CHAPTER 6

Cloning, expression and characterization of keratinolytic protease from Bacillus subtilis P13

Prediction is very difficult, especially about the future.
- Niels Bohr
**6.1 Introduction**

The alkaline proteases from bacteria have been intensively studied. They mainly come from *Bacillus sp.*, such as *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus* and *B. alcalophilus*, and the representative is subtilisin from *B. subtilis*. Genes encoding alkaline proteases have been cloned, sequenced and over-expressed (Jacob et al., 1985; Koide et al., 1986; Jang et al., 1992; Jorgensen et al., 2000; Nakamura et al., 1992). Subtilisins are encoded by aprE gene encoding of 275 amino acid long polypeptide. The expression of bacillar genes is controlled by complex regulatory system, which includes specific regulatory proteins (spoO, AbrB, CepA, DegU, sinR, Hpr and others). Most of the species of Bacilli secrete proteases with two maxima of activity, one in the early (24hr of growth) and the other in late (44hr of growth) stationary phase. Many of the genes of the adaptive response pathway are responsible for the sporulation, but the selection of the pathway, depends on the availability of nutritional sources (Fig. 6.1).

![Fig. 6.1: Network of the different genetics pathways related to cell differentiation in *Bacillus subtilis*. Genes related specifically for each differentiation process are located within the specific frame (Lopez et al., 2008)](image_url)

Many regulatory factors influence aprE expression, acting via 5' regions in cis. In some cases, direct contact with aprE DNA has been demonstrated, and target sites defined, e.g., the transition-state regulator Hpr, affecting subtilisin production negatively interacts. The main reported regulators that control AprE production are DegU/DegS (Henner et al., 1988b; Kunst et al., 1988; Msadek et al., 1990), AbrB
Chapter 6: Cloning, expression and characterization of keratinolytic protease from Bacillus subtilis P13

(Strauch et al. 1989), Hpr (also known as ScoC) (Kallio et al.1991), and SinR (Gaur et al., 1991; Mandic-Mulec et al.1992; Bai et al. 1992). Furthermore, direct binding of AbrB, Hpr and SinR to the aprE regulatory region has been demonstrated by in vitro gel retardation assays and/or footprinting analysis (Strauch et al., 1989; Gaur et al., 1991; Kallio et al., 1991). SinR binds to the aprE promoter at a region between 217 and 263 bp with respect to the transcription start point (Gaur et al., 1991). Furthermore, it is known that high levels of SinR repress production of extracellular proteases and the expression of an aprE-lacZ fusion and is generally assumed that SinR is a repressor directly acting on aprE (Gaur et al., 1986, 1991).

The LCMS analysis and MASCOT search revealed the similarity of keratinolytic serine protease produced by B. subtilis P13 to nattokinase (Section 5.3.11). Nattokinase is an enzyme, (E.C. 3.4.21.62), produced by B. subtilis (natto) isolated from natto, a fermented soybean Japanese food. Nattokinase has potent fibrinolytic activity and has attracted worldwide attention because of its health benefits, such as reducing blood cholesterol level, decreasing blood pressure, preventing atherosclerosis and inhibiting osteoporosis (Yokota et al., 1996; Tsukamoto et al., 2000; Milner and Makise, 2002). Three main mechanisms for the breakdown of fibrin by nattokinase have been suggested (Milner and Makise, 2002). Action of nattokinase was demonstrated in two stages; consist of direct degradation of fibrin strands, followed by the activation of urokinase, enhancing the transformation of plasminogen to plasmin which then breaks down fibrin. Third, nattokinase enhances the production of tissue plasminogen activator that promotes the transformation of plasminogen to plasmin, thus fibrin is degraded (Milner and Makise, 2002).

Recently, many fibrinolytic enzymes have been identified from different fermented soybean foods, such as subtilisin DFE (Peng et al., 2003) and subtilisin FS33 (Wang et al; 2006) from douchi in China, subtilisin DJ-4 from doen-jiang (Koide et al., 1986), and subtilisin CK 11-4 from chungkook-jang (Kim et al., 1996) in Korea. It was reported that nattokinase action could be enhanced and prolonged in the plasma when it was taken orally (Sumi et al., 1990; Suzukhi et al., 2003).

Since E. coli has a short generation time, an established fermentation procedure, a high production of foreign proteins, it has become the most widely used prokaryotic organisms for recombinant protein production. Cloning and heterologous expression studies carried for many proteases find application in pre-tanning, especially in dehairing. DHAP, dehairing alkaline protease, produced extracellularly by Bacillus
**Chapter 6: Cloning, expression and characterization of keratinolytic protease from *Bacillus subtilis***

*pumilus* UN-31-C-42 comes from a wildtype strain BA (06), which was isolated from biological waste in Cheng Du, P. R. China was purified, the gene cloned and its expression in *E. coli* and *B. subtilis* WB60 (Pan et al; 2004).

Subtilisins are synthesized as preprosubtilisin precursors consisting of a typical signal peptid of 29 residues followed by the propeptide of 77 residues and then a mature subtilisin segment of 275 residues (Vasantha et al., 1984; Stahl and Ferrari, 1984). When the DNA sequences coding for the subtilisin signal peptide have been fused to the gene encoding the mature protein A from *Staphylococcus aureus* (Vasantha and Thompson, 1986) and to the TEM β – lactamase derived from pBR322 (Wong et al., 1995), then the resulting fusion proteins were processed by signal peptidase, and the mature products were secreted into external medium by *B. subtilis*. But when the OmpA (*E.coli* outer membrane protein) signal peptide was directly fused to the mature subtilisin sequence (without the propeptide), protease activity was not detected (Ikemura et al., 1987). So propeptide is essential for the correct folding of the enzyme and not for the release from the plasma membrane (Ikemura and Inouy, 1988). Liang et al., (2008) reported a nattokinase producing bacterium, *B. subtilis* YF38, was isolated from douchi, using the fibrin plate method. The gene encoding this enzyme was cloned by polymerase chain reaction (PCR). Cytoplasmic expression of this enzyme in *E. coli* resulted in inactive inclusion bodies. But with the help of two different signal peptides, the native signal peptide of nattokinase and the signal peptide of PelB, active nattokinase was successfully expressed in *E. coli* with periplasmic secretion, and the nattokinase in culture medium displayed high fibrinolytic activity. Cloning and over expression of a keratinolytic protease having potential tannery applications from *B. subtilis* P13 and the exploration of the proposed fibrinolytic activity of the protein are described in the current chapter.
6.2 Materials and Methods

6.2.1: Bacteria and strains of plasmid used

Table 6.1 shows the plasmids used in the study along with the bacterial strains and transformants used for the cloning and over-expression of keratinolytic protease from *B. subtilis* P13. All bacteria were routinely grown in Luria-Bertani (LB) medium. Solid medium was prepared by addition of 2.5% agar to LB broth. Ampicillin was used at a concentration of 100μg/ml for the cultivation of *E. coli* DH5α containing pJET1.2 clones, while 50 μg/ml ampicillin was added for the growth of *E. coli* strains bearing pET22b(+).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus subtilis</strong> P13</td>
<td>Lab isolate from Vrajeswari hotspring, Mumbai, India. Moderately thermophilic with a growth optima of 40°C</td>
<td>Present work (Pillai and Archana, 2008)</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 DlacU(f80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi 1 relA1</td>
<td>Lab. stock (Sambrook and Russell, 2001)</td>
</tr>
<tr>
<td>BL-21(DE3)</td>
<td>F-ompT gal(dcm)(lon)hsdS8(θB-θB-θB) an E.coli B strain )with DE3,a λ prophage carrying the T7 polymerase gene</td>
<td>Lab. stock (Sambrook and Russell, 2001)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJET1.2 (Fig. 6.2)</td>
<td>Blunt ended cloning vector with Ampicillin as the selection marker and a lethal gene eco471 enabling positive selection.</td>
<td>MBI Fermentas</td>
</tr>
<tr>
<td>pET22b(+) (Fig. 6.3)</td>
<td>N-terminal pelB signal sequence and an optional C-terminal 6X His Tag with Ampicillin as a selection marker. No blue white selection</td>
<td>Novagen</td>
</tr>
</tbody>
</table>
Chapter 6: Cloning, expression and characterization of keratinolytic protease from *Bacillus subtilis* P13

**Fig. 6.2**: Vector map of pJET1.2

**Fig. 6.3**: Vector map of pET22b (+)
6.2.2: Molecular biology techniques

Media constituents, agar and agarose were purchased from Hi-Media Pvt. Ltd. India. Inorganic salts, buffers and other chemicals were of analytical grade. Restriction enzymes and PCR reagents like dNTPs, *Pfu* and *Taq* polymerases were purchased from Bangalore Genei, Bangalore, India. Oligonucleotide primers were procured from Sigma–Aldrich, USA.

6.2.2.1: Genomic and plasmid DNA isolation

Genomic DNA extraction from *B. subtilis* P13 was carried out by CTAB method (Sambrook and Russell, 2001). The culture pellet of 12h old culture was resuspended in 68 μl of Tris.EDTA buffer, pH 8.0 (10:1) and 2 mg lysozyme was added followed by incubation at 50°C for 1 h. Protinase k, 3 μl from 100μg/ml stock and 10 μl of 10% sodium dodecyl sulfate(SDS) and of was added and incubated at 50°C for another 1 h. After incubation.100 μl of 5M NaCl and 16 μl of 10% hexadeccyltrimethyl ammonium bromide(C-TAB) were added and incubated at 65°C for 10 min. The solutions were then extracted with an equal volume of phenol:chloroform: isoamylalcohol (25:24:1) followed by extraction using chloroform:isoamylalcohol (24:1). Double the volume of ice-cold ethanol was added to precipitate nucleic acids. After centrifugation at (12,000 x g for 5 min at RT).the pellets were washed with 70% ethanol, air dried and dissolved in 30μl of autoclaved distilled water at 65°C for 10 min.

Plasmid Preparation from *E.coli* both miniscale and maxiscale were obtained by alkaline lysis method described by Sambrook and Russel, 2001.

6.2.2.2: Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% agarose (containing 1μg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer at 5v/cm for 0.5-2h. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator (Alpha Imager HP).
6.2.2.3: Restriction enzyme digestion analysis

DNA samples (0.5-1.0μg) were used for each restriction enzyme digestion. 1-3U of the restriction endonuclease (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10μl. The reaction mixture was incubated overnight at 37°C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and were subsequently photographed. In case of double digestion, a compatible buffer for the two REs was essentially checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers.

6.2.2.4: Ligation

The ligation reaction was usually done in 10μl volume containing the following constituents: Purified vector and insert DNA (volume varied depending on the respective concentrations); 10X T4 DNA Ligase buffer, 1μl; T4 DNA ligase (MBI Fermentas), 0.5-1.0U and sterile double distilled water to make up the volume. The cohesive end ligation reaction was carried out at 15°C for 12-16h. The vector to insert molar ratio (molar concentrations calculated by the under mentioned formula) of 1:4 was maintained, with a total of 50-100ng of DNA in each ligation system.

\[
p\text{moles of DNA} = \frac{\text{Amount of DNA (μg)} \times 1,515}{\text{Size of DNA fragment (No. of base pairs)}}
\]

6.2.2.5: Gel elution and purification

From the sample electrophoresed on 0.8% agarose gels, the DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band The PCR products were purified using the tube in tube protocol described by Sambrook and Russell, (2001). The homemade Sephadex G-10 spin columns consist of a 2 ml and a 0.5 ml micro centrifuge tube without caps and a tiny bit of sterilized glass wool to make such a spin column. A needle or a pin, was heated with a Bunsen burner, the heated needle was lightly stabbed into the bottom of a 0.5 ml micro centrifuge tube to make a small hole, no bigger than 1 mm. The 0.5 ml tube was put into a 2 ml tube to form a homemade spin column. The band(s) of interest were cut out with a clean surgical blade under long wavelength UV illumination. The gel slice was transferred into the small tube and the gel slice was
smashed against the tube. The column was centrifuged for 10 minutes at the maximum speed of the bench-top micro centrifuge at 12,000xg. The DNA solution was transferred to a clean 1.5 ml tube, 1/10 volume of 3 M sodium acetate (pH 5.2) was added and double the volume ice-cold ethanol was used to precipitate the DNA. The DNA pellets were washed twice with ice-cold 70% ethanol and the DNA was dissolved in autoclaved distilled water (D/W). The eluted DNA was analysed on 1% agarose gel.

6.2.2.6: Transformation of E. coli

Plasmid transformations of E.coli strains were carried out by preparing competent cells using CaCl₂ method and heat shock method was used as mentioned by Sambrook and Russell, 2001. Before transforming the main ligated product, the purity of the competent cells was checked. A mock transformation was performed with known concentration of p BlueScript (pBS) and the efficiency of transformation was calculated. The ligated product was transformed only if the efficiency was ~ 10⁵ transformants/μg of DNA.

6.2.2.7: SDS-PAGE

SDS-PAGE slab gel electrophoresis was carried out using 12% acrylamide gel by following the procedures described by Sambrook and Russell, (2001). After electrophoresis the gel was stained using the staining solution (Appendix I) for about 1h and then de-stained with de-staining solution (Appendix I) by incubating overnight under mild shaking conditions. Result was recorded by direct scanning of gel.

6.2.2.8: Polymerase Chain Reaction (PCR)

The chromosomal DNA from B. subtilis P 13 was used as a template for the PCR. PCR amplifications were performed in thermocycler, (Applied biosystem, AB: 2720). The amplification reaction was carried out in a total volume of 25μl containing 10X Pfu buffer, 0.8mM dNTPs, 1μl of each primer from 20 pmoles stock, approximately 20 ng of genomic DNA and 1.5 U of Pfu Polymerase. The PCR conditions were as follows: initial denaturation at 95°C for 5min; 30 cycles of denaturation at 94°C for 30s, annealing at 45°C for 30s followed by an extension at 72°C for 1:30s; final extension at 72°C for 10 min.
Chapter 6: Cloning, expression and characterization of keratinolytic protease from Bacillus subtilis P13

The first set of primers, NK1 and NK2 (Table 6.2) were used to validate the similarity of keratinolytic protease from B. subtilis P13 obtained by LC-MS analysis as nattokinase. To express nattokinase in E.coli, expression vector was constructed using the PCR amplified product using primers NK3 and NK4 (Table 6.2) having unique restriction sites of BspHI and BamHI on forward and reverse primers respectively. The amplification of coding sequence of nattokinase was carried out by PCR in a total volume of 25μl and Pfu polymerase. The PCR conditions were similar, except the annealing condition of 50°C for 30s.

Table 6.2: Details of primers used

<table>
<thead>
<tr>
<th>Primer details</th>
<th>RE site (underlined nucleotides)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK1: 5’-GCGGTCGACGTATGAAAAATAGTTA</td>
<td>EcoRI</td>
<td>Xiaobo et al, 2007</td>
</tr>
<tr>
<td>NK2: 5’-GTAGAATTCTCCGGTGCTTGTGAA</td>
<td>SalI</td>
<td></td>
</tr>
<tr>
<td>NK3: 5’ATTCATGACGAAAAAGCAGTACAGAA</td>
<td>BspHI</td>
<td></td>
</tr>
<tr>
<td>NK4: 5’GTACAAGCAGCTGACAATCGGATCCGATA</td>
<td>BamHI</td>
<td></td>
</tr>
</tbody>
</table>

6.2.2.9: Sub-cloning of nattokinase like protease in pET22b+

Since the protease gene to be cloned was having an internal site of NcoI, BspHI, a restriction enzyme which produces compatible ends to NcoI, was incorporated in the forward primer. These primers are reported to amplify the 1100 bp coding region of nattokinase (Xiaobo et al., 2007). The amplified fragment of 1100bp was initially cloned into blunt cloning vector pJET 1.2. Insert of expected size was released from pJET-Kpr using vector specific enzyme BglII and digested with BspHI and BamHI. Subsequently, the DNA fragment of pET22b was digested with NcoI and BamHI and subcloned into pET22b+. The construct pET-AJ-Kpr carries only the propeptide and mature enzyme gene under the control of the T7 promoter and lac operator. The pelB in the vector pET22b+ codes for signal peptide and assist the secretion of the expressed protein to periplasm. The pJET1.2 clones and pET22b (+) clones were confirmed by using the gene specific primers. The PCR cycling conditions were kept the same as described in Section 6.2.2.8. The 1.1Kb amplicon was analysed on 1% agarose gel. The sequence analysis of the selected clone was carried out at the
facility available at Bangalore Genei Ltd. The construct was further transformed into protease null strain of E.coli, *Escherichia coli* BL-21 (DE3) for expression analysis.

**6.2.2.10: Expression of keratinolytic protease protein**

*Escherichia coli* BL-21 DE3 cells harbouring the expression construct were grown to an OD of approximately 0.4 to 0.5 at 600nm. Gene expression was induced by treatment with 0.7 mM isopropyl-1-thio-L-D-galactopyranoside (IPTG) and cells were further incubated for 18 h at 25°C. The cells were centrifuged to remove the supernatant and washed with sterile Phosphate Buffered Saline (PBS). PBS 1M solution of pH 7.4 contain 137mM NaCl, 2.7mM KCl, 10mm Na₂HPO₄ and 2mM KH₂PO₄. Cells were stored as frozen pellets at -20°C. The thawed cells were disrupted by sonication in 10mM PBS, centrifuged at 12,000xg for 10 min. The supernatant was collected and mixed with 5X gel loading dye, boiled for 2-3 min and analysed on 12% SDS-PAGE for the expression of recombinant protein with against un-induced cultures. Protein estimation was done by measuring absorbance at 280nm against BSA standard.

**6.2.3: Nattokinase/ Fibrinolytic activity**

Fibrin plate was used to determine the enzyme activity, which was described by Choi and Kim, (2001), with some modifications. Five millilitres of 6mg/ml(w/v) fibrinogen solution in a 50 mM Tris-Cl buffer (pH 7.5), 5 ml of 2.5% (w/v) agarose solution and 0.1 ml of thrombin solution (1mg/ml) were mixed well in a petri dish and was placed at room temperature for 1 h, to allow the thrombin to convert fibrinogen to fibrin. The culture supernatants were diluted appropriately and 10µl of the diluted supernatant was dropped into a well made in the plate and incubated for 18 h at 37°C. The fibrinolytic activity was observed by measuring the diameter of the clear zone.

Fibrinolytic activity was quantified according to the following protocol (Sigma protocol). Fibrinogen (0.4ml of 6mg/ml) and thrombin 0.1ml of (1mg/ml) were mixed and incubated at 37°C for 10 min under shaking to form the fibrin. A 0.1ml aliquot of appropriately diluted enzyme was added to the above mixture and whole cocktail was incubated at 37°C for 20 minutes. The enzyme reaction was
stopped by adding 0.4ml of 10% trichloro acetic acid (TCA). The enzyme blank was prepared by adding TCA to the mixture prior to the addition of the enzyme. The difference in absorbance at 275nm for test and blank were observed. The standard Urokinase Enzyme Solution was diluted in ice cold 50mM Tris.C.l (Immediately before use, prepare a solution. One unit will activate that amount of porcine plasminogen which will produce an A275nm of 1.0 per ml per minute at pH 7.5 at 37°C, when measuring perchloric acid soluble products from a-casein (1 cm light path). Urokinase from Win medicor, NewDelhi, India was used as the standard.

6.2.4: Purification and characterization of cloned keratinolytic protease

Over expressed enzyme after IPTG induction was recovered by cell lysis as mentioned in Section 6.2.2.10. The cells were separated by centrifugation (12,000g for 15 min at 4 °C) and the supernatant was concentrated by ultrafiltration (MW cut off, 50 kDa, Amicon,Millipore Inc.USA) and charged on casein-affinity column and purified in the similar manner as described in section 5.2.1. The purified enzyme was evaluated for caseinolytic and keratinolytic activity, preference to synthetic peptides, hide depilation and hydrolysis of the milled feather as described in sections 2.2.10 and 2.2.12.

6.2.5: Modelling and phylogenetic analysis of cloned protease

Translated Protein sequence of protease obtained from gene sequencing, was subjected to BLASTP with PDB database to search homologs. Structure of protease was modeled with the best match using MODELLER 9. Modeled structure of cloned keratinolytic protease as validated with Ramachandran plot using PROCHECK and was used to determine structural features of the protein. Protein sequence of protease from *B. subtilis* P13 was subjected to BLASTP to search homologs for multiple sequence alignment. 50 homologs were taken and multiple sequence alignment and phylogenetic analysis was done using phylogeny.fr server.

6.3: Results

6.3.1: PCR amplification of keratinolytic protease from *B. subtilis* P13

The protease gene showing homology to nattokinase was amplified by PCR using primers NK1 and NK2 from the total DNA of the isolated bacterium *B. subtilis* P13.
A single band of about 1,500 bp (Fig. 6.4a) was obtained. pJET1.2 vector which is an advanced positive selection system for the highest efficiency cloning of PCR products generated with *Pfu* DNA polymerase. This vector pJET1.2 contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site (Fig. 6.2), resulting the propagation of cells only with recombinant plasmids. The PCR product was purified by tube in tube method and cloned in pJET1.2 vector and cloned in pJET1.2. Ten colonies were randomly selected and were further analysed by digesting with with Bgl II sites for which flank the insert and 1.5Kb fragment was released (Fig. 6.4b). Fig. 6.5 shows the map of the recombinant plasmid, pJET-AJ-Kpr. A total of 30 colonies were obtained after cloning in pJET1.2 which indicated the putative clones Confirmation of the selected clone (Fig. 6.4c) was carried out using Primers NK1 and NK2 (Fig. 6.5) and was sequenced at the sequencing facility of Exceleris Ltd. Ahemadabad, India.. RE map of the cloned gene is depicted in Fig. 6.6. The sequenced DNA was analyzed via BLAST at NCBI, which revealed that the obtained DNA sequence exhibits 100% homology with the subtilisin NAT (aprN) gene (Appendix VI & VII) (Nakamura et al., 1992). The sequence revealed one open reading frame (ORF) of 1,143 bp, with the start codon ATG.

---

**Fig. 6.4 Cloning of keratinolytic protease gene from *B. subtilis* P13.**

a. PCR amplification of protease gene using NK1 and NK2; b. RE digestion of pJET-AJ-Kpr clone with vector specific enzyme BglII; c. PCR one confirmation of putative clone from blunt ended cloning in pJET vector. Lane1 in Fig. 6.4a&b: 500bp ladder and lane.2: 1.5 kb amplicon and vice versa in Fig. 6.4b
Chapter 6: Cloning, expression and characterization of keratinolytic protease from Bacillus subtilis P13

Fig. 6.5: Map of pJET AJ KPr construct harbouring nattokinase like keratinolytic protease gene from B. subtilis P13

Fig. 6.6: Primer binding sites and RE map of PCR amplified gene for nattokinase like protease from B. subtilis P13

PCR amplification of coding region of keratinolytic protease gene having homology to nattokinase (1100bp) was carried out from the genomic DNA of B.
subtilis P13 using primers NK3 and NK4 (Fig. 6.7a). Amplicon was cloned in blunt end cloning vector pJET 1.2 and putative clones were confirmed by the insert release of 1.1Kb using vector specific RE,BglII (Fig. 6.7b). Clone pJET –AJ-Kpr4 was sequenced and the sequence deposited at the NCBI gene bank (accession #:JF921199). On BLAST analysis, the cloned gene revealed 100% homology to aprN gene, which codes for a fibrinolytic enzyme (Appendix VIII and IX). The deduced protein of the ORF contained 381 amino acids. The sequence of the protein-coding region of this fibrinolytic enzyme was found to include a 29-residue signal peptide, a 77-residue propeptide, and a 275-residue mature enzyme.

Fig. 6.7: PCR amplification of clones of coding region of nattokinase (a) and confirmation of putative Keratinolytic protease by BglIII digestion (b)

6.3.2 Sub-Cloning of pJET Kpr harbouring the coding region of nattokinase like protease in pET22b (+)

Cloning of gene fragment of B. subtilis P13 protease in pET22b(+) was carried out for its expression in E.coli under the strong T7 promoter at the NcoI and BamHI sites in the MCS. The map of the recombinant construct pET-AJ-Kpr is depicted in Fig. 6.8. The recombinant protein is expected to have a 6X His tag at the N-terminus. The vector was digested with Nco I and BamHI (Fig. 6.8). Since the gene has an internal site for NcoI, (Fig. 6.5) BspHI an enzyme produces compatible ends with NcoI was used for
cloning. Insert was digested BspHI and BamHI (Fig. 6.9) and ligation in to digested pET22b (+) yielded 30 putative clones. Thirty putative clones obtained from the pET22b (+), plasmids were extracted and analysed by using two approaches, digestion with XbaI and Hind III, expecting the the release of of 1.1Kb insert. Except in five cases, two fragments of around 700 and 500bp. were obtained on the gel, (Fig. 6.10). The reason for the above said restriction pattern was the presence of HindIII site at the 447th position (Fig. 6.5) of the cloned NK gene. Clones were also further confirmed by PCR using gene specific primers NK3 and NK4 (Fig. 6.11). An amplicon 1.1Kb confirmed the clones.

---

**Fig. 6.8: Map of the pET 22+ construct, pET-AJ-Kpr harbouring protease gene**
Fig. 6.9: pET 22b (+)plasmid preparation (a) and digestion and linearization (b)
Linearized plasmid from lane 2 was used for subcloning

Fig. 6.10. Double digested (BspHI and BamHI) pET-AJ- Kpr.
Insert released after the digestion with BgIII was subjected to double digestion.
Lane.1:500bp ladder and lane.2 : insert released
Chapter 6: Cloning, expression and characterization of keratinolytic protease from Bacillus subtilis

Fig. 6.11: RE digestion of putative clones using XbaI and Hind III, showing the release of two fragments of 460 and 640 bp.

Fig. 6.12: PCR confirmation of selected clones.
Clones from lane # 7,8,12 and 13 of fig.6.11 are considered as pET-AJ-Kpr1,2,3 and 4 respectively.

6.3.3: Expression analysis keratinolytic protease in construct pET-AJ-Kpr
The transformants were called as pET-AJ-Kpr series. The protease expression level of clone pET-AJ-Kpr1,2 and 3 were compared with vector control. All 3 clones showed the presence of a 40 kDa band in induced cultures in contrast to vector control and un-induced culture (Fig. 6.13). thus it indicated that cloned protein was expressed in these clones. pET-AJ-Kpr3 clone in lane 4 was selected for sequencing and further characterization of the protein (Fig. 6.14). Functional
analysis based on caseinolytic activity carried with pET-AJ-KPr3. No protease activity was observed in the extracellular fractions of the clones after induction, activity with respect to different duration of induction was depicted in Fig. 6.15. Nil activity was recorded for induced vector control. The molecular weight of the over expressed protein was around 40kDa. The protein band of about 40 kDa appeared in the intracellular lysate, which might be the unprocessed nattokinase containing the signal peptide PelB, the propeptide and the mature peptide of nattokinase according to the deduced molecular weight.

The N-terminal sequence of the secreted protein was confirmed as AQSVPYGISQ by Edman degradation, and LC-MS analysis of the purified protein. The first 10 aminoacid of the of the over expressed protein on translation (from 45th-55th aminoacids) (Appendix X.) showed 100% homology with the N-terminal sequences of the processed extracellular keratinolytic protease of B. subtilis P13. First 44 Sequences were showing exact homology to the reported propeptide sequence of subtilisin-nattokinase (Appendix XII).

![Fig. 6.13: SDS PAGE of NK clones on IPTG induction](image)

Lane.1,2 and 4: IPTG induced pET-AJNK Lane.3. uninduced pET-AJ-NK;Lane.5: vector control induced;Lane.6: vector control uninduced and Lane7: MW marker (medium range)
Chapter 6: Cloning, expression and characterization of keratinolytic protease from Bacillus subtilis P13

Fig. 6.14: pET-AJ-Kpr 3: clone selected for sequencing
Lane.1 IPTG induced; lane.2: uninduced and lane.3: high Mw marker

Fig. 6.15: Protease expression levels of pET-AJ-Kpr3 during different durations of IPTG induction

6.3.4: Purification and characterization of keratinolytic protease cloned from B. subtilis P13

Cloned keratinolytic protease from E.coli BL21DE3 was prepared in the crude form as mentioned in Section 6.2.4 and concentrated by ultrafiltration. The filtrate from 50KDa cut-off was purified to 69.4 fold with a 41.13% recovery by casein affinity chromatography. Purification details are summarised in Table 6.4. SDS-PAGE analysis and gelatin zymogram of the purified protease is depicted in Fig.6.16 and 6.17.
respectively. A band of proteolysis corresponding to 40kDa was observed on the zymogram (Fig. 6.17).

Table 6.4: summary of purification of recombinant keratinolytic protease

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Fold Purification</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>26.5</td>
<td>90</td>
<td>0.294</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Ultrafiltration (50kDa filtrate)</td>
<td>22.2</td>
<td>8</td>
<td>2.775</td>
<td>9.4</td>
<td>83.7</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>10.9</td>
<td>0.434</td>
<td>25.1</td>
<td>85.4</td>
<td>41.13</td>
</tr>
</tbody>
</table>

Fig. 6.16. Purification profile of the cloned and expressed protein by casein affinity chromatography

(A): Different fractions from column chromatography

(B): Pooled purified fraction showing a protein band of 40kDa
6.3.5: Biochemical properties of cloned keratinolytic protease

Caseinolytic, keratinolytic, collagenase activity and affinity towards synthetic peptides were compared for crude, purified, over expressed and purified enzyme from *B. subtilis* P13 (Table 6.5). Other biochemical parameteres like temperature optimum and pH optimum was also performed the biochemical identity of the overexpressed protein sequence.

Table 6.5: Comparison of the activity of crude, purified and cloned keratinolytic and protease from *B. subtilis* P13

<table>
<thead>
<tr>
<th>Activity (U/mg protein)</th>
<th>Culture supernatant from <em>B. subtilis</em> P13</th>
<th>Purified protease from <em>B. subtilis</em> P13</th>
<th>Lysate from <em>E.coli</em> BL21 (vector control)</th>
<th>Overexpressed protease from <em>E.coli</em> BL21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caseinolytic</td>
<td>0.6± 0.056</td>
<td>4.5±1.06</td>
<td>ND*</td>
<td>5.3±0.987</td>
</tr>
<tr>
<td>Keratinase</td>
<td>0.51±0.064</td>
<td>5.1±2.10</td>
<td>ND*</td>
<td>5.19±1.89</td>
</tr>
<tr>
<td>Collagenase</td>
<td>0.09±0.012</td>
<td>ND</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Fibrinolytic</td>
<td>53 ± 2.34</td>
<td>349±5.79</td>
<td>ND*</td>
<td>572±5.054</td>
</tr>
<tr>
<td>pNP acetate</td>
<td>0.68±0.12</td>
<td>4.89±1.10</td>
<td>ND*</td>
<td>4.29±3.37</td>
</tr>
<tr>
<td>AAA-PFpNA</td>
<td>12.9±3.4</td>
<td>135±6.23</td>
<td>ND*</td>
<td>146±4.91</td>
</tr>
<tr>
<td>AAVpNA</td>
<td>0.945±0.897</td>
<td>15±  3.41</td>
<td>ND*</td>
<td>18±  2.53</td>
</tr>
</tbody>
</table>

*Not detected
6.3.6: Evaluation of the cloned enzyme for tannery applications

To confirm functional similarity of the over expressed protein with respect to its native counter part, soaking, dehairing, milled feather degradation and hydrolysis of thermally denatured chromeshavings etc. were compared among crude, purified and overexpressed-purified enzyme (Table 6.6). Efficiency to perform above applications were more or less similar with same quantity of purified and overexpressed enzyme, but the whole process was faster with lesser amount of enzyme in comparison to the crude enzyme.

Table 6.6: Comparision of application efficiency of crude, purified and over expressed protease from B. subtilis P13

<table>
<thead>
<tr>
<th>Properties studied</th>
<th>Culture supernatant From B. subtilis P13</th>
<th>Purified protease from B. subtilis P13</th>
<th>Overexpressed protease from pAJKpr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units</td>
<td>Duration</td>
<td>Efficiency</td>
</tr>
<tr>
<td>Soaking</td>
<td>2</td>
<td>18h</td>
<td>19% swelling</td>
</tr>
<tr>
<td>Dehairing</td>
<td>2</td>
<td>16h</td>
<td>100% dehairing</td>
</tr>
<tr>
<td>Feather hydrolysis in presence of 0.1% βME</td>
<td>2</td>
<td>12h</td>
<td>95% weight reduction</td>
</tr>
<tr>
<td>Feather hydrolysis</td>
<td>2</td>
<td>24h</td>
<td>98% Weight reduction</td>
</tr>
<tr>
<td>Hydrolysis of CS</td>
<td>2</td>
<td>24h</td>
<td>98.46% Weight reduction</td>
</tr>
</tbody>
</table>

6.3.7: Nattokinase activity of cloned keratinolytic protease from B. subtilis P13

The fibrinolytic activity of cloned keratinolytic protease to nattokinase was evaluated by urokinase activity (Fig. 6.18) and fibrin plate assay (Fig. 6.19). The N-terminal sequence of the cloned keratinolytic protease was compared with other reported nattokinases from Bacillus.sp.(Table 6.7). The first 10 amino acids of the nattokinase like keratinolytic protease from B. subtilis P13 showed 100% homology with all, except B. subtilis CK and B.amyloliquefaciens
Chapter 6: Cloning, expression and characterization of keratinolytic protease from Bacillus subtilis P13

Fig. 6.18: Comparison of fibrinolytic activity of keratinolytic protease

Fig. 6.19: Fibrin plate assay. Clear zone around the wells are due to fibrinolysis
Table 6.7: Comparision N-terminal sequence of keratinolytic protease from \textit{B. subtilis} P13 with other reported nattokinase from \textit{Bacillus} sp

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Source</th>
<th>Name of the enzyme</th>
<th>MW.pI. Opt.Temp pH</th>
<th>N-terminal sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{B. natto}</td>
<td>Natto, Japan</td>
<td>Nattokinase, NK</td>
<td>27.7 kDa, pI 8.6</td>
<td>AQSVPYGISQIKA PALHSQGYTGS</td>
<td>Fujita et al., 1993</td>
</tr>
<tr>
<td>\textit{B. amyloliq}</td>
<td>Douchi, China</td>
<td>Subtilisin DFE</td>
<td>28 kDa, pH 9.0, 48°C</td>
<td>AQSVPYGVSQIK APALHSQGFTGS</td>
<td>Peng et al., 2003</td>
</tr>
<tr>
<td>\textit{Bacillus sp. CK}</td>
<td>Chungkook, Korea</td>
<td>CK</td>
<td>28.2 kDa, pH 10, 70°C</td>
<td>AQTVPYGIPLIKAD</td>
<td>Kim et al., 1996</td>
</tr>
<tr>
<td>\textit{Bacillus sp. DJ-4}</td>
<td>Doenjang, Korea</td>
<td>Subtilisin DJ-4</td>
<td>29 kDa, pH 10.0, 40°C</td>
<td>AQSVPYGVSQIK AP</td>
<td>Kim and Choi, 2000</td>
</tr>
<tr>
<td>\textit{B. subtilis} QK02</td>
<td>Fermented soybean and QK-2</td>
<td>Keratinolytic serine protease</td>
<td>28 kDa, pH 8.5, 55°C</td>
<td>AQSVPYGISQIAKAPALHSQG</td>
<td>Ko et al., 2004</td>
</tr>
<tr>
<td>\textit{B. subtilis} P13</td>
<td>Hot spring isolate</td>
<td>Keratinolytic serine protease</td>
<td>28 kDa, pH 7.2, 60°C</td>
<td>AQSVPYGISQ</td>
<td>Present study</td>
</tr>
</tbody>
</table>

6.3.8: Modelling of cloned keratinolytic protease

All protein structures were modeled by comparative modelling techniques with Discovery studio 3.1. Protein BLAST of pET-AJ-Kpr protein shows high homology to subtilisin proteins having similarity/identity ranging from 98-100%. The homolog which has been chosen for homology modeling of the cloned protein is (PDB ID: 1MEE, 1MEE sequence identity= %) having sequence identity of 99%. The given sequence identity shows the most nearby/best homolog of subtilisin. Homology modeling of the protein was on the basis of 1MEE results in the highly accurate modeled structure with RMSD xyzA0 (Fig. 6.20).
Fig. 6.20: Schematic representation of the secondary structure topology of cloned keratinolytic protease from *B. subtilis* P13

α-helices shown as cylinders and β-sheet strands as arrows. Substrate pocket is in the vicinity of catalytic triad ASP32 – HIS64 – SER221

Model deduced along with 44 sequences of propeptide showed a proper attachment to the subtilisin as separate structure (Fig. 6.20).

Energy minimization of modeled subtilisin protein made it energetically stable and orientation of each amino acids became stable and accurate. Ramachandran plot of modeled subtilisin showed that all amino acids of modeled subtilisin fall in allowed regions. These result indicated that the conformation of all amino acids are accurate/standard which provides proper conformation of modeled pAJNK protein. Thus, the results obtained showed that the structure is conformationally accurate.

Structural characteristics/features were determined on the basis of 1MEE. The catalytic triad of ASP32 – HIS64 – SER221 and substrate specificity pocket was identified. These catalytic triad and substrate specificity pocket are very important structural features of the protein as it contributes to its activity (Fig. 6.21).
Fig. 6.21: Structure of protease with propeptide attached to it.

Propeptide is having autocatalytic property and gets cleaved once the protease folded into its right conformation.

6.3.9: Phylogeny of cloned keratinolytic protease

Phylogenetic tree of nattokinase like keratinolytic protease protein showed, the similarity of a protein with other proteins having the structural and functional relationship. BLASTP of the protein resulted in highly identical homologs. 50 homologs were taken which shows the similarity with subtilisin protein. Phylogenetic tree reveals that the one complete group of 8 to 10 proteins having 99% relationship with subtilisin protein which would have similarity in structure and function (Fig. 6.22). The analysis shows that the isolated subtilisin protein has maximum evolutionary relationship with subtilisin proteins from B. subtilis. Thus, structural and functional similarity analysis of the over expressed protein with other proteins of phylogenetic groups provide the information regarding the function and structural characteristics of the subtilisin protein. The most evolutionary related subtilisin proteins are B. subtilis thermostable alkaline serine protease and B. subtilis fibrinolytic enzyme AprE8. This observation is reconfirms the similar observation obtained from LCMS analysis and also the accuracy of the structure which has been predicted. The other proteins have approx. 80% similarity with subtilisin protein which indicates the functional evolution of subtilisin.
Chapter 6: Cloning, expression and characterization of keratinolytic protease from Bacillus subtilis P13

Fig. 6.22: Phylogenetic tree of cloned keratinolytic protease from B. subtilis P13 where the enzyme is positioned according to its functional homology

6.4: Discussion

Keratinolytic protease having fibrinolytic activity was successfully expressed in recombinant E.coli-BL21 (pEt-AJ-NK) under T7 promoter. The leader peptide was pelB and maximum activity was observed in the cell lysate. Different induction conditions were tried in presence of IPTG and maximum specific activity of 5.3 caseinolytic units and 500 urokinase units observed after induction at 25°C for 18h. The pelB signal peptide was from Yerwinia and shown to have the capability of directing the synthesized foreign protein experiments through the cytoplasmic membrane of E. coli (Lucic et al., 1998). Even though the production of nattokinase
in *B. subtilis* is considerable, the long growth cycle, high endogenous proteases and the excessive byproducts of *B. subtilis* are not favourable for the development of this enzyme into therapeutic medicine for thrombosis. The *E. coli* expression system, by contrast, could surmount such disadvantages, and can be successfully used in the production of heterologous proteins Periplasmic expression of nattokinase in presence of pELB and secretory expression in presence of native promoter was reported by Laing et al., (2007).

The over expressed keratinolytic serine protease from *B. subtilis* P13 showed 100% homology to fibrinolytic nattokinase, subtilisin NAT (Tsukamoto et al. 2000). Xiaobo et al, 2007, reported the heterologous expression of fibrinolytic protease, which also showed homology to subtilisin NAT except a serine at 258. The difference between fibrinolytic protease aprE and subtilisin NAT (Tsukamoto et al. 2000) consists only in one amino acid at 258, which is serine in the former instead of asparagine in the latter. The yield of active nattokinase from *B. subtilis* (natto) (Tsukamoto et al. 2000) in the culture of the recombinant *E. coli* is related to the temperature. However, at a relatively low temperature a higher yield of active nattokinase was detected. Pfeifer (Pfeifer et al 2001) proposed that a moderate decrease of culture temperature could improve the expression of recombinant proteins in *E. coli*, and Missiakas and Raina, (1997) suggested that lower temperatures could help propeptide to fold properly. The propeptide of subtilisin (Fu et al 2000) and a-lytic protease (Baker et al 1992) was also reported to be necessary for active expression. Compared to the extracellularly produced protease by the wild strain (0.6U/mg protein for crude lysate from *B. subtilis* P13 and 5.3 U/mg protein for the *E.coli* BL21 clone,pET-AJ-NK), protease activity was 8 fold higher.

Other biochemical properties were all compared and confirmed that the serine protease produced by *B. subtilis* P13 is a thermostable keratinolytic enzyme having fibrinolytic activity. There are many reports on recombinant keratinases. In recombinant *P. pastoris*X33 (pPZK3), the *ker* gene was successfully expressed under alcohol oxidase (*AOXI*) methanol inducible promoter and keratinasewas secreted using the *Saccharomyces cerevisiae* a-factor signal sequence. Compared with wild type strain *B. licheniformis* MKU3, recombinant *Pichia* strain has shown 2.9-fold increased keratinase production. Similarly, recombinant *P. Pastoris* strain expressed the *Thermomyceslanuginosus* xylanase (XynA) at high yield (3-fold) after
Chapter 6: Cloning, expression and characterization of keratinolytic protease from *Bacillus subtilis* P13

96 h of induction (Radha and Gunasekaran, 2009). However, *P. Pastoris* transformants carrying the keratinase gene from *B. licheniformis* PWD-1 has shown steady increase in keratinase activity (285 U/ml) up to 144 h after the induction (Porres et al., 2002). *Aspergillus fumigates* ker gene in *P. pastoris* produced maximum keratinase (1 U/ml) in 72 h of growth (Noronha et al., 2002). The heterologously expressed keratinolytic protease from *B. subtilis* P13 was purified to 85.4 fold purity by casein affinity chromatography. Purification of recombinant keratinase from *P. pastoris* (pPZK3) through nickel affinity chromatography with 85.55 fold purity was reported by Radha and Gunasekaran, 2009. The same research group reported a recombinant strain of *B. megaterium* produced keratinase constitutively under amylase promoter (3-fold) higher than the wild type strain (Radha and Gunasekaran, 2008).

An alkaline protease gene, named the AP gene) was cloned from *B. pumilus* UN-31-C-42 and the N-terminal amino acid sequence of the deduced mature protease is identical to that of the purified dehairing protease DHAP (Pan et al., 2004). The AP gene was expressed in *B. subtilis* WB600 successfully and the expressed product had an identical molecular weight to the protease DHAP, which also had alkaline protease activity and dehairing activity (Huang et al., 2003). Aoyama et al., (2000) have reported an extracellular alkaline serine protease, APRP, which can coagulate soybean milk, and the gene aprP from *B. pumilus* TYO-67. However, they have not identified the dehairing effect of this protease.

Cloned keratinolytic protease having fibrinolytic property is a high value protein, par with commercially available fibrinolytic enzyme, urokinase. The microbial fibrinolytic enzymes, especially those from food-grade microorganisms, have the potential to be developed as functional food additives and drugs to prevent or cure cardiovascular diseases. NK has already been developed as drugs in the market, including Nattokinase NSKSD, Jarrow NattoMax JR-154, and Natto-K. (Sherry, 1987). Development of other microbial fibrinolytic enzymes is still ongoing, and much work needs to be done, especially concerning thrombolytic effects in vivo. The new trend for improving thrombolytic agents is to increase the efficacy and fibrin specificity, focusing on developing effective targeted thrombolytic agents. Several reports have illustrated successful construction of the chimeric proteins, in which a thrombus-specific polypeptide or antibody was attached to the plasminogen activator...
Chapter 6: Cloning, expression and characterization of keratinolytic protease from *Bacillus subtilis* P13

to enhance the thrombolytic specificity (Ruppert et al., 2003; Tait et al., 1995). These advances direct the way of future research on microbial fibrinolytic enzymes. Keratinolytic protease from *B. subtilis* P13 can also be explored further to develop it further as a food additive or drug, which could be administered with ease. The modelled structure appeared similar to that of reported structure of the subtilisins (Siezen and Leunissen, 1997). The structure suggests that the triad architecture of ser-His-Asp shares the catalytic site with other serine proteases. Protein engineering can be employed to improve the catalytic property and also to adapt it to low temperature.

6.5: Conclusion

Keratinolytic protease from *B. subtilis* p13 was expressed to 9.5 fold higher levels heterologusly in *E.coli* BL21 under T7 promotor. The protein was purified to 85.4% homogeneity by casein affinity chromatography. The expressed protein showed a molecular weight of 40kDa on SDS-PAGE and the same was confirmed on gelatine zymogram. The same keratinolytic protease secreted out by the wild type is of 28kDa. The higher molecular weight of the ecloned protein is attributed to the additional 77aminoacids coding for the propeptide. Propeptide is required for the proper folding of the protein and is autotctalytic and gets cleaved before secretion. The potential of the enzyme for soaking, dehairing, feather hydrolysis and the hydrolysis of chromeshavings were confirmed. The identity of the protein to nattokinase was confirmed by sequencing of the cloned gene and the functionality was confirmed with respect to urokinase activity, and in terms of its phylogenetic position, the enzyme branched along with fibrinolytic enzymes. The 3-D modelled structure was exactly matching with subtilisin having a catalytic traid of ASP32 – HIS64 – SER221.