Materials & Methods
Chapter 3

MATERIALS AND METHODS

This section describes the methodology followed in the present study. The methods for each section of Chapter 4 (Observations and Results) have been covered separately.

Analytical procedures

Source of keratin substrate

Chicken feather which were used as keratin substrate were procured from local poultry plants. They were thoroughly washed with 1% (w/v) triton x-100 and rinsed with distilled water followed by autoclaving at 15 psi. Thereafter, feather were chopped into small pieces, dried in an oven at 60°C for 1h and passed through a sieve of mesh number 10 having a pore size of 2.0 mm. The feather powder thus obtained was used to perform the keratinase assay.

Keratinase assay

The assay mixture containing 1 ml of appropriately diluted enzyme, 4 ml of glycine-NaOH buffer (50mM, pH 9.0), and 20 mg of feather powder was incubated at 60°C for 1h. The reaction was terminated by addition of 4ml of 5% (w/v) TCA (trichloroacetic acid) and the tubes were left undisturbed at room temperature (25±1°C) for 1h to allow settling of the precipitate. Feather and insoluble residues were removed by filtration through glass wool. The filtrate thus obtained was centrifuged at 8000 rpm for 10min and proteolytic products in the supernatant were determined by reading the absorbance at 280nm against appropriate blank. The enzyme and substrate controls were also prepared. Enzyme control was prepared by addition of 1ml of appropriately diluted enzyme sample, 1ml of TCA and 3ml glycine-NaOH buffer. Substrate control was prepared by addition of 5ml buffer and 20mg feather.

One unit of keratinase is defined as the amount of enzyme required to bring an increase in absorbance (A280) of 0.01 under standard assay conditions.
**Protease assay / Caseinolytic assay**

Casein was used as a substrate for protease assay. One milliliter of appropriately diluted enzyme was incubated with 1 ml of 1% w/v primed casein solution (prepared in glycine-NaOH buffer, pH 10.0) for 20 min at 60°C. The reaction was terminated by addition of 4 ml of 5% w/v TCA and left undisturbed at room temperature (25°C ± 1°C) to allow settling of unhydrolyzed substrate. The contents were centrifuged at 5000 rpm for 10 min. 1 ml of the clear supernatant was added to 5 ml of 0.4 M Na₂CO₃ followed by addition of 0.5 ml diluted Folin Ciocalteau’s reagent. The tubes were incubated in dark for 30 min. The optical density of the samples was measured at 660 nm against appropriate reagent blank. The enzyme and substrate controls were also prepared. Enzyme control was prepared by addition of 1 ml TCA (5% w/v) to 1 ml of appropriately diluted enzyme. Substrate control was prepared by addition of 1 ml casein (1% w/v) to 1 ml glycine- NaOH buffer.

**Calculation of protease activity**

One unit of protease was equivalent to the amount of enzyme required to release 1 µg of tyrosine/ml/min under standard assay conditions.

The tyrosine concentration was calculated using the regression equation obtained from the standard curve of tyrosine.

\[
\text{Tyrosine concentration (µg/ml)} = \frac{\text{Test} - \text{Control} + 0.285}{0.00926}
\]

Protease activity (U/ml) = \( \frac{\text{µg of tyrosine} \times \text{dilution factor} \times \text{total volume}}{\text{Reaction time}} \)

**Protein content estimation**

**Lowry’s method** (Lowry et al., 1951)

**Reagents:**

- **Reagent A:** 2% Na₂CO₃ in 0.1 N NaOH
- **Reagent B:** 0.5% CuSO₄.5H₂O in distilled water
- **Reagent C:** 1% potassium sodium tartrate in distilled water
Reagent D: 98ml of reagent A was mixed with 1ml of reagent B and 1ml reagent C (prepared afresh at the time of use)

Reagent E: Folin Ciocalteau’s reagent (AR grade, SRL, India) prepared afresh by diluting with distilled water in the ratio 1:1

Protocol

To 1ml of the appropriately diluted protein sample, 5ml of reagent D was added and the mixture was incubated at room temperature (25±1°C) for 10min. To this, 0.5ml of freshly prepared reagent E was added and incubated in the dark for 30min. Absorbance was measured at 660nm against appropriate reagent blank.

Calculation of protein content

The protein content was calculated using the regression equation prepared from a standard curve of bovine serum albumin (BSA) in the concentration range 100-1000μg/ml. The amount of protein was estimated as μg protein/ml of sample.

Protein conc. (μg/ml) = A660 +0.05 X dilution factor

0.002

Keratinase production

Inoculum preparation

A loopful of culture from nutrient agar was inoculated in 50ml nutrient broth in 250ml Erlenmeyer flask and incubated at 37°C, 250 rpm in a New Brunswick Scientific shaker (Edison, New Jersey, USA) for 18h. A 2% v/v inoculum (8 X 10⁸ cfu/ml) of this culture was used to inoculate the production medium.

Enzyme production

The keratinase was produced in the feather-peptone medium (Feather: 0.5%; Peptone: 0.5%; Glucose: 0.5%; KH₂PO₄: 0.1%; K₂HPO₄: 0.3%; pH 7.0). The medium (50ml in a 250ml Erlenmeyer flask) was inoculated with 2% v/v of 18h old seed culture and incubated at 37°C at 200 rpm in a New Brunswick Scientific shaker (Edison, New Jersey, USA).
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**Harvesting and concentration of enzyme**

The culture broth obtained was centrifuged at 10000rpm for 15 min at 4°C in a Sigma centrifuge (Sigma 3K30). The supernatant obtained was concentrated by passing through 10kDa centricons. The concentrated enzyme was then filtered through a 0.2µ membrane syringe filter (mdi, India). This microfiltered, concentrated enzyme preparation was used for screening experiments.

**Measurement of feather degradation by dry weight method** *(Bertsch and Coello 2005)*

To determine the percent feather degradation, the whole contents of the culture broth were filtered through a pre-weighed Whatman filter paper no.1 to retain non-degraded feathers. The residue remaining on the filter was washed twice with distilled water to remove cells and dried in an oven at 60°C until constant weight. Feather degradation was determined by subtracting the weight of feathers in un-inoculated flasks, used as control, from the weight of feather left over after fermentation. Percentage dry weight of feather left over after degradation was calculated.

**I. Screening, selection and identification of a potential keratinase producer**

**Screening and selection of keratinase producer**

Fifty feather degrading bacterial isolates were procured from the laboratory culture collection. The isolates were revived from glycerol stocks (50% v/v) in nutrient broth at 37°C, 200rpm. They were screened for their keratinolytic potential by testing for their ability to degrade chicken feather. Basal medium *(Varela et al., 1997)* containing 0.5% w/v feather was used to inoculate 2% v/v of overnight grown cultures prepared in nutrient broth. All the isolates were observed for complete feather degradation. The degradation was observed for upto four days.

**Evaluation of keratinase from selected isolates to degrade chicken feather**

Keratinase from the selected isolates was produced and concentrated as described above. Equal units (5000 U) of all the microfiltered, concentrated enzymes was then added to 0.5% chicken feather suspended in 50 ml phosphate buffer (pH 7) and incubated at 37°C, 100 rpm for 16 h. Feather degradation was studied by dry
weight method. All the selected isolates were identified based on partial 16S sequencing.

Selection based on degradation of surrogate yeast prion protein, Sup 35NM

Overexpression of Sup 35NM

The plasmid pJC25NM stop harbouring the yeast surrogate prion protein (Sup35 NM) was purchased from North Carolina University, USA. It was transformed in *E. coli* BL21 CODON PLUS competent cells and the clones were selected on LB agar medium containing 100mg/ml ampicillin. Positive clone was grown overnight at 37°C, 200 rpm in LB-Amp medium. 1% of this overnight grown culture was inoculated into 1L of fresh LB-Amp medium and incubated till an OD$_{600}$ of 0.5. 1 mM IPTG was then added to the culture to induce expression of prion protein at 37°C, 200 rpm for 3h. After induction, the cells were harvested and resuspended in 50 ml of lysis buffer (10 mM Tris-HCl, pH 7.2, 1 mM DTT, 1 mM PMSF, 8 M urea) and sonicated by 5 sec pulse on and 5 sec pulse off for 2 min. Lysate was then cleared by centrifugation at 5000 rpm for 30 min at 10°C. The level of expression was checked in the pellet and lysate by 15% SDS-PAGE. Coomassie brilliant blue was used for visualization of the protein bands.

Purification of Sup 35NM

Sup 35NM was purified using the protocol of Chen et al., 2005. The cleared supernatant from cell lysis was applied to a 20-ml Q Sepharose Fast Flow column (Pharmacia) pre-equilibrated with lysis buffer at a flow rate of 3 ml/min. The column was washed with 5x bed volumes of Q wash buffer I [10mM Tris-HCl, pH 7.2, 1 mM DTT, 1mM PMSF, 8 M urea, 85 mM NaCl], and 5x volumes of Q wash buffer II [10mM Tris-HCl, pH 7.2, 8 M urea, 150 mM NaCl]. The protein was eluted in three volumes of Q elution buffer [10mM Tris-HCl, pH 7.2, 8 M urea, 200 mM NaCl]. The eluate from the Q Sepharose was then loaded directly onto a 25-ml Macro Prep Ceramic Hydroxyapatite Type I 40-um column (Bio-Rad) pre-equilibrated with Q elution buffer. The column was washed with 2x volumes of HA wash buffer I [1 mM potassium phosphate, pH 6.8, 8 M urea, 1 M NaCl] and then with 2x volumes of HA wash buffer II [25 mM potassium phosphate, pH 6.8, 8 M urea]. The protein was eluted using a step gradient of 75 mM and 125 mM potassium phosphate, pH 6.8 in
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8M urea. Fractions (5 ml) were analyzed by 15% SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250.

Quantitation of Sup 35NM

Purified Sup 35NM was diluted in 6M guanidium hydrochloride (1:1) for 30 min followed by checking the absorbance at 280nm. Protein concentrations were determined with the calculated extinction coefficient of 0.90 for a 1 mg/ml NM solution at 280nm absorbance (Gill and Von Hippel, 1989). Further, molar concentration of Sup 35NM was calculated taking molecular weight of monomeric Sup 35NM as 35kDa.

Concentration and storage of purified Sup 35NM

For long-term storage, Sup 35NM was methanol precipitated to remove urea and the precipitate stored at –80°C. Anhydrous methanol (100%) was added to eluates containing Sup 35NM on ice at a ratio of 5:1. The mixture was incubated on ice for 30 min, and the precipitate was collected by centrifugation at 14,000g for 30 min at 4°C. The pellet was then washed with 100% methanol (1/2 volume of supernatant) and collected by centrifugation again. The supernatant was removed and the pellet was stored in 70% (v/v) methanol (1/2 volume of supernatant) at –80°C.

Aggregation and deaggregation of Sup 35NM

Methanol precipitated NM was collected by centrifugation at 14,000g for 30 min at 4°C. The methanol was removed and the pellet was air-dried for 5 min. The protein precipitate was then resuspended in congo red binding buffer (CRBB: 5mM potassium phosphate, pH 7.4, 150 mM NaCl). 200 µl of 25µM resuspended Sup 35NM was incubated at temperatures ranging from 10 to 90°C for 10 min to check aggregation and de-aggregation.

Congo red binding assay

Congo red binding is a sensitive probe for fibril formation (Klunk et al., 1989). 10 mM congo red (Sigma Aldrich, USA) prepared in CRBB was added to Sup 35NM and incubated at room temperature for 5 min followed by centrifugation at 14,000 rpm for 10 min. Absorbance of free congo red in the supernatant was monitored between 300 nm to 700 nm using UV-1800 Schimadzu spectrophotometer (UV-Probe Version 2.3 software).
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Enzymatic degradation of Sup 35NM

Five hundred units of keratinase of each of the selected strain were added to 25µM of aggregated Sup 35NM resuspended in CRBB and incubated at 37°C for 16h. After incubation, congo red binding assay was performed as described above. Reaction with inactivated enzyme was taken as control.

Identification of the selected bacterium

Identification on the basis of biochemical characteristics

The selected strains were streaked on nutrient agar plates and loopful of inoculum was transferred to nutrient broth. The plates and broth were incubated at 37°C for 18h. The detailed biochemical characteristics were studied by BIOLOG at Central instrumentation facility, University of Delhi, South Campus for the finally selected strain.

Identification on the basis of 16S rRNA gene sequencing

The taxonomic status of the selected strain was confirmed by 16S rRNA gene sequencing. Partial 16S rRNA sequencing was done for all the screened strains and complete was done for the finally selected strain.

The genomic DNA was isolated from a freshly grown culture using the Qiagen genomic DNA extraction kit (Qiagen, Germany). It was used as template for PCR amplification of 1.4kb 16S rRNA gene. The forward and reverse primer sequences used for the amplification were as follows:

Forward primer: 5’ AGAGTTTGATCCTGGCTCAG 3’
Reverse primer: 5’ TACGGCTACCTTTGTTACGACTT 3’

The PCR reaction mix and the thermocycler conditions were as follows: 50ng of template DNA, 0.2mM dNTPs, 5µl reaction buffer (10X, pH 9.0), 10pmoles of each primer, 2U Taq DNA polymerase (Banglore Genei, India). The thermocycler conditions were as follows: initial denaturation of 95°C for 5min, 30 cycles of 94°C for 30s, 50°C for 30s and 72°C for 1min; final extension at 72°C for 10min. Amplicons were sequenced for all strains selected after preliminary screening. However, amplicon of the finally selected strain was further ligated into pGEMT-Easy vector for 1h at 22°C. The ligation mix was transformed into E. coli DH5α
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competent cells and they were plated on LB-Amp plates having X-gal and IPTG for blue white selection. The positive clone was further sequenced at Chromous Biotech, India. The sequence thus obtained was blasted in the NCBI databank and the taxonomic status of the bacterium was ascertained. The complete 16S rRNA gene sequence has been submitted to NCBI GenBank and the culture has been deposited to microbial type culture collection (MTCC), Chandigarh.

II. Purification and biochemical characterization of keratinase

Production of keratinase

For keratinase production, feather peptone medium (0.5% feather, 0.5% peptone, 0.5% glucose, 0.1% KH₂PO₄ and 0.3% K₂HPO₄ (pH 7.0)) was used. 50 ml of the medium was dispensed in 250 ml Erlenmeyer flasks and sterilized by autoclaving at 121°C for 20 min. Two percent of a 24 h old culture grown in nutrient broth was used as inoculums. The flasks were incubated at 37°C under shaking (200 rpm) in a New Brunswick Scientific shaker (Edison, New Jersey). After 24 h of incubation, the cells were harvested by centrifugation at 8000 g (Sigma Centrifuge, Germany) for 10 min and the supernatant was subjected to further purification of keratinase.

Purification of keratinase

Acetone precipitation

The keratinase was precipitated from the culture supernatant by adding acetone at a final concentration of 50% v/v. The solution was left at 4°C for 1 h and the precipitate collected by centrifugation at 10000 rpm. The precipitate obtained was air dried and dissolved in minimum amount of Tris-HCl buffer (10 mM, pH 8.0). The protein concentration and keratinase activity were determined.

Q- sepharose column chromatography

Packing of the column

Wetted glass wool was inserted at the bottom of a 20ml syringe with the help of forceps or glass rod. 5ml of pre-swollen anion exchange matrix- Q-sepharose
(Pharmacia Biotechnology, Upsala, Sweden) was packed in the column. It was pre-equilibrated with Tris-HCl buffer (10mM, pH 8.0).

**Loading and elution of the sample enzyme**

Two millilitre of the concentrated protein (14 mg) was loaded onto the column and the flow rate adjusted to 30 ml/h.

The enzyme was eluted from the column using a continuous NaCl gradient (0-5.0 M) prepared in Tris-HCl buffer (10mM, pH 8.0). 160 fractions of 2ml each were collected. The protein content in each of the fractions was monitored by using a UV-Vis spectrophotometer (UV-1700 Shimadzu, Japan) at 280nm. The keratinase activity was determined in the fractions. Fractions containing major keratinase peaks were pooled and concentrated by spinning under vacuum in a speed vac evaporator (EYELA, Tokyo, Japan). HPLC and SDS-PAGE were used to check the protein purity and to determine the molecular weight of the protein.

**Column washing – matrix regeneration and storage**

The column was washed with decreasing salt gradient (5M-0.1M) and equilibrated with Tris-HCl buffer (pH 8.0). For storage, it was washed with 0.01% sodium azide solution and overlaid with approximately 10ml of buffer to avoid drying, cracking and microbial growth.

**HPLC analysis**

The purified keratinases KP1 and KP2 were run on HPLC using C18 column (Shimadzu, Japan) with acetonitrile: water (90:10) as the mobile phase and a flow rate of 0.5ml/min. The protein was detected at A$_{280}$ using UV detector.

**Polyacrylamide gel electrophoresis**

The purified keratinases were subjected to SDS-PAGE in BioRad Mini Protean® 3 system using 12% w/v acrylamide gel (Table 3.1). Protein sample was prepared by mixing appropriate amount of protein with the sample buffer and boiled for 5min at 100°C before loading onto the gel. Electrophoresis was carried out at a constant voltage of 80V for stacking gel and 100V for resolving gel till the tracking dye reached the bottom of the gel. The relative molecular mass of the protein was determined using standard protein markers (Sigma-Aldrich, USA) run simultaneously.
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The bands were visualized by Coomasie blue. The enzyme was also subjected to native PAGE and activity was checked by performing zymogram analysis.

**Stock solutions for SDS-PAGE** (Sambrook *et al.*, 1989)

**30% Acrylamide solution**

29g of acrylamide and 1g of N, N’ methylene bis acrylamide was dissolved in distilled water (pH 7.0) up to a total volume of 100ml. The solution was stored in a dark bottle at 4°C and used within 30 days.

**Resolving gel buffer (1.5M Tris, pH 8.8)**

Prepared by dissolving 91g Tris in 300ml distilled water. The pH of the solution was adjusted to 8.8 using 1N HCl and volume was made up to 500ml with distilled water. The solution was stored at 4°C.

**Stacking gel buffer (0.5M Tris, pH 6.8)**

Prepared by dissolving 6.05g Tris in 40ml distilled water. The pH of the solution was adjusted to 6.8 using 1N HCl and volume was made up to 100ml with distilled water. The solution was stored at 4°C.

**Ammonium per sulfate (APS- 10% w/v)**

Prepared by dissolving 0.1g of APS in 1ml of distilled water. The solution was prepared afresh each time.

**Sodium dodecyl sulfate (SDS 10% w/v)**

Prepared by dissolving 10g SDS in 100ml of distilled water.

**Electrophoresis buffer (5X stock solution)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>15.1g</td>
</tr>
<tr>
<td>Glycine</td>
<td>72g</td>
</tr>
<tr>
<td>SDS</td>
<td>5g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>8.3</td>
</tr>
</tbody>
</table>

The solution was diluted to 1X concentration at the time of use by adding 100 ml of electrophoresis buffer (5X) to 400ml distilled water.
SDS sample buffer or tracking dye (4X)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (200mM, pH 6.8)</td>
<td>4ml</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>0.4ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>4ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.4g</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.02g</td>
</tr>
</tbody>
</table>

The volume of the buffer was made up to 10ml using distilled water. The protein samples were mixed with the tracking dye in the ratio 4:1 and heated at 100°C for 5min and allowed to cool before loading. For native PAGE, β-mercaptoethanol was not added in the tracking dye and samples were not boiled.

**Table 3.1: Composition of resolving and stacking gels**

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Resolving gel (12%)</th>
<th>Stacking gel (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3.3</td>
<td>2.7</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>4.0</td>
<td>0.67</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>1.5 M Tris (pH 6.8)</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>APS (10%)</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004</td>
<td>0.004</td>
</tr>
</tbody>
</table>

**Staining procedure**

**Coomasie blue staining**

The gel was stained with Coomasie brilliant blue R-250 solution prepared by dissolving 1g Coomasie blue in 500ml of isopropanol. To this, 200ml of glacial acetic acid was added and the volume made up to 1 L with distilled water. The solution was filtered using Whatman filter paper no1.

Staining was carried out for 2h under gentle shaking followed by overnight destaining using 10% glacial acetic acid.
Non-denaturing polyacrylamide gel electrophoresis (Native PAGE)

The composition and volume of Native PAGE was same as described for SDS-PAGE except that it did not contain SDS in any of the buffers. The samples for native PAGE were not subjected to heat treatment.

Zymogram analysis

The keratinase samples were mixed with the electrophoresis sample buffer without heat denaturation prior to electrophoresis. Native-PAGE was carried out using 12% polyacrylamide gel. After electrophoresis, the gel was equilibrated in Tris-HCl pH 8.0 buffer for 10min and placed onto agar plate. 10 mM N-Suc-ala-Ala-Pro-Phe-pNA was flooded onto the plate and it was incubated at 45°C till yellow colored band appeared onto the gel. Further gel was dipped for 5 min in 0.1% (w/v) sodium nitrite solution in 1N HCl followed by 0.5% ammonium sulfamate solution in 1N HCl for 5min. Gel was placed in N-1-N’ (naphthyl) ethylene diamine solution in 1N HCl for 1-5min, till the pink colored band appeared.

Isoelectric focusing

Reagents

Monomer concentrate

24.25g of acrylamide and 0.75g of N-N’ methylene-bis-acrylamide was dissolved in distilled water (pH 7.0) up to a total volume of 100ml. The solution was stored in dark bottle and used within 30 days.

Ammonium per sulfate (APS-10% w/v)

Prepared by dissolving 0.1g of APS in 1ml of distilled water. The solution was prepared afresh each time.

25% w/v glycerol

25ml glycerol was added to 50ml distilled water. The volume was made up to 100ml with distilled water.

Monomer- ampholyte solution

Monomer concentrate 2.0ml
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- 25% glycerol  
  2.0ml
- Ampholyte*  
  0.5ml
- APS (10%)  
  15μl
- TEMED  
  3μl
- Distilled water  
  1000ml

*Bio- Lyte® 3/10 ampholyte with pH operating range 3.7-9.3.

**Fixative**

- 12.5% trichloroacetic acid (TCA)
- 30% methanol

**Staining solution**

- 27% isopropanol
- 10% acetic acid
- 0.04% Coomasie brilliant blue
- 0.5% CuSO₄

CuSO₄ was dissolved in water before adding isopropanol.

**Destaining solution I**

- 12% isopropanol
- 7% acetic acid
- 0.5% CuSO₄

CuSO₄ was dissolved in water before adding isopropanol.

**Destaining solution II**

- 25% isopropanol
- 7% acetic acid

**Protocol**

The purified keratinases were subjected to isoelectric focusing using the BioRad Mini IEF Cell Model 111. A few drops of water were pipetted onto the glass plate and the hydrophobic surface of the gel supporting film was placed onto the plate. The gel support film-plate was placed on the casting tray with the gel support film facing downwards. The monomeric ampholyte solute was pipetted between the glass plate and the casting tray and allowed to set for...
1h. 2μl of the sample was applied onto the gel using the template and the samples allowed to diffuse into the gel for 5min. The template was removed carefully and focusing was carried out under constant voltage conditions. Initial voltage conditions were 100V for 15min followed by 200V for 15min. The voltage was finally increased to 450V for 60min. The focusing was carried out at 4°C. After the focusing the protein was detected by fixing, staining and destaining at room temperature (25±1°C).

**MALDI-TOF and N-terminal analysis of keratinases**

Purified keratinases were identified by using mass spectroscopy *i.e.* LC-MS/MS at The Centre of Genomics Application (TCGA), Okhla, New Delhi, India. Protein bands were cut from the SDS PAGE and send for LC-MS/MS analysis using Nano LC MS (Thermofinnnigan LCQ Deca).

**Biochemical characterization of keratinases**

**Effect of pH on the activity and stability of keratinases**

The enzyme activity was assayed at 60°C in the pH range of 2.0-11.0 using buffers (50mM) of varying pH (KCl-HCl buffer (pH 2.0), citrate phosphate buffer (pH 3.0-6.0), phosphate buffer (pH 7.0), tris-HCl buffer (pH 8.0-9.0), glycine-NaOH buffer (pH 10.0), phosphate hydroxide buffer (pH 11.0)). 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 (2.0-11.0) for 1h at room temperature (25±1°C) and thereafter the residual activity was determined at optimum pH and temperature.

**Effect of temperature on the activity and stability of keratinases**

Effect of temperature on keratinase activity was determined by incubating the reaction mixture at different temperatures ranging from 40-80°C under standard assay conditions. Activity was expressed as percentage relative activity with respect to temperature optima which was considered as 100%.
The temperature stability was determined by incubating the enzyme samples at various temperatures ranging from 50-80°C for different time intervals up to 2h (30, 60, 90 and 120 min), the residual activity was determined at optimum pH and temperature.

**Effect of metal ions**

The effect of various metal ions viz. Ba\(^{2+}\), Ca\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), and Zn\(^{2+}\) on keratinase activity was studied by incubating the enzyme with various metal ions at a final concentration of 10mM at room temperature (25±1°C) for 1h and then determining the residual activity at their respective pH and temperature optima. Residual activity expressed in terms of percentage activity against control without the metal ions was taken as 100%.

**Effect of inhibitors**

The effect of various inhibitors viz. Phenyl methyl sulfonyl fluoride (PMSF), ethylenediamine tetraacetate (EDTA), bromo acetic acid, iodoacetic acid, β-mercaptoethanol, dithiothreitol (DTT), 5,5’ dithio-bis(2-nitrobenzoic acid) (DTNB), phosphorhamidon, bestatin (Sigma-Aldrich, USA; ICN chemicals, USA) on keratinase activity was determined by incubating the enzymes with the inhibitors at a final concentration of 10mM for 10 min. at room temperature (25±1°C). The residual activity was measured at their respective pH and temperature optima. Residual activity was expressed in terms of percentage activity against control without the inhibitor which was taken as 100%.

In case of inhibition by EDTA, reversal of keratinase activity was checked by addition of metal ions viz. Ba\(^{2+}\), Ca\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\). 5 mM of various metal ions was added to EDTA inhibited enzyme for 1h followed by keratinase assay using standard assay protocol.

**Substrate specificity of keratinases**

**Activity on complex substrates**

The substrate specificity of the keratinases was studied using both soluble and insoluble substrates viz. casein, gelatin, elastin, feather keratin, fibrin, meat protein, nail-hoof keratin (substrates were either locally available from CDH and SRL or
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purchased from Sigma-Aldrich, USA). 20mg of each of the substrates was added to 1 ml of appropriately diluted enzyme at optimum pH and temperature for 1 h. The reaction was stopped by the addition of 1 ml of 5% (w/v) trichloroacetic acid. The contents were centrifuged after 1 h at 1,006 g for 10 min. Protein release was recorded by Folin-Lowry method. Folin–Ciocalteau’s reagent (0.5 ml) was added to 1 ml of the supernatant and the optical density of the samples was recorded at 660 nm against appropriate substrate and enzyme controls. Enzyme control was prepared by addition of 1 ml appropriately diluted enzyme sample and 1 ml of 5% (w/v) trichloroacetic acid (TCA). Substrate control was prepared by addition of 20mg substrate in 1ml of optimum pH buffer and 1 ml of 5% (w/v) trichloroacetic acid (TCA). Protein release was recorded as

Amidolytic activity

Amidolytic activity of keratinases was also examined by checking its ability to hydrolyze synthetic $p$-nitroanilide substrates viz. N-Suc-Ala-Ala-Pro-Phe-$p$NA, D-Val-Leu-Lys-$p$NA, N-Suc-Ala-Ala-Ala-$p$NA, N-Suc-Ala-Ala-Pro-Leu-$p$NA, N-Suc-Gly-Gly-Phe-$p$NA, N-Benzyol-DL-Val-Gly-Arg-$p$NA, N-Benzyol-L-tyrosine-$p$NA, N-Suc-L-Phe-$p$NA (purchased from Sigma-Aldrich, USA; ICN chemicals, USA). 100mM stock solutions of the peptides were prepared in DMSO. In a total reaction volume of 1 ml, 10mM of the substrates was added to appropriately diluted enzyme prepared in phosphate buffer (50mM, pH 8.0) buffer. The reaction mix was incubated at their optimum temperature for 10min. The hydrolyzed product was measured at 405nm using a UV-Vis spectrophotometer (UV 1700 Shimadzu, Japan). The concentration of the product released was calculated according to the following equation (Bressollier et al., 1999).

\[ \text{Ab} = \varepsilon \times c \times L \]

where Ab is absorbance, $\varepsilon$ is extinction coefficient (9900 M$^{-1}$cm$^{-1}$), c is concentration of the product released, L is the path length.

Determination of kinetic constants

The kinetic constant for keratinases were determined on casein and N-Suc-Ala-Ala-Pro-Phe-$p$NA (AAPF). Varying concentrations of casein (1-10 mg/ml) and AAPF (1.0-10 mM) were assayed with appropriately diluted enzyme. The initial
velocities were then determined, and steady state kinetic parameters ($K_m$ and $V_{max}$) for each substrate were calculated using a Lineweaver-Burk plot.

**Hydrolysis of insulin B chain and mass spectrometry**

The substrate specificity of keratinases was also determined on the basis of hydrolysis of insulin B-chain (Sigma, cysteine residues oxidized). 100 µl of appropriately diluted enzymes were incubated with 100 µl of insulin B-chain (1 mg/ml in 10 mM Tris–HCl buffer, pH 9). The mixture was incubated at their respective temperature optima for 16 h, after which 40 µl of 0.1% (v/v) TFA was added to inactivate the enzyme. Next, it was centrifuged at 10,000 rpm for 10 min followed by concentration of the peptides in the supernatant by speed vac evaporator (EYELA, Tokyo, Japan). Further identification of the cleavage products was performed by liquid chromatography-electrospray mass spectrometry (LC-ESI/MS, GenPro Biotech, India). The $m/z$ values ranged between 650 and 2100. Hydrolysis sites were determined using the FindPept, a part of the Expasy software package.

**III. Molecular characterization of keratinases**

**Cloning and expression of keratinases**

**Genomic DNA isolation**

*Pseudomonas aeruginosa* KS-1 was grown into LB medium and genomic DNA was isolated using Qiagen DNA isolation kit following manufacturer’s protocol. DNA was checked using agarose gel electrophoresis.

**PCR amplification of the keratinase gene**

Two keratinases were purified and characterised in the previous section. Keratinase KP1 showed homology with putative aminopeptidase and keratinase KP2 showed homology with pseudolysine from *Pseudomonas aeruginosa* POA-1. Their nucleotide sequences were procured from the NCBI database and gene specific primers were designed for the same based on the N-terminal sequence (KP1F: ATGAGCAACAAGAACAATCTCAGA; KP1R: TTACTTGATGAAGTCTGACC; KP2F: ATGAAGAAGGTTTCTACGCTT; KP2R: CAACCGCGCTCGGCG AGGTCAC).
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PCR amplification was carried out using Taq DNA polymerase. Annealing temperature was first standardized using gradient PCR from 43°C to 55°C. Bulk PCR was carried out with the following conditions: Initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 30 sec, 43°C for 30 sec and 72°C for 2 min followed by final extension at 72°C for 10 min. The PCR products were electrophoresed on agarose gel to check for amplification. Amplicons were purified using Qiagen Qiaquick® gel extraction kit as per manufacturer’s instructions.

**Cloning into pGEMT-Easy vector**

The eluted PCR product was cloned into pGEMT-Easy vector (Promega, USA). One hour ligation was carried out at 22°C. The ligation mix was subsequently transformed into *E. coli* DH5α competent cells. Positive clones were selected on the basis of blue white selection on LB-ampicillin (100μg/ml) agar plate containing X-gal (2% w/v prepared in dimethyl formamide) and IPTG (25μl of 100mM IPTG). Screening of the white colonies was performed using colony PCR. Positive clones were confirmed by colony PCR and sequenced at Central Instrumentation Facility, University of Delhi using T7 and SP6 primers.

**Sequence analysis**

Sequences thus obtained were analyzed for its homology using BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn), amino acid by Translate tool of ExPASY (http://web.expasy.org/translate/), alignment by Clustal W (http://www.ebi.ac.uk/Tools/services/web_clustalw2/toolform.ebi), signal identification by Signal P software (http://www.cbs.dtu.dk/services/SignalP/) and restriction mapping by NEB Cutters tool (http://tools.neb.com/NEBcutter2/). Sequences of rKP1 and rKP2 have been submitted to NCBI GenBank.

**Sub-cloning of rKP1 and rKP2**

**Cloning into pGEMT-Easy vector**

Based on the above sequence analysis, primers were again designed with *EcoRI* and *BamHI* restriction sites incorporated in forward and reverse primer respectively. Sequences of the primers are listed in table 3.2. Cloning into pGEMT-Easy vector was performed as described earlier and positive clones for keratinase KP1 and KP2 were designated as pGEMT-*rKP1* and pGEMT-*rKP2* respectively.
Materials & Methods

Table 3.2: Primers designed to amplify keratinase KP1 and KP2 genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon size (kB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rKP1F</td>
<td>GCBAATTTCATGAGCAACAGAAGAC</td>
<td>1.6</td>
</tr>
<tr>
<td>rKP1R</td>
<td>CGGGATCCATTACTTGTAGAAGT</td>
<td></td>
</tr>
<tr>
<td>rKP2F</td>
<td>GCBAATTTCATGAGAAGGTTTCT</td>
<td>1.4</td>
</tr>
<tr>
<td>rKP2R</td>
<td>CGGGATCCACAACGCGCTCGGCG</td>
<td></td>
</tr>
</tbody>
</table>

Sub-cloning into secretory vector, pEZZ 18

For sub-cloning into pEZZ 18, first plasmid was isolated from the pGEMT-rKP1 and pGEMT-rKP2 clones. It was double digested with Eco RI and Bam HI. pEZZ 18 was also digested with the same restriction enzymes. The fall outs of 1.6 kB and 1.5 kB and linearized pEZZ 18 were purified by gel elution. This was followed by ligation using an optimal insert: vector ratio and T4 DNA ligase at 16°C for overnight. The ligation mixture was transformed into E.coli HB101 competent cells and these were then spread plated on LB-Amp plates having X-gal and IPTG for selection. Colony PCR was performed to select positive clones.

Expression of recombinant keratinases in E. coli HB101

pEZZ 18-E. coli HB101 system is a very useful system for cloning and extracellular expression of recombinant proteins especially proteases. The positive clones were sub-cultured (2% v/v inoculum) from an overnight grown culture into LB-ampicillin (100μg/ml) medium and incubated at 37°C, 300rpm for 24h. Culture broth was centrifuged at 8000rpm for 10 min and extracellular expression was checked in the supernatant by qualitative and quantitative estimation of keratinase activity on synthetic substrate of keratinase i.e. N-Suc-Ala-Ala-Pro-Phe-pNA (AAPF) followed by SDS PAGE analysis. E. coli HB101 containing only pEZZ18 vector (without insert) was taken as control.

Purification of the recombinant keratinases

Extracellular keratinases were produced in LB-ampicillin medium at 37°C, 300rpm for 24h and culture broth was centrifuged at 7000rpm, 4°C for 10min. Cell free supernatant was concentrated with 50% (v/v) chilled acetone and subjected to purification by IgG sepharose chromatography. IgG sepharose 6 Fast flow matrix was
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purchased from GE Healthcare, India. It was equilibrated with three bed volumes each of 0.5M acetic acid, pH 3.4 and TST (50 mM Tris buffer, pH 7.6, 150 mM NaCl and 0.05% Tween 20). Concentrated broth was loaded onto the column. Next, the column was washed with 10 bed volumes of TST and 2 bed volumes of 5mM ammonium acetate (pH 3.4). The samples were eluted with 0.5 M acetic acid, pH 3.4 and the column was re-equilibrated with TST.

The purified IgG-tagged protein eluted was found to be inactive therefore, further purification of the functional protein was performed by Q-sepharose ion exchange chromatography as described earlier. The purity of the recombinant enzymes was checked by SDS-PAGE analysis. Further, MALDI-TOF analysis of the purified proteins was performed as described earlier.

Biochemical characterization of recombinant keratinases

Biochemical characteristics of the recombinant enzymes, rKP1 and rKP2 were studied with respect to their pH, temperature kinetics and substrate specificity as described in the earlier section on biochemical characterization of keratinase. As substrate specificity revealed that both the keratinases effectively cleaved fibrin thus further detailed fibrinolytic activity of both rKP1 and rKP2 were studied.

rKP1 and rKP2 were checked for the following three activities:
1. Fibrinolytic
2. Fibrinogenolytic
3. Plasminogen activating activity

Fibrinolytic activity

Fibrin degradation analysis was performed using method of Wu et al., 2009 with slight modifications. In brief, 200µl of 10mg/ml fibrinogen solution prepared in Tris-HCl (pH 7.4) buffer containing 150 mM NaCl, was mixed with 100µl of thrombin (47 U/mg) dissolved in the same buffer. The fibrin clot was allowed to stand for 1 h at room temperature. 10 µg of purified keratinases was placed on the clot surface and incubated at 37°C for various time intervals. Enzyme was inactivated by addition of 1mM PMSF and the hydrolytic products were analysed on 12% SDS-PAGE. Plasmin from human plasma was used as a positive control.
**Materials & Methods**

**Fibrinogenolytic activity**

Fibrinogenolytic activity was measured by the modified method of Wu et al., 2009. Two hundred microliter of 10mg/ml fibrinogen solution prepared in Tris-HCl (pH 7.4) buffer containing 150 mM NaCl was incubated with 10 µg of purified keratinases at 37°C. After various time intervals, 60 µl of the reaction solution was withdrawn and analysed by SDS-PAGE. Plasmin from human plasma was used as a positive control.

**Plasminogen activating activity**

Plasminogen activating activity was determined by comparing the activity of recombinant keratinases on both plasminogen-free and plasminogen-rich fibrin plate. The fibrin agarose plate contained 1.2% agarose, 4.5 mg/ml fibrinogen and 0.45 U/ml thrombin. First, 20 ml of 1.2% agarose was layered on 35mm diameter petridish. After solidification, the rest of the contents were poured onto the agarose. The clot was allowed to set for 30 min at room temperature. For plasminogen-rich plates, 0.85 U and 4U of plasminogen were added to the above plates. Subsequently, wells were punched in the plates and 10 µg of purified keratinases were added to it. The plates were incubated at 37°C for 16h and plasminogen activating activity was estimated by measuring the dimension of the clear zone obtained.

**Identification of pro-sequence region of keratinase KP2**

*In silico analysis of keratinase KP2*

For multiple sequence alignment, prospective proteases to be aligned with KP2 were selected using the position specific PSI-BLAST algorithm, based on position specific scoring matrices obtained from the NCBI online portal (www.ncbi.nlm.nih.gov). Five proteases above the threshold E value were selected. 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. The alignment was, thereafter, assessed based on their amino acid conservation.
DNA Manipulations and Construction of Plasmids

Genomic DNA of *P. aeruginosa* KS-1 was isolated using Qiagen genomic DNA extraction kit. The nucleotide sequence of the keratinase KP2 gene (NCBI accession no. HM452163) from *P. aeruginosa* KS-1 was used to design the primers for PCR amplification. PCR was performed as described earlier. The DNA fragments encoding the full length and N-terminally truncated forms of KP2 were obtained by amplification from *P. aeruginosa* KS-1 genomic DNA using the primer pairs KP F1-R, KP F2-R, KP F3-R, KP F4-R, KP F5-R (Table 3.3). The PCR products were cloned as *EcoR*I/*BamH*I fragments into the corresponding sites of pEZZ-18 vector to generate pEZZ 18-KP F1, pEZZ 18-KP F2, pEZZ 18-KP F3, pEZZ 18-KP F4, pEZZ 18-KP F5. All the constructs were sequenced at the central instrumentation facility at University of Delhi, India.

**Table 3.3: Primers designed for N-terminal truncations of keratinase KP2**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP2F1</td>
<td>GAATTCGATGAAGAAGGTTTCT</td>
</tr>
<tr>
<td>KP2F2</td>
<td>GAATTCGGCCGACCTGAATC</td>
</tr>
<tr>
<td>KP2F3</td>
<td>GAATTCGGCGATCCGCAGC</td>
</tr>
<tr>
<td>KP2F4</td>
<td>GAATTCGAAGGCCCAGGGC</td>
</tr>
<tr>
<td>KP2F5</td>
<td>GAATTCGCCCGCGAGGGA</td>
</tr>
<tr>
<td>KP2R</td>
<td>GGATCCTTACAACGC</td>
</tr>
</tbody>
</table>

Expression and purification of the recombinant enzymes

To express the recombinant proteins, *E. coli* HB 101 cells harboring pEZZ 18-KP2F1, pEZZ 18-KP2F2, pEZZ 18-KP2F3, pEZZ 18-KP2F4, and pEZZ 18-KP2F5 were grown in LB medium supplemented with ampicillin (100 µg/ml) 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).

**Comparative biochemical characterization of complete and truncated KP2**

Full-length keratinase along with all its truncated forms were purified using Q-sepharose anion exchange chromatography. pH, temperature kinetics and substrate specificity of full length form (KP1) along with truncated forms (KP2, KP3, KP4 and KP5) were studied as described earlier. Further, steady state kinetics (K_m and V_max) on insoluble (casein and haemoglobin) and soluble (N-Suc-Ala-Ala-Pro-Phe-pNA and N-Suc-Ala-Ala-Pro-Leu-pNA) were determined.

**IV. Structural analysis of keratinases using in silico approaches**

Molecular characterization of both the keratinases revealed KP1 to share homology with putative aminopeptidase from *Pseudomonas aeruginosa* POA-1 and KP2 to share complete homology with pseudolysine of *Pseudomonas aeruginosa*. As pseudolysine is a well-characterized protein here structural characterization of only KP1 was carried out. Keratinase KP1 though shared homology with putative aminopeptidase but was found to have protease activity leading to keratinolytic property. Hence, through *in silico* approaches including homology modeling and docking analysis attempt has been made to explain its endopeptidase function.

**Protein translation and homology analysis**

The nucleotide sequence of keratinase KP1 which has been submitted to NCBI database under the gene accession number GenBank: ADP00719.1 was translated using online available expasy translate tool (http://web.expasy.org/cgi-bin/translate/dna_aa).
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The protein sequence of KP1 was submitted for PSI-BLAST analysis against Protein Database (pdb) using default parameters (Altschul et al., 1997). Two criteria were used for selection of target sequences i.e. proteins belonging to the same superfamily and sequence identity greater than 30%.

Multiple sequence alignment

The multiple sequence alignment of homology sequences identified by PSI-BLAST search against KP1 was performed by MultAlin (http://multalin.toulouse.inra.fr/multalin/) and ESPript (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). Catalytic residues using MSA were predicted based on available data about aminopeptidase from *Streptomyces grisues* (1CP7) (Gilboa et al., 2000).

Homology modelling

Three dimensional model of KP1 was built using MODELLOR 9.10 tool taking *Streptomyces* and *Aneurinibacillus* aminopeptidase as template. Further, energy minimization was done using YASARA force field (Krieger et al., 2009). Final model was allowed for PROCHECK (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) program for Ramchandran plot analysis. Functional validation was checked by superimposition of target and template via Pymol.

Binding pocket analysis of KP1

Pocket Finder tool was used to identify the most probable binding pocket of rKP1 (http://www.modelling.leeds.ac.uk/pocketfinder/). Superimposition of complete pocket of KP1 over *Streptomyces* aminopeptidase was carried out by Pymol. Next, superimposition of active site residues of both was also performed.

Binding mode of keratinase KP1

Docking analysis of KP1 with various substrate and inhibitors was carried out using online available PATCHDOCK tool (http://bioinfo3d.cs.tau.ac.il/PatchDock/). Docking of KP1 was performed with substrates of aminopeptidases i.e. leucine, methionine and inhibitors of proteases i.e. rivastigmine, N-Benzylhexoycarbonyl-L-serine-Betalactone and kazal. For docking of KP1 with kazal, Maestro 9.2 was using the following protocol:

Ligand preparation: PDB structure of kazal was retrieved from online available Protein Data Bank. Energy minimization of kazal was performed by Impact
module inbuilt in Maestro using OPLS2005 as force field anduncated Newton as algorithm. Minimized structure was used for further docking studies in maestro.

Protein preparation: Protein preparation wizard inbuilt in Maestro 9.2 package was used for protein preparation and bond angle and bond length energy minimization using default parameters and OPLS2005 as force field.

Grid Generation: Glide module was used for grid generation and docking study. Grid was set surrounding the predicted active site residues for KP1. Grid box was set at 25 Å as main box size and 14 Å as ligand diameter midpoint box size to set xyz coordinates.

Docking: Extra precision (XP) docking study was used done by Glide module inbuilt in Maestro9.2.

*Functional validation by site directed mutagenesis*

Based on the above results eleven residues namely His296, Asp308, Glu340, Glu341, Asp369, Met370, Tyr381, Arg422, Ser423, Tyr466, His467 were predicted to constitute the catalytic pocket of KP1. Among these, Glu340, Glu341 and Arg422 were directly involved in hydrogen bond formation with leucine and methionine in case of rKP1 and Tyr466 formed hydrogen bond with leucine in case of aminopeptidase from *Streptomyces griseus*. Thus, these residues were selected for functional validation. Further, three other residues namely His296, Ser423 and His467 were also mutated. Site directed mutagenesis was carried out individually for each residue mutating it to alanine using QuickChange™ Site Directed Mutagenesis kit (Stratagene). The list of primers used for site directed mutagenesis is given in Table 3.4. The mutants were confirmed by sequencing at central instrumentation facility, University of Delhi, South Campus.
Table 3.4: List of primers used for site directed mutagenesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu340 F</td>
<td>TGGTGGGGCGCCGCCGGAAGGCCCGCTG</td>
</tr>
<tr>
<td>Glu340 R</td>
<td>CAGGCGGCTTCGGCAGGCCGCCCCACCA</td>
</tr>
<tr>
<td>Glu341 F</td>
<td>TGGGGCGCCGCCAGGCCGCTGGTG</td>
</tr>
<tr>
<td>Glu341 R</td>
<td>CACCAGGCGCGCGCCTCGGGCGCCCCCA</td>
</tr>
<tr>
<td>Arg422 F</td>
<td>GAGATCGACTTCCGCTCAGACTACGCGCC</td>
</tr>
<tr>
<td>Arg422 R</td>
<td>GCCGTAGTCCGAGGCCGAAAGTCGATCTC</td>
</tr>
<tr>
<td>Tyr466 F</td>
<td>TACGACGAGTGCGCCACAGCAAGTCGCGCC</td>
</tr>
<tr>
<td>Tyr466 R</td>
<td>GCACTTGCTGTGGGCCGACTCGTCGTC</td>
</tr>
<tr>
<td>His296 F</td>
<td>ATGGTCCGCGCCGCTCCGACTCGACCTCGTC</td>
</tr>
<tr>
<td>His296 R</td>
<td>GACCGAGTCGAGGCCGCCGCGAGCAT</td>
</tr>
<tr>
<td>Ser423 F</td>
<td>ATCGACTTCCGCGCCGACTACGCCAG</td>
</tr>
<tr>
<td>Ser423 R</td>
<td>CTCGCGTAGTCCGCGCCGAGTCGAT</td>
</tr>
<tr>
<td>His467 F</td>
<td>GCCCTGGAGATCGCGCCAGCGAGCCCCGATCGTC</td>
</tr>
<tr>
<td>His467 R</td>
<td>CATGCGCGCTCGCGCATCGTCGACCGGC</td>
</tr>
</tbody>
</table>

Fluorescence spectroscopy and enzyme kinetics

Wild and mutant rKP1 were purified by Q-sepharose anion exchange chromatography as described earlier. Purified protein was dialyzed against pH 7 phosphate buffer to remove salt and concentrated upto 200µg/ml using speed vac (Eyla, Japan). Intrinsic fluorescence spectrum of all four mutant rKP1 was analyzed using Fluorospectrophotometer (RF 5301PC, Spectrofluorophotometer, Shimadzu,) at Department of Electronics, University of Delhi. Wavelength of 280nm was selected for the excitation of protein sample and emission spectrum was checked. Fluorescence spectrum of mutant rKP1 was compared with wild rKP1. Enzyme activity of wild and mutant rKP1 was compared on N-Suc-Ala-Ala-Pro-Phe-pNA as substrate using protocol described earlier.
V. Feather degradation by recombinant *E. coli*

Both the recombinant strains of *E. coli* HB101 harboring rKP1 and rKP2 were used to study feather degradation. *E.coli* HB101 harboring pEZZ18 only was used as a control strain.

*Feather degradation potential of recombinant E. coli*

Feather degradation by both recombinant strains was checked by growing overnight grown cultures of *E. coli* in LB-Amp medium containing 2% chicken feather at 37°C and 300 rpm. Degradation was checked after 24 h by dry weight method as described earlier. *E. coli* HB101 (with and without vector pEZZ 18) and *E. coli* ATCC 35421 were taken as controls. Protease activity using casein as a substrate and keratinase activity using chicken feather as a substrate was also checked in all the cases.

*Structural changes during feather degradation*

Scanning electron microscopy (SEM) of feather at different time intervals ranging from 0 to 18 h was performed for both recombinants alongwith control *E. coli* strains. Feather samples were withdrawn at time intervals of 0, 3, 6, 9, and 18 and fixation was done in 2.5% (v/v) glutaraldehyde and 2% (v/v) formaldehyde prepared in sodium phosphate buffer (0.1 M, pH 7.4) for 6-12h at 4°C. The fixed specimens were then transferred to phosphate buffer (50mM, pH 7.0). The feather samples were trimmed into 1.0-1.5mm thick pieces and put on stubs. The stubs were then gold-coated for 60sec with an SPI sputter coater (SPI supplies, West Chester, Pennsylvania). Samples were examined with a LEO 435VP SEM (Carl Zeiss NTS, GmbH, Germany) at an accelerating voltage of 30kV. All scanning studies were performed at Department of Anatomy, AIIMS, New Delhi.

*Is colonization pre-requisite for degradation by keratinase?*

Autoclaved feather was considered as un-colonized feather while for obtaining pre-colonized feather, 2% chicken feather in 50 ml LB-Amp medium was inoculated with control *E. coli* strain and incubated for 12 h at 37°C, 200 rpm. After 12 h, 0.2% sodium azide was added to arrest further growth of *E. coli*. Finally, feather was harvested and washed with Tris-HCl buffer (50 mM, pH 9.0) twice.
2% of both un-colonized and pre-colonized feather was suspended in 50 ml of Tris-HCl buffer (50 mM, pH 9.0). To this, 500 U of each keratinase were added and the flasks were incubated at 37°C, 100 rpm for 12 h. Feather degradation was also analyzed by dry weight method.

Availability of free sulfydryl group upon colonization was compared by DTNB assay. Un-colonized and pre-colonized feather were soaked in 10 mM DTNB (5, 5′- dithiobis-2-nitrobenzoic acid) for 10 min. Subsequently, feather were filtered through glass wool and free cysteinyl group was measured by taking absorbance at 420 nm against DTNB blank. Cysteinyl group concentration was calculated using molar extinction coefficient ($A_m$) of 1.4X 10^3.

**Feather degradation is a property of keratinases only**

The above experiment was repeated with the same units of a non-keratinolytic enzyme, trypsin (SRL, India) using pre-colonised feather. Feather degradation was observed visually and by dry weight method after 12 h at 37°C, 200 rpm.

**Involvement of free cysteinyl group during feather degradation by recombinant E. coli**

Feather degradation by recombinants was studied in LB-Amp medium containing 2% chicken feather as a function of DTNB concentration (5mM to 50mM). LB-Amp feather without DTNB was considered as control. Degradation was studied after a period of 12 h at 37°C, 300 rpm. Feather degradation was studied by dry weight method.

**Degradation of feather by E. coli GGT (γ-glutamyltranspeptidase) knockout recombinant strain**

pEZZ 18-rKP1/rKP2 were transformed into E. coli HB101 ggt⁻ knockout strain developed in the laboratory to obtain ggt⁻ recombinants (rKP1/rKP2 ggt⁻). Feather degradation was studied by these strains at 37°C, 300 rpm. These recombinants were grown in LB-Amp-feather medium for a period of 24 h to study colonization, degradation and keratinase production. E. coli ggt⁺ recombinants served as control.

**Effect of GGT-GSH redox on keratinase activity**

Activity of recombinant keratinases was measured in presence and absence of GGT-GSH (γ-glutamyltranspeptidase-glutathione) as a source of redox. Purified GGT
of *E. coli* HB101 was available in the laboratory. Standard keratinase assay was performed with slight modifications. Experiment was carried out in two sets:

**Set 1: Single step**

A. 20mg chicken feather + 1 ml of appropriately diluted keratinase + Tris-HCl buffer (pH 9.0)
B. 20mg chicken feather + 1 ml of appropriately diluted keratinase + 100 µl of appropriately diluted GGT + 20mM GSH + Tris-HCl buffer (pH 9.0)
C. 20mg chicken feather + 1 ml of appropriately diluted keratinase + 100 µl of appropriately diluted GGT + Tris-HCl buffer (pH 9.0)

**Set 2: Two step**

A. 20 mg chicken feather + appropriately diluted GGT + GSH incubated for 1h at 37°C followed by addition of 1mM azaserine (pre-treated feather).
B. Appropriately diluted keratinases added to A (pre-treated feather)

All the sets of experiments were incubated at 50°C for 1h. The reactions were terminated by addition of 5% (w/v) TCA followed by centrifugation at 8000 rpm for 10 min. Finally protein release was measured by absorbance at 280 nm.

**VI. Degradation of Sup 35NM by keratinases**

The major objective of this section was to achieve complete degradation of surrogate yeast prion protein (Sup 35NM) using rKP1 and rKP2 under ambient conditions (37°C, pH 7.0). Sup 35NM was overexpressed and purified as described earlier in Section I. The degradation studies were done by western blot analysis.

**Western blot analysis**

N-region of Sup 35NM is responsible for its aggregation and infectivity. Peptide of this region (Ac-QGGYQQYNDAGYQ-amide) was synthesised from Techno Concept (India). It was used to generate anti-Sup35NM polyclonal anti-peptides against amino acids 55-68 (anti-N) at Imegenex (Orissa, India). Sup35NM was run on 15% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). The membranes were blocked in 3% bovine serum albumin for 1 h in PBS buffer. Primary antibody (1:5000) was added and the membrane was incubated for 1 h at mild shaking, then washed three times for 10 min with PBS buffer with 0.1% Tween 20.
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(PBST). Secondary anti-rabbit antibody (1:5000) conjugated with alkaline phosphatase was then added, and the membrane was incubated for 1 h. The membrane was again washed three times with PBST. The proteins were visualized by addition of 1 ml developer i.e. substrate for alkaline phosphatase (Promega, USA).

Degradation of Sup 35NM using different concentration of keratinases

50µg of purified Sup 35NM was incubated with different concentrations of rKP1 and rKP2 (2-10 µg) in pH 7.0 buffer (Tris-HCl, 50 mM) at 37°C for 18h. After incubation, the enzyme was inhibited by addition of 10 mM PMSF and degradation of Sup 35NM was analysed using western blot.

Effect of additives on degradation of Sup 35NM using rKP1 and rKP2

Various additives such as Triton X-100, Tween 80, SDS, sodium carbonate and β-mercaptoethanol were added to the reaction mixture containing 50µg Sup 35NM and 10 µg of rKP1/rKP2 at a final concentration of 0.5% and degradation was checked after 18h by western blot analysis. rKP2 completely degraded Sup 35NM in presence of SDS and thus it was selected for further studies.

Next, the reaction time of degradation of Sup 35NM by rKP1-SDS was checked from 15 min to 60 min and the concentration of SDS was standardised by varying it from 0.05% to 0.5%. All reactions were performed at 37°C, pH 7.

Destabilization of Sup 35NM in presence of SDS

To study the effect of SDS on destabilisation of Sup 35NM, conformational changes induced upon their co-incubation were monitored by circular dichroism (CD). 0.1% (v/v) SDS was incubated with Sup35NM and secondary structure changes were analyzed using Jasco-715 (Jasco Inc., Easton, MD, USA) till 20 min. The CD spectrum of each sample was recorded at 30°C, and for each CD spectrum, three scans were accumulated using a step resolution of 1nm and bandwidth of 1 nm, response time of 2 s, a scan speed of 100nm.min, and a high sensitivity. The CD spectrum of the buffer was subtracted from that of each sample to eliminate interferences from the optical equipment.

Comparative analysis of degradation of Sup 35NM

The efficiency of the formulation was compared with proteinase K in presence and absence of SDS. 50 µg of Sup 35NM was digested with 10 µg of each enzyme at
37°C, pH 7 for 15 min. In one set, the reaction was supplemented with 0.1% SDS. The digestion was followed by SDS gel electrophoresis and western blot analysis.

**Degradation of Sup 35NM by rKP2-GGT-GSH combination**

Enzymatic degradation of 50 µg of Sup 35NM was checked at 37°C, pH 7 for 15 min under the following conditions:

- Sup 35NM + rKP2
- Sup 35NM + rKP2 + GSH
- Sup 35NM + rKP2 + GSH + GGT
- Sup 35NM + Trypsin + GSH + GGT
- Sup 35NM + rKP2 + GSH + GGT + DTNB

10µg of rKP2, 5U of GGT (γ-glutamyl transpeptidase), 20 mM GSH (glutathione) and 50 mM DTNB (5, 5'-Dithio-bis (2-nitrobenzoic acid) was used for the above experiment. After 15 min, the enzyme was inhibited by addition of 10 mM PMSF and degradation of Sup 35NM was analysed using western blot.

Another set of reactions was set up with 10µg of proteinase K (PK) as a positive control under the following conditions:

- Sup 35NM + PK
- Sup 35NM + PK + GSH
- Sup 35NM + PK + GSH + GGT