Swapping of pro-sequences between keratinases of Bacillus licheniformis and Bacillus pumilus: Altered substrate specificity and thermostability

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Pro-sequences were swapped in cis between keratinases from Bacillus licheniformis (Ker BL) and Bacillus pumilus (Ker BP) to construct Ker ProBP–BL and Ker ProBL–BP, respectively. Expression of these keratinases was carried out constitutively by E. coli HB101-pEZZ18 system. They were characterized with respect to their parent enzymes, Ker BL and Ker BP, respectively. Ker ProBP–BL became more thermostable with a t\(_{1/2}\) of 45 min at 80 °C contrary to Ker BL which was not stable beyond 60 °C. Similarly, the activity of Ker ProBL–BP on keratin and casein substrate, i.e. K:C ratio increased to 1.2 in comparison to 0.1 for Ker BL. Hydrolysis of insulin B-chain revealed that the cleavage sites increased to six from four in case of Ker ProBP–BL in comparison to Ker BL. However, cleavage sites decreased from seven to four in case of Ker ProBL–BP in comparison to the parent keratinase, Ker BP. Likewise, Ker ProBL–BP revealed altered pH and temperature kinetics with optima at pH 10 and 60 °C in comparison to Ker BP which had optima at pH 9 and 70 °C. It also cleaved soluble substrates with better efficiency in comparison to Ker BP with K:C ratio of 1.6. Pro-sequence mediated conformational changes were also observed in trans and were almost similar to the features acquired by the chimeras constructed in cis by swapping the pro-sequence region.

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1. Introduction

Keratinases are subtilisin or metallo-proteases which have the capacity to act on recalcitrant proteins such as keratin. Their potential to act on β-keratin of chicken feather makes them prospective candidate for hydrolyzing proteinase-K resistant β-amyloid and prion plaques [1]. However, limited availability of efficient keratinases has boosted the search for better keratinases with improved catalytic efficiency for attacking recalcitrant proteins. At present, protein engineering techniques involving site-directed mutagenesis and DNA shuffling of core protein are used to develop better catalysts with improved substrate specificity and thermostability [2,3].

Proteases are expressed in pre pro form where pre-sequences are signal peptides responsible for mobilizing the proteases along with their pro-sequence across the cytoplasmic membrane. Pro-sequence region acts as chaperone leading active conformation in proteases in Bacillus [3]. Pro-sequences are subsequently autoprocessed or degraded by proteases to yield active protein into the extracellular medium [3]. Pro-sequences have been well recognized as hot spots for mutagenesis to develop conformational variants through pro-sequence engineering [3]. There are several reports where such variants have been developed by utilizing chaperone functions of an exogenous pro-sequence i.e. in trans or in vitro. However, their use in cis exchanges where protease folding mediated by an exchanged pro-sequence, has been reported to result in functionally less active proteins [4].

Here, we report the effect of pro-sequence exchange on biochemical properties of keratinases from two closely related species, Bacillus licheniformis and Bacillus pumilus. The enzymes from these two species have been previously reported to possess contrasting features with respect to thermostability and substrate specificity [5,6].

2. Materials and methods

Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs (Beverly, USA). The oligonucleotides were synthesized by Sigma–Aldrich (USA). Genomic DNA extraction, plasmid extraction and gel elution kits were purchased from Qagen, Hilden, Germany. Expression vector pEZZ18 was purchased from GE Healthcare Science (India). E. coli HB101 was used as the expression host. All bacterial strains were grown on Luria-Bertani (LB) medium supplemented with 1% tryptone, 0.5% yeast extract, and 1% NaCl (pH 7.2). When required, ampicillin was added to the medium to a final concentration of 100 μg/mL. Q-Sepharose, DEAE-Sepharose and various synthetic substrates were purchased from Sigma–Aldrich (USA).
Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Ker BP</td>
<td>EcoRI 5’ GAATTCCAGGCTCTCAGAAA 3’</td>
</tr>
<tr>
<td>Ker BP*</td>
<td>BamHI 5’ GGATCCGCTATCAGATGGC 3’</td>
</tr>
<tr>
<td>Ker BL</td>
<td>ScaI 5’ GAGCTCCGTCACGGGCGAAAAAT 3’</td>
</tr>
<tr>
<td>Ker BLR</td>
<td>KpnI 5’ GGAACCTTATTGACCAGACCTCGCA 3’</td>
</tr>
</tbody>
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Italic bases stands for restriction sites.

2.1. In silico analysis of Ker BP and Ker BL

Pair-wise sequence alignment of Ker BL (GenBank accession no. AY590140) and Ker BP (GenBank accession no. HM219183) was done using the PSI-BLAST pre-profile processing (Homology-extended alignment) available from the PRALINE online resource portal (http://www.ncbi.nlm.nih.gov/praline/blast) which uses an optimized heuristic with a gap opening penalty of 12 and an extension penalty of 1 [7-8]. The alignment was thereafter assessed based on their amino acid conservation and the motifs identified were analyzed with those already available in literature.

2.2. Cloning of Ker ProBP–BL and Ker ProBL–BP

Keratinases with swapped pro-sequences were created utilizing Ker BL and Ker BP clones which were already available in the laboratory in pEZZ18 vector [5,6]. pEZZ18-Ker BL was restricted with ScaI/KpnI to obtain Ker BL and pEZZ18-Ker BP was restricted with EcoRI/BamHI to obtain Ker BP. Restriction enzyme with single common restriction site of Ker BL and Ker BP gene was determined using the NEB cutter tool. Possible restriction site was recognized in both Ker BL and Ker BP close to the pro-sequence region. Fallouts of Ker BL and Ker BP were thereby digested with PsiI and the digestion mixtures were mixed in a ratio of 1:1 to create a chimeric ligation mix. The ligation mix was then further ligated into SacI/BamHI and EcoRI/KpnI digested pEZZ18 and transformed into E. coli HB101 to obtain chimeric pEZZ18-ProBP–BL (pro-sequence of Ker BP and mature protein from Ker BL) and pEZZ18-ProBL–BP (pro-sequence of Ker BL and mature protein from Ker BP), respectively. Positive clones were reconfirmed by colony PCR using a set of gene specific primer, Ker BPf-Ker BLr for Ker ProBP–BL and Ker BLf-Ker BPr for Ker ProBL–BP. The clones were subsequently sequenced at the Central Instrumentation Facility, University of Delhi. The primer sequences have been tabulated in Table 1 and the cloning strategy to form chimeric keratinases has been schematically presented in Fig. 1.

2.3. Expression of Ker ProBP–BL and Ker ProBL–BP

Extracellular expression of recombinant keratinases was carried out constitutively by E. coli HB 101-pEZZ18 system. The vector pEZZ18 has a spa promoter and protein A signal alongside “ZZ” domain based on IgG binding sites [5]. Under the direction of protein A signal, the expressed protein gets secreted as a fusion protein with “ZZ” peptides under non-inducible condition [5]. E. coli HB101 cells harboring pEZZ18-Ker ProBP–BL and pEZZ18-Ker ProBL–BP were grown in LB medium supplemented with ampicillin at 37 °C, 300 rpm. After 18 h, the cells were separated by centrifugation at 7441 × g for 10 min and expression was checked in the extracellular broth by keratinase assay and SDS-PAGE analysis. Simultaneously, original keratinases Ker BL and Ker BP were also produced in the same manner [5,6].

2.4. Purification of Ker ProBP–BL and Ker ProBL–BP

The cell free culture broth was concentrated 10 times using ultrafiltration by 10 KDa molecular cut-off cassette. The retentate was applied to anion exchanger columns i.e. Q-Sepharose and DEAE-Sepharose pre-equilibrated with 10 mM Tris/HCl buffer, pH 8. The column was washed with the same buffer and 15 L fraction was collected at a flow rate of 2 mL/min. Bound protein was eluted in a step gradient of sodium chloride (0.1–1 M NaCl). Purity of the protein was determined by SDS-PAGE analysis with the parent proteins i.e. Ker BP and Ker BL. Protein was concentrated with 50% (w/v) trichloroacetic acid (TCA) before loading onto the gel. The gel was stained with Coomasie brilliant blue R-250 solution.

2.5. N-terminal sequencing of Ker ProBP–BL and Ker ProBL–BP

After swapping the pro-sequences between keratinases, Ker BL and Ker BP their processing was confirmed by N-terminal analysis. The N-terminal sequence of the purified proteins, Ker ProBP–BL and Ker ProBL–BP was analyzed at the Protein Facility of Iowa State University, USA by automated Edman degradation performed with a 494 Procise Protein Sequencer/140C Analyzer (Applied Biosystems, Inc.).

2.5.1. Source of keratin substrate

Chicken feather was obtained from local poultry plants. They were washed thoroughly with triton X-100 (1%, w/v) and rinsed with distilled water followed by autoclaving at 15 psi. Thereafter, feather were dried in an oven at 60 °C for overnight and passed through a sieve of mesh no. 10 having pore size of 2 mm. Feather powder as a substrate was used to perform the keratinase assay.

2.5.2. Keratinase assay and protein estimation

Keratinase activity was measured as described by Dozie et al. [9] with some modifications. The assay mixture containing 1 mL of appropriately diluted enzyme, 4 mL of 50 mM glycine–NaOH buffer at optimum pH and 20 mg feather powder was incubated at optimum temperature for 1 h. The reaction was terminated by adding 4 mL of 5% (w/v) TCA and tubes were incubated at room temperature (25 ± 1 °C) for 1 h. Insoluble residues were removed by filtration through glass wool, and the filtrate was centrifuged at 7441 × g for 5 min. Control was set up by adding 20 mg feather powder, 1 mL of 5% trichloroacetic acid and 1 mL enzyme diluted in 3 mL of glycine–NaOH buffer. Proteolytic products in the supernatant were monitored at 280 nm. An increase in absorbance of 0.01 at 280 nm was considered as 1 U enzyme activity (1 U = 1000 U).

The total protein was estimated by Bradford [10] taking bovine serum albumin (BSA) as the standard protein.

2.6. Biochemical characterization of Ker ProBP–BL and Ker ProBL–BP vs Ker BL and Ker BP

2.6.1. Effect of pH and temperature on activity and stability of Ker ProBP–BL and Ker ProBL–BP

The effect of pH was studied by performing the keratinase assay at different pH values ranging from pH 4 to 12 using 50 mM of each buffer including citrate phosphate (pH 4–6), sodium phosphate (pH 7), Tris–HCl (pH 8), glycine–NaOH (pH

Fig. 1. Strategy for construction of keratinases Ker ProBP–BL and Ker ProBL–BP by swapping pro-sequence between keratinase from B. licheniformis (Ker BL) and B. pumilus (Ker BP) in cis.
9–10), phosphate hydroxide (pH 11) and hydroxide–chloride buffer (pH 12). Subsequently, the effect of temperature was determined by performing the keratinase assay at different temperatures ranging from 30 to 90 °C at optimum pH. The activity was expressed as the percentage relative activity with respect to maximum activity which was considered as 100%.

The stability was studied over a broad range of pH and temperature by pre-incubating the enzyme in buffers of 10 mM of varying pH (4–12) for 1 h at 25 ± 1 °C. The temperature stability was determined by incubating the enzyme samples at temperatures from 50 to 90 °C for different time intervals. The activity was expressed as percentage residual activity against the control which was taken as 100%.

2.6.2. Substrate specificity of Ker ProBP–BL and Ker ProBL–BP

Proteolytic activities of keratinases, Ker ProBP–BL and Ker ProBL–BP alongwith their parent keratinases, Ker BL and Ker BP were determined over a variety of soluble (soluble in buffer, viz. azocasein, BSA and casein), and insoluble (not soluble in buffer, viz. elastin, feather keratin, fibrin, gelatin, hemoglobin, keratin azure and meat protein) substrates. 20 mg of each substrate was incubated with 1 mg enzyme at optimum pH and temperature for 1 h. Proteolytic products were measured by Folin Ciocalteau’s reagent against BSA standard [11]. However, for azocasein, proteolytic products were measured directly at 420 nm as described by Secades and Guijarro [12] with some modifications, where one enzyme unit was defined as the amount of enzyme yielded an increase in A420 of 0.01 in 1 h under optimum conditions. For keratin azure, product release was measured at 595 nm according to Bressollier et al. [13], where an increase in absorbance of 0.01 at 595 nm was considered as 1 U enzyme activity under defined conditions. Control reaction was set up in the same manner by adding 5% (w/v) trichloroacetic acid immediately at the time of addition of the enzyme.

2.6.3. Determination of kinetic parameters of Ker ProBP–BL and Ker ProBL–BP

Kinetic studies of keratinases were carried out at varying concentrations of azocasein and casein (10–100 mg/mL). $K_{cat}/K_m$ was calculated by Lineweaver-Burk plot using Sigma Plot software.

2.6.4. Hydrolysis of insulin B-chain and mass spectrometry of Ker ProBP–BL and Ker ProBL–BP

The substrate specificity of keratinases was also compared for the hydrolysis of insulin B-chain (Sigma, cysteine residues oxidized). 100 μL of insulin B-chain (1 mg/mL in 10 mM Tris/HCl buffer, pH 9) was mixed with 100 μL of the enzyme
and mixture was incubated at 37 °C for 16 h. After incubation, 40 μL of 0.1% (v/v) TFA was added to the reaction mixture to inactivate the enzyme. Hydrolysis of insulin B-chain by enzyme was analyzed by liquid chromatography-electron spray mass spectrometry (LC-ESI/MS, GenPro Biotech, India). Cleavage sites were determined using FindPept, a part of the ExPasy software package.

2.7. Folding and biochemical characterization of keratinases in presence of pro-sequence (Pro) in trans or in vitro (protease folding mediated by an exogenous pro-sequence)

2.7.1. Cloning of Pro BL and Pro BP

Pro-sequence of B. licheniformis (Pro BL) and B. pumilus (Pro BP) were cloned in E. coli BL21 as a fusion with Glutathione-S-transferase (GST) in pGEX-4T-1 expression vector to study their role on structural conformation of keratinases, Ker BL and Ker BP. Pro BL was constructed using the forward primer (Pro-BL) 5′ BamHI GAATCCCAGGAAAATGTTGA 3′ and reverse primer (Pro-BLR) 5′ EcoRI ATGCTTCTGC 3′ and BP using the forward primer (Pro-BP) 5′ BamHI CAAITCCGCTTCTCCAAA 3′ and reverse primer (Pro-BPR) 5′ Xhol GTC-GACATATGCTTCTGCG 3′, respectively. Further, cloning was performed using the same strategy adopted in Section 2.2 under the heading “Cloning of Ker ProBP–BL and Ker ProBL–BP.”

2.7.2. Expression of Pro BL and Pro BP

E. coli BL21 cells harboring pGEX-4T-1-Pro BL and pGEX-4T-1-Pro BP were grown in LB medium supplemented with ampicillin at 37 °C, 200 rpm. After 18 h, the cells were separated by centrifugation at 7441 × g for 10 min and harvested. 1 g cell pellet was resuspended in 10 mL ice cold 1× PBS and lysed on ice by sonication at 3 s pulse on and 6 s pulse off for 10 min and the lysate clarified by centrifugation and expression was checked in the clear lysate by SDS-PAGE analysis.

2.7.3. Purification of Pro BL and Pro BP

Fusion proteins, GST-Pro BL and GST-Pro BP were produced from E. coli BL21 (DE3) cells carrying the pGEX-4T-1 plasmid, after induction with 1 mM IPTG. Harvested 1 g cell pellet was resuspended in 10 mL ice cold 1× PBS and lysed on ice by sonication at 3 s pulse on and 6 s pulse off for 10 min and the lysate clarified by centrifugation at 18°C. The clear lysate was loaded onto a resin GSH-agarose (0.5 mL). The column was washed with 10 column volumes of 1× PBS, and elution of bound protein was performed with 5 column volumes of elution buffer (50 mM Tris/HCL, pH 8 and 20 mM reduced glutathione). The purified fusion protein was collected and treated with 10U of thrombin to release the GST tag from the fusion. A second passage through the affinity column was performed to remove the GST tag and the pure protein was collected. The purified protein was then treated with 1 M guanidine hydrochloride in 1:1 ratio for 30 min at room temperature and total protein was estimated at 280 nm. The purity of protein was checked by HPSEC on C-18 column (Shimadzu, Japan) using acetonitrile-water (90:10) as mobile phase with flow rate of 0.5 mL/min. The protein was detected at 280 nm using UV detector.

2.7.4. Unfolding and folding of Ker BL and Ker BP in presence of an exogenous pro-sequence

Original proteins, Ker BL and Ker BP (without pro-sequence) at a final concentration of 1 mg/mL were incubated at 50 °C at their optimum pH for 1 h. These proteins were cooled down in presence of equimolar concentration (0.3 mg/mL) of their respective pro-sequence to generate the parent enzymes and also with exchanged pro-sequence to develop the conformational variants i.e. Ker BLAP and Ker BPAP.

2.7.5. Biochemical characterization of keratinases, Ker BLAP and Ker BPAP

2.7.5.1. Thermostability of Ker BLAP and Ker BPAP.

Temperature stability of Ker BLAP and Ker BPAP at 70 °C was compared with their counterparts i.e. Ker BL and Ker BP. Activity was performed on azocasein at optimum conditions.

2.7.5.2. Substrate specificity of Ker BLAP and Ker BPAP.

Proteolytic activities of conformational variants, Ker BLAP and Ker BPAP were compared to their respective parent enzymes, Ker BL and Ker BP on soluble (azocasein and casein) and insoluble (feather keratin) protein substrates. Proteolytic products were measured as described in Section 2.6.2 under the heading “Substrate specificity of Ker ProBP–BL and Ker ProBL–BP.”

2.8. Statistical analysis of data

All the above experiments were repeated twice in triplicate and the final values have been presented as mean ± standard deviation.

3. Results

Keratinase from B. licheniformis (Ker BL) and B. pumilus (Ker BP) are subtilisins which have already been recognized for their keratinolytic potential. Keratinases Ker ProBP–BL and Ker ProBL–BP were constructed by swapping the pro-sequence in cis between Ker BL and Ker BP. Moreover, conformational variants, Ker BLAP and Ker BPAP have also been developed by using chaperone functions of pro-sequence in trans.

3.1. Pairwise sequence alignment analysis of Ker BL and Ker BP

Sequence alignment between Ker BL and Ker BP revealed a highly conserved mature protein region and a variable N-terminal region (Fig. 2A). Looking into the alignment there were 31 positions of variations in the first 76 amino acids sequences (pro-sequence) compared to 74 positions of variation in the mature protein region of 374 amino acids. The pro-sequence was thereby characterized as Motif N1, Motif N2 and variable regions according to Takagi and Takahashi [5] and presented in Fig. 2B.

3.2. Expression of Ker ProBP–BL and Ker ProBL–BP

The expression of keratinases (Ker ProBP–BL and Ker ProBL–BP) was achieved in E. coli HB101 using pEZ18, as the expression vector after 18 h of incubation at 37 °C and 300 rpm with high specific activity of 391 and 401 U/mg protein.

3.3. Purification of Ker ProBP–BL and Ker ProBL–BP

Single step purification of the keratinases Ker ProBP–BL and Ker ProBL–BP, was achieved using Q-Sepharose and DEAE-Sepharose columns, respectively. The homogeneity of purified keratinases was revealed by SDS-PAGE, where certain shift in the molecular weight of Ker ProBP–BL and Ker ProBL–BP was observed with respect to their parent keratinases, Ker BL and Ker BP (Fig. 3).

3.4. N-terminal sequencing of Ker ProBP–BL and Ker ProBL–BP

N-terminal analysis revealed that after swapping the pro-sequence between closely related keratinases, Ker BL and Ker BP, the processing had occurred in the same manner as that of original proteins. The N-terminal for Ker ProBP–BL was recovered with a sequence of “AQTVPYGIPQ” and for Ker ProBL–BP “AQTVPYGIPL”, respectively.

3.5. Biochemical characterization of Ker ProBP–BL and Ker ProBL–BP vs Ker BL and Ker BP

Keratinase Ker ProBP–BL with core protein of B. licheniformis and pro-sequence swapped from B. pumilus shared most of the
properties with Ker BP. It was active within the same pH range to that of Ker BL which was active from pH 4 to 12, however, its optima shifted to pH 9 similar to Ker BP from where the pro-sequence was swapped (Fig. 4A). Noteworthy, keratinase Ker ProBP–BL became highly thermostable with a $t_{1/2}$ of $>2$ h at 60 °C, $>1$ h at 70 °C and 45 min at 80 °C as compared with the original protein derived from B. licheniformis having a $t_{1/2}$ of $>1$ h at 50 °C and 10 min at 60 °C (Fig. 4C). Likewise, keratinase Ker ProBL–BP with core protein from B. pumilus and pro-sequence swapped from B. licheniformis, was active and stable in the pH range, 7–11 same as that of original
protein derived from Ker BP of *B. pumilus*, however, its optima shifted to pH 10 which was similar to Ker BL from where the pro-sequence was swapped (Fig. 4A). Likewise, its temperature optima was also altered and shifted to 60 °C which was again in contrast to the original protein, where optima was at 70 °C (Fig. 4B). However, major differences were observed with respect to their thermostability, where keratinase Ker BP was highly thermostable with a $t_{1/2}$ of >2 h at 80 °C and 30 min at 90 °C, respectively, while keratinase Ker ProBL–BP became comparatively thermostable with a $t_{1/2}$ of >2 h at 50 °C and 1 h at 60 °C (Fig. 4C). Overall, analysis revealed that the keratinase Ker ProBL–BP was much closer to the keratinase of *B. licheniformis*, Ker BL from where the pro-sequence was swapped. Overall, both keratinases Ker ProBP–BL and Ker ProBL–BP had acquired similar pH-temperature kinetics from where the pro-sequence was swapped.

3.5.2. Substrate specificity of Ker ProBP–BL and Ker ProBL–BP
Keratinases Ker ProBP–BL and Ker ProBL–BP hydrolyzed a large array of soluble and insoluble protein substrates as their original counterparts (Fig. 5). However, altered substrate specificity was observed after pro-sequence swapping. Substrate specificity of Ker ProBP–BL was much more improved for all insoluble substrates over soluble substrates. There was almost 50% reduction in the hydrolysis of both azocasein and casein after pro-sequence exchanges from Ker BP in comparison to the original enzyme. Its substrate specificity was again similar to the source of pro-sequence i.e. Ker BP which preferably hydrolyzed insoluble proteins.

Likewise, Ker ProBL–BP hydrolyzed soluble proteins like azocasein and casein with 2.7 and 2.1 fold enhancement over Ker BP.

Further, there was 25–30% reduction in hydrolysis of insoluble substrates compared to Ker BP. Here, again it was observed that the increased affinity for soluble proteins was acquired from the source of pro-sequence i.e. keratinase of *B. licheniformis*. Further, ratio of activity on feather keratin (K) and that on casein (C) decreased from 2.9 for Ker ProBL–BP as against the original keratinase BP. Ker ProBP–BL was converted to a highly keratinolytic enzyme with K/C ratio of 1.2 as against 0.1 of Ker BL by swapping the pro-sequence from a keratinolytic protease, Ker BP.

3.5.3. Steady state kinetics of Ker ProBP–BL and Ker ProBL–BP
Steady state kinetics of keratinases Ker ProBP–BL and Ker ProBL–BP revealed kinetic modulation with respect to $K_{cat}/K_m$ for azocasein and casein. Kinetic studies of Ker ProBP–BL revealed decreased catalytic efficiency for both the soluble substrates with $7.7 \times 10^4 \text{min}^{-1} \text{mM}^{-1}$ and $9.6 \times 10^6 \text{min}^{-1} \text{mM}^{-1}$ in comparison to its original enzyme Ker BL, which had catalytic efficiency of $9.5 \times 10^5 \text{min}^{-1} \text{mM}^{-1}$ and $2.27 \times 10^6 \text{min}^{-1} \text{mM}^{-1}$ on azocasein and casein, respectively. On the contrary, catalytic efficiency of Ker ProBL–BP for azocasein and casein was increased with $K_{cat}/K_m$ of $1.22 \times 10^2 \text{min}^{-1} \text{mM}^{-1}$ and $1.03 \times 10^4 \text{min}^{-1} \text{mM}^{-1}$, respectively which was in contrast to the original enzyme Ker BP with $1.09 \times 10^3 \text{min}^{-1} \text{mM}^{-1}$ and $6.3 \times 10^4 \text{min}^{-1} \text{mM}^{-1}$, respectively. Thus, the catalytic efficiency of Ker ProBL–BP was considerably improved for azocasein and casein by swapping the pro-sequence from *B. licheniformis* which was known to hydrolyzed soluble proteins better than insoluble one.

3.5.4. Hydrolysis of insulin B-chain and mass spectrometry of Ker ProBP–BL and Ker ProBL–BP
LC-ESI/MS analysis of oxidized insulin B-chain hydrolysis by Ker ProBP–BL and Ker ProBL–BP was compared to Ker BL and Ker BP. The mass spectra of hydrolyzed B-chain of insulin revealed that Ker ProBP–BL hydrolyzed oxidized insulin-B chain at six sites between Val12–Glu13, Cys19–Gly20, Arg22–Gly23, Gly23–Phe24, Phe25–Tyr26 and Tyr26–Pro27 residues (Fig. 6). Ker ProBL–BP revealed only four cleavage sites between Cys7–Gly8, His10–Leu11, Gly23–Phe24 and Tyr26–Pro27 whereas Ker BP hydrolyzed peptide bonds at seven sites after hydrophobic amino acid between Leu6–Cys7, Cys7–Gly8, Tyr16–Leu17, Cys19–Gly20, Gly23–Phe24, Phe24–Phe25 and Phe25–Tyr26 residues (Fig. 6).

3.6. Folding and biochemical characterization of keratinases in presence of pro-sequence in trans

3.6.1. Expression of Pro BL and Pro BP
Expression of Pro BL and Pro BP was achieved in *E. coli* BL21 (DE3) cells harboring pGEX-4T-1 as the expression vector after 18 h of growth in LB medium supplemented with ampicillin at 37 °C, 200 rpm. After 18 h, the cells were separated by centrifugation at 7441 × g for 10 min and expression was checked on SDS-PAGE where Pro BL and Pro BP had molecular weight of 37.1 kDa and 37.4 kDa, respectively (Fig. 7).

![Fig. 5. Comparative substrate specificity of Ker ProBP–BL and Ker ProBL–BP generated by pro-sequence in cis swapping to their respective parent enzymes Ker BL from *B. licheniformis* and Ker BP from *B. pumilus*.](image-url)
3.6.2. Purification of Pro BL and Pro BP

Single step purification of fusion proteins, GST-Pro BL and GST-Pro BP was achieved using affinity resin GSH-agarose. The homogeneity of purified protein was revealed by HPLC analysis on C-18 column.

3.6.3. Unfolding and folding of Ker BL and Ker BP in presence of an exogenous pro-sequence

Keratinases, Ker BL<sub>N</sub> and Ker BP<sub>N</sub>, were generated by allowing the folding of Ker BL in presence of Pro BP and Ker BP in pro-sequence of Pro BL, respectively. These conformational variants were found active and biochemically characterized with respect to thermostability and substrate specificity which was compared further with their parent enzymes, Ker BL and Ker BP. The biochemical features with respect to thermostability and substrate specificity acquired by these variants, Ker BL<sub>NP</sub> and Ker BP<sub>N</sub> were the same to those acquired by chimeras, Ker ProBP–BL and Ker ProBL–BP constructed in cis (data not shown).

4. Discussion

Keratinases from <i>B. licheniformis</i> (Ker BL) and <i>B. pumilus</i> (Ker BP) are subtilisins with established keratinolytic property [5,6]. Pairwise amino acid sequence alignment of these keratinases revealed 89% homology. Irrespective of their high homology it was interesting to note that the enzymes exhibited drastically different properties with Ker BP being more thermostable than Ker BL and having a higher keratinolytic:caseinolytic (K:C) ratio. Dissecting the sequence alignment further, a high variability of 66% was observed in the N-terminal pro-sequence region and the core protein was 90% conserved. Earlier, Shinde et al. [14] had schematically divided the pro–sequence of subtilisins into two conserved hydrophobic motifs viz. Motif N1 and N2 with flanking variable region, which were also observed in the pro-sequences of Ker BP and Ker BL. The motifs N1 and N2 contain hydrophobic residues and have been documented to initiate the folding process through a “hydrophobic collapse” [15] and the variable region is reported for interaction with associated protease domains during protein folding, as well as for inhibition of functional activity of the enzyme [16]. Thus, conformational variations are a result of this variable region that flanks between motif N1 and N2. The variable region in Ker BP stretched from Ala<sub>17</sub> to Asn<sub>532</sub> whereas in Ker BL the stretch was from Ser<sub>17</sub> to Ser<sub>332</sub>. It was thus hypothesized that this N-terminal variable region could be responsible for the drastic difference in the biochemical properties the two enzymes. To prove our hypothesis, an attempt at swapping the pro-sequences between Ker BP and Ker BL in cis was made. The mature proteins were recovered where exchanged pro-sequences were cleaved as observed by N-terminal analysis which was the same as that of original mature proteins. This is in confirmation with the results of Takagi and Takahashi [3].

Utilizing a common restriction site close to the C-terminal of the pro-sequence in both Ker BP and Ker BL, chimeric functionally active constructs Ker ProBP–BL and Ker ProBL–BP were obtained. This is in contrast to earlier reports where pro-sequence exchange in cis has resulted in decreased enzyme activity. A cis experiment wherein ProA was covalently linked to the subtilisin E protease domain has been reported to result in poor activity [4]. This might be due to low sequence identity between ProS and ProA while the present exchange has been done between closely related enzymes. However, there have been successful reports of pro-sequence swapping in trans and the pro-sequence of aqualysin I (ProA) has been reported to refold denatured subtilisin despite it being a weak inhibitor of subtilisin and having low sequence identity with subtilisin pro-sequence (ProS) [17–19].

Kinetics of enzymes Ker ProBP–BL and Ker ProBL–BP were compared to their parent keratinases. pH and temperature optima of Ker ProBL–BP were 10 and 60 °C which was similar to Ker BL whereas the optima of Ker ProBP–BL was pH 9 and 70 °C similar to Ker BP. Ker ProBL–BP was comparatively thermostable than Ker BP and Ker ProBP–BL was highly thermostable in contrast to Ker BL. With respect to substrate specificity, K:C (keratinolytic:caseinolytic) ratio is considered as a criterion for distinguishing keratinases from conventional proteases where ratio >0.5 makes an enzyme a potential keratinolytic enzyme [20]. In this perspective, Ker ProBP–BL was highly keratinolytic with K:C ratio of 1.2 as against 0.1 of Ker BL while Ker ProBL–BP had K:C ratio of 1.6 as against 2.9 of Ker BP. In addition, the substrate specificity of Ker ProBP–BL widened in comparison to Ker BL. The pro-sequence seems to be the nucleus of information of the protease and through its chaperoning function transfers this information to the mature protein. This in confirmation with earlier reports wherein the mutated pro-sequence resulted in altered folding and thus altered enzyme characteristics [14].

Also, conformational variants were generated by utilizing chaperone functions of an exogenous pro-sequence in trans. Keratinase, Ker BL acquired the features of the keratinase of <i>B. pumilus</i> in presence of Pro BP and likewise Ker BP was almost similar to the keratinase of <i>B. licheniformis</i> in presence of Pro BL similar to the changes observed in cis exchanges. Analysis of folding experiments suggests that the pro-sequence is instrumental in influencing the folding of enzyme not only in cis but also in trans conditions.
5. Conclusions

Pro-sequence swapping appears to be an effective molecular breeding method for engineering of proteases. Pro-sequence mediated conformational changes can be achieved by utilizing chaperone functions both in cis and trans. In the present case, wherein the two enzymes were closely related such swapping was possible both in cis and trans strategy without any inhibition.

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