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Molecular Characterization of N-Terminal Pro-sequence of Keratinase Ker P from *Pseudomonas aeruginosa*: Identification of Region with Chaperone Activity

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Abstract In silico analysis of keratinase Ker P from *Pseudomonas aeruginosa* revealed that its full gene of 1,497 bp constituted of a 72-bp signal sequence along with a long 520 bp pro-sequence and 905 bp core region. Position specific multiple sequence alignment of Ker P protein with other distant proteases revealed high variability within their N-terminal regions while the core protein was considerably conserved. Ker P (F1) and its four N-terminal truncations (F2-F5) lacking 72, 177, 405, 507 bp, respectively, were cloned and constitutively expressed as extracellular protein in pEZZ-18 secretory vector with *Escherichia coli* HB101 as the expression host. Ker P F1, Ker P F2, Ker P F3 and Ker P F4 products were active whereas no keratinolytic activity was obtained in Ker P F5. Further analysis revealed that only 187 bp pro-sequence region is required for correct folding of the protein into its active conformation and, thus, has chaperone-like activity. Further, comparative biochemical characterization revealed that the full-length keratinase Ker P F1 was catalytically more efficient than the truncated forms. Among the truncated enzymes, keratinase Ker P F4 exhibited better thermostability than Ker P F2 with a $t_{1/2}$ of >1 h at 60 °C. It also had a higher V_{max} and K_m on casein as compared with Ker P F2. However, no significant variation was observed with respect to kinetics on synthetic substrates.

Keywords Keratinase · N-terminal truncation · *Pseudomonas aeruginosa* · Pro-sequence · pEZZ 18

Introduction

Keratinases are a special class of proteases with the ability to degrade recalcitrant proteins. Like other proteases, keratinases are first synthesized as pre-pro-proteins before they attain their catalytically active form. Pre-sequences (signal peptides) are required for the secretion of the pro-enzyme across the cytoplasmic membrane whereas pro-sequences are found essential for correct folding of the associated protein. Finally, the pro-sequences are removed either autocatalytically or by existing active protease to produce active mature enzyme. In addition to their action as molecular chaperones, pro-sequences also play a role

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in protein sorting, act as inhibitor/activator of proteins, and aid in interaction of the protein with other molecules [1]. The length of pro-sequences generally varies between short stretches of 77 amino acids in case of subtilisin to 177 residues in case of neutral metalloprotease [2]. Pro-sequences have a major role in determining the final conformation and function of proteases and thus pro-sequence engineering including site-directed and/or random mutagenesis, chimeras and gene shuffling has emerged as a major prospective area for improving the function and altering the catalytic potential of autoprocessing proteases. Pro-sequence engineering is expected to construct proteases with altered substrate specificity, high activity, and high stability as reported in case of *Streptomyces griseus* protease B where a point mutation in the pro-sequence led to modification of its substrate specificity [3]. Thus, with the final aim to produce catalytically efficient mutants of keratinase Ker P from *Pseudomonas aeruginosa* KS-1 by pro-sequence engineering, experiments were designed to first molecularly characterize its pro-sequence. As it has a 173 aa long pro-sequence, efforts have been directed to ascertain the minimum length of pro-sequence required for functional expression of keratinase Ker P by N-terminal pro-sequence truncations and to study the effect of varying pro-sequence length on the kinetics of the enzyme.

Materials and Methods

Materials, Strains, and Growth Conditions

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 Qiagen (Hilden, Germany). Expression vector, pEZZ-18 was purchased from GE Healthcare Science (India). *Escherichia coli* HB 101 was used as the expression host. All bacterial strains were grown on Luria-Bertani (LB) medium consisting of 1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl (pH 7.2). When required, ampicillin was added to the medium to a final concentration of 100 μg/mL. Q-sepharose and various synthetic substrates were purchased from Sigma-Aldrich (USA).

N-Terminal Analysis of Keratinase Ker P

Keratinase Ker P was produced and purified as described earlier [4]. The purified band of keratinase Ker P from SDS-PAGE was subjected to tryptic digestion and analyzed by MALDI-TOF/TOF analysis at The Center for Genomics Application, India. The N-terminal of the Keratinase Ker P was determined by overlapping the results of the MALDI analysis with the known database.

In Silico Analysis of Keratinase Ker P from *P. aeruginosa*

Signal sequence of the keratinase Ker P was identified using the Signal P software [5]. For multiple sequence alignment, prospective proteases to be aligned with Ker P were selected using the position specific PSI-BLAST algorithm, based on position specific scoring matrices [6, 7] obtained from the NCBI online portal (www.ncbi.nlm.nih.gov). Five proteases above the threshold E value were selected were then multiple sequence alignment was done using the PSI-BLAST pre-profile processing (Homology-extended alignment) available from the PRALINE online resource portal (http://www.ibi.vu.nl/programs/pralinewww/) which uses an optimized heuristic with a gap opening penalty of 12 and an extension penalty of 1 [8]. The alignment was, thereafter, assessed based on their amino acid conservation.

DNA Manipulations and Construction of Plasmids

Genomic DNA of *P. aeruginosa* KS-1 (MTCC no. 10775) was isolated using a genomic DNA extraction kit. PCR was performed as described earlier [9]. The nucleotide sequence of the keratinase Ker P gene (NCBI accession no. HM452163) from *P. aeruginosa* KS-1 was used to design the primers for PCR amplification. The DNA fragments encoding the full length and N-terminally truncated forms of Ker P were obtained by amplification from *P. aeruginosa* KS-1 genome using the following primer pairs: Ker P F1-R, Ker P F2-R, Ker P F3-R, Ker P F5-R, Ker P F7-R. The sequence of the primers is as follows: Ker P F1 (5′ GAATTCGATGAAGAAGGTTTCT 3′); Ker P F2 (5′ GAATTCGGCCGACCTGAATC 3′); Ker P F3 (5′ GAATTCGGCGATCCGCAGC 3′); Ker P F4 (5′ GAATTCGAAGGCC CAGGGC 3′); Ker P F5 (5′ GAATTCGCCCGGCGAGGGA 3′); Ker P R (5′GGATCCTTA CAACGCGCT 3′). The PCR products were cloned as EcoRI/BamHI fragments into the corresponding sites of pEZZ-18 vector to generate pEZZ 18-Ker P F1, pEZZ 18-Ker P F2, pEZZ 18-Ker P F3, pEZZ 18-Ker P F4, pEZZ 18-Ker P F5. Details of the truncation are presented in Fig. 1. All the constructs were sequenced at the central instrumentation facility at University of Delhi, India.

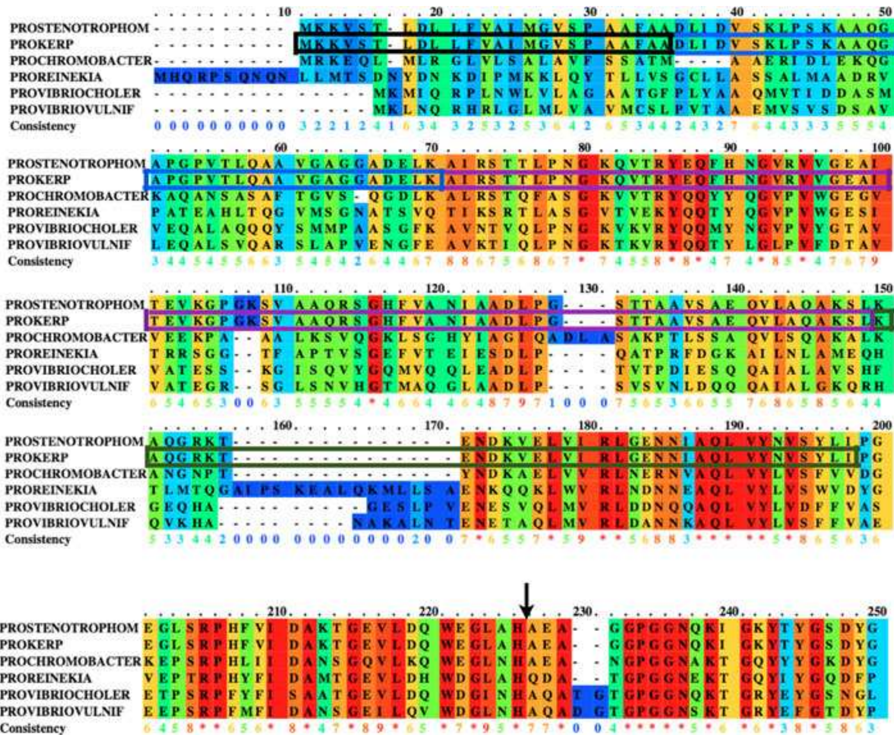

Fig. 1 Sequence alignment of keratinase Ker P of *P. aeruginosa* with proteases of different bacteria. The truncations are marked in boxes and the N-367 terminal is marked by an arrow. The sources of the sequences are as follows: 368 PROSTENOTROPHOM is the elastase LasB precursor form *Stenotrophomonas maltophilia* ; PROCHROMOBACTER is the class 4 metalloprotease from *Chromobacterium violaceum* ATCC 12472; PROREINEKEA is the zinc metalloprotease from *Reinekea* sp.; PROVIBRIOCHOLER is hemagglutinin/ protease of *Vibrio cholerae* RC385; PROVIBRIOVULNIF is the metalloprotease of *Vibrio vulnificus*

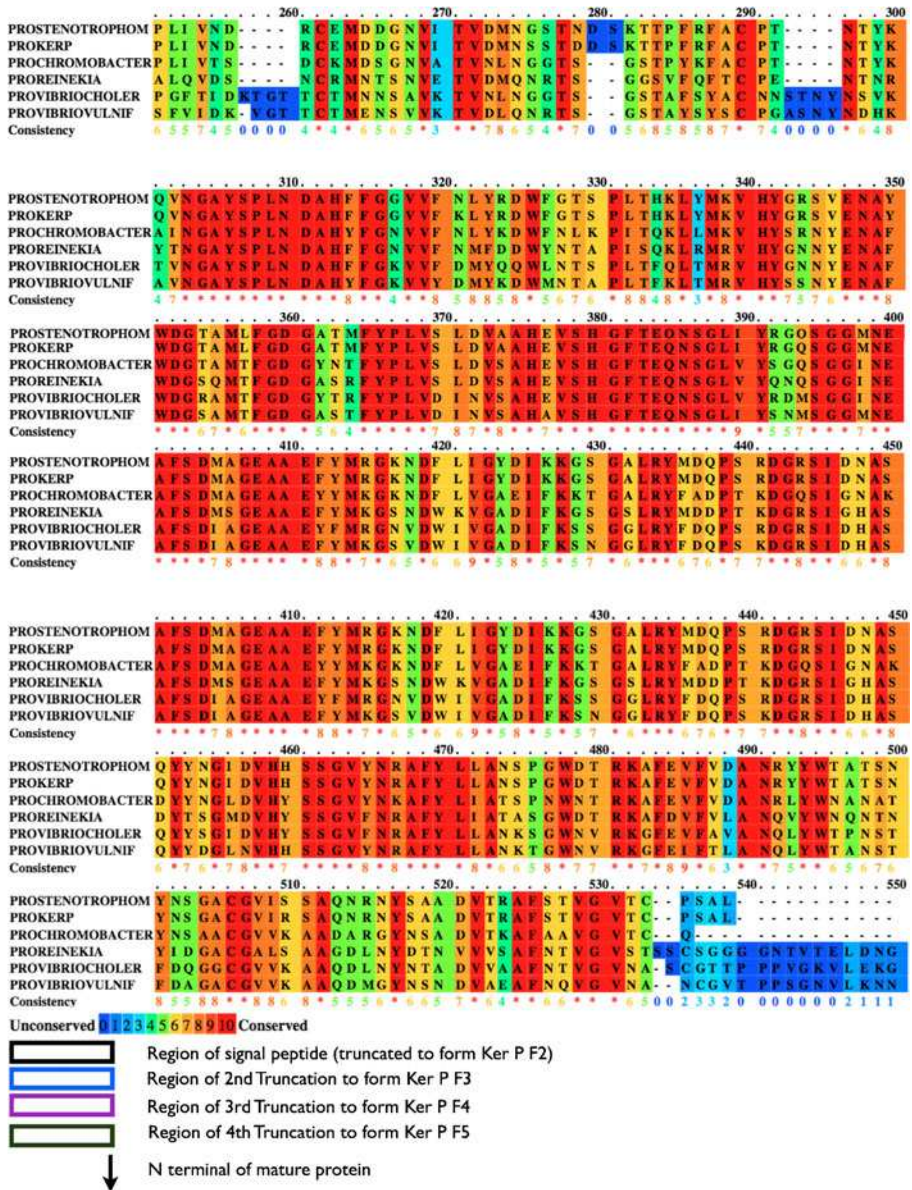

Fig. 1 (continued)

Expression and Purification of the Recombinant Enzymes

To express the recombinant proteins, *E. coli* HB 101 cells harboring pEZZ 18-Ker P F1, pEZZ 18-Ker P F2, pEZZ 18-Ker P F3, pEZZ 18-Ker P F4, and pEZZ 18-Ker P F5 were grown in LB medium supplemented with ampicillin at 37 °C, 300 rpm. After 18 h of incubation, the cells were separated by centrifugation at 7,400×*g* for 10 min, and expression was checked in the extracellular broth by keratinase assay and SDS-PAGE analysis. For

purification, the culture supernatant was concentrated ten times using ultrafiltration by 10 kDa molecular cut-off cassette. The retentate was applied to the Q-sepharose anion exchange column pre-equilibrated with 10 mM Tris/HCl buffer, pH 8. The column was washed with the same buffer and, a 15-ml fraction was collected at a flow rate of 2 ml/min. Bound protein was eluted in a linear salt gradient (0.1 M-1 M NaCl). Protein elution was monitored by measuring absorbance at 280 nm and keratinase activity was determined. Purity of the protein was determined by SDS-PAGE analysis and by HPLC (Shimadzu, Japan; C18 column, mobile-phase acetonitrile: water 90:10, flow rate: 1 ml/min, UV detector).

Keratinase Assay and Protein Estimation

Keratinase activity was measured as described by Dozie et al. [10] with some modifications. The assay mixture containing 1 mL of appropriately diluted enzyme, 4 mL glycine-NaOH buffer (50 mM, pH 10), and 20 mg of chicken feathers was incubated at 60 °C for 60 min. The reaction was terminated by adding 4 ml of 5% (*w*/*v*) trichloroacetic acid, and the tubes were incubated at room temperature (25 °C) for 1 h. Feather and insoluble residues were removed by filtration through glass wool, and the filtrate was centrifuged at 5,000×*g* for 5 min. An enzyme control was prepared in a similar manner, except that 1 ml of 5% trichloroacetic acid and 3 ml of the buffer were added instead of 4 ml of the buffer used in the test. Substrate control was prepared by adding only 20 mg feather in 5 ml buffer. Proteolytic products in the supernatant were determined by absorbance at 280 nm. An increase in absorbance of 0.01 was considered as 1 U enzyme activity (1 KU=1,000 U).

Biochemical Characterization of Keratinases

Effect of pH and Temperature on the Activity and Stability of Keratinases

The effect of pH on the keratinases was studied by carrying out the keratinase assay in the pH range of 5.0–11.0. Similarly, the effect of temperature on keratinase activity was determined by incubating the reaction mixture at different temperatures ranging from 40 to 70 °C at pH 9. Activity was expressed as percentage relative activity with respect to maximum activity, which was considered as 100%. pH stability was determined by incubating the enzyme in buffers of varying pH (4.0–11.0) for 1 h at room temperature (25±1 °C) and thereafter the residual activity was determined at the optimum pH and temperature. The temperature stability was determined by incubating the enzyme samples at various temperatures ranging from 50 to 70 °C for different time intervals. The residual activity was determined at pH 9 and 50 °C.

Substrate Specificity of Recombinant Keratinases

The proteolytic activity of the full-length keratinase along with its truncated forms was studied using both soluble and insoluble substrates. The reaction mixture was setup using 20 mg of each of the complex substrates in 1 ml of Tris–HCl buffer (50 mM, pH 9) and 1 ml of appropriately diluted enzyme prepared in the same buffer. The reaction was incubated at 50 °C for 1 h. The reaction was stopped by the addition of 4 mL of 5% (*w*/*v*) trichloroacetic acid. The contents were centrifuged after 1 h at 1,006×*g* for 10 min. Folin Ciocalteau's reagent (0.5 mL) was added to 1 mL of the supernatant and the optical density of the samples was taken at 660 nm against appropriate substrate and enzyme blanks. One

unit of protease was equivalent to the amount of enzyme required to release 1 μg of tyrosine mL^{-1} h^{-1} under standard assay conditions.

Activity on Synthetic Substrates

Activity was also examined using 10 mM stock solutions of *p*-nitroanilide substrates. Reaction mixture contained 100 μl of synthetic substrate and 900 μl of appropriately diluted enzyme prepared in Tris–HCl (50 mM, pH 10.0) buffer. The reaction mix was incubated at optimum temperature for 10 min. The hydrolyzed product was measured at 405 nm using a UV–vis spectrophotometer (UV 1700 Shimadzu, Japan). The molar extinction coefficient for *p*NA was taken to be 9,900 M^{-1} cm^{-1}. In addition, kinetic constants of the full-length recombinant keratinase along with all its truncated forms were determined on various complex and synthetic substrates. Kinetics of the native keratinase produced from *P. aeruginosa* KS-1 was also determined. The native keratinase was produced and purified as described earlier by Sharma and Gupta [4].

Results

Molecular Analysis of Keratinase Ker P

A 24 amino acid signal sequence was predicted by the signal peptide software. The N-terminal sequence was AEAGGP indicating a long pro-sequence of 173 amino acids. PSI-BLAST of the Ker P gene (NCBI accession no. HM452163) resulted in a number of proteases with E value better than threshold from which five sequences were selected. The sequences were the elastase LasB precursor from *Stenotrophomonas maltophilia* (NCBI accession no. ADM89079.1 renamed to PROSTENOTROPHOM) which had 100% query converage, class 4 metalloprotease from *Chromobacterium violaceum* ATCC 12472 (NCBI accession no. NP_899727.1 renamed to PROCHROMOBACTER) which had 97% query coverage, zinc metalloprotease from *Reinekea* sp. (NCBI accession no. ZP_01112982.1 renamed to PROREINEKEA) which had 98% query coverage, hemagglutinin/protease of *Vibrio cholerae* RC385 (NCBI accession no. ZP_06942058.1 renamed to PROVIBRIOCHOLER) which had 87% query coverage and the metalloprotease of *Vibrio vulnificus* (NCBI accession no. AAC72410.1 renamed to PROVIBRIOVULNIF) which shared 97% query coverage.

The five selected sequences and the Ker P sequence were then used as query sequences for the position specific multiple sequence alignment, and the amino acid conservation analysis of the six sequences resulted in a considerably variable N-terminal region of 200 aa and a surprisingly highly conserved core region (Fig. 1). Though variable, the N-terminal region however contained interspersed stretches of conservation (Fig. 1: region 80–100, 120–130, 180–190) and truncations were made randomly in this region to study the effect of deletions (truncations have been represented in Fig. 2) in the N-terminal pro-sequence.

Cloning and Expression of N-Terminal Truncated Enzymes

Keratinase Ker P was truncated by 72 bp (signal peptide) and other truncations of 72, 177, 405, and 507 bp were made after analyzing the N-terminal pro-sequence. The size of the various PCR amplicons was 1,425, 1,320, 1,092, and 990 bp, respectively, along with the

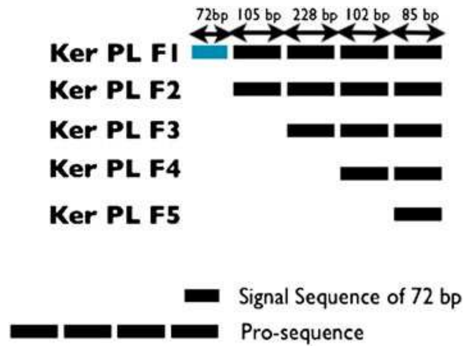

Fig. 2 **a** Cloning strategy used to study the N-terminal pro-sequence of Ker P

full-length gene of 1,497 bp. All these were cloned in pEZZ-18 vector and transformed into *E. coli* HB101. Positive clones were checked for expression in LB supplemented with ampicillin at 37 °C, 300 rpm after 18 h of incubation under uninduced conditions. The full length as well as the truncated enzymes, i.e., Ker P F2, Ker P F3, and Ker P F4 were secreted as active enzyme whereas no activity was detected in case of Ker P F5 by which it can be inferred that the minimum length of pro-sequence required for the functional expression of Ker P is 187 bp.

The specific activity of Ker P F1, Ker P F2, Ker P F3, and Ker P F4 was 5.8, 5.5, 5.3, and 5.0 kU/mg protein, respectively. The enzymes were largely secreted as active proteins (without IgG tags) with a molecular mass of 33 kDa (Fig. 3).

Biochemical Characterization of Ker P F2 and Ker P F4

Full-length keratinase along with all its truncated forms were purified using Q-sepharose anion exchange chromatography. The purity of the protein was analyzed by HPLC and a single peak with retention time of 5 min was observed at 280 nm (data not shown). All the keratinases were found to be active and stable over a pH range of 5–11 with optimal activity at pH 9 (data not shown). Their temperature optima were also same with maximal activity at 50 °C (data not shown). Noteworthy was the better thermostability of the truncated enzyme Ker P F4 over Ker P F2 which had the complete pro-sequence at 60 °C. Ker P F4 had a $t_{1/2}$ of >1 h and 20 min at 60 and 70 °C, respectively, compared with 20 and 5 min at 60 and 70 °C, respectively, for Ker P F2 (Fig. 4).

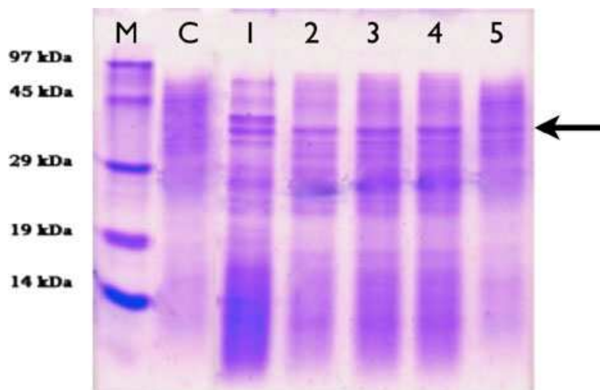

Fig. 3 Expression of the full-length and truncated forms of Ker P. *Lane M*, marker; *lane C*, vector (Pezz only) without insert control; *lane 1*, Ker P F1; *lane 2*, Ker P F2; *lane 3*, Ker P F3; *lane 4*, Ker P F4; and *lane 5*, Ker P F5

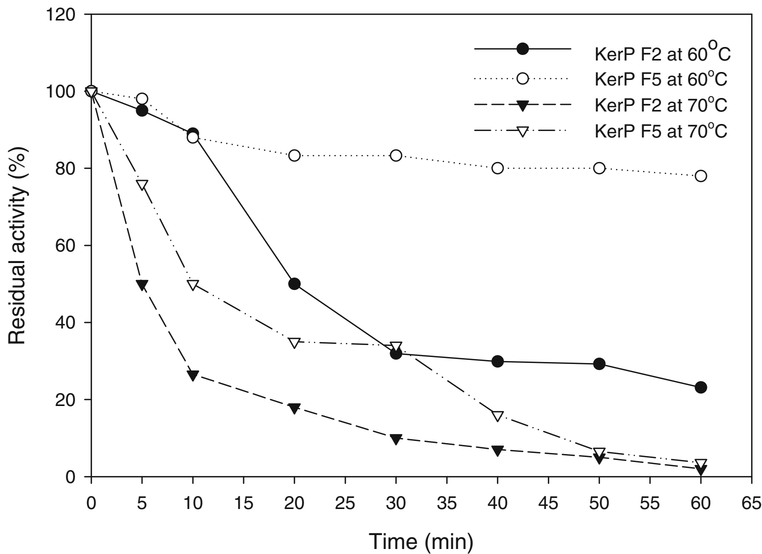

Fig. 4 Thermostability of Ker P F2 and Ker P F4 at 60 and 70 °C. 100% corresponds to 5.0 kU/mg protein on N-Suc-Ala-Ala-Pro-Phe-pNA as substrate

Substrate specificity was also studied and it was found that all the forms of keratinases had similar substrate specificity hydrolysing a variety of complex as well as synthetic substrates. With respect to complex substrates, they exhibited maximum cleavage of haemoglobin followed by fibrin>feather keratin>casein>meat protein. Among synthetic substrates, maximum cleavage was observed for *N*-Suc-Ala-Pro-Phe-*p*NA followed by 60% relative activity on *N*-Suc-Ala-Ala-Pro-Leu-*p*NA. N-Suc-Ala-Ala-Ala-*p*NA was the least hydrolysed (data not shown).

Kinetic comparison of the native and recombinant enzyme revealed that the native keratinase from *P. aeruginosa* KS-1 had a much higher V_{max} of 2×10^3 µg/mg/min on casein as compared with the full-length recombinant keratinase which had a V_{max} of just 185 µg mg^{-1} min^{-1}. However, on haemoglobin and other synthetic substrates, the V_{max} of the native enzyme were lesser than that of the recombinant enzyme (Table 1). Amongst the full length and the truncated forms of Ker P, the full-length Ker P F1 exhibited lower K_m and higher V_{max} for all the substrates. In addition, the kinetics of Ker P F4 was almost two times higher than that of Ker P F2 on casein. However, not much difference was observed in case of haemoglobin. In case of synthetic substrates, there was a decrease in K_m of Ker P F4 from 0.66 to 0.55 mM, 1.00 to 0.71, and 0.83 to 0.71 mM in case of *N-Suc-Ala-Ala-Pro-Phe*-pNA, *N-Suc-Ala-Ala-Pro-Leu*-pNA, and *N-Suc-Ala-Ala-Ala*-pNA, respectively, although no significant variation in V_{max} was observed.

Discussion

Pro-sequences are like architects of the protein and are known to contribute immensely in determining the final confirmation as well as function of the mature protein. In the present study, position specific alignment of keratinase Ker P from *P. aeruginosa* KS-1 along with proteases of various other distantly related counterparts viz. *Stenotrophomonas* sp., *Chromobacterium* sp., *Reinkia* sp., and *Vibrio* sp. was carried out and it was observed

Table 1 Steady-state kinetics of native and recombinant keratinases

Complex substrates	Casein		Haemoglobin	
	K_m (mg/ml)	V_{max} (μg mg^{-1} min^{-1})	K_m (mg/ml)	V_{max} (μg mg^{-1} min^{-1})
Ker P F1	4	185	7	2.16×10^4
Ker P F2	5	125	9	1.66×10^4
Ker P F3	7	167	8	1.83×10^4
Ker P F4	9	204	9	1.53×10^4
Native Ker P	12	2×10^3	8.3	5×10^2
Synthetic substrates	*N*-Suc-Ala-Ala-Pro-Phe-*p*NA		*N*-Suc-Ala-Ala-Pro-Leu-*p*NA	
	K_m (mM)	V_{max} (μmol mg^{-1} min^{-1})	K_m (mM)	V_{max} (μmol mg^{-1} min^{-1})
Ker P F1	0.35	7.9×10^3	0.75	3.2×10^3
Ker P F2	0.66	5.5×10^3	1.00	2.5×10^3
Ker P F3	0.55	5.7×10^3	1.00	1.9×10^3
Ker P F4	0.55	5.0×10^3	0.71	1.17×10^3
Native Ker P	1.25	2.5×10^2	1.70	0.85×10^2

that the total alignment among the 5 selected proteases and Ker P was around 59.8%. However, the detailed analysis revealed that while the core protein was highly conserved with around 80% alignment, the N-terminal pro-sequence region was highly variable with an alignment percentage of just 54%. This is in conformation with chymotrypsin like proteases, which have 63% homology in core protein and just 23–49% homology in pro-sequence region [11]. This much higher heterogeneity of pro-sequence compared with catalytic domain is a clear indicative that variations in the pro-sequence influence the structure and function of the mature protein. Thus, for developing enzyme variants, these pro-sequence regions can be considered as hot spots for modifications. Hence, in the present study the detailed analysis of the pro-sequence was carried out.

pEZZ 18-*E. coli* HB101 system was used for expression of all the constructs of Ker P. This system has been already documented by our group for extracellular expression of keratinases [9, 12]. It has a spa promoter and protein-A signal along with "ZZ" domain of IgG binding sites. Under the direction of protein-A signal, the expressed protein gets secreted as a fusion protein with "ZZ" peptides under non-inducible system which may get cleaved along with pro-sequence processing. First, the full-length Ker P gene (Ker P F1), including the signal peptide sequence was expressed as an active protein which suggests that additional signal does not hinder the expression of keratinase Ker P in heterologous host *E. coli*.

Subsequently, truncations of Ker P gene from the variable N-terminal were made and it was observed that among all the N-terminal truncations Ker P F2, Ker P F3, and Ker P F4 were functional, whereas no activity was observed in case of Ker P F5 carrying only 85 bp of the pro-sequence. This indicates that the length of pro-sequence required for the functional expression of Ker P was around 187 bp (102+85 bp) out of the total 520 bp. It can be put forth that this region, might be associated with a chaperone or foldase like activity, responsible for driving Ker P into its correct, functionally active conformation. This is in confirmation with an earlier report on elastase of *P. aeruginosa* where downstream regions of the pro-sequence, i.e., Pro C domain has been documented to be involved in the folding of the mature elastase [13]. This is also supported by the fact that amongst various pro-sequence regions of distant proteases maximum conserved stretches like $R_{181}L_{182}$ and $A_{188}QLVYXV_{194}$ were observed in this region.

Among the total of 173 aa residues of the pro-sequence, the chaperone activity lies in only 62 aa residues. The rest of the sequence is redundant during heterologous expression in *E. coli*. However, earlier reports have documented that the region preceding this, i.e. Pro M is required for secretion of elastase in *P. aeruginosa* [13].

Comparison of the biochemical characteristics of all the forms of keratinases revealed no significant changes with respect to their substrate specificity and pH and temperature optima however, Ker P F4 exhibited a better thermostability than Ker P F2. It can be inferred that keratinase with a smaller pro-sequence makes it more thermostable without changing the substrate specificity making it biotechnologically more viable.

Kinetic comparison of the native keratinase from *P. aeruginosa* with the recombinant keratinase revealed a higher K_m and V_{max} of the native protein on casein. However, a lower V_{max} was obtained on haemoglobin and other synthetic substrates. These differences in the characteristics of the enzyme can be attributed to the variation in conformation of the recombinant protein when expressed in heterologous host. Amongst the full-length and truncated forms of keratinase Ker P, the full-length Ker P F1 was catalytically better than the rest exhibiting the lowest K_m and highest V_{max} for all the substrates except casein on which Ker P F4 exhibited a higher K_m and V_{max}. On synthetic substrates, decrease in the length of pro-sequence led to a slight decrease in the V_{max} of the enzyme. All these differences indicate the variable folding of the proteins as a result of the difference in size of the pro-sequence.

Thus, through N-terminal truncation studies it can be concluded that the size of the pro-sequence has a direct impact on the conformation of the protein hence leading to altered kinetics of the enzyme. Also, out of the total 520 bp long pro-sequence of Ker P, only 187 bp region is the minimum length required for the correct folding of the enzyme.

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