CHAPTER-V

PURIFICATION AND CHARACTERIZATION STUDIES
PURIFICATION AND CHARACTERIZATION OF ALKALINE PROTEASE

Experimental

Chemicals

Agarose, acrylamide, bis-acrylamide, sodium dodecyl sulphate (SDS), TEMED, ammonium per sulphate and other chemicals for polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma Chemical Co., U.S.A.

Microorganism

A strain of GAS-4 isolated by us in our laboratory was used in the study.

Preparation of cell suspension

The inoculum was prepared as described earlier and used for the fermentation.

Production of alkaline protease in a 2 litre fermenter

The alkaline protease production was carried out in a 2 litre fermenter (B.Braun Biotech International, Micro DCU-200) as shown in Fig. 5.0 containing 1.5 L modified production medium. The composition of modified production medium was (g/L): Glucose, 10; Soyabean meal, 20; CaCl₂, 0.4; MgCl₂, 2.0 with pH 10. A 10% (v/v) level of inoculum was added and the fermenter was run at 37°C for 48 hours. After the completion of fermentation, the whole fermentation broth was centrifuged using Sor vall RC 5C centrifuge at 10,000rpm at 4°C and the clear supernatant was separated. The supernatant (crude enzyme) was subjected to recovery and purification process.
Enzyme recovery and purification procedure

Ammonium sulphate precipitation

A trial was run to determine the optimal concentration required for the enzyme precipitation with various concentrations of Ammonium sulphate. For this purpose, the supernatant obtained after centrifugation was subjected to ammonium sulphate fractionation. Ammonium sulphate was added at different concentrations ranging from 40 to 80% saturation. The precipitates so obtained were suspended in cold saline solution (2ml) and tested for protease activity and total protein content. The salting out concentration of the crude enzyme was established to be 60% on the basis of enzyme activity. To obtain complete precipitation of the crude enzyme, the remaining harvest fluid was subjected to ammonium sulphate precipitation at 60% saturation. For this purpose, solid ammonium sulphate (195g) was added gradually with mechanical stirring to harvest fluid (2x500ml) at 4°C to a saturation of 60%. The precipitate so formed was separated by centrifugation (8000g) for 15min., resuspended in cold saline solution (100ml) and dialyzed in cold against 1L of 0.05M Tris-HCl-0.1M NaCl (pH 10) for 20 hrs. After dialysis, the solution was centrifuged and supernatant obtained was designated as fraction -I.
The dialyzed enzyme (fraction-II) was centrifuged at 8000g for 15min. and supernatant was chromatographed on a column of Sephadex G-200. The sample (fraction-II) was loaded on to a column of Sephadex G-200 (1.5cmx24cm) equilibrated with 0.05M Tris-HCl-0.1M NaCl (pH10). The column was eluted at a slow rate of 1.0ml/hr with a discontinuous gradient from 0.1M to 0.8M NaCl in the same buffer. A total of 40 fractions were collected. A typical chromatogram is shown. From the elution profile it was observed that the protein was eluted as a well resolved peak of Caseinase activity coinciding with single protein peak at a NaCl concentration of 0.4M. Fractions (15-18) with high protease activity were pooled together, dialyzed and concentrated by lyophilization and used for further studies. It was labeled as fraction-III(Deyl.z,1979).
ION-EXCHANGE CHROMATOGRAPHY:

The dialyzed enzyme subjected to ion exchange chromatography containing DEAE-Cellulose. The resin was poured to the column and equilibrated with 10mM Tris–Cl buffer (pH-7.0). The dialyzed sample was loaded to the column and its was eluted from column by using gradient elution.

Preparation of elution buffer (gradient elution)

<table>
<thead>
<tr>
<th>S.NO</th>
<th>1M TRIS –Cl,(10ml)pH-7.0</th>
<th>1M NaCl (10 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>250</td>
<td>750</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>1000</td>
</tr>
<tr>
<td>4</td>
<td>250</td>
<td>1250</td>
</tr>
<tr>
<td>5</td>
<td>250</td>
<td>1500</td>
</tr>
<tr>
<td>6</td>
<td>250</td>
<td>1750</td>
</tr>
</tbody>
</table>

The above are in micro liter. The final volume in each tube was made to 10 ml with distilled water. All the six tubes were estimated for protein. The tube containing highest protein was assayed for enzyme.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

After Sephadex G-200 column chromatography, the fractions (15-18) showing the highest specific activity was dialyzed, lyophilized and then subjected to SDS-PAGE. The SDS-PAGE was performed according to Laemmli (1970) using 10% acrylamide.

The gels were cast by mixing the ingredients as detailed below:
The separating gel mixture was cast in a slab gel apparatus (Minigel, Genei, Bangalore, India) and overlaid with water. This was left for several hours to allow for polymerization to occur. Then the water layer was removed and the stacking gel was cast on top of the separating gel. To run the SDS gel, the electrophoresis buffer containing 25mM Tris, and 250mM glycine (electrophoresis grade) with pH 10 was used. The SDS was added to a final concentration of 0.1% to the electrophoresis buffer. Protein samples containing the five molecular weight markers Bovine serum albumin (67KD), a crude broth and fractions obtained after dialysis and ion exchange chromatography were dissolved in sample buffer containing 10mM Tris HCl (pH 6.8), 4% SDS, 20% glycerol, 0.002% β-mercaptoethanol and 0.002% bromophenol blue as the tracking dye and boil for 10min. to denature the protein. The treated samples were loaded in the wells of the slab gel and electrophoresis was started by applying 60 Volts per gel. When the dye front reached the separating gel the voltage was increased to 120V and the electrophoresis was continued till the tracking time reached the lower end of the gel. After the run was over, the gel was soaked overnight (about 16 h) in a fixative solution containing 50% (v/v) methanol and 12% (v/v) acetic acid. After taking out the gel from the fixative solution, it was stained by Coomassie Blue R-250 solution. The results were shown in the Fig. 5.2.

**Gel staining**

Coomassie blue staining

The gel was stained for 1h with 0.25% Coomassie Blue R-250 in methanol/water/acetic acid (50:40:10) and the gel was finally destined in a detaining solution containing water/acetic acid/methanol (87.5:7.5:5).
Native PAGE:

Here the gel electrophoresis is run in the absence of SDS and DTT. The electrophoretic mobility in SDS-PAGE depends on the molecular mass, while in native PAGE the mobility depends on both protein's charge and its hydrodynamic size (Deyl, Z., 1979). Native PAGE serves as an excellent tool to study conformation, self-association or aggregation, and the binding of other proteins or compounds in neutral pH conditions (Hames, B.D., 1990). Thus it is a powerful technique to study structure and composition of proteins since both conformation and biological activity remain intact during the process. (Laemmli., 1970).

PROCEDURE:

- Thoroughly clean and dry the glass plates and spacers and insert within bulldog clips. Fix the chamber in the upright level position.

- Prepare 10ml Separating Gel Mixture

<p>| | |</p>
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide:Bis Solution (37.5:1)</td>
<td>1ml</td>
</tr>
<tr>
<td>4x Separating Gel Buffer</td>
<td>2.5ml</td>
</tr>
<tr>
<td>50% Glycerol</td>
<td>2.5ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>4ml</td>
</tr>
</tbody>
</table>

- Degas the solution and then add:

  10% Ammonium Persulphate 50ul

  TEMED (N,N,N\textsubscript{1},N\textsubscript{1} tetramethyl-ethylene diamine) 10ul

- Mix gently and use immediately to avoid polymerization reaction. Carefully pour the solution into the chambers without formation of air bubbles.
- Carefully add acrylamide solution with water saturated n-butanol as an overlayer without mixing to remove oxygen and generate flat surface on the gel.

- Polymerize acrylamide layer for an hour.

- Prepare 4ml of Stacking gel solution

<table>
<thead>
<tr>
<th>40% Acrylamide:Bis solution (37.5:1)</th>
<th>0.4ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x Stacking gel buffer</td>
<td>1.0ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.6ml</td>
</tr>
</tbody>
</table>

- Degas the Stacking gel solution and then add:

  10% Ammonium Persulphate 20ul
  TEMED 5ul

- Mix gently and use the mixture immediately. Discard the n-butanol from the polymerized gel and wash with water to remove the remnants. Fill the voids with Stacking Gel mixture and insert the comb.

- Polymerize the acrylamide for an hour.

- After polymerization remove the comb and clips without disturbing plates. Install the gel in the apparatus.

- The apparatus is filled with Reservoir Buffer. Gently remove any air bubbles from top and underneath the gel using spacers. Use the gel immediately.
SAMPLE PREPARATION:

- Dissolve the protein sample in same volume of Sample buffer. The sample concentration is adjusted such that it gives sufficient amount of protein in a volume not greater than size of the sample well.

- Load the gel with 10-30ul of Protein sample solution by pipette.

- Electrophoresis is carried out. The bromophenol dye front takes 3hours to reach the bottom of the gel. Application of greater voltages enhances the speed of electrophoresis but may generate heat.

- Remove the gel from the glass plates.

- Stain the gel in Staining Solution for 2-3hours. The composition of Staining solution is given below:

<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Coomassie Brilliant Blue R250</td>
<td>0.25g</td>
</tr>
<tr>
<td>Methanol</td>
<td>125ml</td>
</tr>
<tr>
<td>Glacial Acetic acid</td>
<td>25ml</td>
</tr>
<tr>
<td>Deionized H₂O</td>
<td>100ml</td>
</tr>
</tbody>
</table>

- Remove the dye that is unbound to protein using Destaining Solution.

<p>| | |</p>
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>100ml</td>
</tr>
<tr>
<td>Glacial Acetic acid</td>
<td>100ml</td>
</tr>
<tr>
<td>Deionized H₂O</td>
<td>800ml</td>
</tr>
</tbody>
</table>
After 24 hours the gel background becomes colorless and leaves blue, purple or red colored protein bands. The results were shown in Fig. 5.3.

ZYMOGRAPHY METHOD:

Zymography is the latest method being used to analyze Matrix Metalloproteinases (MMP) and Tissue Inhibitors of Metalloporteinases (TIMP) in biological samples. This technique is rather simple, sensitive, accurate and quantifiable. In zymography, the proteins are separated by gel electrophoresis where separation occurs in polyacrylamide gel. (Patricia A.M. et al. 2005).

REAGENTS USED IN ZYMOGRAM:

- 1% Gelatin
- 1% Casein
- SDS-PAGE gel stock without urea
- Calcium Chloride
- Tris-Acetic acid
- Tris-Base
- Glycine
- Triton X-100
- 30% Acrylamide
• 0.8% Bis-acrylamide

• TEMED

• Ammonium persulphate

• Sodium Dodecyl sulphate

• Coomassie Brilliant Blue R-250

**PRINCIPLE OF ZYMOGRAPHY:**

• Gelatin is retained on the gel during electrophoresis.

• The activity of MMP is reversibly inhibited by SDS during electrophoresis.

• It also enables the separation of MMP and TIMP complexes. Thus, both MMP and TIMP can be detected independently.

An advantage of using Zymography is that both proenzymes and active forms of MMPs can be distinguished on the basis of their molecular weight.

**PREPARATION & PROCEDURE:**

• Samples are prepared in the standard SDS-PAGE treatment buffer without reducing agent (in order to keep the enzyme in the native state).

• A suitable gel is placed in the resolving gel during the preparation of acrylamide gel.

• Electrophoresis is carried out. The SDS is removed from the gel by incubation in unbuffered Triton-X-100.
• The gels are later incubated in digestion buffer for a specified period of time at 37°C.

• The zymogram is subsequently stained using Amido black, Coomassie brilliant blue dyes.

• The areas of digestion appear on clear bands against darkly stained background where the substrate has been degraded by the enzyme. The results were shown in the Fig. 5.4.

**BIOINFORMATICS MODELING AND INVITER PRODUCTION LABELS**

Proteases are the group enzymes involved in hydrolysis of peptide bonds. Proteases are grouped into four different classes: the cysteine, serine proteases, metallo and aspartic acid proteases. Based on the structural similarities alkaline proteases have been grouped into 20 families with six clan subdivisions. Alkaline proteases hydrolyse the peptide bond containing tyrosine, phenylalanine or leucine at the carboxyl end of the splitting bond (Vivek Kumar Morya et al. 2011).

The DNA and protein sequence homology have widespread use now-a-days. The nucleotide and amino acid sequences of number of proteases have been determined and the results find use in elucidating structure-function relationship. Availability of genome sequences from several *Streptomyces* species found their use in identification of putative secondary metabolism genes and gene clusters which were not known previously. However, functional analysis is necessary in certain putative secondary metabolism genes which may not be expressed at a level sufficient to detect products. This difficulty can be overcome by manipulating structural and regulatory genes to obtain expression or by experimentation on various strains of the same species since expression may be strain dependant (Rabbani Syed et al. 2012). The results were shown in Fig. 5.5.
Analytical methods

Determination of alkaline protease activity

Alkaline protease activity was determined as described earlier.

Protein assay

Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was calculated from the absorbance at 280 nm.


Effect of substrate concentration on Alkaline protease activity

In order to characterize the alkaline protease produced by the strain of GAS-4, the enzyme (1mg/ml) was incubated at a time interval of 30 min with different concentrations of protease. The protease concentration was shown in Table 5.1.1 and Fig.5.7.0. From the graph it was observed that at 15mg concentration, protease showed the maximum velocity. Further increment of protease concentration did not enhance the activity significantly.
**Characterization of purified enzyme**

*Effect of pH on purified enzyme activity and stability*

Activity of the purified protease was measured at different pH values to study the effect of different pH values on activity. The pH was adjusted using the following buffers (0.05 M): phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0) and glycine-NaOH (pH 9.0-12.0). Reaction mixtures were incubated at 55°C for 30 min. and the activity of the enzyme was measured. The results are shown in Fig. 5.6.

To determine the stability of enzyme at different pH values, the purified enzyme was diluted in different relevant buffers (pH 6.0-12.0) and incubated at 37°C for 2 and 20 h. The relative activity at each exposure was measured as per assay procedure and the results are shown in Fig. 5.6.

*Effect of temperature on enzyme activity and stability*

The activity of the purified enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30 to 100°C for 30 min. in the absence and presence of 10 mM CaCl₂. The results are shown in Fig. 5.7.1.

To determine the enzyme stability with changes in temperature, purified enzyme was incubated for 30 min. at different temperatures (60, 70, 80 and 90°C) in the presence of 10 mM CaCl₂ and relative protease activities were assayed at standard assay conditions. The results are shown in Fig. 5.8.
Effect of protease inhibitors and chelators on enzyme activity

The effect of various protease inhibitors (at 5mM), such as serine inhibitors [Phenylnethylsulphonyl fluoride (PMSF) and Diisopropyl fluorophosphate (DFP)], cysteine-inhibitors [p-chloromercuric benzoate (pCMB) and β-mercaptoethanol (β-ME), and a chelator of divalent cations [Ethylene diamine tetra acetic acid (EDTA)]] were determined by preincubation with the enzyme solution for 30 min at 37°C before the addition of substrate. The relative protease activity was measured. The results are shown in the Fig. 5.9.

Effect of various metal ions on protease activity

The effects of different metal ions viz., Ca$^{2+}$, Mg$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Fe$^{3+}$, Na$^{+}$, Zn$^{2+}$ and Cu$^{2+}$ (10 mM) were investigated by adding them into the reaction mixture. The mixture was incubated for 30 min. at 37°C and the relative protease activities were measured. The results are shown in the Fig. 5.10.

Hydrolysis of protein substrates

Protease activity with various protein substrates such as bovine serum albumin (BSA), casein, egg albumin and gelatin was assayed by mixing 100ng of the purified enzyme and 200μl of assay buffer containing the protein substrates (2 mg/ml). After incubation at 37°C for 30 min, the reaction was stopped by adding 200μl of 10% TCA (w/v) and allowed to stand at room temperature for 10 min. The undigested protein was removed by centrifugation and the released peptides were assayed. The specific protease activity towards casein was taken as a control. The results are shown in the Fig. 5.11.

Enzyme stability in presence of detergents

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The compatibility of GAS-4 protease with local laundry detergents was investigated in the presence of 10 mM CaCl$_2$ and glycine. The following detergents were used: Nirma (Nirma Chemical, India), Henko (SPIC, India), Surf Excel, Super Wheel, Rin (Hindustan Lever Ltd, India) and Ariel (Procter and Gamble, India). The detergents were diluted in distilled water (0.7% w/v), incubated with 0.1 ml of enzyme (364U/ml) for 4h at 37°C and the residual activity was determined. The enzyme activity of a control sample (without any detergent) was taken as 100%. The results are presented in the Table 5.2. Our protease showed good stability and compatibility in the presence of Ariel. As such, the compatibility of our enzyme was studied with Ariel in presence of 10 mM CaCl$_2$ and 1M glycine for different time periods (0.5–3h) at 60°C. The results are shown in Fig. 5.12.

Washing test with protease preparation

Application of protease as a detergent additive was studied on white cotton cloth pieces (4 x 4 cm) stained with blood (0.1ml) and kept aside for 1h. The stained cloth pieces were taken in separate flasks. The following sets were prepared and studied:

1. Flask with distilled water (100 ml) + stained cloth (stained with blood).

2. Flask with distilled water (100 ml) + stained cloth (stained with blood) + 1 ml ariel detergent (7mg/ml).

3. Flask with distilled water (100 ml) + stained cloth (stained with blood) + 1 ml ariel detergent (7mg/ml) + 2 ml enzyme solution.

The above flasks were incubated at 37°C for 15 min. After incubation, cloth pieces were taken out, rinsed with water and dried. Visual examination of various pieces
exhibited the effect of enzyme in removal of stains (Fig. 5.13). Untreated cloth pieces stained with blood were taken as control.

*Dehairing of animal skin with protease*

The dehairing capacities of the crude broth and purified enzyme were studied on fresh goat skins. For this purpose, enzyme solutions (20 ml) were applied as a paste with kaolin (10g) and streptomycin sulphate (100mg) on the flesh side of freshly slaughtered paired goatskin pieces. The skins were kept aside for 6h. A control was also kept using water instead of enzyme solution. After 6h contact time, the ease of dehairing was noted by removing the hairs with a blunt scalpel. The results are shown in Fig.5.14.
RESULTS AND DISCUSSION:

Purification of alkaline protease of *Streptomyces indicus* GAS-4:

The enzyme production was carried in a 2L fermenter as per the general procedure. The clear fermentation broth containing the crude enzyme was subjected to purification.

*Sephadex G-200 gel filtration chromatography*

The crude broth obtained after fermentation was subjected to ammonium sulfate precipitation at 60% followed. The pellet obtained was dialyzed in 0.1 M Tris-HCl buffer. After dialysis, the dialyzed enzyme was subjected to ion exchange chromatography on a DEAE-Cellulose column. The elution profile shown in the Fig. 5.2.

It was observed that the protease was eluted as a well resolved single peak of caseinase activity coinciding with a single protein peak at NaCl concentrations of 0.4 M. Fractions (15-18) with high protease activities were pooled, dialyzed and concentrated by lyophilization and used for further studies. The summary of purification steps involved for alkaline protease is reported in the Table 5.1.

*Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PA GE)*

The crude broth, (precipitate obtained after ammonium sulphate precipitation) and the purified protease along with standard molecular weight markers were run on SDS-PAGE. Several bands were observed in the case of ammonium sulphate precipitate (Fig. 5.2) while purified protease showed a single band on SDS-PAGE, indicating a homogeneous preparation. The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins. The molecular mass standards used were bovine

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serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsinogen (24 kDa) and α-lactalbumin (14 kDa). The molecular weight was measured by interpolation from a linear semi-logarithmic plot of relative molecular mass versus the Rf value (relative mobility) (data not shown). Depending on the relative mobility, the molecular weight of the protein band was calculated to be around 60 kDa. Thus it was concluded that our alkaline protease enzyme has a molecular weight of 60 kDa.

Many reports had been published on purification of different microbial proteases using ammonium sulphate precipitate and anion exchange chromatography method (Yamamoto et al. 1987). The molecular weight of purified enzyme of St.halstedii Salh-12 and St.endus Salh -40 were 60 and 35 kDa.
Table 5.1 Summary of purification steps of alkaline protease from S.indicus GAS-4

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Activity (IU)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity</th>
<th>Fold Purification</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>306400</td>
<td>1000.2</td>
<td>306.4</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>AMM</td>
<td>249930</td>
<td>701.6</td>
<td>356.2</td>
<td>1.162</td>
<td>81.56</td>
</tr>
<tr>
<td>PPT</td>
<td>Dialysis</td>
<td>200700</td>
<td>400.1</td>
<td>501.7</td>
<td>1.637</td>
</tr>
<tr>
<td>Gel</td>
<td>IEC</td>
<td>184048</td>
<td>80</td>
<td>2300.6</td>
<td>7.508</td>
</tr>
<tr>
<td>Filtration</td>
<td>135422</td>
<td>28</td>
<td>4836.5</td>
<td>15.78</td>
<td>44.1</td>
</tr>
</tbody>
</table>

The purification profile indicated that the enzyme was purified 15.78 fold with an yield of 44%. 

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Fig. 5.1 Elution profile of alkaline protease from Streptomyces indicus GAS-4.
Fig. 5.2 SDS-PAGE of alkaline protease from *Streptomyces indicus* GAS-4.
Fig. 5.3 Native PAGE of alkaline protease from *Streptomyces indicus* GAS-4.
Zymography method:

Fig. 5.4 Zymograph of alkaline protease from *Streptomyces indicus* GAS-4

The arrow mark showing colourless patches is indicative of protease activity.
Fig. 5.5. (A) Production template; (B) 3D Protease respect to template; (C) Observed structure of protease.
Comparative modeling predicts the 3-D structure of alkaline protease model as a given protein sequence (target) based on the template. The hypothetical 3D structures of template and the model are given in the above Fig 5.5.

**Characterization of purified enzyme**

**pH optimum and pH stability**

For the determination of the pH optimum, phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0) and glycine-NaOH (pH 9.0-12.0) buffers were used. The highest protease activity was found to be at pH 9.0 using glycine-NaOH buffer. The results are shown in Fig. 5.6. The stability of the purified protease was also determined by the preincubation of the enzyme in various buffers of different pH values. In the case of 2 h preincubation group, the enzyme was stable over a broad range of pH 8-10. On the other hand, in the case of 20 h preincubation group, the enzyme was stable between pH 8 and pH 9 (Fig.5.6).

**Effect of substrate concentration on protease activity**

The substrate profiles of protease activity were shown in Fig. 5.7.0 and Table 5.1.1. From the graph it was observed that at 15mg concentration, protease showed the maximum velocity.
Temperature optimum and thermal stability

The activity of the purified enzyme was determined at different temperatures ranging from 30° to 90°C in the absence and presence of 10 mM CaCl₂. The optimum temperature recorded was at 37°C for protease activity. The enzyme activity was gradually declined at temperatures beyond 40°C (Fujiwara and Yamamoto, 1987). The results are shown in Fig.5.7.

The GAS-4 protease had a half-life of 250 min. and less than 50 min at 70°C and 80°C respectively. The enzyme was almost 100% stable at 37°C even after 350 min of incubation (Dhandapani and Vijayaragavan, 1994). The results are shown in the Fig. 5.8.

Effect of inhibitors and chelators

Inhibition studies primarily give an insight of the nature of enzyme, its cofactor requirements and the nature of the active center (Sigma and Mooser, 1975). The effect of different inhibitors on the enzyme activity of the purified protease was studied and the results are presented in the Fig.5.9. Of the inhibitors tested (at 5 mM conc.), PMSF was able to inhibit the protease completely while DFP exhibited 94% inhibition. In this regard, PMSF sulphonates the essential serine residue in the active site of the protease and has been reported to result in the complete loss of enzyme activity (Gold and Fahrney, 1964). Our result were similar to that of Yamagata et al. (1989), who reported complete in inhibiting protease by PMSF. This indicated that it is a serine alkaline protease. In the case of other inhibitors, the protease was not inhibited by EDTA, while a slight inhibition was observed with pCMB and β – ME.
Fig. 5.6 Effect of pH on the activity of alkaline protease

Optimum pH: 9.0
Table 5.1.1. Effect of substrate concentration on protease production.

<table>
<thead>
<tr>
<th>Substrate concentration [S], µg</th>
<th>Protease activity (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.16</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
</tr>
<tr>
<td>5</td>
<td>0.24</td>
</tr>
<tr>
<td>7</td>
<td>0.28</td>
</tr>
<tr>
<td>9</td>
<td>0.31</td>
</tr>
<tr>
<td>10</td>
<td>0.33</td>
</tr>
<tr>
<td>12</td>
<td>0.38</td>
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<td>13</td>
<td>0.44</td>
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<td>15</td>
<td>0.46</td>
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<tr>
<td>17</td>
<td>0.48</td>
</tr>
<tr>
<td>19</td>
<td>0.51</td>
</tr>
<tr>
<td>20</td>
<td>0.52</td>
</tr>
<tr>
<td>22</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Fig. 5.7.0 Effect of substrate concentration on protease activity.
Optimum temperature: $37^0\text{C}$
Fig. 5.8 Effect of temperature on the stability at 37°C
Fig. 5.9 Effect of protease inhibitors/chelators on enzyme activity.
Effect of metal ions

Some of the metal ions tested had slight stimulatory effect (Ca$^{2+}$, and Na$^{+}$) or slight inhibitory effect (other ions) on enzyme activity. The results are presented in the Fig. 5.10. Addition of the metal ions Ca$^{2+}$, and Na$^{+}$ increased and stabilized the protease activity of enzyme probably because of the activation of the enzyme by these metal ions. These cations also have been reported to increase the thermal stability of alkaline proteases from Bacillus sp. (Rahman et al. 1994; Paliwal et al. 1994). Other metal ions such as Zn$^{2+}$, Cu$^{2+}$, Co$^{2+}$, Cd$^{2+}$, and EDTA did not shown any appreciable effect on enzyme activity. The residual protease activity was greater than the control when the enzyme was exposed to Ca$^{++}$ and Na$^{+}$ ions and same as the control when exposed to Mn$^{2+}$. Similar results were given by Tsuchiya K. et al who reported that Calcium divalent cation increased pH and Heat stability. This is beneficial when the enzyme is used industrially.

Hydrolysis of protein substrates

When assayed with native proteins as substrates, the protease showed a high level of hydrolytic activity against casein and moderate hydrolysis of BSA and egg albumin. The hydrolysis of gelatin was significant and slightly lower compared to casein. The results are presented in the Fig.5.11.
Compatibility with detergents

Besides pH, a good detergent protease is expected to be stable in the presence of commercial detergents. The protease from GAS-4 showed excellent stability and compatibility in the presence of locally available detergents (Tide, Wheel, Mr. White, Surf Excel, Ariel and Rin). The results are presented in the Table 5.2.

Fig. 5.10 Effect of various metal ions on alkaline protease activity.

![Graph showing the effect of various metal ions on alkaline protease activity.](image-url)
Fig. 5.11 Alkaline protease activity against different natural substrates.
Table 5.2 Compatibility of alkaline protease activity with commercial detergents in the presence of CaCl$_2$ and glycine.

<table>
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<th>Time (h)</th>
<th>Control</th>
<th>Ariel</th>
<th>Surf Excel</th>
<th>Tide</th>
<th>Rin</th>
<th>Mr. White</th>
<th>Wheel</th>
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</table>
The protease showed stability and compatibility with the above commercial detergents at 37°C in the presence of CaCl$_2$ and glycine as stabilizers. Our protease showed good stability and compatibility in the presence of Ariel detergent powder. The enzyme retained more than 50% activity with most of the detergents tested even after 3 hr incubation at 37°C after the supplementation of CaCl$_2$ and glycine (Table 5.2).

Our enzyme was found to be stable in commercial detergents. As our protease showed good stability and compatibility in presence of ariel detergent powder, a detailed study was conducted with Ariel in the presence of 10mM CaCl$_2$ and 1M glycine for different periods (0.5 to 3 h) at 37°C. The results are shown in Fig. 5.12. The enzyme retained about 60% activity after 1.5 hr in the presence of Ariel at 37°C and was almost inactivated after 3 hr in the absence of stabilizer. However, the addition of CaCl$_2$ (10 mM) and glycine (1M), individually and in combination, was very effective in improving the stability where it retained 50% activity even after 3 h.

As the protease produced by our isolate GAS-4 was stable over a pH range of 8-10 values and also showed good compatibility with various commercial detergents tested, it can be used as an additive in detergents. To check the contribution of the enzyme in improving the washing performance of the detergent, supplementation of the enzyme preparation with detergent i.e. Ariel significantly improved the removal of blood stains. The results are shown in Fig. 5.13.
Dehairing of animal skin with protease

The dehairing capacities of the crude and purified enzyme were studied. Freshly slaughtered paired goat skin pieces were treated with crude enzyme and purified enzyme. The skins were kept aside for 6h. A control was also kept using water instead of enzyme solution. After 6h contact time, the ease of dehauling was determined by removing the hairs with a blunt scalpel. It was observed that the purified enzyme could dehair with greater ease than the crude enzyme. The results are shown in Fig. 5.14.

Fig. 5.12 Compatibility of alkaline protease with Ariel in the presence of CaCl₂ and Glycine

![Graph showing the compatibility of alkaline protease with Ariel in the presence of CaCl₂ and Glycine.](image)
The isolate GAS-4 producing an alkaline phosphate was identified as *Streptomyces indicus* var GAS-4. The purified enzyme was studied in pH range 8-10 with maximum activity at pH 9. The enzyme was completely inhibited by Phenylmethysulphonyl fluoride (PMSF) indicate that it is a serine alkaline protease. Ca$^{2+}$ and Na$^{+}$ metal ions increased and stabilized the protease activity. Stability and activity in the presence of these metal ions is industrially very useful where high concentration of these metal ions are present. The protease strain GAS-4 showed excellent stability and compatibility in the presence of locally available detergents. The blood strain removing and dehairing experiments showed that the enzyme improved the washing capability of the detergent.
Fig. 5.14 Comparison of proteolytic activities on dehairing of goat skin before (A) and after (B) addition of the enzyme
REFERENCES


