A STUDY ON SERRATIAPEPTIDASE ENZYME PRODUCTION FROM SERRATIA SPECIES

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ABSTRACT

Serratiapeptidase is an endopeptidase, having molecular weight of about 60 KDa. It absorbs strongly at 275-280nm. Serratiapeptidase is a stronger caseinolytic agent than any other known alkaline or neutral protease. Production of an anti-inflammatory enzyme Serratiapeptidase by fermentation with Serratia species was studied to ascertain optimal nutritional conditions for shake flask production. The optimized medium contain carbon source, nitrogen source, inorganic nitrogen source, and the optimized fermentation conditions were pH 7.0, temperature 37°C and for a duration of 48hr. The fermentation conditions produced a maximum Serratiapeptidase at the end of 24 Hr and the molecular weight of the purified Serratiapeptidase was found to be 51kD. The purification fold was 2.39 after dialysis.

Key Words: Serratiapeptidase, Therapeutic, Enzyme, Fermentation, Dialysis, Purification

INTRODUCTION

Enzymes have many roles in the pharmaceutical and diagnostic industries. There are many applications to cover but typical applications include Enzymes are direct pharmaceutical products such as in the treatment of genetic disorders leading to a specific enzyme deficiency. Extraction of medicinally important compounds such as heparin. Combinational biocatalysts. Manufacture of pharmaceuticals chemically where enzymes are used for inter conversion of chemical intermediates and removal of unwanted products. Enzymes are widely used in agriculture, animal feeds, baking, brewing, detergent industry, starch industry, pharmaceuticals and diagnostics. Serrapeptase is a proteolytic enzyme with many favourable biological properties like anti-inflammatory, analgesic, anti-bacterial, fibrinolytic properties and hence, is widely used in clinical practice for the treatment of many diseases. The enzyme was also an effective mucolytic in the treatment of various disorders related to viscous sputum or pus, and their efficacies have been warranted to be more potent and reliable than those of a chymotrypsin and others. Therefore, they have widely been used not only in Japan but also in some other countries. The use of enzyme with fibrinolytic, proteolytic and anti-endemic activities has gained increasing support in recent years for the treatment of inflammatory ear, nose and throat (ENT) conditions. Serratiopeptidase is used mainly in post-traumatic and post-operative inflammatory conditions (after any minor and major oral surgical procedures), orodental infections.

Serratiopeptidase can help in conditions like Fibro myalgia - Arthritis - Chronic Joint Pain, Chronic Fatigue, Chronic Pain - Inflammation, Clogged Arteries - Fibroids Spider Veins - Viral infection, Circulatory disorders and Systemic yeast Infection, Autoimmune Diseases, Post-Operate Scarring, Fibrocystic Breast Disease, Bladder Infections. Serratiopeptidase has many clinical uses including: An anti-inflammatory agent (particularly for post traumatic swelling) For Fibrocystic breast disease For Bronchitis (Serratiopeptidase loosens and expels mucous) Serratiopeptidase digests dead tissue, blood clots, cysts, and arterial plaque. Horse gram (Microtylonauniflorum) is a novel substrate for the production of serratiopeptidase by using a novel source.

*Author for correspondence
Streptomyces under solid state fermentation

A simple, precise and accurate isocratic liquid chromatographic method has been developed for quantitative determination of Serratiopeptidase in the fermentation broth, bulk drug and pharmaceutical dosage forms. Many bacteria have proven to be beneficial sources of this enzyme.

**Aim of study**
The main aim of this particular study is to optimize the production of Serratiapeptidase from Serratia species and lab production and purification of the enzyme. In this regard the society can be immensely benefitted by the therapeutic value of Serratiapeptidase which is in great demand.

**OBJECTIVES**
- Testing the serratia species for the Production of the enzyme under certain fermentation parameters
- Purification and characterization of enzyme to some extent.

**MATERIAL AND METHODS**

**Strain**
Bacterial culture used for the production of Serratiapeptidase enzyme was Serratia species. The pure culture of the Serratia species was obtained from RVCE soil samples. The strain labelled as RV1001 and was used for the present study.

**Cultivation and Sub culture**
The LB (Luria Bertani) medium was prepared and sterilized at 121°C for 15 minutes. The test tubes (for agar slants) and petriplates were also sterilized. The sterilized medium was poured in sterilized test tubes and petriplates. The poured medium was allowed to solidify. The microorganism was inoculated and subsequent sub culturing was performed and the agar slants were stored at 37°C for further use.

**Estimation of caseinolytic activity of Serratia species (Casein assay)**
The caseinolytic activity of the organism was observed on casein agar medium.

**Method of inoculation**
The petriplates and the medium were sterilized. The sterilized medium containing tryptone, sodium chloride, yeast extract haem and agar was poured on sterile petriplates. Since haem cannot be autoclaved, it was pasteurized at 75°C for 30 minutes. 0.1% haem was added to the medium. The medium was allowed to solidify. The organism was inoculated on the solidified medium with a single streak. The inoculated medium was incubated at 37°C for 24 hrs. After incubation, the caseinolytic activity was observed by observing the clear zone around the colony.

**Estimation of hemolytic activity of Serratia species (heme assay)**
The hemolytic activity of the organism was observed on haem agar medium.

**Enzyme Assay**
Before proceeding for production steps, the specific activity of crude enzyme had to be found out, hence Protease and Lowry assay were performed. These were done for 24 hour inoculated media and 48 hour inoculated media. A loopful of micro-organisms was inoculated in LB media and incubated in an orbital shaker. After 24 hours, 10 mL of the inoculated media was centrifuged at 6000 rpm for 10 minutes. Supernatant was collected. Then, after 48 hours, another 10 mL sample was centrifuged at 6000 rpm for 10 minutes and the Supernatant was collected. The bacterial cells were successfully separated from the fermentation broth. (Table 2) Reagents used:
- 50 mM Sodium Phosphate Buffer, pH 7.5.
- 0.65% (w/v) Casein Solution. Dissolve in [1].
- 6.1 N Trichloroacetic Acid Reagent (TCA).
- Folin and Ciocalteu’s Phenol Reagent (F-C).
- 500 mM Sodium Carbonate Solution.
- 1.1 mM L-Tyrosine Standard (Standard solution).
- Enzyme Solution (sample)
Table 1: Protease assay pipetted (in mL)

<table>
<thead>
<tr>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Enzyme</td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

L– Tyrosine standard (Working standard: 1mg in 5mL)

Table 2: L-Tyrosine standard solution preparation

<table>
<thead>
<tr>
<th>Tyrosine mL</th>
<th>Distilled water, mL</th>
<th>Reagent E mL</th>
<th>Reagent D mL</th>
<th>Incubation in dark for 30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.0</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.8</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>0.6</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>0.4</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>0.2</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.0</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Protein analysis (Protein analysis was done by the procedure of Lowry et al., 1951)

Ammonium sulphate precipitation
A loopful of microorganism was inoculated in 150mL LB media and incubated at 37°C for 24 hours. After 24 hours, it was centrifuged at 6000 rpm for 10 minutes. The STP enzyme was partially purified by ammonium sulphate precipitation and dialysis. Supernatant was used for further purification strategies. 70% saturation was done for precipitation. 37.6 g of ammonium sulphate was weighed and slowly added to about 87mL of supernatant under ice cold conditions with constant stirring. Followed by centrifugation step (at 6000 rpm for 10 mins). The pellet was dissolved in 10mM Tris buffer for dialysis. Protease assay and Lowry assay was performed to determine specific activity of the protein obtained.

Dialysis
After purification of protein was done by Ammonium Sulphate precipitation, dialysis was performed. The sample containing enzyme was filled in dialysis bags for activation. Activation of dialysis bags were done by heating with 0.1M EDTA in a boiling water bath. After activation the dialysis bags are washed in running tap water and were stored in distilled water in a refrigerator. The Dialysis bags are taken with gloves and the sample containing enzyme was filled. They are dialyzed at 40°C in 1.5liters 10mM phosphate buffer at pH 6.0 for approximately 5hrs and continued dialysis overnight, with 1.5 litres of fresh buffer. The dialyzed enzyme solution was used for assay and protein estimation.

Characterization of Serratiopeptidase by SDS-PAGE
SDS-PAGE was performed to identify and determine the molecular weight of Serratiapeptidase.

Sample preparation
Equal amount of sample loading buffer was added to the sample (extracted enzyme) and placed in boiling water bath for 3-5 minutes.

Staining and destaining
The glass sandwich was then dismantled and the gel was placed in Coomassie Brilliant Blue staining solution for overnight. And then destained in 7% acetic acid for about 2 hours. The formation of bands was examined.

RESULTS AND DISCUSSION
Cultivation and sub-culture
Sub culturing of the organism was done for every 10 days. Nutrient agar was universally used as the medium for sub culturing of organism because it does not contain inhibitors for the growth of organism. The sub culturing
maintains the organism in a viable state for further studies.

**Estimation of caseinolytic activity of Serratia species (casein assay)**

A clear zone of caseinolytic around the growth of the organism appeared in casein agar medium after 24 hours, incubation at 37°C. The caseinolytic activity of organism was determined by inoculating on casein agar medium on incubation gave a clear zone due to the secretion of peptidase by the organism which degraded the casein present in the medium. This reveals that the organism is a peptidase producing organism. The clear zone formation took a long time for the formation; hence it reveals that the peptidase was an endopeptidase. (Fig. 1)

![Fig. 1: Clear zone formation on casein agar media](image1)

**Estimation of hemolytic activity of Serratia species (heme assay)**

A clear zone of hemolysis around the growth of the organism appeared in heme agar medium after 24 hours, incubation at 37°C. The hemolytic activity of organism was determined by inoculating on heme agar medium on incubation gave a clear zone due to the secretion of peptidase by the organism which degraded the casein present in the medium. This reveals that the organism is a peptidase producing organism. The clear zone formation took a long time for the formation; hence it reveals that the peptidase was an endopeptidase. (Fig. 2)

![Fig. 2: Clear zone formation on haem agar media](image2)
Enzyme Assays
The standard graph was plotted. The standard was L-Tyrosine standard.

Table 3: Absorbance values

<table>
<thead>
<tr>
<th>Concentration of tyrosine (microgram/mL)</th>
<th>Absorbance at 660nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>40</td>
<td>0.272</td>
</tr>
<tr>
<td>80</td>
<td>0.5</td>
</tr>
<tr>
<td>120</td>
<td>0.761</td>
</tr>
<tr>
<td>160</td>
<td>0.906</td>
</tr>
<tr>
<td>200</td>
<td>1.219</td>
</tr>
</tbody>
</table>

The standard graph was plotted between concentration (x-axis) and absorbance (y axis).

Fig. 3: Standard graph

From the standard graph micromole of tyrosine liberated was calculated by extrapolation the absorbance values of protease assay and hence finding out the activity of protein at 24 hours and 48 hours interval. (Table 4) The absorbance value at 24 hour production was 0.731. The absorbance value at 48 hour production was 0.794.

Table 4: OD values (Lowry’s method)

<table>
<thead>
<tr>
<th>S/N</th>
<th>Concentration of BSA (microgram/mL)</th>
<th>Absorbance at 660nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>0.095</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>0.113</td>
</tr>
<tr>
<td>3</td>
<td>120</td>
<td>0.241</td>
</tr>
<tr>
<td>4</td>
<td>160</td>
<td>0.435</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>0.455</td>
</tr>
<tr>
<td>Test 1 (24 hours)</td>
<td>from graph</td>
<td>0.412</td>
</tr>
<tr>
<td>Test 2 (48 hours)</td>
<td>from graph</td>
<td>0.317</td>
</tr>
</tbody>
</table>

The activity of enzyme was found out to be:
For 24 hour interval: 233.425 units/mL
For 48 hour interval: 208.325 units/mL

Protein analysis
To find out the total protein content and hence to find out specific activity from the protein content, Lowry assay was performed. (Table 4)
Fig. 4: Standard graph for protein estimation

From the graph, the total protein content was found out to be:
- For 24 hour interval: 168 microgram/mL
- For 48 hour interval: 139 microgram/mL

Specific activity of enzyme:
- For 24 hrs: 1688.40 µmol min\(^{-1}\) mg\(^{-1}\)
- For 48 hrs: 1507.24 µmol min\(^{-1}\) mg\(^{-1}\)

It was found that the activity of enzyme produced was higher in 24 hour inoculated media than 48 hour inoculated media. Hence for purification, 24 hours was chosen as optimum time since it showed higher activity.

**Purification of Serratiapeptidase**

**Ammonium sulphate precipitation:** The purified enzyme was obtained by Ammonium Sulphate precipitation method. The purified enzyme was further used for characterization.

**Dialysis** The purified concentrate of enzyme was obtained by dialysis. The dialysis was carried out in dialysis membrane which was tied into dialysis bag. The dialysis was carried out in dialysis bag with phosphate buffer by applying mild shaking. The dialysis membrane was activated by boiling with 0.1mM EDTA and was thoroughly washed and converted into dialysis bag. The concentrated enzyme was further used for protein profile estimation. (Table 5)

**Table 5: Fold purification of protein obtained after each step of purification**

<table>
<thead>
<tr>
<th></th>
<th>Activity Units</th>
<th>Protein concentration mg/mL</th>
<th>Specific activity Units/mg/mL</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>233.425</td>
<td>0.168</td>
<td>1688.40</td>
<td>1</td>
</tr>
<tr>
<td>Salt precipitation</td>
<td>512.65</td>
<td>0.205</td>
<td>2500.73</td>
<td>1.48</td>
</tr>
<tr>
<td>Dialysis</td>
<td>1020.48</td>
<td>0.252</td>
<td>4049.523</td>
<td>2.39</td>
</tr>
</tbody>
</table>

**Characterization of Serratiapeptidase**

**SDS – PAGE**

The enzyme sample showed distinct bands when stained with CBB upon Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis. The sample was found to be in par with the standard marker at 51KDa. Hence the molecular weight of the sample was approximately found to be 51KDa. (Fig. 5) The molecular weight was in correspondence with about approximately 52 KDa which is the molecular weight of the standard STP as demonstrated in SDS-PAGE. The result is an indication of the confirmed production of STP enzyme and the molecular weight determination completes the characterization of the enzyme.
CONCLUSION

Serratia species designated as RV1001 was found to produce of Serratiapeptidase, a medically important therapeutic enzyme. After subsequent subculturing, it was subjected for screening tests such as casein and haem assay and they were positive, which confirmed the presence of endopeptidase.

After positive results of the above screening tests, the optimum time for enzyme production was found out by using protease assay and protein assay (Lowry’s assay). The specific activity of enzyme of 24 hr inoculated media was higher than the 48 hr inoculated media. Hence, 24 hr was the optimum time for enzyme production.

The purification of protein was enhanced by ammonium sulphate precipitation followed by dialysis. The characterization of protein was done by SDS-PAGE. The enzyme purification increased by 2.39 fold after dialysis. The average molecular weight of the enzyme was found to be 51 kDa which confirms the presence of Serratiapeptidase.

Scope for future studies:
Optimization of commercial media for maximum production of the enzyme and scalingup of the process.

ACKNOWLEDGEMENT

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REFERENCES

7. A. Mohan Kumar, R. Krishna Raj, Production and characterization of serratiopeptidase enzyme from Serratia


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**Environment is God's gift, preserve it**

If U

Protect

Nature

Nature

will

Protect U

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**Grow tree, Feel the Environment free**