Tris, 30% Glycerol, 5% β mercaptoethanol, and 0.02% bromophenol blue) was added. The samples were boiled at 100 °C for 10 min. Approximately 0.1 OD (20 µL) of uninduced and induced samples were loaded in each well of the 12.5% SDS PAGE.[13]

The induced sample was sonicated to estimate the protein present in the soluble fraction. 1.5 OD cells with 240 µL of PBS was subjected to sonication using Q Sonica sonicator with 10 seconds on and 10 seconds off cycle at an amplitude of 10 μm. After 10 min of the sonication cycle, the sample was spun, after collecting the supernatant separately, the pellet was resuspended in 240 µL of 1X PBS and 60 µL of 5X SSB was added into it. The samples were boiled at 100 °C for 10 min. The sonicated, uninduced and induced samples of 0.1 OD (20 µL) were loaded on Tricine SDS PAGE.[14]

Fed-batch fermentation

The production of TrpE trypsin was carried out in a 2 L bench-scale bioreactor. Luria broth was inoculated with 1 mL glycerol stock culture. Once the seed medium reached an OD of 3.0, 50 mL of inoculum was aseptically transferred into the bioreactor loaded with an initial volume of 750 mL. The bioreactor medium contained 4.8 g/L ammonium sulfate, 10 g/L dipotassium hydrogen orthophosphate, 9 g/L magnesium sulfate, 35 g/L yeast extract, 20 g/L dextrose, and 20 g/L trace salts. The batch phase ended once the pH started increasing slowly and was followed by the fed-batch phase.[15] The latter phase involved the addition of feed mixture containing 400 g/L dextrose monohydrate, 70 g/L yeast extract and 15 g/L peptone at regular intervals to adapt the cells to the increasing nutrient concentration and attain higher biomass within a short span of time. Once 350 g/L wet cell weight was attained, the broth was induced for 6 h with 1 mM IPTG based on the existing volume in the bioreactor . During induction, the feed addition was adjusted such that the accumulation of glucose or acetate was restricted in the medium by setting it in the pH-stat mode.[15] The dissolved oxygen level was maintained above 20%. The temperature was held at 37 °C during the growth and induction phases.[16] The volume at harvest was 1.4 L.

Cell lysis, isolation and solubilization of the inclusion bodies

The harvested broth of 1 L was centrifuged at 10,000 g for 20 min at 4 °C. Around 9 mL of lysis buffer (100 mM Tris and 10 mM ethylenediamine tetra-acetic acid (EDTA) (pH-8.0)) was used for every 1 g of cell to achieve efficient lysis. The pelleted cells of 350 grams were lysed using a high-pressure homogenizer at 1000 bar pressure [17] and was centrifuged at 10,000 g for 30 min. Lysis efficiency was measured using O. D at 600 nm. O. D_{600} before lysis was 2.18 and it reduced to 0.8 after 3 passes in the homogenizer. For the washing steps, 5 volume of wash buffer (100 mM Tris, 10 mM EDTA (pH 8.0) and 0.5% Triton X-100) was added to every 1 gram of inclusion bodies. After each wash, the sample was centrifuged at 10,000 g for 30 min. The supernatant was discarded, pellet was weighed and resuspended in wash buffer according to its current weight. The washing step was repeated using the same buffer devoid of Triton X-100, and each step was followed by centrifugation. The supernatant was discarded, and the pellet containing the inclusion bodies were retained. Once inclusion bodies were obtained, they were solubilized with a buffer containing 6 M guanidine HCl and 100 mM Tris (pH 8.0) at a ratio of 1:60 (w/v) and incubated at 4 °C with gentle mixing.

Denaturation and refolding

The solubilization and refolding protocol suggested by Ahsan et al. was applied.[18] Reduced inclusion bodies were added in a pulsated manner into the precooled (10 °C) refolding buffer under the stirring condition at a ratio of 1:40 (final ratio). The concentration of the protein during refolding was maintained in between 0.6 and 1 mg/mL to increase the efficiency of refolding. The refolded protein solution was later concentrated upto 100 times using an

Amicon 10 kDa ultrafiltration membrane and later dialyzed against 100 mM Tris buffer (pH 9.0) containing 100 mM NaCl, 0.4 M guanidine-HCl and 1 mM of both cysteine and cystine. This refolding mixture was kept under gentle stirring for 72 h after dialysis. Subsequently, the protein was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 5 mM benzamidine-HCl with 4 mM Calcium chloride for activation.

Activation of trypsin

The dialyzed protein mixture was subjected to centrifugation at 15,000 g, following which the supernatant was activated to separate pure trypsin from the tagged protein. To the renatured protein mixture, (50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 5 mM benzamidine-HCl, 4 mM Calcium chloride) 1 unit of enterokinase was added. The activation mixture was incubated at room temperature for 3 h to trigger the self-activation of trypsin.

The protein with fusion tag is in its zymogen form. When favourable conditions are provided, such as pH 7.5–8.5[19] and activation by the addition of enterokinase or trypsin, trypsinogen can get converted into trypsin in the presence of calcium ions.

Purification and storage

The process flow for chromatography was as per the protocol given by Santos et al.[20] Once trypsin was activated, it was selectively purified by cation-exchange chromatography with a column internal diameter of 85.0 × 2.2 cm and packed with Sepharose SP resin at 4 °C. The column was equilibrated with 4 column volumes of 10 mM phosphate buffer (pH-6.5) treated as buffer A during elution, and sodium chloride was considered as buffer B for gradient elution. Once the activated protein was loaded, washing was done with 4 column volumes of 10 mM phosphate buffer (pH 6.5) containing 100 mM NaCl. As our desired protein binds to the column, it was eluted using 7 column volumes of elution buffer B with a gradient of 0–100%. The eluted fractions were monitored by measuring the absorbance at 280 nm.[21] The protein-containing fractions were pooled and the buffer was exchanged against 10 mM HCl (pH 3.0), 100 mM NaCl and 10 mM $CaCl_2$ and frozen at −20 °C until further use.

Estimation of protein concentration

The concentration of the desired protein was estimated using the bicinchoninic acid (BCA) method.[22] Bovine serum albumin (BSA) of various concentrations were used as a standard for the assay, and it was subjected to same condition as that of the sample.

Enzymatic assay for activity study

The final product was assayed for enzyme activity using the protocol of Bergmeyer et al.[23] The volumes taken for the assay have been shown in Table 2.

Table 2. Volume of reagents used for the trypsin assay.

Reagent	Blank (mL)	Test (mL)
Substrate	3	3
HCl solution	0.2	–
Trypsin	–	0.2

The standard enzyme amounting to 40–100 units was diluted to 200 µL and added to the reaction mixture. The absorbance at 253 nm was read at 1-min intervals. Fifteen data points were noted down, and the same procedure was carried out for the samples. The specific activity of the in-house sample was estimated in units/mL by using the formula. This formula was used for both standard and the sample and units of enzyme was estimated by the difference obtained between the initial and final value.

$$\text{Trypsin Units} = (A1 - A2)/(0.003.T.W)$$

A1 – Absorbance straight line final reading; A2 – Absorbance straight line Initial reading; 0.003 – One trypsin unit is the activity causing a change in absorbance of 0.003 per minute under the conditions specified in the assay; T – Time elapsed in minutes, between initial and final reading; W – Weight, in mg, or volume, in mL of trypsin in the volume of solution used in determining the absorbance.

Substrate reaction assay with in-house trypsin

The glargine gene expressed with an intact single chain "B chain RR connecting peptide A chain" (BCA) was used for trypsin substrate activity, and it contained an arginine recognition site in between the chain to detect the separation of A and B chains. In our experiment, 2 µg of intact glargine molecule was digested using 1 unit of in-house trypsin in the activation buffer (50 mM Tris-HCl, 20 mM calcium chloride, pH-8) was incubated at 25 °C for 16–18 h. Another reaction mixture devoid of the enzyme was set as a substrate blank, and the amount of intact glargine was kept constant. This reaction was stopped by adding sample solubilization buffer; 25 µL of the samples were loaded on Tricine SDS PAGE[14] and analyzed by the silver staining method.[24]

Results and discussion

Construction of the expression vector and cloning of the gene

The TrpE trypsin gene was cloned into pET41b (+) vector to evaluate the expression levels. The clone was found to be positive based on the restriction digestion pattern obtained with the desired size of release using three different combination of enzymes (*NdeI*+*BamHI*, *PvuII* and *PstI*) during agarose gel electrophoresis (Figure 3). Clone number 1 was selected for BL21 (DE3) transformation after sequencing the plasmid with T7 forward and reverse primers. The nucleotide sequence of the sequenced gene was matching with the original sequence of TrpE trypsin.

Small-scale induction and expression studies

The protein samples obtained after induction were electrophoresed on 12.5% SDS PAGE (Figure 4). Expression of the desired protein with the fusion tag was observed in the induced samples at 25.84 kDa; however, no expression was evident in the uninduced samples. TrpE trypsin was expressed in pET41b (+) vector in the form of inclusion bodies. As shown in figure number 4, the proportions of the desired and host cell proteins were analyzed using the Bio-Rad EZ imager quantification tool, which revealed that inclusion bodies made up the major part of the cell. Nearly 50–65% of the protein in the induced sample was TrpE trypsin, while the remaining was host cell protein.

The sonicated sample, induced and uninduced samples were electrophoresed on Tricine SDS PAGE to estimate the quantity of desired protein in the soluble fraction (Figure 5). The gel results infer that almost equal amount of TrpE trypsin is present in the sonicated pellet and induced sample when an equal amount of protein was loaded. The sonicated supernatant showed neither the HCP's nor the desired protein in it (data not shown). The results demonstrated that the overexpressed protein was present in the inclusion bodies form and no protein in the soluble fraction.

TrpE fusion tag[25] has been used for proteins lacking methionine as their first codon, such as transforming growth factor-alpha.[26] The high level of expression obtained was mainly due to the hydrophobic core of the tag, which enhanced the production of inclusion bodies in the cell. TrpE fusion tag helps in enhancing the protein production, as inferred from the accentuated induction levels of the desired protein. The expression levels were increased when compared with the native protein expressed in *E. coli* without any fusion tag. Based on the expression levels obtained at shake flask levels, clone 2 was selected for fermentation studies in a 2 L bench-scale bioreactor.

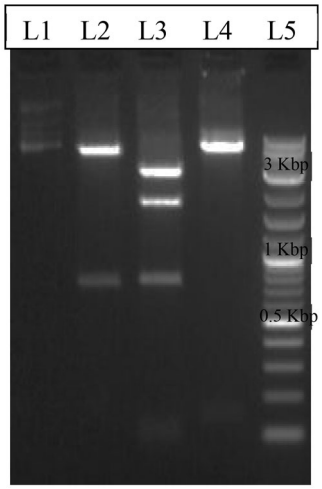

Figure 3. Restriction digestion of TrpE trypsin electrophoresed on 1.8% agarose gel. Lane 1 – TrpE trypsin/pET41b (+) Uncut, Lane 2 – TrpE trypsin/pET41b (+) NdeI + BamHI (5081bp +744 bp), Lane 3 – TrpE trypsin/pET41b (+) PvuII (3032bp + 1821bp + 762 + 117 + 93 bp), Lane 4 – TrpE trypsin/pET41b (+) PstI (5680 + 145 bp), Lane 5 – Quick 2- log purple DNA ladder.

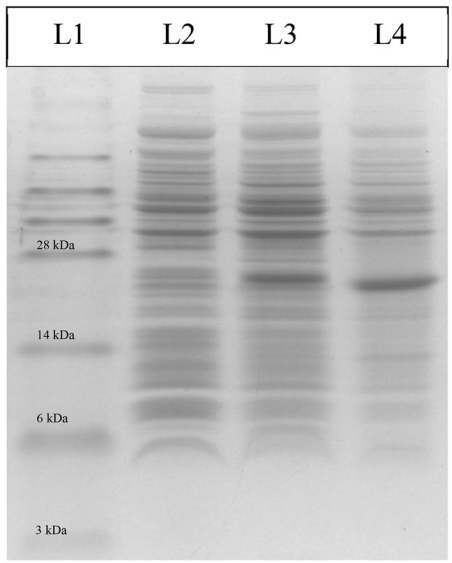

Figure 5. TrpE trypsin protein in the insoluble fraction of the cells loaded on Tricine SDS PAGE. Lane 1: See blue prestained Protein Ladder, Lane 2: Uninduced sample, Lane 3: Induced sample, Lane 4: Sonicated induced sample.

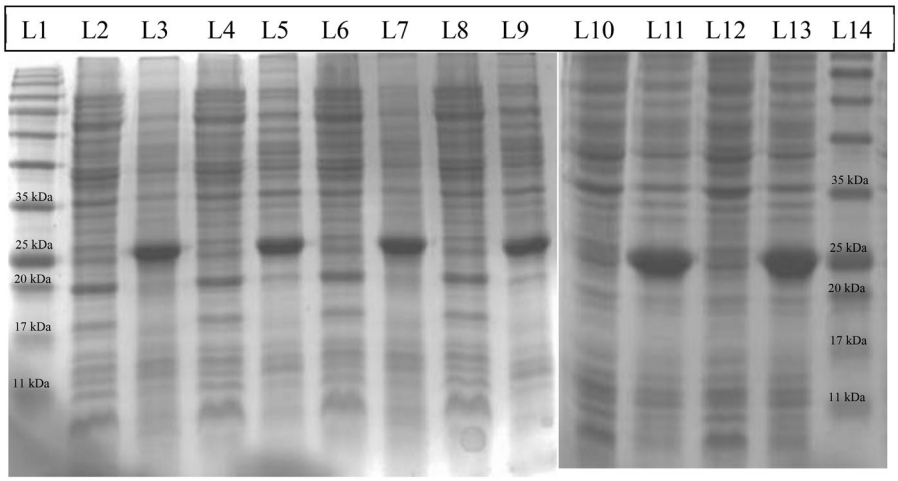

Figure 4. TrpE Trypsin expression in E. coli BL21 (DE3) cells on 12.5% SDS PAGE. Lanes 2, 4, 6, 8, 10 and 12: Un-induced samples of clones 1, 2, 3, 4, 5 and 6, respectively, Lanes 3, 5, 7, 9, 11 and 13: Induced samples of clones 1, 2, 3, 4, 5 and 6, respectively, Lanes 1 & 14: HI Media pre-stained protein ladder.

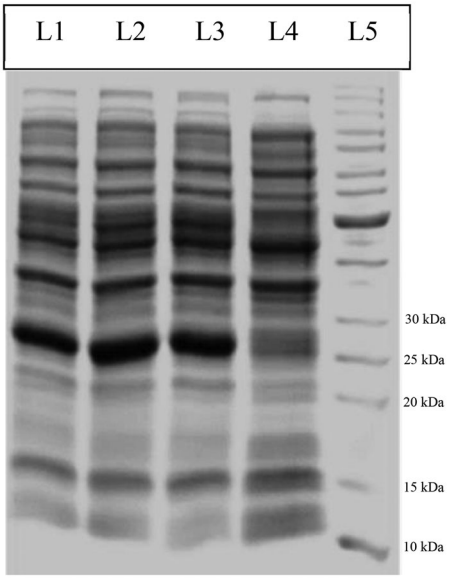

Figure 6. TrpE trypsin production by *E. coli* BL21 (DE3) in a 2 L bench-scale bioreactor on 12.5% SDS PAGE. Lane 1: 2-h induction, Lane 2: 4-h induction, Lane 3: 6-h induction, Lane 4: Uninduced sample, Lane 5: Page ruler unstained protein ladder.

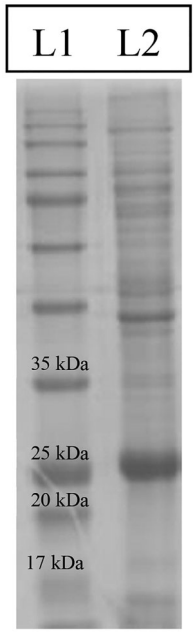

Figure 7. TrpE trypsin inclusion bodies after cell lysis and consecutive washes loaded on 12.5% SDS PAGE. Lane 1:Hi media pre stained protein ladder, Lane 2: Inclusion bodies after lysis and wash.

Fed-batch fermentation

Samples were collected from the bioreactor every 2 h during induction and electrophoresed on SDS PAGE, which demonstrated an exponential increase in the expression of TrpE trypsin. Increase in protein expression was observed from the second hour to the sixth hour induction (Figure 6). Based on the promising expression levels obtained at the bench level, it appears that the production process could be scaled-up.

As expected, the desired protein titer reduced after the sixth hour of induction; hence, the broth was harvested at that point. Similar trends were obtained with the optimization batches taken at bench-scale bioreactor. The expression levels achieved from the bioreactor studies were superior to those obtained from the shake-flask studies. The overall time taken for a batch of fermentation was about 18–20 h, thereby reducing the cost of operating the bioreactor for a long time to achieve high biomass and the required product yield. The OD of the inoculum before loading it in the bioreactor, wet cell weight prior to induction, temperature, optimum pH and media composition for growth, as well as feed and induction conditions were the significant factors that positively influenced the expression levels. Optimum conditions were arrived based on the fermentation trials taken for optimization. The conditions for IPTG addition were optimized at shake-flask level and the same was replicated at the bioreactor level, which helped us in obtaining a commercially viable titer.

Cell lysis, isolation and solubilization of inclusion bodies

We ensured complete lysis of cells by measuring O. D_{600} after each pass in the homogenizer where the OD reduced from 2.18 to 0.8 after three consecutive washes. Three hundred and fifty grams of cells were processed for cell lysis, and 27.8 g of inclusion bodies were obtained. The proportion of inclusion bodies was found to be 7.94%. The isolated inclusion bodies were loaded on SDS PAGE, which showed almost complete cell lysis. Only minimal amounts of host cell proteins were observed (Figure 7). As the solubilized sample contained a high concentration of denaturing agents, resolving the samples on SDS PAGE was not effective.

Denaturation and refolding

The solubilized protein was passed through a membrane filter of pore size 0.45 µm in order to obtain the protein devoid of cell debris and other particulates, which resulted in the removal of host cell proteins. Reducing the concentration of guanidine gradually by dilution with the refolding buffer comprising of 100 mM Tris buffer (pH 9.0) containing 100 mM NaCl, 0.4 M guanidine-HCl and 1 mM of cysteine and cystine helped the protein to refold. The concentration of protein in the refolding solution was in between 0.6 and 1 mg/mL to increase the efficiency of refolding process. No autocatalytic activity was observed during refolding as the protein was buffer exchanged with Tris of pH 9, at this point the enzyme remained inactive.

As there are chances of the protein becoming active at the refolding stage if favorable conditions are provided, the pH was maintained at 9.0. This step helped in the activation of the total trypsin only during the actual activation step. In this paper, the protein refolding step was crucial as it played a major role in increasing the overall yield of trypsin.

Activation of trypsin

The cleavage of the fusion tag was possible because of the favorable conditions, including pH. Auto activation was

observed after the addition of enterokinase, resulting in the removal of fusion tag. The fusion tag of 16 amino acids did not interfere in the folding of the 223 amino acids; hence, refolding and cleavage of the fusion tag was easy to perform.

Purification and storage

Protein of 98% purity was obtained using cation exchange chromatography, and it was validated by RP-HPLC (Data not shown). Separation of trypsin was based on its exclusive binding to the column. The bound fractions were eluted using 50 mM Tris (pH 8.0) buffer with 0.4–0.8 M NaCl. It was immediately buffer exchanged against 10 mM HCl (pH 3.0), 100 mM sodium chloride and 10 mM calcium chloride at 4 °C and frozen at −20 °C until further use. The pure trypsin thus obtained was loaded on Tricine SDS PAGE, and it showed a single, clear band comparable to the standard trypsin (Figure 8). Storage in a buffer of pH 3.0, which contained HCl, helped the protein to be stable until the activation buffer was added to it.

Estimation of protein concentration

After fusion tag removal, the concentration of the protein was estimated using BCA method, and the value was found to be 417 µg/mL by comparison with the BSA standard. The final titer of pure trypsin obtained per liter was 209 mg/L of the fermentation broth (Table 3). We have produced trypsin of high purity by enhancing the expression level with the use of novel fusion tag and a robust fermentation process, which eliminated the constraints pertaining to the production of trypsin at a large scale.

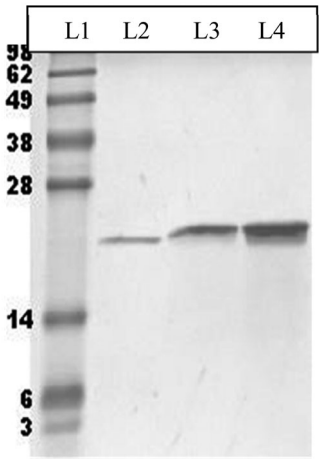

Figure 8. Trypsin purified samples compared with trypsin standard loaded on Tricine SDS PAGE. Lane 1: See Blue Pre-Stained Protein Ladder, Lane 2: Trypsin standard, Lane 3: Trypsin after cation exchange chromatography, Lane 4: Trypsin finished product.

The main purpose of choosing *E.coli* as host for expression of trypsin was to achieve higher yield, shorter fermentation time, and its advantage of producing inclusion bodies by keeping the enzyme inactive until activated by addition of an inducing enzyme. So, these advantages have played a major role and the protein recovery achieved was higher than any of the previous reports. In spite of protein loss during the solubilization step, increased inclusion bodies, and refolding efficiency maximized the product recovery (Table 3). No evidence of trypsin activation was noticed before the addition of enterokinase into the refolded protein mixture resulted in maximum trypsin activation after enterokinase addition. Optimization of enterokinase volume and incubation time for trypsin activation amplified the recovery percentage. Ion exchange chromatography with optimal buffer concentration enhanced retrieval of around 60% of the protein-loaded in the column and increased the yield by 3–4 times when compared to the previous yield of 30–60 mg/L.[6–9] Overall, the concentration of trypsin in the formulation buffer was 209 mg/L. Evidently, the results suggest that usage of *E.coli* as host, hydrophobic fusion tag, and the fermentation strategy played a crucial role in increasing the final product recovery.

Enzymatic assay for activity study

The activity of the in-house trypsin was found to be 1.94 USP units/µL or 1940 USP units/mL when compared to the standard (2.5 USP units/µL or 2500 USP units/mL). The average of difference for the standard and sample was 0.019 and 0.015. We observed that the linearity difference between the values of trypsin sample obtained was nearly constant. The difference in the values of the sample noted every minute ranged from 0.013 to 0.017, indicating the maintenance of enzyme efficiency until the 15th minute. The value after enzyme addition was almost linear and had an R-square value of 0.99, which shows that the enzyme was fully active for the initial 15 min.

The cleavage of trypsin mainly depends on its free amino terminus without fusion tag or methionine. Reasons of lower activity of enzymes are due to inefficient removal of fusion tags and unproductive refolding procedures. In our case, the activity of the enzyme has showed a positive signal by correlating with the quantity of trypsin.

Table 3. Recovery and loss percentage of protein during purification steps.

Purification Steps	Recovery (%) step wise	Loss step wise (%)	Total yield (%)	Trypsin (g/L)
Inclusion bodies	100	0	100	27.80
Solubilization	6	94	5.97	1.66
Refolding	54	46	3.24	0.90
Dialysis	82	18	2.63	0.73
Enterokinase Enzyme reaction	59	41	1.55	0.43
Ion Exchange Chromatography	60	40	0.94	0.26
Overall Recovery (Storage buffer after dialysis)	80	20	0.75	0.209

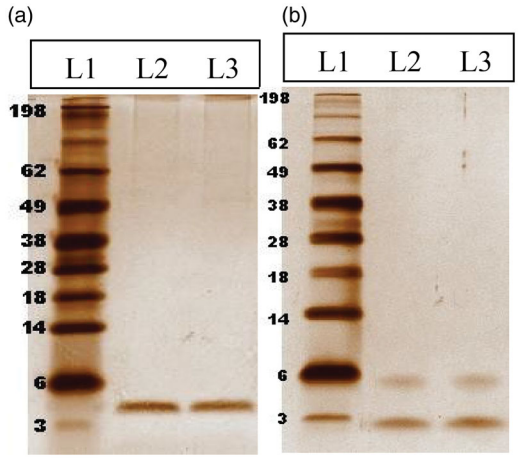

Figure 9. Substrate cleavage reaction of trypsin on glargine as substrate (Silver stained) loaded on Tricine SDS PAGE Figure 9a. shows glargine samples loaded on silver stained Tricine SDS-PAGE, while 9b shows glargine samples digested with trypsin and loaded on silver stained Tricine SDS-PAGE. 9a Lane 1: See blue pre-stained protein ladder, Lanes 2 & 3: Undigested glargine samples (2 µg), 9b Lane 1: See blue pre-stained protein ladder, Lanes 2 & 3: Glargine treated with trypsin (2 µg).

Substrate reaction assay using in-house trypsin

One unit of in-house trypsin cleaved 2 µg of intact glargine molecule within 16–18 h, leading to 75–85% separation of A and B chains. As the protein concentration was not noticeable on Tricine SDS PAGE upon cleavage, silver staining was done to detect minute amounts of protein. Digested bands close to 3 kDa can be clearly differentiated (Figure 9(b)) from the undigested band near 6 kDa (Figure 9(a)). There was not much difference between the molecular weights of A and B chains. Hence, two bands of glargine after cleavage with trypsin could not be differentiated individually on silver-stained Tricine SDS-PAGEand both overlapped with each other.

Optimization of trypsin addition and protein concentration in the reaction mixture led to an increase in the percentage of protein being cleaved. Therefore, reaction conditions need to be optimized to achieve maximum cleavage of the product.

Conclusion

In conclusion, overexpression of bio products is highly crucial for commercial production. In our study we have successfully expressed trypsin with high efficiency in *E.coli* expression system producing 27.8 g/L of inclusion bodies yielding about 209 mg/L of active trypsin detected in the final product after cation exchange chromatography yielded protein of high quality and purity. This study also confirms the application of TrpE fusion tag with novel fermentation process developed in-house. This tag enhanced trypsin expression by 3–4 fold when compared to the expression without fusion tag, this is mainly due to its hydrophobic nature. Overall process optimization led to the production of cheaper trypsin at a commercially viable scale. Moreover, the same approach can be exploited to express different recombinant enzymes of commercial interest.

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Ethical approval

This article does not contain any studies with human participants.

Disclosure statement

The authors of this paper declare that they have no conflict of interest in context of this research paper submitted to the journal.

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