International Research Journal of Pharmaceutical and Applied Sciences (IRJPAS)
Available online at **www.irjpas.com**
Int. Res J Pharm. App Sci., 2013; 3(4):95-98

Research Article

PRODUCTION, CHARACTERIZATION & OPTIMIZATION OF POTENT PROTEASE (SERRATIOPEPTIDASE) FROM *SERRATIA MARCESCENS* E 15

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Abstract: Production, characterization & optimization of serratiopeptidase from Serratia marcescens E 15 was the base for this study. Serratia marcescens E 15 was allowed to grow in trypticase soy broth. The serratiopeptidase(STP) enzyme activity was determined according to the gelatin clearing zone. Enzyme assay was carried out &protein content was determined. A standard serratiopeptidase tablet "Serric-5" (5mg) containing 10000 Units of STP enzyme was used for the assay. Protease assay was carried out to determine the Units of protease in STP. Optimum conditions for STP production were 37oC temperature, 25hrs. Incubation period & pH=7.3. The STP enzyme was partially purified by ammonium sulphate precipitation & dialysis. STP enzyme activity increased with the increase in enzyme concentration.

Key**words:** Production, Characterization, Serratiopeptidase, Serratia marcescens E 15

INTRODUCTION

Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of total worldwide sale of enzyme. *Serratia marcescens* E15 was originally isolated in the late 1960s from intestine of silkworm *Bombyx mori L*. It is used by silkworm to dissolve the cocoon and reemerge as a moth. It was found to excrete a potent proteolytic enzyme which was named as Serratiopeptidase (STP) from the reason of its origin.[1-2]

Serratiopeptidase is an extracellular metalloproteinase with three zinc ligands and one active site[11]. This structure was predicted by comparing the structure of enzyme with those of thermolysin and *Bacillus subtilis* neutral protease. Zinc atom is an essential element for the proteolytic activity.[3]

Serratiopeptidase binds to $\propto_2$ – macroglobulin in the blood in a ratio 1:1. This helps to mask its antigenicity but retains its enzymatic activity. [3]This endopeptidase has absorption maxima at 275-280 nm. Serratiopeptidase induces fragmentation of fibrinose aggregate and reduces viscosity of exudates facilitating drainage of the products of the inflammatory response and promoting tissue repair process. Pain is one of the most sterling inflammations. Acute pain produced by cellular chemical reaction is part of body's natural inflammatory healing response. STP has an ability to reduce pain through its ability to block the release of pain inducing amines from inflamed tissues without any internal bleeding.[4]

Mechanism of action of serratiopeptidase appears to be hydrolysis of histamine, bradykinin and serotonin. It reduces capillary permeability induced by histamine, bradykinin and serotonin breaks down abnormal exudates and proteins and facilitates the absorption of decomposed products through blood and lymphatics,; thus promoting healing and repair and restores the skin temperature of burns, trauma or inflammation to normal[5].

Serratiopeptidase has a proteolytic and fibrinolytic effect which is achieved by dissoloving the complement (specific proteins responsible for inflammation) and increasing the plasmin activity by inhibiting the plasmin inactivators. The addition of metal ions like zinc, manganese and cobalt to serratiopeptidase stabilizes and ensures its efficacy. It is completely inhibited by heating at 55^0C for 15 min [6] Serratiopeptidase has been called the "miracle enzyme" or "super enzyme" due to its wide range of actions in human body.

MATERIALS AND METHODS

Culture Collection and Innoculum Preparation.

A non-pathogenic culture of *Serratia marcescens* strain E15 procured from NCIM, Pune was used for STP production. Stock cultures were maintained in nutrient broth. A loopful of bacterial suspension was transferred to 50ml sterile trypticase soy broth as per the composition[7] and allowed to grow overnight at 37^0C before being used for fermentation as an innoculum.

Production, Extraction and Purification of STP.

Crude STP enzyme was produced by adding 5% of inoculums in to sterile trypticase soy broth. The contents of the flask were then mixed thoroughly and incubated at 37^0C for 24 hrs for enzyme production. The fermented broth containing STP enzyme was centrifuged at 10,000 rpm for 20 minutes and the supernatant was filtered twice through the Whatmann filter paper No.1 and then through the membrane filter. The extracted enzyme was preserved in the refrigerator at 4^0C and used as a crude STP enzyme[8]. The 100ml of crude enzyme was saturated with solid ammonium sulphatein a beaker placed on a magnetic stirrer. The precipitated proteins were separated by centrifugation at

5000 rpm for 15 min. The pellet was then dissolved in 2.5ml phosphate buffer of pH 7 while supernatant was discarded[9]. The pellet then introduced in to dialysis bag and dialyzed against distilled water for 3 hours and against phosphate buffer at pH -7. Purified STP enzyme was used for enzyme assay.

Enzyme assay:

Gelatin clearing zone technique:

The STP enzyme activity was determined according to the gelatin clearing zone **(**GCZ) technique[2].

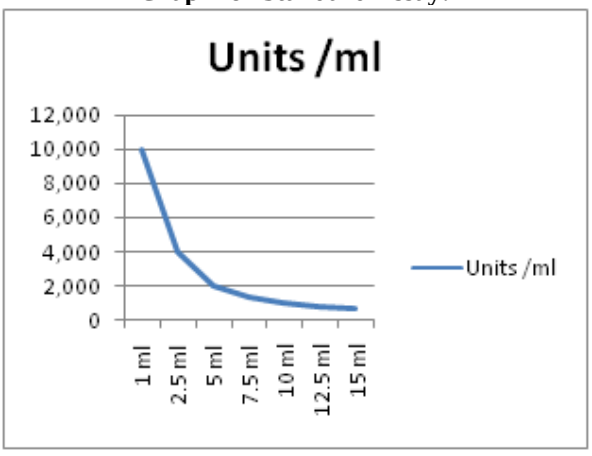
Standard Assay:

A standard Serratiopeptidase tablet "Serric-5" (5mg) was used for the assay consisting of 10,000 U of STP enzyme. One Unit of Serratiopeptidase is equivalent to one micro – gram of tyrosine that is produced by one milligram of Serratiopeptidase from casein substrate in one minute (J.C-Biotech- Serratiopeptidase

Seven different dilutions containing 1 "Serric-5" tablet each were made respectively and the Units per dilution were as follows.

D/W	Tablet	Units /ml
1 ml	1 "Serric-5" Tablet each	10,000
2.5 ml		4000
5 ml		2000
7.5 ml		1333.33
10 ml		1000
12.5 ml		800
15 ml		666.66

Graph for Standard Assay:



Wells were made in the sterile gelatin agar plates and 0.1 ml of each of these dilutions was added to the respective wells and after diffusion for 30 min. plates were incubated at 37^0C for 24 hrs.

Determination of protein content

The calibration curve for casein substrate was obtained by Biuret experiment[11].

Protocol for protein estimation by Biuret Method.

Test tube no.	Conc. of Standard protein sol.^{n} (ml)	D/W (ml)	Biuret reagent (ml)	
Blank	0.0	2.0	3	**Mix well & keep boiling water both for 15 min**
1	0.2	1.8	3	
2	0.4	1.6	3	
3	0.6	1.4	3	
4	0.8	1.2	3	
5	1.0	1.0	3	
6	1.2	0.8	3	
7	1.4	0.6	3	
8	1.6	0.4	3	
9	1.8	0.2	3	
10	2.0	0.0	3	
STP	1.0	1.0	3	

Protease Assay:

Protease assay was carried out to determine the Units of protease in STP. The standard set was carried out using casein as a substrate and the calibration curve was obtained. A stock of 10 mg /ml of substrate in distilled water was prepared. Further procedure was followed according to the protocol and the readings for the control tube as well as the test sample (STP enzyme) were obtained and further calculated.

Protocol for Protease Assay (Calibration curve)[11]

Test Tube No.	Protein Conc. (mg)	Abs_{520nm}
Blank	0	0.00
1	1	0.03
2	2	0.06
3	3	0.09
4	4	0.12
5	5	0.15
6	6	0.18
7	7	0.21
8	8	0.24
9	9	0.27
10	10	0.30

Tube Type	Substrate (ml)	Enzyme (ml)	Incubation at 37^0C for 60 min	Inactivator (ml)	Enzyme (ml)	Biuret reagent (ml)	Incubation at 37^0C	Abs. at 520 nm
Control	1.0	-		0.8	0.2	3.0		0.09
Test	1.0	0.2		0.8	-	3.0		0.06

Optimization of Enzyme production:
Effect of media ingredients:

All four ingredients (casein, digest, soyabean meal, K_2HPO_4 and glucose) of the fermentation media (trypticase soy broth) were subjected to a process of elimination; one ingredient at a time.

Effect of incubation temperature:

The fermentation medium was inoculated and incubated at different incubation temperatures as 10^0C, 28^0C, 37^0C 40^0C and 50^0C respectively.

Effect of incubation period:

The fermentation is carried out for different incubation periods as 10 hrs, 18 hrs, 24 hrs, 36 hrs and 45 hrs. at 37^0C.

Effect of pH:

Phosphate buffer of different pH values as 6.5, 6.8, 7.3, 7.7, and 8.0was prepared. The pH of production medium was adjusted using the buffer and the effect of pH studied.

Study of antimicrobial activity of STP enzyme.

Antimicrobial activity of Serratiopeptidase was studied by using the agar diffusion method. The test organisms were used as*Pseudomonas putida, Klebsiella pneumoniae, Salmonella typhi, Staphylococcus aureus, Bacillus spp., E.coli* andfungal spp. like *Aspergillus niger, Penicillium notatum and Mucor* were used.

RESULTS AND DISCUSSION
Serratiopeptidase production:

The extracellular STP enzyme was produced by *Serratia marcescens.* The results obtained in this work revealed the ability of *Serratia marcescens* E15 strain to produce the extracellular protease enzyme.

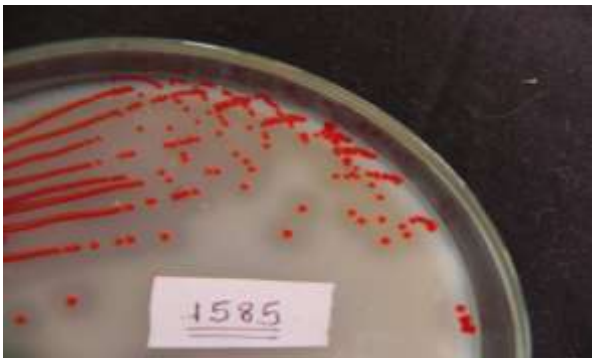
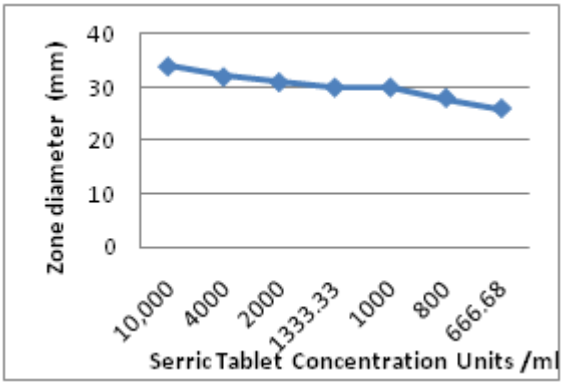


Figure 1-Extracellular protease by *Serratia marcescens* E15 on milk agar.

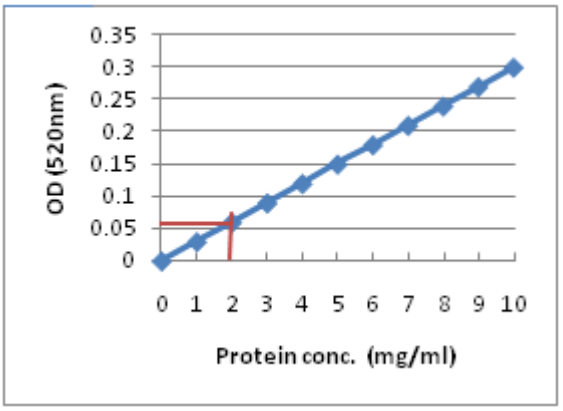
Enzyme assay
Zone diameters of enzyme assay.

Sr. No	Enzyme Source	GCZ (mm)
1	Crude Enzyme	26
2	Purified Enzyme	34

Enzyme assay as compared to Serric tablet

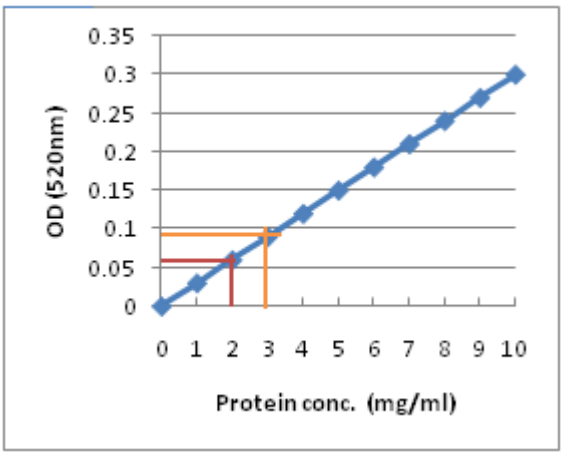


Estimation of protein content:
Protein estimation by Biuret method



Amount of protein estimated in the STP enzyme was **2mg**.

Protease Assay:



Calculations:-
P_1 = Test = **B**= 2mg/ml
P_0 = Substrate control = **A**= 3 mg/ml

Thus; **1mg/ml** of protein was transformed during the enzyme reaction.

$$\therefore \textbf{Amount of protein} = \frac{P_o - P_1}{\text{Amt.of enzyme X min.}}$$

$$= \frac{3-2}{0.2 \times 60}$$

$= \frac{1}{0.2 \times 60}$

$= \frac{1}{12}$

∴ Amount of protein hydroysed / ml of enzyme / minute = **0.083 mg.**

In Units; Amount of protein hydrolyzed in $\mu g/ml$ of enzyme within one minute

= 0.083 x 1000

= **83 Units**

Thus, 83 U of protease are present in 0.2 ml of STP used as a test sample.

Optimization of Enzyme Production.

Effect of elimination of one ingredient in fermentation media on STP production

Sr. No	Eliminated ingredient	GCZ (mm)
1	Pancreatic digest of casein	18
2	Soya bean meal	21
3	K_2HPO_4	25
4	Glucose	31

Effect of incubation temperature on STP production.

Sr. No	Incubation temperature (^{0}C)	GCZ (mm)
1	10	20
2	28	31
3	37	35
4	40	29
5	50	25

Effect of incubation period on STP production.

Sr. No	Incubation period (hrs.)	GCZ (mm)
1	10	23
2	18	26
3	25	34
4	36	28
5	45	26

Effect of pH on STP production.

Sr. No	Different pH values	GCZ (mm)
1	6.5	20
2	6.8	23
3	7.3	32
4	7.7	29
5	8.0	26

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