PURIFICATION AND CHARACTERIZATION OF FIBRINOLYTIC ENZYME FROM STAPHYLOCOCCUS HOMINIS NGP2 ASSOCIATED FROM ULVA LACTUCA

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***Abstract:** This study was conducted to investigate the fibrinolytic activity of Staphylococcus hominis NGP2 associated from Ulva lactuca seaweed. The presence of fibrinolytic enzyme in Staphylococcus hominis NGP2 was confirmed by quantitative fibrin degrading assay. The protein was precipitated by ammonium sulphate followed by dialysis. The protein content was estimated 2 mg/ml. The fibrinolytic enzyme was subjected to preparative High Performance Liquid Chromatography. Four fractions were collected and fibrinolytic activity was checked for all fractions. The fruction I showed highest fibrinolytic activity (64±0.24%). So the fraction I was considered as an active fraction and further characterization was done with fraction I. The molecular weight of the fibrinolytic enzyme was determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis as 63 kDa.*

***Key words:** Seaweed associated bacteria, Ulva lactuca, Staphylococcus hominis NGP2, Fibrinolysis.*

INTRODUCTION

The macro algae and marine bacteria are an inexhaustible source of bioactive compounds.[3]Since late 1980s more than 50,000 natural products have been discovered from marine microorganisms. Among them more than 10,000 had biological activity.[3,11,13] In marine environment, the microorganisms will colonize on living substances like seaweeds sponge's etc. more than fungus Bacteria is often found on the surface of seaweeds.[10,5,8]Normally the bacteria found on the surface of seaweeds are non photogenic to seaweeds.[4,6,9]In fact the seaweeds and these bacteria are reported to have a symbiotic relationship. The relationship between seaweed and its associated microorganism are not well studied; however some seaweed associated microorganisms are reports to improve the seaweeds ability to tolerate abiotic stresses.[3,7] In addition, seaweed surface associated microorganisms have proved to play very significant role in the production of some antiviral, antibacterial and antifungal compounds produced from seaweeds.[3]So the seaweed associated microorganisms have high pharmacological importance.[25]From different seaweeds approximately two thousand chemical compounds have been characterized and reported.[3,7,9] The seaweed surface associated microorganisms are directly or indirectly helping the seaweeds to produce these chemical compounds even though not even 1% of seaweed associated microorganisms has been studied. So this study is an attempt to find out biological active compounds especially fibrinolytic enzyme from seaweed associated microorganisms. The currently using synthetic drugs for fibrinolysis have been reported hemorrhagic side effects. So the pharmacological industries are focusing to develop a fibrinolytic agent from a natural source. There were several plants and few microorganisms were reported fibrinolytic activity. But fibrinolytic enzyme from seaweed surface bacteria is not studied much. So the objective of the study was to isolation of fibrinolytic enzyme from *Staphylococcus hominis* NGP2 bacteria associated from *Ulva lactuca* seaweed.

MATERIALS AND METHODS

Collection of the Seaweeds

The seaweed *Ulva lactuca* (figure 1) was collected from Mandapam (Palk Bay, 9˚16´N, 79˚7´E) Tamilnadu, India. The collected seaweed was put into a ziplock plastic bag and placed in an ice bag and 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 marine agar plate.[26] 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[16] with slight modification. 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.

Molecular Identification

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: MH021846).

Fibrinolytic Enzyme Production

The strain *Staphylococcus hominis* NGP2 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[2]. The saturation percentage which exhibited maximum activity was selected for further study.

Analytical and Preparative Reverse Phase-High Performance Liquid Chromatography (RPHPLC)

The fibrinolytic enzyme was subjected to preparative HPLC (Shimadzu C-18) using UV detector at 220nm. Water is used in Pump A and acetonitrile is used in pump B in the ratio of 20:80 and the mixture of these solvents act as mobile phase. Both the solvents (water and acetonitrile) were filtered using 0.22-µm syringe filter before use and de-gassing were performed using inbuilt method. The pump is used to deliver the mobile phase through the liquid chromatography at a specific flow rate (1ml/minute) and a stable baseline was obtained by using UV detector at 220nm to ensure proper equilibration of the column. The auto sampler was used to introduce the sample into the flow stream of the mobile phase. The detector (UV detector) helps to detect the individual molecule that elute from the column. It provides an output to a recorder or computer that result in the liquid chromatogram. Totally 70 minutes took for the elution of the sample. Four fractions were collected automatically at regular intervals and were evaporated. The fibrinolytic activity and protein content were checked for obtained fractions. The fraction which showed the highest inhibitory activity among the four fractions was considered as the active fraction and the fraction was subjected to analytical HPLC and single peak was obtained in the chromatogram.

SDS-PAGE Analysis

SDS-PAGE was carried out with 10% separating gel and 5% stacking gel by the method of Laemmli.[15] the protein sample mixed with loading dye was loaded into each lane along with the protein marker of 10-245kDa in the adjacent well. The gel was for proteins with Coomassie Brilliant Blue R-250 and the destained gel was visualized for the bands.

Cell Cytotoxicity Assay

The cell cytotoxicity was checked using Vero cell line. The MTT (dimethyl thiazolyltetrazolium bromide) assay was done using the method of Yang *et al* [27]. The cells in suspension containing approximately 1×10^6 were added to each well of a 96-well culture plate and were incubated for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . Vero cell line was treated three different concentration of (50µg, 100µg, 150µg) purified fibrinolytic enzyme. Control cultures were treated with DMSO. After 24-36 h, 20 µl of MTT solutions were added to each well and the cultures were further incubated for 4 h and then 200 µl of DMSO was added. The formed crystals were dissolved gently by pipetting two to three times slowly. The absorbance at 570 nm was measured using plate reader.

Per cent inhibition was calculated using the following formula

Control OD-Test OD/ Control OD X100

RESULTS ANDDISCUSSION

Identification of Seaweed

Ulva lactuca 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, Tamilnadu, India.

Figure 1: *Ulva lactuca*

Bacterial isolation and Fibrinolytic Activity

Totally 7 different *Ulva lactuca* associated bacteria were isolated and named as SDU1, SDU2,SDU3,SDU4 and SDU5, and all the bacterial isolates were screened for fibrinolytic activity, among 7 isolates SDU3 showed potent fibrinolytic activity. 500µl of cell free supernatant of showed 73.4±0.58% (2mg/ml) of fibrinolytic activity and positive control showed 94±1.34% fibrinolytic activity (30,000 I.U). The negative control showed only 4±0.97% of fibrinolytic activity (table 1).

S.No	Isolates	% of fibrinolytic activity
1	SDU1	11±0.43
2	SDU2	15±1
3	SDU3	73.4±0.58
4	SDU4	10±0.39
5	SDU5	18.1±0.25
6	SDU6	20.4±1.1
7	SDU7	13.5±0.82
8	streptokinase	94±1.34
9	Negative control	4±0.97

Table 1: Quantitative fibrin degrading test

Identification

The bacterium which showed promising fibrinolytic activity was identified as *Staphylococcus hominis* NGP2 by 16S rDNA sequencing. And the phylogenetic tree was constructed for identified bacterium.

GACGTTAGCGGCGGACGGGTCGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACC
GGATAATATTTCGAACCGCATGGTTCGATAGTGAAAGATGGCTCTGCTATCACTTATAGATGGACCTGCGCCGTATTAGCTAGTT
GGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTT
CGGATCGTAAAACTCTGTTATTAGGGAAGAACAAACGTGTAAGTAACTGTGCACGTCTTGACGGTACCTAATCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGTCGTAAACCTCGCGTAGGCGTTT
TTTTAAGTCTGATGTGAAAACCCACGGCTCTACCGTGGAGG

Figure 2: 16S rDNA Sequence of *Staphylococcus hominis* NGP2

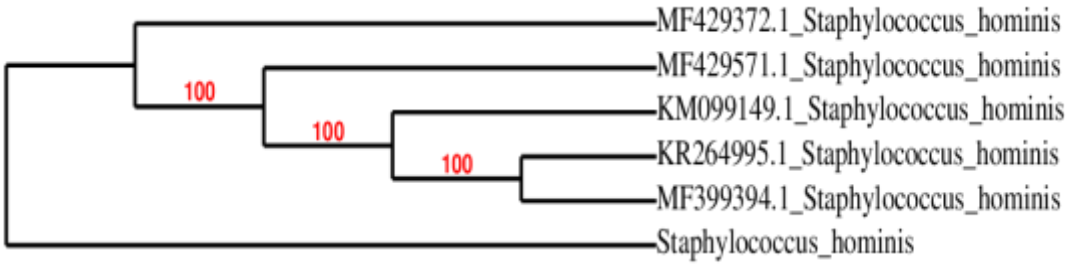


Figure 3: Phylogenetic tree of *Staphylococcus hominis* NGP2

Production and Purifications of the Enzyme

The protein precipitation was done by ammonium sulphate followed by dialysis. In 60% saturation the sample showed high fibrinolytic activity so further study was done with 60% of ammonium sulphate saturation. The protein concentration of dialyzed fibrinolytic enzyme was estimated as 2mg/ml. The preparative HPLC was used for the purification of the enzyme produced from *Staphylococcus hominis* NGP2. Four fractions were collected from preparative HPLC. Fraction I showed 64±0.24% of fibrinolytic activity whereas other fractions showed only 20±0.29, 15±0.41 and 10±0.22 percentage of fibrinolytic activity respectively. Hence fruction I was considered as an active fraction and the further study was concentrated with fraction I. Fraction I was subjected to analytical HPLC for analyzing the purity. A single peak was obtained in the chromatogram with the retention time of 3.603 (figure 5). The protein concentration of fraction I was estimated as 1mg/ml. the molecular weight of the protein was determined as 63kDa (figure 6).

Figure 4: Quantitative fibrinolytic activity assay for fraction I

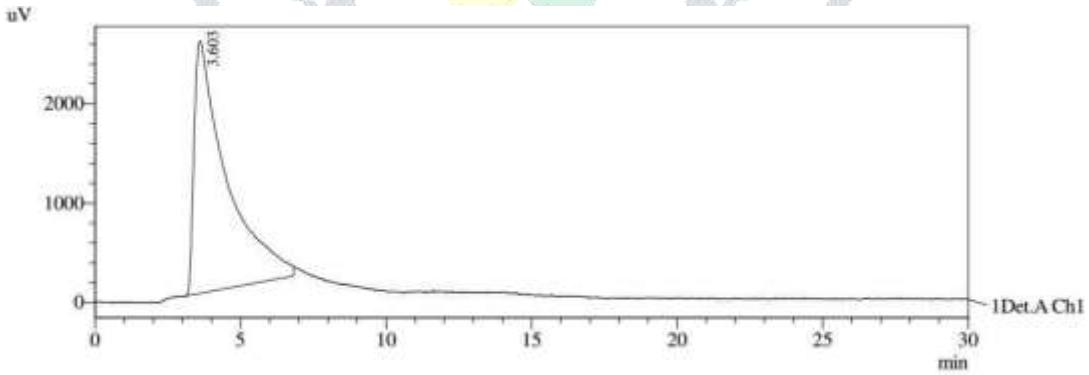


Figure 5: HPLC Chromatogram of fibrinolytic protease from *Staphylococcus hominis* NGP2

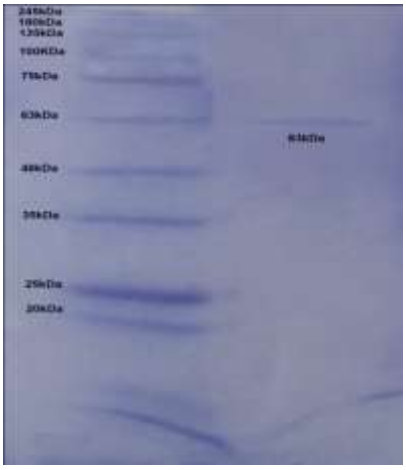
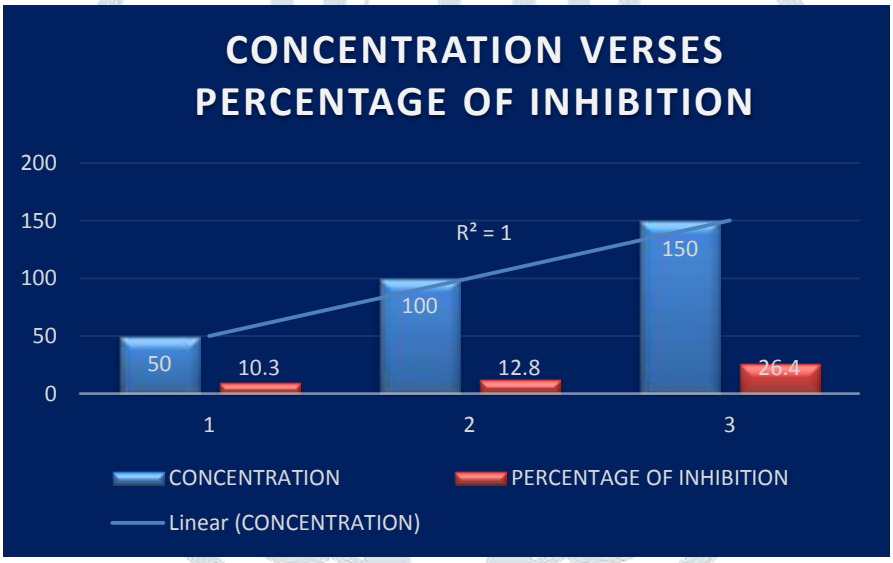


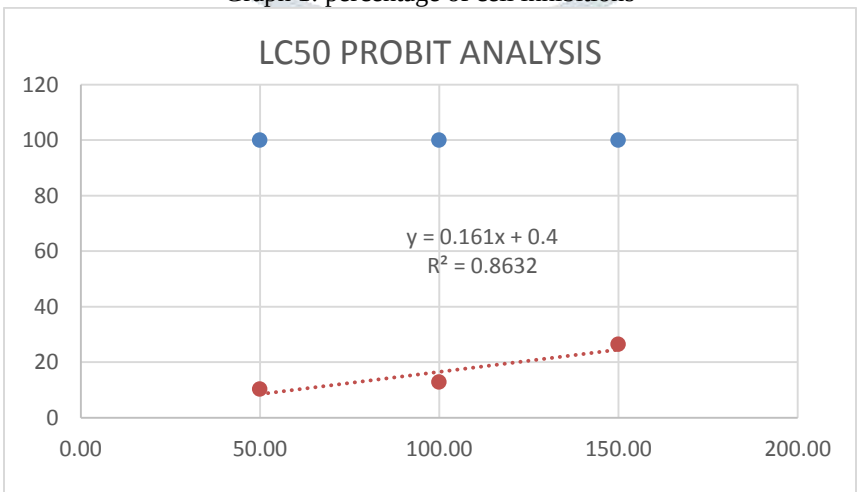
Figure 6: SDS PAGE Analysis of fibrinolytic protease from *Staphylococcus hominis* NGP2

Cell Cytotoxicity Assay

The cell cytotoxicity was performed with 3 different concentrations 50µg, 100µg, and 150µg and cell inhibitions were found as 10.3%, 12.8% and 26.4% respectively, there was a the enzyme was found nontoxic to the cells. The LC50 of the enzyme was calculated as 308.1µg/ml.



Graph 1: percentage of cell inhibitions



y axis inhibition: x axis concentrations

Graph 2: LC50 analysis

DISCUSSIONS

Myocardial infarction is a major plight of contemporary society. The main reason for myocardial infarction is fibrin clot. Normally fibrin will produced to prevent the blood loss during injury. Once the bleeding get stopped the plasminogen get activated and produce plasmin. The plasmin will remove the fibrin clot from blood. But in some case the plasmin will not lyse the fibrin clot completely; the unsolved clot by plasmin will lead to the cardiovascular disease. There are many drugs using for the fibrinolytic therapy, like urokinase, streptokinase, tissue- type plassminogen activators etc. among these drugs streptokinase and urokinase are first generation fibrinolytic agents these drugs are reported have a problem with normal blood clotting process. Streptokinase is also an immunogenic so it will lead to the drug resistance. The second generation agent is tissue- type plasminogen activators. The generation thrombolytic drugs are reported of increase risk of intracranial hemorrhage.[26]Another big drawback of these drugs is its high expense and large dosage. So a safer and cheaper fibrinolytic agent from a natural source is the only way to solve the problem causing by the synthetic drugs. Few microorganisms plants and some mushrooms are reported fibrinolytic activity with low molecular weight to high molecular weight compounds.[17, 18,19,21,22] While comparing with plants the fibrinolytic compound from microbial source is a best option because the productions of microorganisms are very easy and microorganisms are highly available so the cost of the fibrinolytic compound developed from the microorganisms will be cheap. In this study we have try to develop a fibrinolytic drug from *Staphylococcus hominis* NGP2 since it's a bacteria the production cost of the drug will be less and in our study we have taken very less concentration of sample (1 mg/ml) after that also we have observed 64±0.24% of fibrinolytic activity, if we could develop a drug from this enzyme the dosage of the drug will be less. So the further study of fibrinolytic enzyme developed from *Staphylococcus hominis* NGP2 may lead to the production of new potent drug for cardiovascular diseases

CONCLUSION

The fibrinolytic enzyme from *Staphylococcus hominis* NGP2 associated from *Ulva lactuca* seaweed showed significant fibrinolytic activity. Further characterization of bioactive compound will put light into its hidden fibrinolytic molecular mechanisms.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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