

PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE SERINE ALKALINE PROTEASE

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ABSTRACT

The purpose of the research was to study the purification and characterization of thermo stable serine alkaline protease. The enzyme was purified in a two step procedure involving ammonium sulphate precipitate and HIC (Hydrophobic interaction chromatography). The enzyme was shown to have a relative low molecular weight by SDS polyacrylamide gel electrophoresis. It was most active below 50 degree. It was stable at pH 8. It was strongly activated by metal ions Ca^{++} . The aim of the research was to optimize the conditions for the production of protease.

Keywords: HIC (Hydrophobic interaction chromatography), alkaline protease

1. INTRODUCTION

Proteases belong to one of the single class of enzymes that occupies a pivotal position with respect to their application in both physiological and commercial fields. These enzymes account for 60% of total world wide sale of enzyme with two third among them of microbial origin. Proteases catalysis the breakdown of proteins. These are involved in a multitude of physiological reactions from simple digestion of ingested proteins to highly regulated reactions cascades. Proteases possess activity and stability over a high range of pH and temp. The most important proteases are alkaline proteases used in detergents formulations and acid proteases.

Alkaline proteases secreted by both neutrophilic and alkalophilic bacteria are important and represent a major source of proteolytic enzymes. X-ray films contains a great deal of silver and good amount of it remains in the film even after it has been used this silver is generally recovered by burning films. A few studies have reported the use of alkaline proteases to recover silver from used x-ray film efficiently.

With the recent introduction of enzyme based detergents in Indian market, there is an increasing need for development of an indigenous process for production of alkaline protease for use in detergents.

2. MATERIALS AND METHODS:

2.1 Materials:

{Chemicals}: Peptone, yeast extract, glucose, beef extract, NaCl, NaOH, biopeptone, agar, skimmed milk,

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NaCO₃, starch, soya, peptone, MgSO₄, KH₂PO₄.

{Composition of media}: HK media for 100 ml: glucose = 1.gm, biopeptone = 0.5 gm, yeast extract = 0.5 gm, KH₂PO₄ = 0.25 gm, MgSO₄ = .05 gm.

{Strach-soyatoase media for 100 ml}: Starch = 0.5 gm, soyatose = 0.5 gm, MgSO₄ = 0.2 gm / liter = 0.1 gm, KH₂PO₄ = 0.1 gm / liter = 0.5 gm.

{Starch-soya media}: Starch = 0.5gm, soya = 0.5gm, MgSO₄ = 0.1gm, KH₂PO₄ = 0.5gm.

{Preparation of NASM plates for 250 ml}: Skimmed milk = 2.5gm in 100.ml dist. H₂O, NaCl = 1.25gm, peptone = 1.25gm, beef extract = 0.375gm, yeast extract = 0.375gm, make up the final vol and add 3.75gm of agar to it, 10% Na₂CO₃

{Tris HCl buffer (0.2M HCl)}: weighed 1.21gm of buffer and dissolve it in 50 ml of dist. H₂O add to it 1.ml conc. HCl and 9.ml of distl H₂O after that make up the final vol. to 40.ml take 26.8.ml of its part for the pH 8 after that add tris buffer and make up the final vol. to 200.ml

{Phosphate buffer}: NaH₂PO₄ (monobasic) (A) 6.89.gm in 250.ml dist H₂O NaH₂PO₄ (dibasic) (B) 13.412gm in 250ml dist H₂O take 39.ml part of A + 61.ml part of B mix it.

{Glycine-NaOH buffer}: A. preparation of 0.2M glycine NaOH solution: weigh 0.75 glycine in 50.ml dist H₂O. B. 2M NaOH solution: weigh 8gm in 100ml of dist H₂O.

Take 50ml part of A+32ml part of B make up the final vol. to 200 ml.

2.2 METHODS:

Procedure adopted to optimize media conditions for protease production:

One loop full of glycerol stock is transferred to 20 ml of Horikoshi medium after that incubate it for 24 hours at 200 rpm for 30°C in the shaker. Take loop full of culture and streak it over the skimmed milk nutrient agar plates again incubate it for 36 hr at 200 rpm at 30°C in a shaker pick single colony from the plate and inoculate it in the 50 ml of Horikoshi medium for another 24 hr. After 24 hr take out the sample, centrifuge it for five minutes at 10,000 rpm. Discard the pellet and collect the supernatant for further assay.

2.3 Enzyme assay for enzyme activity:

The proteolytic activity was estimated by using azocasein as a substrate, using a slightly modified method of samal. Reaction mixture contain 20 ml azocasein and 47 ml of assay buffer, preincubated at 50 degree for five minutes. Reaction was initiated by the addition of 10 ml of suitably diluted enzyme solution and the above solution was incubated at 50 degree for 30 minutes. After 30 minutes the reaction was terminated by the addition of 500 ml of 10% trichloroacetic acid. These sample were kept on ice for 15 minutes to completely precipitate the unhydrolysed proteins. After precipitation these were centrifuged at 10,000 rpm for five minutes. 800 ml of supernatant was mixed with 200 ml of 1.8 NaOH and the absorbance was measured at 420 nm.

3. PURIFICATION STUDIES:

1. Ammonium sulphate precipitation:

The organism was grown for 48 hours cells are separated by centrifugation at 10,000 rpm for 15 minutes and the supernatant was fractionated by

precipitation with ammonium sulphate between 30-80% of saturation. All the steps are carried out at 4° the pellet was resuspended in 0.05N tris HCl buffer of pH 8.

Step Involved:

Harvesting: The culture was taken in centrifuge bottles, centrifuge at 10,000 rpm for 10 minutes and the supernatant was collected and discard the pellet. Enzyme activity was measured by casein azocasein assay. Salt precipitation of protease, using ammonium sulphate was carried out at 40% saturation level. The amount of salt to be added for 40% saturation was calculated by:

$$\text{Ammonium Sulphate Added} = 533 (S_2 - S_1) / 100 - 0.3 S_2$$

Where S_2 = Final % of saturation of ammonium sulphate

S_1 = Initial saturation

The required amount of ammonium sulphate was dissolved in the supernatant collected previously. Addition of salt carried out on to ensure constant stirring under chilled condition. After all ammonium sulphate was dissolved, stirred for another two hour to allow complete equilibration between dissolved and aggregated protein. A range of ammonium sulphate saturation level was used to find out in which percentage saturation is maximum and protease is precipitating out. The saturation level tried was from 50 to 100%.

2. Solid – Liquid separation by centrifugation:

As alkaline protease was secreted extracellularly therefore sample was centrifuged to separate out cell pellet.

For this centrifugation was done at 10,000 rpm for 20-25 minutes.

3. Purification by Hydrophobic Interaction Chromatography (HIC):

HIC separates molecules based on their hydrophobicity. Sample molecules containing hydrophobic and hydrophilic characteristics are applied to HIC column in a high ionic strength (high salt) buffer. The salt in the buffer promotes a favorable environment for binding by interacting with water molecules to reduce the solvation of the other molecules in the solution. As solvation decreases, exposure of hydrophobic regions in the molecules increases and these molecules are absorbed by the beads. The more hydrophobic the molecule, the less salt needed to be promote binding. Sample elution is done with the help of organic solution.

The whole process was carried out in following steps:

Column Equilibration:

It was done by washing the column with equilibration buffer (2M NaCl, 0.05 M tris HCl pH 8) with flow rate of 1 ml / minute.

Sample preparation:

2 ml sample was prepared by mixing 2 ml crude protein with 16 ml 5M NaCl, 8 ml tris HCl and 16 ml water.

Sample loading:

Sample was loaded with flow rate of 0.5ml / minutes.

Washing the column:

When the whole sample was through the column, column was washed with washing buffer (2M NaCl, 0.05 M tris HCl pH 8) to wash out unbound protein.

Elution of the sample:

Sample was eluted by washing the column with buffer.

4. Purification Analysis :

Gel Electrophoresis of protein samples (Principle):

Electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of proteins into their individual polypeptide subunits and that minimize aggregation.

Anionic detergent SDS along with reducing agent (beta mercaptoethanol) and heat is used to dissociate the protein before they are loaded. The denatured polypeptide binds SDS and become negatively charged. The amount of SDS bound is directly proportional to molecular weight of polypeptides and independent of its sequences. SDS polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptides. By using markers of known molecular weight, estimate the molecular weight of polypeptide chains. After migrating through a stacking gel of high porosity, the complexes are deposited in a very thin zone on the surface of resolving zone.

The polyacrylamide gels are composed of chains of polymerized acrylamide that are cross-linked by a bi-functional agent as N,N-methylenebisacrylamide which

gives rigidity and tensile strength to gel and forms pores.

The effective range of separation of SDS polyacrylamide gels depends on the concentration of polyacrylamide used to cast the gel and on the amount of cross-linking. The sieving properties of the gels are determined by the size of the pores, which is a function of the absolute concentration of acrylamide and bis acrylamide to cast the gel.

REAGENTS:

- Gel loading buffer: Composition of gel loading dye (5x)
Tris HCl (6.8) = 1ml
10% SDS = 4ml
Glycerol = 2ml
Bromophenol blue = 0.02g
Distilled water = 10ml
β mercaptoethanol was added per ml of this dye solution just before using.
- 5X Running buffer: g Tris-base and g Glycine and SDS was dissolved in water and volume was made up to 1 litre.

METHOD:

Gel of 1.5mm thickness and 10 cm Gel length was casted using a pair of glass plates, a pair of spacers, a gasket, a teflon comb and a pair of casting clamps with the following composition (Table 1)

Table 1:

Reagents	Resolving Gel (10%) = 15ml	Stacking Gel (5%)=5ml
Sterile DDW	5.9 ml	3.4 ml
Acrylamide (30%) mix	5.0 ml	0.83 ml
Upper Tris 1.5M,pH 8.8	3.8 ml	-
Lower Tris 1.0M,pH 6.8	-	0.13 ml
10% SDS	0.15 ml	0.05 ml
10% APS	0.15 ml	0.05 ml
TEMED	0.006 ml	0.005 ml

Samples were adding 50µl of dye to 50µl of sample and boiled for three minutes then cooled and centrifuged at 10,000 rpm for five minutes at 4 degree Celsius. After casting the gel, 10µl-20µl of samples were loaded in to the wells and the gel was run at a constant current of 10Ma, which was increased to 13mA as a tracking dye (bromophenol blue) comes out of stacking gel. When the dye reaches the end, current was stopped and proceeded further.

Staining SDS-polyacrylamide gels: The SDS-PAGE gels were stained using either of the following procedure.

Staining with Coomassie Brilliant Blue:

Polypeptides separated by SDS-polyacrylamide gel electrophoresis can be fixed with methanol and glacial acetic acid and stained with coomassie brilliant blue R250, a triphenylmethane textile dye also called acid blue 83.

Method involves various steps:

The gel was immersed in the staining solution for two hours on shaker at room temperature.

Relative activity % =

Effect of metal ions:

Purified protease was dissolved in tris HCl buffer of pH 8 with different metal ions. Solutions of different metal ions with different conc were added to assay buffer. This solution was used in enzyme assay to check protease activity in

Gel transferred from staining solution to destaining solution.

The gel was destained by 90ml methanol and water (1:1v/v)+10ml glacial acetic acid by keeping gel for 4-8 hours at room temperature on a shaker at 40 rpm (Changing the destain solution 3-4 times). Destaining for 14-18 hours usually allow as little as 0.1 µg of protein in a single band to appear.

4. PHYSIOLOGICAL CHARACTERIZATION:

Effect of temperature:

Study the effect of different temp. 45, 30, 37, 50, 55, 60° C over the activity of the purified enzyme, the reaction mixture was incubated at different temp. ranging from 20 to 80° and the activity was determined. Then check enzyme stability at different temp, purified enzyme was dissolved in tris HCl buffer pre incubated at different temp.

Relative activity % at the given temp calculated as:

Activity at given temp X 100
Activity at opt. Temp.

presence of metal ions. Assay buffer any metal ion was used as control.

Relative activity calculated as: % of the activity in the control set was taken as 100%

Effect of detergents:

Equal vol of suitably dil enzyme solution and detergent at different conc were mixed and assay buffer was used instead of detergent component solution in control. Residual enzyme activity was checked by enzyme assay. Residual activity in presence of detergent component was calculated as % of residual activity in control set which taken as 100%.

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RESULTS AND DISCUSSIONS:

Effect of temp on enzyme activity:

The purified enzyme shows good activity over a wide range of temp. graph illustrates the relative activity maximum (Table 2 and Figure 1).

Table 2:

TEMP	O.D
30	70.48
27	119.84
45	127.84
50	206.48
55	47.52
60	90.96

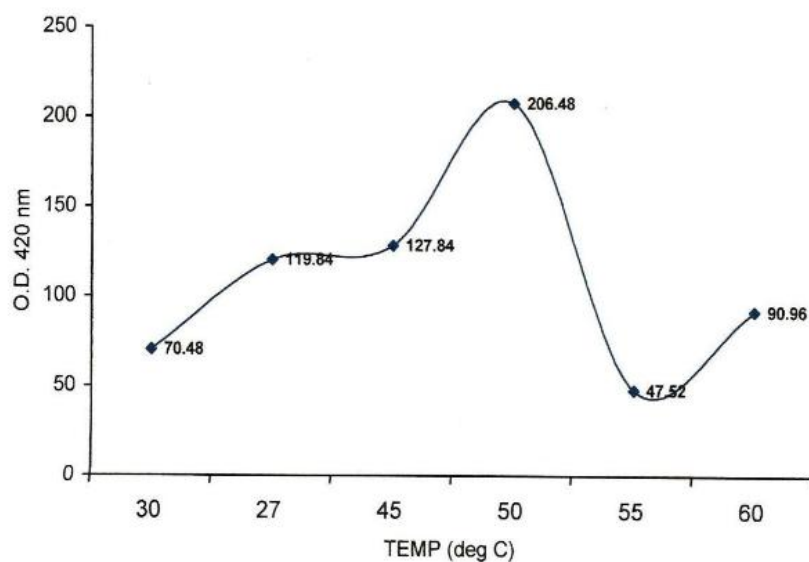


Fig 1: shows good activity over a wide range of temperature

Effect of metal ions:

The effect of metal ion the activity of purified enzyme is maximum in case of

Ca^{++} enhanced the enzyme activity slightly. While heavy metals Co^{++} almost inactivates the enzyme (Figure 2).

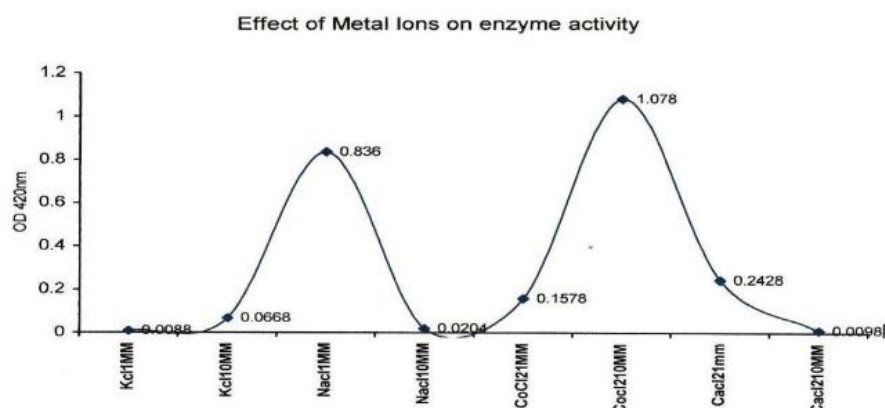


Figure 2: shows effect of metal ions on enzyme activity

Effect of detergents:

Enzyme is completely stable in presence of detergents and the activity is also

enhanced, maxi in case of nirma (commercial detergent) (Figure 3).

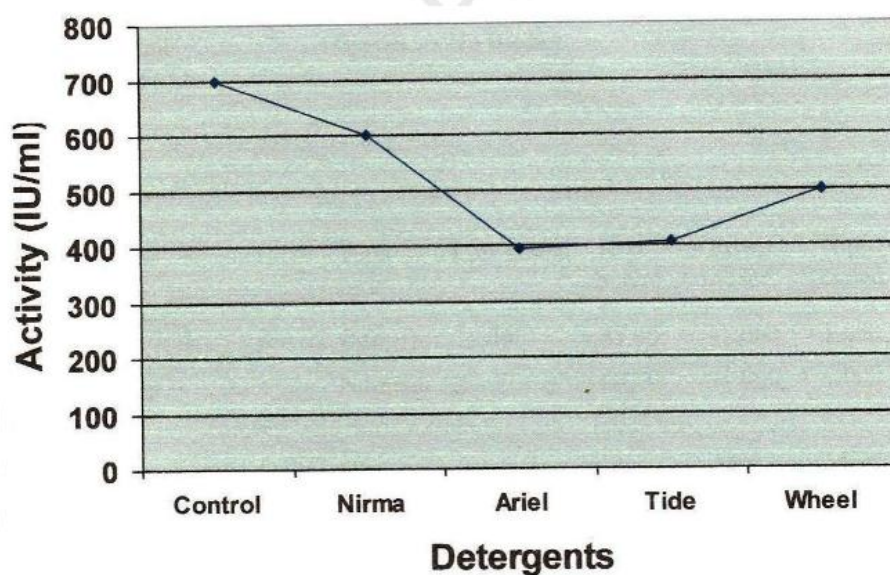


Figure 3: shows the activity of detergents

CONCLUSION:

It was concluded that this newly isolated *Serratia marcescens* produces an

alkaline protease, using a relatively less expensive production medium, which seems to a very good industrial potential for a variety of applications.

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