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Research Article

Strategies towards the Functionalization of Subtilisin E from *Bacillus subtilis* for Wool Finishing Applications

Subtilisin E is an alkaline serine protease secreted by the Gram positive bacterium *Bacillus subtilis* and widely used in industry as a biocatalyst for various processes. The most common application of subtilisins is in laundry detergents. However, due to environmental concerns, the application of subtilisins to treat wool, is under study. There are some reports regarding the attempts to substitute the conventional chlorine treatment by an enzymatic process capable of providing the same characteristics to the fabric, like anti-shrinking and better uptake and fixation of the dyestuff. However, the degree of uncontrolled hydrolysis due to diffusion of the enzyme inside the wool fiber causes unacceptable losses of strength. To overcome this fact, and taking advantage of the x-ray crystallographic structure, the authors have modified subtilisin E genetically, increasing its molecular weight, to restrict the hydrolysis to the surface of the wool fibers. Therefore, three genetically modified enzymes with a molecular weight 2-fold to 4-fold higher than the native subtilisin E were produced and assessed for activity. The prokaryotic expression systems, pET25b (+), pET11b and pBAD C, were explored for the production of recombinant enzymes. The results demonstrated that regardless the expression system or strain used, chimeric subtilisins were not expressed with the correct folding. No active and soluble recombinant protein was recovered under the testing conditions. Despite this drawback, a novel approach was described to increase the molecular weight of subtilisin. The reported results are noteworthy and can indicate good guidelines for future work aiming at the solubilization of recombinant chimeric subtilisins.

Keywords: In vitro refolding, Protein engineering, Subtilisin E, Wool hydrolysis

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1 Introduction

Subtilisins are a family of alkaline serine proteases generally secreted by a variety of *Bacillus* species. There are also some reports about the production of subtilisins by *Flavobacterium* [1]. Subtilisins are characterized by a common three-layer $\alpha/\beta/\alpha$ tertiary structure and a catalytic triad of aspartate, histidine and serine residues [2] and their molecular weight ranges from 15 to 30 KDa, with few exceptions, like a 90 KDa subtilisin from *Bacillus subtilis (natto)* [3]. Subtilisins have an optimum pH range between 10.0 and 12.5 and an isoelectric point (pI) near 9.0 [4]. Subtilisins from *Bacillus* sp. are quite stable at high temperatures and the addition of Ca^{2+} enhances enzyme thermostability [5]. They are strongly inhibited by phenyl methyl sulphonyl fluoride (PMSF), diisopropyl-fluorophosphate (DFP) and potato inhibitor [6, 7].

Due to their widespread distribution, availability and broad substrate specificity, subtilisins are useful as biocatalysts for the detergent industry, leather processing, silver recovery in photographic industry, for management of industrial and household waste, for food and feed processing, as well as for medical purposes and chemical industry [8, 9]. Regarding the cited industrial applications, subtilisins have been extensively investigated as promising targets for protein engineering.

Among subtilisins, the subtilisin E from *B. subtilis* is one of the best studied alkaline serine proteases. Subtilisin E has been

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first synthesized as a membrane-associated precursor preprosubtilisin [10]. The NH_2-terminal prepeptide, consisting of 29 amino acid residues is a typical signal peptide that is required for the secretion of prosubtilisin across the plasma membrane. The propeptide located between the prepeptide and mature sequence has 77 amino acids and is essential for producing active subtilisin in vivo, as well as in vitro. It acts as an intramolecular chaperone required for the correct folding of a mature enzyme [11–14]. The mechanism of maturation by propeptide consists of three steps: *(i)* folding of the mature region mediated by its propeptide; *(ii)* cleavage of the peptide bond between propeptide and subtilisin and *(iii)* removal of the propeptide by an auto-proteolytic degradative process [14–18]. Degradation is required, because the propeptide can inhibit the active site of the subtilisin forming a stable and inactive propeptide-subtilisin complex [19–21]. The 3D structure of subtilisin E has been used to develop protein engineering strategies, aiming at the enhancement of catalytic activity and thermostability, as well as substrate specificity and oxidation resistance.

Catalytic efficiency of subtilisin E was increased 2–6 fold after changing the isoleucine at position 31 by a leucine, using site-directed mutagenesis (SDM) [22]. The same group constructed a novel subtilisin E with high specificity, activity and productivity through three cumulative amino acid substitutions [23]. Sroga and Dordick (2001) performed protein engineering to convert subtilisin E into an enzyme with broader esterase activity as opposed to its native amidase activity [24].

Improvement of thermal stability was first achieved by Takagi and collaborators (1990) with the introduction of an additional disulfide bond linkage between cysteines 61 and 98 in subtilisin E [25]. SDM was used to introduce an N218S mutation that increased the thermostability of the enzyme [26]. SDM was also used by Yang and collaborators to generate a S236C mutant subtilisin E with a half-life (at 60 °C), 4-fold longer than that of native subtilisin E. Using this mutant, thermostability could also be increased, by forming a disulfide bridge between two molecules of S236C subtilisin E [27]. The same group used random mutagenesis PCR technique to develop a thermal stable and oxidation-resistant mutant. The new M222A/N118S subtilisin E was 5-fold more thermally stable than the native enzyme [28]. In another report, the thermal stability of subtilisin E was increased using directed evolution to convert *B. subtilis* subtilisin E into an enzyme functionally equivalent to its thermophilic homolog thermitase from *Thermoactinomyces vulgaris* [29].

Proteases, like subtilisin E, can be used for wool fiber modification. Since wool mainly consists of proteins and lipids, proteases and lipases have been extensively studied in order to achieve more environmentally friendly processes [30]. Wool cuticle treatment with subtilisin improves anti-shrinkage properties, leads to a reduced felting tendency and an increased dyeing affinity [30]. However, due to its small size, the enzyme is able to penetrate into the fiber cortex which causes the destruction of the inner parts of wool structure [31]. Several reports show that the increase of the molecular weight of the enzyme by attaching synthetic polymers, such as polyethylene glycol (PEG) or by crosslinking with glutaraldehyde (GTA), is effectively avoiding enzyme penetration and the consequent reduction of strength and weight loss [32, 33]. Pre-treatment of wool fibers with hydrogen peroxide at alkaline pH in the presence of high concentrations of salts also targets the enzymatic activity on the outer surface of wool by improving the susceptibility of cuticle for proteolytic degradation [34].

Surfactant protein D (SP-D) is a member of the C-type lectin superfamily [35]. It is synthesized and secreted by alveolar and bronchiolar epithelial cells and participates in the innate response to inhaled microorganisms and organic antigens. It also contributes to the immune and inflammatory regulation within the lung [35]. Each SP-D subunit (43 KDa) consists of four major domains: an N-terminal cross-linking domain, an uninterrupted triple helical collagen domain, a trimeric coiled-coil or neck domain and a C-type lectin carbohydrate recognition domain. The neck domain of SP-D is the unit responsible for driving the trimerization of the three polypeptide chains of SP-D and it was demonstrated that the presence of this sequence permits a spontaneous and stable non-covalent association of a heterologous type IIA pro-collagen amino propeptide sequence [36]. SP-D neckdomain (SPDnd) was used for the possible formation of subtilisin E trimers.

Increasing the molecular weight of subtilisin is crucial for its successful application in wool finishing. The main objective of this work was to provide an alternative to the chemical modification of subtilisin, by expressing a genetically modified subtilisin E with an increased molecular weight, to be used for wool finishing applications. Two novel approaches were followed, the construction of two polysubtilisins, (pro2subtilisin and pro4subtilisin) and the formation of a subtilisin trimer by the fusion of native prosubtilisin with SP-D neckdomain. The authors were able to express the three modified enzymes although no activity was recovered for these enzymes yet. Both the expression systems tested and the fermentation conditions (that could increase the solubility of recombinant proteins) are presented in detail.

2 Materials and Methods

2.1 Bacterial Strains, Plasmids and Enzymes

The *Escherichia coli* strains BL21(DE3), BL21(DE3)pLysS and Tuner and the T7 plasmids pET25b (+) and pET11b were purchased from Novagen (Madison, WI, USA). Plasmid pBAD C and *E. coli* strains TOP 10 and LMG194 were from Invitrogen (Carlsbad, CA, USA). The genetic sequences coding for native prosequence, subtilisin E and prosubtilisin E were PCR-amplified with the primers listed in Tab. 1 and the vector pET11a was used as a DNA template. It contained the full sequence coding for pro-subtilisin E from *Bacillus subtilis* (kindly provided by Professor Masayori Inouye, Robert Wood Johnson Medical School, University of Medicine and Dentistry, Piscataway, New Jersey) [37]. Oligonucleotides (0.01 and 0.05 µmol scale) were purchased from MWG Biotech (Germany). Restriction and modification enzymes were from Roche Applied Science (Germany). The theoretical molecular masses of recombinant proteins were calculated using the Compute pI/Mw application from Expasy (http://www.expasy.ch/tools).

Table 1. The primers used to amplify the gene prosequence, subtilisin E and prosubtilisin E.

Gene	Primer (5′→3′)	bp	GC [%]
Prosequence	ProF CGC GGA TCC CAT GGC CGG AAA AAG CAG TAC AG	32	59.4
	ProR GGA AGA TCT CCA TAT TCA TGT GCA ATA TGA T	31	35.5
Subtilisin E	SubF CGC GGA TCC CAT GGC GCA AAG CTT TCC TTA TG	32	56.3
	SubR GGA AGA TCT CCT TGT GCA GCT GCT TGT ACG TTG	33	51.5
proSubtilisin E	ProF CGC GGA TCC CAT GGC CGG AAA AAG CAG TAC AG	32	59.4
	SubR GGA AGA TCT CCT TGT GCA GCT GCT TGT ACG TTG	33	51.5

Unless specifically stated, all the other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Transformation and DNA Sequencing

All the vectors constructed were first established in *E. coli* XL1-Blue strain, according to SEM method [38]. The correct plasmid constructs were verified by a restriction map analysis followed by DNA sequencing with an ABI PRISM 310 Genetic Analyzer, using the method of Sanger [39]. DNA cloning and manipulation were performed according to the standard protocols [40].

2.3 The Design of Chimeric Subtilisin Genes and the Construction of Expression Vectors Based on the pET System

The PCR products purified from a 2 % [w/v] agarose gel electrophoresis were first cloned into the p-GEM T-easy cloning system (Promega, USA), resulting in the following vectors: pGEM:prosequence, pGEM:subtilisin E and pGEM:prosubtilisin E. For the construction of the pro2subtilisin E chimeric gene, containing two subtilisin coding sequences cloned in frame, the *Bam*HI/*Bgl*II fragment of pGEM:subtilisinE was ligated with the *Bgl*II linearized pGEM:proSubtilisinE (see Fig. 1). An identical strategy was used for the construction of the chimeric gene pro4subtilisin E: the pGEM-pro2subtilisin E construction, linearized with *Bgl*II, and the chimeric gene *Bam*HI-2subtilisin-*Bgl*II, were ligated.

The gene corresponding to prosubtilisinE-SPDnd was chemically synthesized by EpochBiolabs, Texas, USA.

Flanked by *Bam*HI and *Bgl*II restriction sites, the entire DNA coding sequences for the native and the three chimeric subtilisins, were subcloned into *Bam*HI digested and dephosphorilated pET25b (+) and pET11b (see Tab. 2).

2.4 Site-Directed Mutagenesis of pBAD C Plasmid and Construction of pBAD Expression Vectors

The pBAD C plasmid was modified by site-directed mutagenesis (SDM), using recombinant PCR technique [41], in order

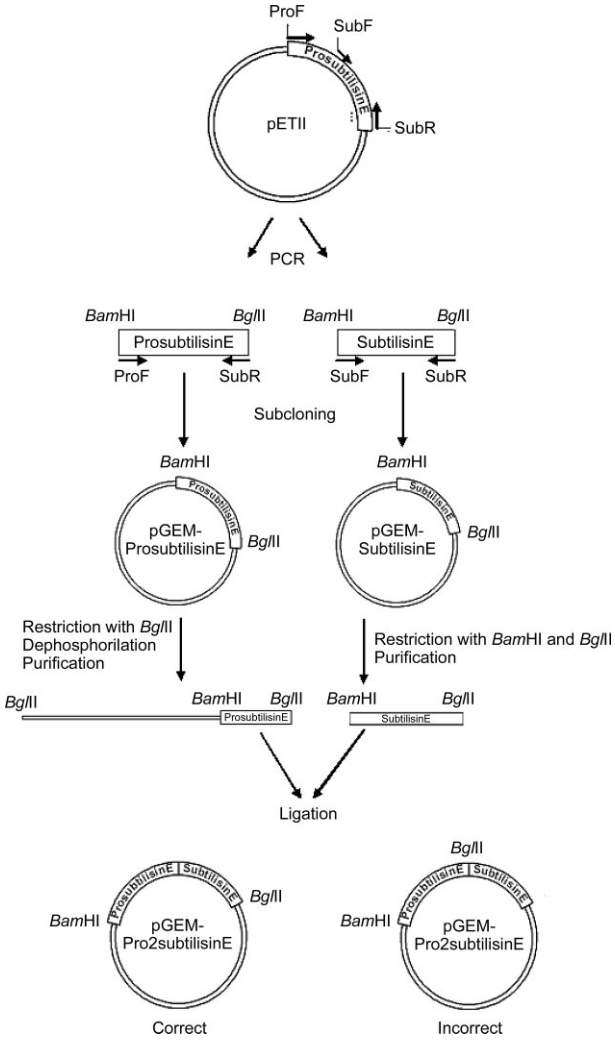

Figure 1. Strategy used for the construction of chimeric gene pro2subtilisin E in pGEM T-easy. PCR was carried out using pET11a:prosubtilisin [37] containing the native prosubtilisin E as a template. PCR products were subcloned into pGEM T-easy. Construction pGEM-subtilisin E was digested with *Bam*HI and *Bgl*II to recover the *Bam*HI-subtilisinE-*Bgl*II fragment, which was then ligated to the *Bgl*II linearized pGEM-prosubtilisin E. *Bam*HI and *Bgl*II recognize different restriction sites generating compatible sticky-ends.

to allow the introduction of the inserts in frame with C-*myc* and His6 tags. The primers were designed to remove an adenine at the end of the pBAD C multiple cloning site (the overlapping regions are underlined), and the mutation is indicated by an asterisk):

BADmutF(5'-TCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATC-3');
BADmutR(5'-TTCAGATCCTCTTCTGAGATGAGTTTTTGTTC*AGAAAGCTTCGAATTCC-3').

The mutation eliminates a recognition site for *Xba*I (TCTAGA → TCT*GA) which permitted to check the insertion of this mutation. Subtilisins coding sequences were recovered from cloning vectors by digestion with restriction enzymes *Bam*HI and *Bgl*II, and further cloned into dephosphorilated pBAD C* linearized with *Bgl*II (see Tab. 2).

The original *Bacillus subtilis* presequence, described by Ikemura and collaborators [13], was synthesized in vitro using the self-annealing oligonucleotides Fpres and Rpres, overlapping in 33 bp (see Tab. 3). The full DNA sequence was obtained in a PCR of 20 s at 94 °C and 20 s at 72 °C, for the Accuzyme (Bioline, Germany) extension, for 30 cycles. Primers Famp and Ramp (flanked with the *Xho*I restriction site) were further used to amplify the presequence (see Tab. 3). The PCR product was digested with the *Xho*I, purified from a 2 % [w/v] agarose gel electrophoresis and cloned into the *Xho*I digested and dephosphorilated pBAD C* constructions, resulting in the final expression vectors pBAD-pre-prosubtilisin E, pBAD-pre-pro2subtilisin E, pBAD-pre-pro4subtilisin E and pBAD-pre-prosubtilisinE-SPDnd (see Tab. 2).

Table 2. The heterologous protein expression systems used: *E. coli* strains and recombinant vectors.

E. coli strain/ vector	Constructs
BL21(DE3)/ pET25b (+)	prosubtilisin E pro2subtilisin E pro4subtilisin E
Tuner/ pET25b (+)	prosubtilisin E pro2subtilisin E pro4subtilisin E
BL21(DE3)pLysS/ pET25b (+)	prosubtilisin E pro2subtilisin E pro4subtilisin E
BL21(DE3)/ pET11b	prosubtilisin E pro2subtilisin E pro4subtilisin E prosubtilisinE-SPDnd
TOP10/ pBAD C*	prosubtilisin E pro2subtilisin E pro4subtilisin E prosubtilisinE-SPDnd
LMG194/ pBAD C*	prosequence prosubtilisin E pro2subtilisin E pro4subtilisin E prosubtilisinE-SPDnd pre-prosubtilisin E pre-pro2subtilisin E pre-pro4subtilisin E pre-prosubtilisinE-SPDnd

2.5 Induction Conditions for Protein Expression

Expression host strains BL21(DE3), BL21(DE3)pLysS and Tuner, transformed with pET25 constructions, were used for protein expression. Cells were grown in Luria-Broth (LB) medium containing 100 μg/μL ampicillin, and induced according to the conditions described in Tab. 4.

Cells of the strain BL21(DE3) containing pET11 constructions were grown in LB medium/ 100 μg/μL ampicillin, at 37 °C and induced with isopropyl *β*-D-1-thiogalactopyranoside (IPTG), 1 mM, at 18 °C.

E. coli strains TOP10 carrying the pBAD C* constructions were grown in Complete Minimal (CM) medium supplemented with 20 amino acids (40 μg/mL) and vitamin B1 (5 mg/L). Glycerol was used at a concentration of 0.20 % [w/v]. Cells of the strain LMG194 were grown in RM medium. In both cases, ampicillin was used at a concentration of 100 μg/μL. Cells were induced according to the conditions described in Tab. 4.

2.6 Cell Fractionation

Overnight cell cultures from all the strains transformed with pET25 and pBAD C* constructions were harvested by centrifugation (5000 rpm for 15 minutes) and resuspended in Osmotic Solution I (OS I: 20 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 2 mM $CaCl_2$, sucrose 20 %, w/v) to an OD_{600} of 5.00. Cells resuspended in OS I were incubated on ice for 10 min and centrifuged at 4 °C. Supernatants were decanted and the cell pellets resuspended in the same volume of Osmotic Solution II (OS II: 20 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 2 mM $CaCl_2$). The suspension was incubated for 20 min on ice and centrifuged at 4 °C. The supernatants (periplasmic fractions) were stored. The pellet samples (cellular fractions) were resuspended in phosphate buffered saline (PBS) solution (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.4). Ultrasonic treatment of bacterial cells was performed at 20 KHz with a 13 mm probe in an Ultrasonic Processor GEX 400. Four 2 min pulses, with 2 min on ice between each pulse, were performed. The lysates were centrifuged for 30 min at 14000 rpm at 4 °C.

The supernatants, soluble fractions, were decanted and stored. The pellets, insoluble fractions, were resuspended in PBS solution and stored.

Table 3. Primers used to generate and amplify *B. subtilis* presequence.

Gene	Primer (5′→3′)	bp	GC [%]
Presequence	Fpres CTC GAG TGA GAA GCA AAA AAT TGT GGA TCA GCT TGT TGT TTG CGT TAA CGT TAA TCT TTA CGA	63	38.1
	Rpres CTC GAG CCT GCG CAG ACA TGT TGC TGA ACG CCA TCG TAA AAG TTA ACG TTA AGC CAA ACA ACA	63	47.6
	Famp CCC TCG AGT GAG AAG CAA AA	20	50
	Ramp CCC TCG AGC CTG CGC AGA CA	20	70

The XhoI restriction site is underlined.

Table 4. Fermentation conditions performed for *E. coli* pET 25b (+) and pBAD vectors.

E. coli strain/ Vector	Temperature of growth [°C]	[Inducer]	Temperature of induction [°C]/time
BL21(DE3)/ pET25b (+)	37; 30; 25	IPTG 1.0; 0.5; 0.3 [mM]	30;18; 4 h
BL21(DE3)pLysS/ pET 25b (+)	30; 25	IPTG 1.0; 0.5; 0.3 [mM]	18 h
Tuner/pET 25b (+)	30; 25	IPTG 1.0; 0.5; 0.3 [mM]	18 h
TOP10 and LMG194/pBAD C*	37; 30	Arabinose 0.2; 0.1 [%]	18 h/ON and 3 h

Overnight cell cultures from the BL21(DE3) strain transformed with pET11b constructions were harvested by centrifugation (5000 rpm for 15 minutes) and resuspended in PBS solution to an OD_{600} of 5.00. Cells were broken with ultrasonic treatment and lysates centrifuged for 30 min at 14000 rpm at 4 °C and pellets were stored.

2.7 In Vitro Renaturation of Recombinant Enzymes

The pellets (inclusion bodies) from fermentations of *E. coli* carrying pET11 constructions were solubilized in 6 M urea. After overnight incubation at 4 °C, the insoluble materials were removed by ultracentrifugation at 90000 × g for 40 min. The supernatants were dialyzed against an excess of 50 mM sodium-potassium phosphate buffer (pH 5.0) containing 5 M urea at 4 °C. Renaturation of recombinant proteins was performed by a stepwise dialysis procedure against 10 mM Tris-HCl (pH 7.0), 0.5 M $(NH_4)SO_4$, 1 mM $CaCl_2$, 5 mM β-mercaptoethanol and decreasing amounts of urea. Buffer was changed every 24 h until urea was completely removed.

2.8 Isolation and Purification of the Prosequence

For prosequence purification, an Immobilized Metal Affinity Chromatography (IMAC) system was used with an HiTrap Chelating HP 5 mL column containing 5 mL of Chelating Sepharose High Performance (Amersham Pharmacia Biotech). The HiTrap Chelating HP column was linked to a peristaltic pump. After loading with 2.5 mL 0.1 M $NiSO_4$ in H_2O, equilibration was performed with 10 mM imidazole, 0.5 M NaCl, 20 mM phosphate buffer, pH 7.6.

Samples were applied onto the column at a flow rate of 5 mL/min, followed by washing with the equilibration buffer. Elution was performed with a buffer containing 500 mM imidazole, 0.5 M NaCl and 20 mM phosphate buffer, pH 7.6.

2.9 Prosequence Mediated Folding

Prosequence mediated folding was performed by the addition of IMAC purified prosequence, produced by *E. coli* LMG194, to native and chimeric enzymes, purified from BL21(DE3) inclusion bodies, in a 1:1 molar ratio. The mixture was allowed to incubate for 12 h at 4 °C.

2.10 Analytical Methods for the Enzymes

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS), using a Tris-SDS-glycine buffer system, was employed to monitor the soluble and insoluble fractions [42]. Protein detection was performed using Coomassie Brilliant Blue R250. The total protein concentration was estimated by the Bradford quantitative protein determination assay using bovine serum albumin as a standard [43].

2.11 Activity Assay

The proteolytic activity was determined using azocasein as a substrate, according to Sako and co-workers [44]. The reaction

mixture containing 0.250 mL 50 mM Tris-HCl (pH 8.0), 2 % [w/v] azocasein and 0.150 mL enzyme solution to a final volume of 0.400 mL, was incubated at 25 °C for 30 minutes. The negative control was prepared replacing the enzyme solution with buffer. The reaction was stopped by the addition of 1.2 mL of 10 % trichloroacetic acid (TCA). The solution was mixed thoroughly and allowed to stand for 15 minutes to ensure a complete precipitation of the remaining azocasein and azocasein fragments. After centrifugation at 8000 × g for 5 min, 1.2 mL of the supernatant was transferred to a test tube containing 1.4 mL of 1 M NaOH solution. The absorbance of this solution was measured at 440 nm using a spectrophotometer Genesis 20 (Thermospectronic). The assays were performed in triplicate. One unit of protease activity is defined to be the amount of enzyme required to produce an absorbance change of 1.0 in a 1 cm cuvette, under the conditions of the assay.

3 Results

3.1 Expression of Recombinant Proteins using the pET25b (+) Expression System

All the constructions reported in this work were originally cloned by PCR from the vector pET11a:pro-subtilisin [37]. The final constructions that are described in Tab. 2 were transformed into the appropriate strains. Depending on the vector used, the expression of recombinant proteins was induced with IPTG or with arabinose, as well as by varying the incubation temperature of the cell cultures, as described in *Materials and Methods*.

E. coli BL21(DE3) cells transformed with pET25b (+) constructions (see Tab. 2), grown at 37 °C, were able to express prosubtilisin E, pro2subtilisin E and pro4subtilisin E at a high level in the presence of 1 mM IPTG at 30 °C (see Fig. 2). The molecular mass of prosubtilisin E, estimated as 45.0 KDa, is not in agreement with the expected molecular weight for mature subtilisin E, (30 KDa) [13]. The chimeric proteins pro2subtilisin E and pro4subtilisin E showed a molecular mass of approx. 67 and 125 KDa, respectively, probably due to the unprocessed pelB-prosubtilisin E, pelB-pro2subtilisin E and pelB-pro4subtilisin E. The theoretical molecular mass of the processed proteins according to Expasy is approx. 58 and 116 KDa. The pelB leader signal peptide directs the recombinant proteins to the periplasmic space, where they were mostly expected to be found. However, recombinant proteins were only found in the insoluble fractions (pellets) (see Fig. 2). The same results were obtained for expression strains BL21(DE3)pLysS and Tuner using the same fermentation conditions (data not shown).

In order to increase the solubility of the recombinant proteins, all bacterial strains were grown under different temperature regimes (at 30 and 25 °C) and the induction phase was performed with lower concentrations of IPTG (0.5 and 0.3 mM) at lower incubation temperatures (18 and 4 °C). It was assumed that combining decreasing of temperature with lower concentrations of inducer would prevent overloading the *E. coli* periplasmic transport system and the recombinant enzymes would be able to fold properly. However, none active subtilisin was secreted to the periplasmic space (see Figs. 3 and 4), which revealed that the pET25b (+) pelB leader sequence was not a suitable signal sequence to export this protease, in the set of conditions tested. In Figs. 3 and 4 the representative results obtained for strain BL21(DE3) are shown for all the parameters tested (culture temperature, inducer concentration and induction phase temperature). Similar results were achieved for the strains BL21(DE3)pLysS and Tuner, using the same fermentation conditions.

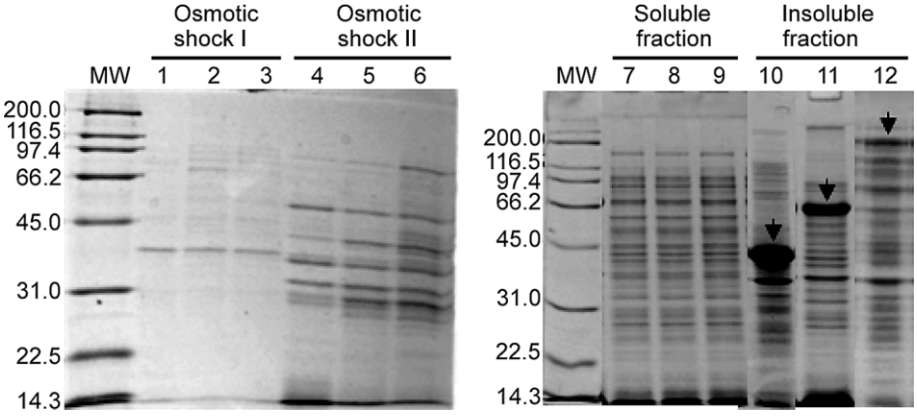

Figure 2. SDS-PAGE of proteins from *E. coli* BL21(DE3) cells grown at 37 °C, induced with IPTG (1 mM) at 30 °C.
Lanes 1, 4, 7 and 10: pET25pro-Subtilisin E.
Lanes 2, 5, 8 and 11: pET25-pro2Subtilisin E.
Lanes 3, 6, 9 and 12: pET25-pro4Subtilisin E.
MW: SDS-PAGE Standard, Broad Range (Bio-Rad).
The *solid arrowheads* indicate the position of recombinant proteins.

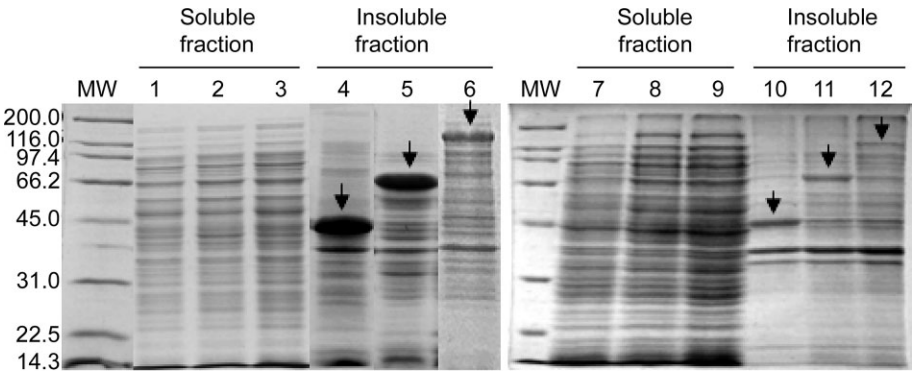

Figure 3. SDS-PAGE of proteins from *E. coli* BL21(DE3) cells grown at 30 °C, induced with 0.5 mM IPTG at 18 °C, and grown at 30 °C, induced with 0.3 mM IPTG at 4 °C.
Lanes 1, 4, 7 and 10: pET25-prosubtilisin E.
Lanes 2, 5, 8 and 11: pET25-pro2subtilisin E.
Lanes 3, 6, 9 and 12: pET25-pro4subtilisin E.
MW: SDS-PAGE Standard, Broad Range (Bio-Rad).
The *solid arrowheads* indicate the position of recombinant proteins.

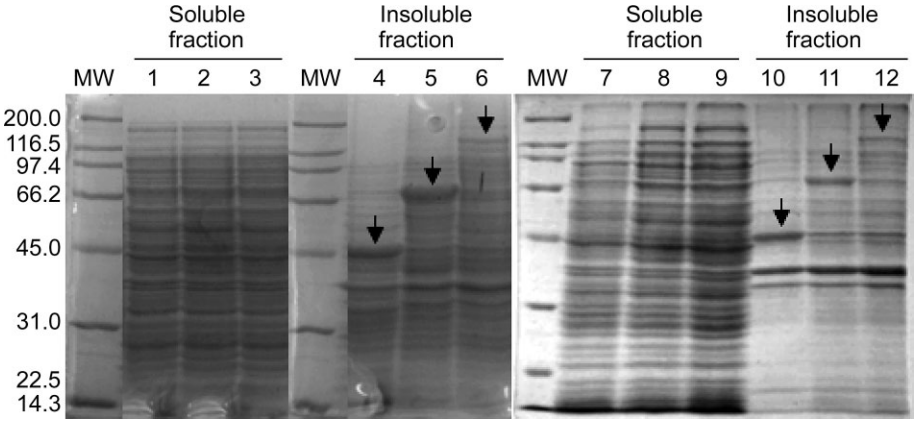

Figure 4. SDS-PAGE of proteins from *E. coli* BL21 (DE3) cells grown at 25 °C, induced with 0.5 mM IPTG at 18 °C, and grown at 25 °C, induced with 0.3 mM IPTG at 4 °C.
Lanes 1, 4, 7 and 10: pET25-prosubtilisin E.
Lanes 2, 5, 8 and 11: pET25-pro2subtilisin E.
Lanes 3, 6, 9 and 12: pET25-pro4subtilisin E.
MW: SDS-PAGE Standard, Broad Range (Bio-Rad).
The *solid arrowheads* indicate the position of recombinant proteins.

3.2 Expression of Recombinant Proteins using pBAD Expression Systems

The *E. coli* pBAD expression system was described to express *B. subtilis* subtilisin E [24, 45], where the entire preprosubtilisin E gene was used and a full functional enzyme was efficiently targeted to the periplasmic space. The pBAD plasmid has a geneIII signal sequence that can be used for the periplasmic expression of recombinant enzymes. SDM by recombinant PCR [41] was performed into the pBAD C vector in order to allow in frame integration of inserts. The native and chimeric genes were cloned into a pBAD C* expression system and the *E. coli* strains TOP10 and LMG194 were used. The TOP 10 *E. coli* did not express any of the recombinant enzymes (data not shown). LMG194 cells, carrying pBAD C* constructions, produced the recombinant proteins in the insoluble fraction under all the conditions tested. Fig. 5 shows the representative results of these assays.

Unlike Sroga and its collaborators [45], the authors were not able to produce active and soluble subtilsin E using the pBAD expression system, since no azocasein activity could be detected in soluble fractions, derived both from cytoplasm or periplasmic space. As previously observed for the pET25b(+) pelB leader sequence, also the pBAD C geneIII leader sequence did not indicate to be a suitable signal peptide to export these recombinant proteins to the periplasmic space. It was assumed that these facts might be explained by the absence of a native *B. subtilis* presequence. Further to this hypothesis, in all the

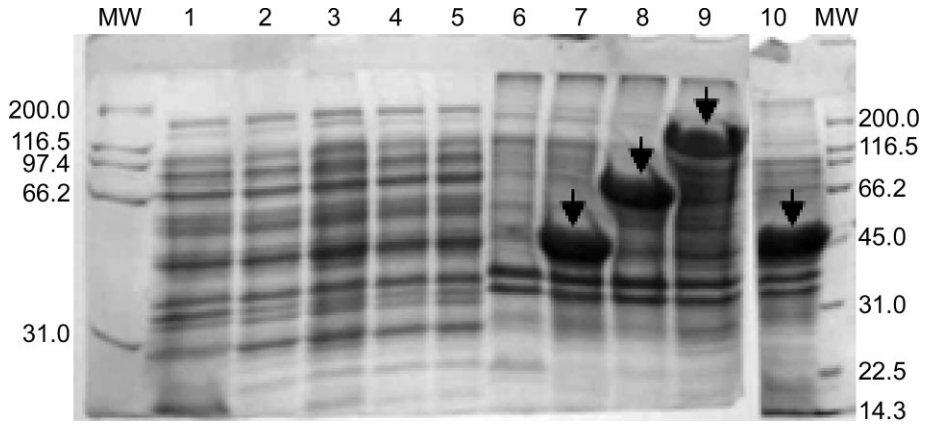

Figure 5. SDS-PAGE of proteins from *E. coli* LMG194 cells grown at 37 °C, induced with 0.2 % arabinose at 18 °C.
Lanes 1 to 5: soluble fractions of the negative control pBAD, pBAD-prosubtilisin E, pBAD-pro2-subtilisin E, pBAD-pro4subtilisin E and pBAD-prosubtilisinE-SPDnd, respectively, and *lanes 6 to 10*: insoluble fractions of the negative control pBAD, pBAD-prosubtilisin E, pBAD-pro2subtilisin E, pBAD-pro4subtilisin E and pBAD-prosubtilisinE-SPDnd.
MW: SDS-PAGE Standard, Broad Range (Bio-Rad).
The *solid arrowheads* indicate the position of recombinant proteins.

pBAD C* constructions, the original *B. subtilis* prepeptide was introduced in frame upstream the pBAD geneIII signal sequence. Cells LMG194 harboring the pBAD-pre-prosubtilisin E, pBAD-pre-pro2subtilisin E, pBAD-pre-pro4subtilisin E and pBAD-pre-prosubtilisin E-SPDnd, were grown and induced as previously described. The results demonstrated that no production of active and soluble recombinant protein using the pBAD/gIII expression system was achieved, even in the presence of the original leader sequence of *B. subtilis* (see Fig. 6).

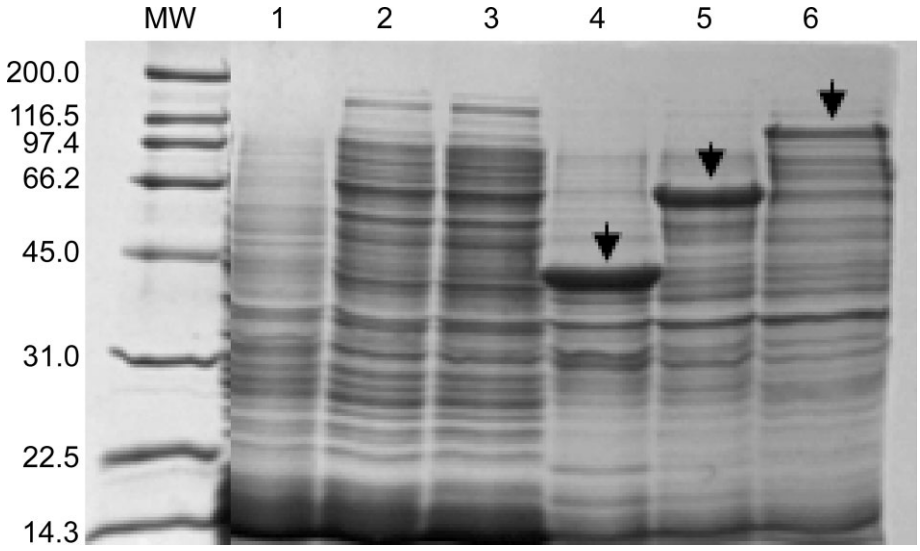

Figure 6. SDS-PAGE of proteins from *E. coli* LMG194 cells grown at 30 °C, induced with 0.2 % arabinose at 18 °C.
Lanes 1 to 3: soluble fractions of pBAD-pre-prosubtilisin E, pBAD-pre-pro2subtilisin E and pBAD-pre-pro4subtilisin E, respectively, and *Lanes 4* to *6*: insoluble fractions of pBAD-pre-prosubtilisin E, pBAD-pre-pro2subtilisin E and pBAD-pre-pro4subtilisin E.
MW: SDS-PAGE Standard, Broad Range (Bio-Rad).The *solid arrowheads* indicate the position of recombinant proteins.

3.3 Expression of Recombinant Proteins using the pET11b Expression System

Since *B. subtilis* subtilisin E was previously expressed in the *E. coli* pET11 system [37], the genes coding for the native enzyme as well as the three chimeric enzymes, pro2subtilisin E, pro4subtilisin E and prosubtilisin E-SPDnd, were cloned into this vector, using *E. coli* BL21(DE3) as a host strain. The transformants carrying pET11 vectors were able to produce all the constructs at a level of almost 80 % of total cellular proteins in the presence of 1 mM IPTG (see Fig. 7).

After cell disruption (using ultra-sonic treatment), the products isolated in the pellets were collected by low-speed centrifugation, indicating that the proteins aggregated to form inclusion bodies. Purification and refolding of native and chimeric enzymes were carried out with the isolated inclusion bodies as described in *Materials and Methods*. All samples were assayed for azocasein activity. Except for native subtilisin E, (3.2 U), no activity was detected for chimeric enzymes pro2subtilisin E, pro4subtilisin E and prosubtilisinE-SPDnd (data not shown).

Mature subtilisin E and pro2subtilisin E purified and renatured from inclusion bodies were used for circular dicroism analysis. Compared to active mature subtilisin E, chimeric pro2subtilisin E presented only 30 % of secondary structure (data not shown), which suggests that in vitro renaturation of chimeric enzymes does not result in the correct folding necessary for enzymatic activity.

3.4 Prosequence Mediated Folding

E. coli LMG194 cells carrying the construction pBAD-prosequence, grown at 30 °C and induced at 18 °C, were able to express the prosequence at a high level in the soluble as well as in the insoluble fractions (see Fig. 8).

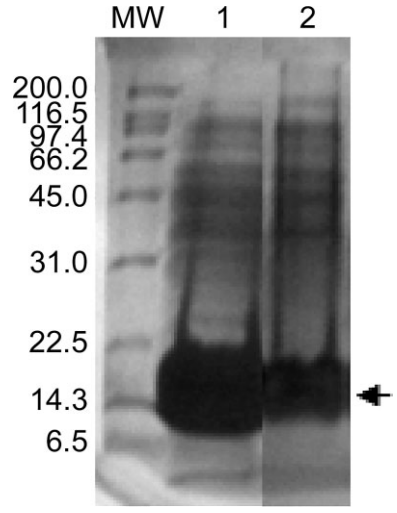

Figure 8. SDS-PAGE of proteins from *E. coli* LMG194 transformed with pBAD C*-prosequence.
Lane 1: soluble fraction and *Lane 2:* insoluble fraction.
MW: SDS-PAGE Standard, Broad Range (Bio-Rad).
The *solid arrowhead* indicates the position of the recombinant prosequence.

The soluble fractions were purified by IMAC, using a nickel column, and employed for refolding procedures. Prosequence-mediated folding was performed by the addition of a prosequence to the recombinant proteins purified from inclusion bodies in a 1:1 molar ratio and incubation at 4 °C for 12 h. All

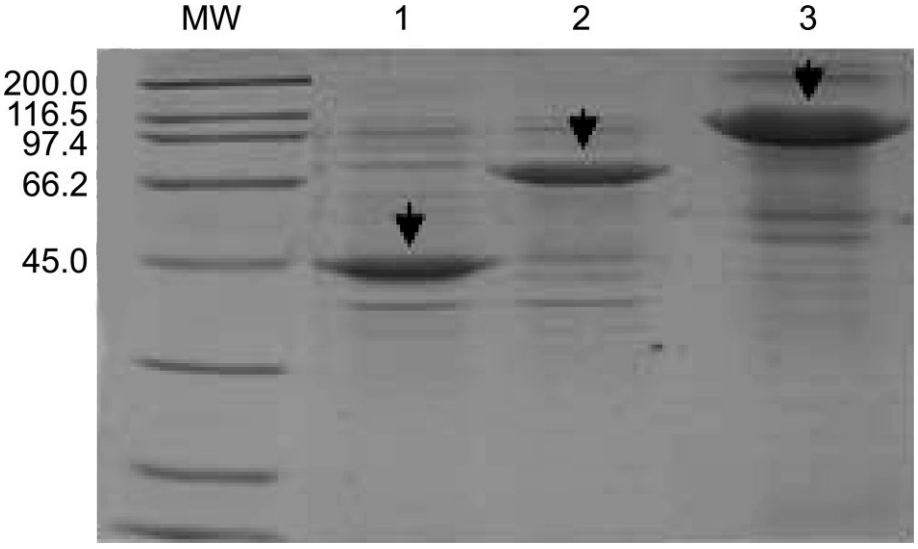

Figure 7. SDS-PAGE of insoluble fractions from *E. coli* BL21 (DE3) cell cultures grown at 37 °C, induced with 1 mM IPTG at 37 °C.
Lane 1: pET11-prosubtilisin E, *Lane 2:* pET11-pro2subtilisin E and *Lane 3*: pET11-pro4subtilisin E.
MW: SDS-PAGE Standard, Broad Range (Bio-Rad).
The *solid arrowheads* indicate the position of recombinant proteins.

the experiments were repeated three times. In a previous work performed by Shinde and its collaborators [16] the refolding of Gdn-HCl-denatured subtilisin E was achieved by an incubation with a prosequence under the same conditions as described above. In all the assays performed refolding of prosubtilisin E was attained, since activity was recovered, but refolding of chimeric pro2subtilisin E, pro4subtilisin E and prosubtilisinE-SPDnd enzymes was not achieved (data not shown).

4 Discussion

The results demonstrated that chimeric subtilisins were not expressed with the correct folding using three different *E. coli* expression systems, pET25b (+), pET11b and pBAD C.

The first objective was to choose an expression system for the periplasmic secretion of recombinant proteins. Compared to cytosolic production, secretory production provides several advantages, for example, the possibility to obtain proteins with authentic N-termini, after cleavage of the signal sequence by a specific signal peptidase; enhanced disulphide-bond formation, because the periplasmic space provides a more oxidative environment than the cytoplasm; decreased proteolysis and minimization of harmful actions of recombinant proteins which are deleterious to the cell [46]. Furthermore, the periplasm contains only about 100 proteins as compared with about 400 proteins in the cytoplasm. Thus recombinant protein purification procedures can be more efficient when proteins are targeted to the periplasm [47].

Although active subtilisin had been expressed in *E. coli* periplasm using the IPTG inducible pIN-III-ompA vector, the amount of functional enzyme obtained was very low [13]. This vector has a strong Ipp promoter, as well as a lac promoter operator fragment to ensure that expression is dependent on the addition of a lac inducer [48]. A disadvantage of this promoter is the absence of complete down-regulation under non-induced conditions, since an early overproduction of chimeric subtilisins could impair cell growth. Therefore, a more tightly regulated IPTG-inducible system, the pET system, was used as an alternative expression system for common lab-scale fermentations. The pET vectors have a T7 promoter which is transcribed only by T7 RNA polymerase and must be used in strains carrying a chromosomal T7 RNA polymerase gene that is under the control of a lac promoter [49, 50]. The pET 25b (+) vector allowed to control the level of chimeric subtilisins expression. Having the pelB leader sequence it is possible to direct the proteins for periplasmic space which, in combination with the addition of a 6 × His tag to the enzyme's C-terminus, improves the purification of recombinant proteins. It also allows an easily immunological detection by adding the C-terminal HSV-epitope tag. Recombinant subtilisins were over-expressed, but in a misfolded and inactive form, associated with *E. coli* insoluble proteins. In order to test another signal sequence, different from the pET25b (+) pelB leader, the genes were cloned into the pBAD/gIII C expression vector. This vector that contains the pBAD promoter from the arabinose operon is tightly regulated and contains the gene III signal sequence utilized for the secretion of the recombinant protein into the periplasmic space. Similarly to the results obtained for pET25b (+), also using the pBAD C expression system, none of the recombinant proteins were secreted to the periplasm. Since periplasmic expression did not reveal to be appropriate for the recombinant proteins under study, chimeric genes were subsequently cloned into pET11b. Using this system, proteins were expressed in the form of inclusion bodies. The dense inclusion bodies could be rapidly recovered by centrifugation and a high purity of protein preparations was obtained. The main disadvantage with a formation of inclusion bodies is the need for solubilization and refolding steps, necessary to achieve the correct folding and activity of recombinant proteins. This strategy was efficient to fold native subtilisin E correctly but not the chimeric enzymes. The authors are currently analyzing the factors affecting the solubility and studying alternative systems for the expression of chimeric enzymes. The co-expression of chimeric proteins with chaperons could be a strategy to promote the correct folding and to increase the solubility of recombinant subtilisins. Different chaperone plasmid sets, able to express multiple molecular chaperons, have been used successfully to increase the recovery of expressed recombinant proteins in the soluble fraction. Such proteins were hardly recoverable using conventional methods due to the formation of inclusion bodies [51–53]. The addition of metal ions to the culture medium could also have a positive effect on the solubilization of recombinant proteins produced by *E. coli* [54]. If the expression of chimeric proteins in *E. coli* is found to be not effective, the genes could be cloned back into bacteria from the genus *Bacillus* [55, 56].

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