STUDIES ON CHARACTERIZATION AND BIOACTIVE POTENTIAL OF THE ENZYME PROTEASE *FROM Vibrio parahaemolyticus*

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ABSTRACT: *In recent years, different screening programs have been performed in saline habitats in order to isolate and characterize novel enzymatic activities with different properties to those of conventional enzymes. Though covering a limited chemical space, vibrios produce compounds with attractive biological activities, including antibacterial, anticancer, and antivirulence activities. In the present study, a halophilic organism named V. parahaemolyticus was isolated from the infected Indian goat fish and characterized which was used for the screening of its production of hydrolases such as proteases. Mass production of the enzyme was carried out and partially purified by fractional precipitation and dialysis. The molecular weight was determined as 29kDa by SDS PAGE. For characterization, the activity of the purified protease was analyzed at various pH, temperature and in presence of various divalent ions. Anticoagulant and antifibrinolytic activity of the enzyme is screened. In the present study, the enzyme with bioactive potential was successfully coated on the layers of porous silicon for improving its efficacy for delivery as a drug by simple surface adsorption which was further subjected to FTIR, SEM and EDX analysis.*

Key Words: *Halophiles, V. parahaemolyticus, protease, anticoagulant, surface adsorption*

Introduction

The concept of the therapeutic enzyme has been around for at least 40 years. For example, a therapeutic enzyme was described as part of replacement therapies for genetic deficiencies [1].

In 1987, the first recombinant enzyme drug, Activase1 (alteplase; recombinant human tissue plasminogen activator), was approved by the Food and Drug Administration (FDA). This was the second recombinant protein drug to be marketed (the first genetically engineered drug was insulin in 1982). Several other enzymes used as anticoagulant or coagulant agents have since been approved by the FDA[2].

In this context, the enzymes derived from halophiles are endowed with unique structural features and catalytic power to sustain the metabolic and physiological processes under high salt conditions. Some of these enzymes have been reported to be active and stable under more than one extreme condition [3][4][5].

Extracellular hydrolytic enzymes such as proteases, amylases, lipases, DNases, pullulanases and xylanases have quite diverse potential usages in different areas such as food industry, feed additive, biomedical sciences and chemical industries[6][7][8][9][10].

Thus in the present study, the enzyme with bioactive potential was successfully screened from a halophilic marine *Vibrio sp.* An initial approach in drug delivery was made by using the layers of porous silicon.

Materials and methods

Isolation and Characterization

Fishes of marine origin were randomly collected from local fish market after immediate fishing at Rameswaram island and the samples were transported to the laboratory in a sterile condition. From the homogenized sample, the organism was isolated. (Banupriya and Arunagiri, 2014). The bacterial isolates were characterized based on their growth on selective media (TCBS agar), colony characteristics, gram staining, motility and a series of biochemical tests such as Hugh- Leifson's test, ONPG (o-nitro phenyl β- D-galactoside), and growth on V. parahaemolyticus sucrose agar, Alkaline peptone water, Tryptone sucrose Tetrazolium agar base (TSTA) and Saline nutrient agar.

Screening and mass production of Protease enzyme

A suitably diluted culture, 0.2ml was spread on TCBS agar plate supplemented with 1% casein and 0.4% gelatin. It was incubated at 37°C for 48 hrs. The culture which was positive for both casein and gelatin hydrolysis was then enriched by inoculating the culture into the using Modified Luria Broth (MLB) in the following composition: (g/ litre)- Tryptone-10.0,Yeast extract -5.0, Magnesium sulphate (MgSO4)-0.5, Sodium chloride (NaCl)-10. A 50ml of culture grown overnight in MLB medium was used as inoculum for mass enzyme production. Mass culture of protease producer was carried out using production medium [11]. 50ml of culture was inoculated in a medium of 1000ml and incubated at 37°C for 72 hours. From the production medium, the enzyme was then partially purified by fractional precipitation and dialysis (reference palanivelu manual). The protein was then separated by SDS- PAGE.

Molecular weight determination by SDS-PAGE

Twenty micro liter of the active fractions was loaded into the gel. The molecular weight of the alkaline protease was estimated by SDS - Polyacrylamide gel electrophoresis with Myosin (205,000 Dalton), phosphorylase b (97,400 Dalton), Bovine Serum Albumin (66,000 Dalton), Ovalbumin (43,000 Dalton), Carbonic Anhydrase (29,000 Dalton), Soyabean Trypsin Inhibitor (20,100 Dalton) and Lysozyme (14,300 Dalton) as molecular markers.

Zymography

Zymogram was performed by simple modification of the method followed by Twining *et al*. (1993). Homogeneity and authenticity of the protease was examined by casein zymography using casein (10 mg/ml) as copolymerized substrate in the resolving gel. Zymography was performed at 50 V in non-reducing conditions. After electrophoresis, gels were incubated in 2.5% Triton X-100 solution for 1 hour and then incubated for 20 hour in buffer (50 mm sodium phosphate buffer, pH 7.4) at 37 °C. A zone of proteolysis was detected by staining overnight with coomassie brilliant blue-R 250. Proteolytic activity was observed as a clear band on a blue back ground. The protease activity of the partially purified sample was determined by Anson's method.

Characterization of protease

Effect of pH on enzyme activity

The effect of pH on enzyme activity was determined by the reaction of 0.1 ml of enzyme solution with one ml of casein substrate (1%, w/v) at various pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) at 37 °C for 30 min and enzyme activity was assayed .

Effect of temperature on enzyme activity

The effect of temperature on enzyme activity was studied by holding the enzyme reaction at various temperatures (30, 40, 50, 60, 70, and 80 °C) for 30 min. The enzyme activity was assayed by standard method.

Effect of metal ions on enzyme activity

The study the effect of divalent ions on enzyme activity, the sample was incubated with 5 mM of the following ions for 30 min. The ions used were Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, and Fe^{2+}. The sample was incubated with substrate for 30 min at 37 °C.

Screening of Bioactive Potential of the Protease enzyme

The bioactive potential of the sample was screened by checking the anti- coagulant and anti-fibrinolytic activities of the sample.

RESULTS

During the course of this study, pathogenic bacteria belonging to the genus *Vibrio* was detected from the diseased fish. Bacterial colony grown on TCBS medium was blue- green in colour, smooth, gram-negative, rod shaped and oxidase positive. The culture was identified as *V. parahaemolyticus*. After 24 hours of incubation, the bacterial colonies were isolated and maintained the culture as in same medium at 20°C.

Biochemical tests revealed that this motile bacteria exhibited positive activity for Hugh -Leif son's test , ONPG (o- nitro phenyl β- D- galactoside) test , *V.parahaemolyticus* sucrose agar, alkaline peptone water , Tryptone sucrose tetrazolium agar base (TSTA), Saline nutrient agar.

Hugh- Leif son's test to carbohydrate fermentable organisms, here *V.parahaemolyticus* utilize glucose as carbon source the colony shown yellow colour in tubes after 24 hours. ONPG (o- nitro phenyl β – D- galactoside) test is for rapid detection of β- galactosidase activity in microorganisms.

The results observed after 6 hours inoculation, showed that β- galactosidase cleaved ONPG to galactose and o- nitro phenyl, a yellow compound. In the presence of two enzymes, required to demonstrate lactose fermentation in a conventional test, these enzymes facilitates to enter into the bacterial cell wall and

the β- galactosidase hydrolyses the lactose to yield glucose and galactose. Positive results showed yellow colour in test tubes containing the liquid culture of marine bacterial isolate.

V.parahaemolyticus sucrose agar used for isolation and enumeration of *V.parahaemolyticus*. *V.parahaemolyticus* could not ferment sucrose and so formed green colonies observed on petriplates after 24 hours which differentiate it from other sucrose fermenting Vibrio species, which indicates positive result.

Alkaline peptone water broth used to isolate Vibro species. Light yellow coloured clear solution without any precipitasion seen in conical flask which indicates positive result observed after 24 hours when the broth inoculated with the cultures isolate.

Tryptone sucrose tetrazolium agar base (TSTA) test with addition of Triphenyl Tetrazolium chloride is needed for isolation Vibrio species. Light yellow coloured with clear zone formation observed after 24 hours in petri plates which indicates the growth the culture isolate. Saline nutrient agar was used for cultivation of *V. parahaemolysis.* Light yellow coloured colonies with clear zone was observed after 24 hours in petriplates.

The isolated and biochemically characterized culture was screened for protease production and enriched for extracellular mass production of enzyme protease. The positive culture was picked again sub cultured in TCBS agar which was used for further studies. Mass culture of protease producer was carried out using production medium supplemented with all the essential nutrients and minerals. The addition of salts was carried out after sterilizing the medium.

For purification of the enzyme, the supernatant was fractional precipitated with ammonium sulphate. After precipitation a white layer was observed which indicates the protein was successfully got precipitated from the sample. The supernatant was carefully removed with the help of the dropper without disturbing the precipitate. After complete removal, the precipitate was stored in 20mM freshly prepared Tris HCl buffer.

Then the sample was subjected to dialysis for further purification. After proper pretreatment of the dialysis membrane, it was used for the process of dialysis. The dialysed sample was stored at -60°C in an ultrafreezer (REMI INSTRUMENTS).

Then the protease was subjected to separation by SDS PAGE. After the completion of electrophoresis, three separate bands were observed in the gel. The result obtained as three different bands suggested the presence of three different proteins in the dialysed sample (figure). The molecular weight of the three different bands of proteins was determined as 29kDa, 56kDa, 63kDa when compared with the ladder of proteins used as marker. The zymography results (figure) suggested that among the three proteins, protein of molecular weight 29kDa was found to be involved in the hydrolysis.

The protease activity of the partially purified sample was determined by Anson's method. Using casein as the substrate, the enzyme activity was determined by using the formulae given below:

$$\text{Unit/ mg or ml} = \frac{\text{Absorption (750 nm) (test sample)- Absorption(750 nm) (blank)}}{10}\ \text{U/ml}$$

$$= \frac{0.28 - 0.16}{10}$$

$$= 0.012\ \text{U/ml}$$

The effect of various pH, ions and temperature was analysed for characterization of the protease enzyme. The effect of pH on enzyme activity was determined by the reaction of 0.1 ml of enzyme solution with one ml of casein substrate (1%, w/v) at various pH values. Among the various pH values, the production of enzyme was found effective at the pH 8. (Table 1)

Table 1. Effect of pH on enzyme activity

pH	Relative activity (%)
4	23
5	28
6	62
7	81
8	100
9	63
10	12.5

The effect of temperature on enzyme activity was studied by holding the enzyme reaction at various temperatures (30, 40, 50, 60, 70, and 80 °C) for 30 min. 40°C was found to be the optimal temperature for effective production and stability of the enzyme. The results were tabulated below (Table 2).

Table 2. Effect of temperature on enzyme activity

Temperature (°C)	Relative activity (%)
30	72.8
40	100
50	63.8
60	52.4
70	40.3
80	9

For the study the effect of divalent ions on enzyme activity, the sample was incubated with 5mM of the following ions for 30 min. The ions used were Ca^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Co^{2+}, and Fe^{2+}. Among the various ions used, in the presence of ion Mn^{2+} the enzyme activity increases up to 89% (Table 3)

Table 3. Effect of divalent ions on enzyme activity

Divalent ions (5mM)	Relative activity (%)
Ca^{2+}	86
Mg^{2+}	72
Mn^{2+}	89
Hg^{2+}	12
Co^{2+}	19
Fe^{2+}	83

In screening of the anticoagulant activity, plasma was prepared from the blood of normal healthy individual. The 100µl of plasma in a test tube was added with 100µl of prothrombin time reagent and the clotting time was noted. In the second tube, 100µl of plasma was added with the purified enzyme and the clotting time was noted. For every increase in concentration of the enzyme from 100µl to 500µl, the clotting time was accurately noted with the help of the stopwatch. The results were tabulated below. (Table 4)

Table 4. Anticoagulant activity of the partially purified enzyme

Concentration of plasma (µl)	Concentration of PT reagent (µl)	Concentration of purified sample (µl)	Clotting time (s)
100	100 (standard)	-	12.5
100	-	100	14
100	-	200	25
100	-	300	39
100	-	400	54
100	-	500	70

The fibrinolytic activity of purified protein was observed by artificial blood clot degradation. An artificial blood clot was made in a glass test tube using fresh human blood obtained from healthy young volunteers. The artificial blood clot was dipped in 0.1 mg/ml of sample solution at room temperature. After 10 minutes of incubation, the dissolution of the clot was observed. No clotting was observed even after the incubation of the aliquot added with the protease enzyme for about 1 hour.

Discussion

The isolation and screening of protease producing bacterial isolate was done on the basis of mean value of zone of gelatin and casein hydrolysis on TCBS agar medium supplemented with casein and gelatin (1%).

Since the enzyme is a protein, their separation was carried out using SDS- PAGE. The molecular weight of the unknown protein (enzyme) is determined by using various proteins with known molecular weight (from 205 kDa to 14 kDa) as markers. On comparison, the molecular weights of three different proteins were found as 29 kDa, 56kDa and 63kDa. The zymography studies suggest that the protein of molecular weight 29 kDa is involved in hydrolysis.

Thus further purification by chromatography is very much essential in order to remove the remaining two proteins of molecular weight 56kDa and 63kDa to get the essential protein of molecular weight 29kDa.

For characterization, the activity of the purified protease was analyzed at various pH, temperature and in presence of various divalent ions. The activity is 100% when the pH is maintained in basic condition

as 8 and on the other hand the acidic pH gradually reduces the activity. Thus the result suggests that the protease which is isolated can be an alkaline protease.

Since the enzyme is isolated from a moderately halophilic organism, their activity is found effective in the temperature of 40°C. Thus the enzyme can tolerate upto the temperature of 40°C. But high temperature to (80°C) reduces the activity. The presence of zinc ions also increases the activity to 89%.

The marine *Vibrio* serves as a source of many new bioactive metabolites with interesting properties. In the present study, the isolated organism exhibit effective anticoagulant activity. Also, adsorption is recognized as an optimal technique for drug loading since it does not require high mechanical energy [15].

Conclusion

Hundreds of new compounds have been discovered from the metabolites of marine organisms every year. Finding new and useful compounds is one of the crucial drivers for the field of research. Bacterial marine natural products serve as an important source of novel lead structures for drug discovery.

In the last few years, different screening programs have been carried out to study the diversity of microorganisms producing hydrolytic enzymes throughout direct plating on agar media supplemented with specific substrates for the enzymes of interest. In the present study, the enzyme protease was successfully screened and an initial approach for drug delivery through surface adsorption has been made.

However, it an acceptable fact that, the adsorption is not feasible for long release periods, since the attachment is weaker than covalent or physical trapping. Hence further approach such as electrostatic adsorption is essential to make it feasible and to improve the efficacy of the enzyme as an effective drug.

In recent years, a great deal of effort has been focused on using nanotechnology to provide advancement of drug delivery and design. The biocompatibility and degradation properties of the material are considered an ideal support for a controlled drug delivery system in which the porous Si device can be implanted and complete dissolution of the delivery device could be obtained *in vivo*. In the present study, all the approaches were made *invitro*.

There are many conditions that affect the lifetime of biomaterials *in vivo*. In order to be considered a good candidate for controlled release in the body, the material has to be able to withstand the harsh biological conditions in the body such as salinity, pH, and enzymatic activity while retaining the physical and chemical properties that deem the material biocompatible. Therefore, the research could be further explored for the development of enzyme as new drugs with effective delivery.

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