ISOLATION OF FIBRINOLYTIC PROTEASE FROM AEROMONAS CAVIAE NGP3 ASSOCIATED WITH SARGASSUM CINCTUM

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Abstract

This study was aimed to investigate the fibrinolytic activity of *Aeromonas caviae* NGP3 associated from *Sargassum cinctum* seaweed. The presence of fibrinolytic protease in *Aeromonas caviae* NGP3 was confirmed by quantitative fibrin degrading assay. The protein was precipitated by ammonium sulphate followed by dialysis. The protein content was estimated 1.4 mg/ml the fibrinolytic protease was characterized by Analytical High Performance Liquid Chromatography.

Key words: *Sargassum cinctum*, *Aeromonas caviae* NGP3, fibrinolytic enzyme**.**

INTRODUCTION

Cardiovascular diseases are one of the major problems facing by our society. According to world health organization in 2015 (WHO) each year 17.7 million death was happening worldwide because of cardiovascular diseases.[1]Mycocardial infraction, stroke, high blood pleasure and venous thrombosis are the main cardiovascular diseases which escalating the mortality rate in the world.[23,7,14] Intravascular thrombosis is a key reason for of cardiovascular disease.[17] Accumulation of the fibrin in the blood will lead to thrombosis. Generally if a fibrin clot happened in the body, plasminogen gets activated and releases the plasmin which will degrade the fibrin clot. But in certain times the clot couldn't lyse completely by plasmin and this will impede the blood flow as a result cerebral and cardiovascular diseases arise to the body.[24] Streptokinase, plasminogen activator (PA) tissue plasminogen activator (t-PA) and urokinase are the currently using fibrinolytic agents or drugs, [16,9] these fibrinolytic agents are able to degrade the fibrin clot from blood but it was also reported to have some undesirable effects such as hemorrhagic gastritis, Blurred vision, wheezing,[20,25] resistance to repercussions, and allergic reactions. Although the drugs which are using to degrade the fibrin clot need large therapeutic doses and they are expensive too. This justifies the search for new fibrinolytic enzymes from natural sources so it can avoid the problems causes by synthetic drug or compounds. Few types of seaweed are reported of fibrinolytic activity. But there hasn't been any report of

fibrinolytic activity of seaweed associated microorganisms. Thus the present work aims to identification and characterizations of fibrinoytic protease from seaweed associated microorganism.

MATERIALS AND METHODS

Collection of seaweed

Sargassum cinctum (figure 1) seaweed is collected from Mandapam (Palk Bay, 9˚16´N, 79˚7´E) Tamilnadu, India. Collected seaweed was aseptically transferred to the laboratory for further analysis.

Isolation of seaweed associated bacteria.

Cotton swabbing was used to isolate the seaweed associated bacteria. Sterile cotton swab bud was applied on seaweed surface and recovered bacteria were inoculated on marine ZoBell 2216E agar plate.[2] The inoculated plates were incubated for 24 h at 28˚C .The bacterial strains were selected based on colony morphology. The selected bacterial strains were purified up to the third generations and maintained in agar slants.

Fibrinolytic activity

All bacterial isolates were screened for fibrinolytic activity, in the method of [3] with slight modification. The micro centrifuge tubes were weighed; into each pre weighed tube 500µl of freshly drowned blood was added and incubated at 37˚C for 90mins for clot formation. After the formation of clot the serum was completely taken out without disturbing the clot. To determine the clot weight, the tubes were again weighed (The clot weight= weight of clot condoning tube- weight of empty tube) and noted down the clot weight. To each micro centrifuge tube of pre weighed clot, 500µl of sample was added. 500µl of milli Q water was used as negative control and streptokinase was used as positive control. The tubes were incubated at 37˚C for 12 hrs and observed for clot lysis. The fluid obtained after the incubation was removed and the clot was again weighed to determine the difference between weight taken before and after clot lysis. All the reactions were carried out in triplicate value. Finally the percentage of clot lysis was calculated using the following formula.

Percentage of clot lysis = (Weight of released clot / Clot weight) x100

The isolates which showed the highest percentage of fibrinolytic activity was taken for further study.

fibrinolytic protease production

The strain *Aeromonas caviae* NGP3 was inoculated in 500ml production medium and incubated at 37˚C for 72 hours. It was then centrifuged at 7,000 rpm for 10 minutes and the cell free supernatant was precipitated by ammonium sulfate at 10-80% saturation and kept for overnight at 4˚C. The precipitated proteins were centrifuged at 12,000 rpm for 30 minutes and the pellet was suspended in 0.02M sodium phosphate buffer

(pH 6.9). Desalting was done by dialysis. The phosphate buffer saline (pH 7.4) was used for the dialysis. Every hour the buffer replaced with fresh phosphate buffer. After dialysis the protein content was estimated by Lowry's method.[4] The saturation percentage which exhibited maximum activity was selected for further study.

Polymer Chain Reaction

DNA was isolated and 5µl of isolated DNA was added into 25µl of PCR reaction solution (12 µl of Taq Master Mix 1.5 µl of forward primer 8F (5'AGAGTTTGATCCTGGCTCAG3') and reverse primer 1541R (5'AAGGAGGTGATCCAGCCGCA3') 5µl of deionized water) and performed Polymer chain reaction using following thermal cycling conditions. Initial denaturation 95˚C for 2 minutes, denaturation 95˚C 30 second, annealing 55˚C 30 second, Extension 72˚C 2 minutes Final extension 72˚C 10 min.

Purification of PCR Production

The Montage PCR Clean up kit (Millipore) was used to remove unincorporated PCR primers and dNTPs from PCR products. The PCR product was sequenced using the primers. Sequencing reactions were performed using ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase.

Sequencing

Single-pass sequencing was performed on each template using 16S rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer.

Identification

The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences. The resulting aligned sequences were cured using the program Gblocks 0.91b.ThisGblocks eliminates poorly aligned positions and divergent regions (removes alignmentnoise) the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. The identified microorganism was submitted into NCBI. (Genbank Accession number: MH021841).

Analytical HPLC

Desalted sample was subjected to analytical HPLC (Shimadzu C-18) for partial purification of proteins by using UV detector at 220 nm with the flow rate of 1ml/minute. Acetonitrile was used as the solvent.

RESULTS AND DISCUSSION

The *Sargassum cinctum* seaweed was collected from, Mandapam (Palk Bay, 9˚16´N, 79˚7´E) Tamilnadu, India. The collected seaweed was identified from Botanical survey of India Coimbatore. Totally 6 different

seaweeds associated bacteria were isolated and named as SSD1,SSD2,SSD3,SSD4,SSD5 and SSD6 and all the bacterial isolates were screened for fibrinolytic activity. Among that SSD5 showed significant fibrinolytic activity, The cell free supernatant of *Aeromonas caviae* NGP3showed 60±1.2% of fibrinolytic activity with protein content (1.8 mg/ml) streptokinase showed 93±0.63% (30,000 I.U). The negative control showed only 4±0.22% of fibrinolytic activity. So the 16S rDNA identification of the bacterium was done and identified as *Aeromonas caviae* NGP3. When compared with other percentage of ammonium sulfate saturations 80% ammonium sulfate saturated protein precipitates showed highest fibrinolytic activity that is 58±0.70% (1.4 mg/ml). The partially purified fibrinolytic protease was then subjected to analytical HPLC at 220nm. The chromatogram reveals the presence of major fraction at the retention time of 3.357, which may be responsible for the observed activity (Figure 3). So far nobody has reported fibrinolytic protease from *Sargassum cinctum* associated *Aeromonas caviae* NGP3. The results of this study cleary explain the fibrinolytic ability of *Aeromonas caviae* NGP3. So the further purification and characterization of the fibrinolytic protease from *Aeromonas caviae* NGP3 may lead the introduction of new fibrinolytic agent.Streptokinase is one of the major drugs using for the fibrinolysis the Streptokinase was produced from *Streptococcus* sp [9] even though the drug has reported lot of adverse effects. So the researchers started looking for a fibrinolytic protease from a better source. Few plants and microorganisms were reported to have a fibrinolytic activity. Apart from these certain fermented food from Asia also been reported fibrinolysis. *Bacillus cereus* isolated from traditional fermented malay sea food has been reported extracellular fibrin degrading activity. [24] The findings of these study correlating the work done study done by Surya *et al*., [28]. Fibrinolytic activity was reported from variety of plants. The metabolic extract of *Byttneria pilosa* showed 46.20% of clot lysis activity [21] Leletha *et al*., reported the fibrinolytic activity of *Tulbaghia violacea* [12] *Hemidesmus indicus* and *Campomanesia xanthocarpa* has been reported thrombolytic activity [15,16] 53.22% of fibrinolytic activity was reported in *Cassia senna*.[17] The bark extracts of *Averrhoa bilimbi* has reported significant fibrinolytic activity. [18] A thrombin degrading enzyme was purified From *Bacillus sphaericus* and the molecular weight of the compound was reported as18.6kDa.[19] Purified nattokinase from mutant strain of *Pseudomonas aeruginosa* was observed the molecular weight of 21kDa.[20] A novel fibrinolytic enzyme was purified from soybean fermented food from china and the enzyme was reported the molecular weight of 30kDa [21] fibrinolytic protease was purified from *Streptomyces* sp and the enzyme showed single protein band of 35kDa in SDS-PAGE. [23] Partially purified fibrinolytic enzyme extracted from *Auricularia polytricha* was detected the molecular weight of 66kDa. [22] The fibrinolytic enzyme purified from *Streptococcus equinus* VIT_VB2 reported to have the molecular weight of 47kDa [14]. The highest molecular weight of fibrinolytic enzyme was reported as 100kDa in *Ganoderma lucidum* [13].So far higher molecular weight to lower molecular weight compounds has been reported fibrinolytic activity. Further characterization and purification of the protease from *Aeromonas caviae* NGP3 will give exact molecular weight and its mode of actions

Figure1
Sargassum cinctum

S.No	Isolates	% of fibrinolytic activity
1	SSD1	15±0.46
2	SSD2	14±1.1
3	SSD3	10±0.63
4	SSD4	12±0.44
5	SSD5	60±1.2%
6	SSD6	9±0.35
7	Negative control	4±0.22
8	Positive control	93±0.63%

Table 1
Quantitative fibrin degrading test

CTGGTAACGCCCTCCCGAAGGTTAAGCTATCTACTTCTGGTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCC
GGGAACGTATTCACCGCAACATTCTGATTTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGA
CTACGACGCGCTTTTTGGGATTCGCTCACTATCGCTAGCTTGCAGCCCTCTGTACGCGCCATTGTAGCACGTGTGTAGCCCTGGC
CGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGGAGGCTCCCTTGAATTGCCACCATTACCTG
GTGGGAACAAAAGACATGGGTTAGGACTCGTTGCGGATTTTAACCCAATTTCTCAGAACAGGAAAGGACGACAGCCATGATCT
GCCGGTGTTCTGATTCCCGAAGGAACACCCGCATCTCTACAGGATTCCAGACATGTCAAGACCAGGTAAGGTTCTTCGCGTTGC
ATCAAATTCAACCACATGCTCCACCGCTTGTGCGTGGCACCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCGCCATGA
GGTCGATTTAACTACGTTCTTCCGAAAGCCACGTCTCACGGACACAGCCTCCAGATCGACATCGTTTACGGCGTGAACTACGAT
GGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCGTTGTCCAGGGGGCAGCCTTCACCACGGGTGTTCCT
CCAGATCTCTACGCATATCTGCGCGACACGTGCAAGTCTACGTGCCTCTACAGATACTACGCTGAACAGTTTAAATGCAATCAC
AGCTGCAGCCTGGAACTTACAGTCAAGTTATCGACCCGCCTGGCTGGCGGTTACATGGCGGTTAATTCCGATGAACGCTGGAC
ACCCTCACCATGACCTCGACATGCTGGCACGTATTAGCAGGTGCTTCATCGGC

Figure 2
16S rDNA Sequence of *Aeromonas caviae* NGP3

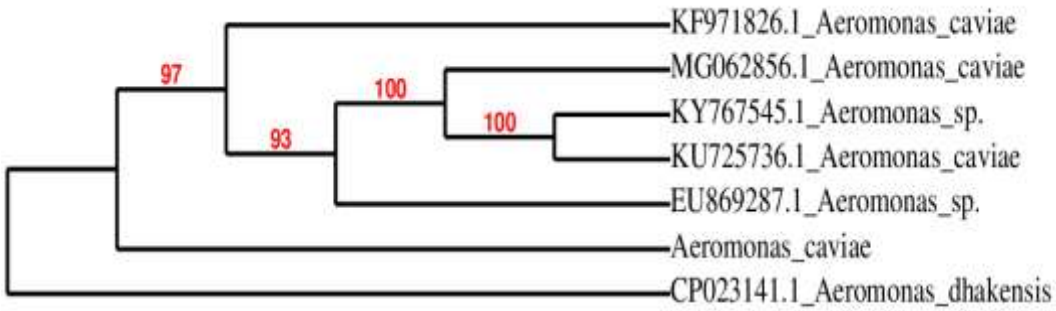

Figure 3
Phylogenetic tree of *Aeromonas caviae* NGP3

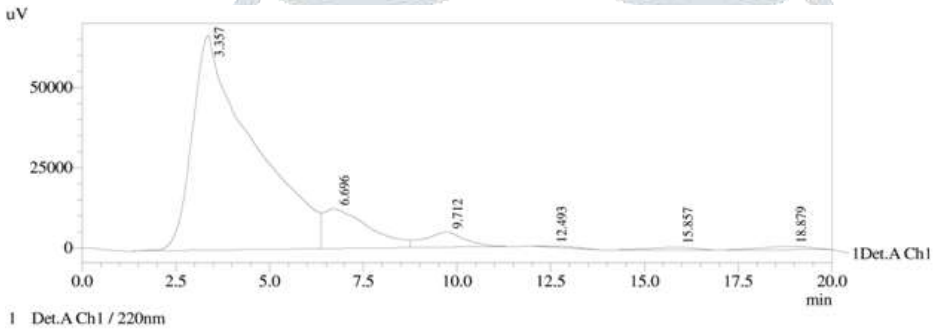

Figure 4
HPLC Chromatogram of fibrinolytic protease from *Aeromonas caviae* NGP3

CONCLUSION

The present drugs using to treat the cardiovascular disease are reported to have adverse effects. So the discovery of a fibrinolytic protease from a better source is one of the top priorities of the world. The partially purified fibrinolytic protease from *Aeromonas caviae* NGP3 showed highly promising fibrinolysis. The further purification and characterization of the compound may lead to the introduction of a new bioactive compound for the cardiovascular disease.

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Conflict of interest declared none.

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