# Purification, Characterization and *in vitro* Anti microbial Activity of Marine Bacterium Proteins: *Pseudomonas sp.*

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## Abstract

The marine water samples were collected from the Kilakarai deep sea, the bacterial colonies were isolated and the dominated colonies were picked out and their morphological characterization was studied. From the biochemical characterization the isolated colony results as *Bacillus sp.* The antimicrobial potential were analysed and the resulted Fraction I, Fraction II, Fraction III shows the greater effect when against with the clinical pathogens. *Staphylococcus aureus, Pseudomonas sp,* Fraction III shows greater zone of inhibition in various concentration of (25 µl, 50 µl, 75 µl, 100 µl). The higher concentration of 100 µl of sample shows the greater results. The high concentration of the 100 µl of the crude sample exhibit higher zone of inhibition against the *Staphylococcus sp* (14mm), *Pseudomonas* (13mm), The results suggested that the fraction-III warranted further purification in order to unveil the active components of Pseudomonas sp. From the curve of elution in DEAE Sepharose Fast Flow chromatography Three A280 nm peak fractions, named G-1, G-2, G-3 were collected respectively. The antimicrobial potential of the marine microbes the G-1, G-2, G-3 shows the greater effect when against with the clinical pathogens. *Staphylococcus aureus, Pseudomonas sp*. Among that G-3 shows the maximum results of 18.2 mm and 15.4mm against *Staphylococcus aureus, Pseudomonas sp* respectively. The present study crude extract of marine bacillus showed anticancer activity against HT-15and HT-29 cell line in a dose dependant manner. The crude extract of of marine Bacillus sp showed activity of 7.88 at 400µg/ml. Whereas, the purified crude of fraction I, fraction II and Fraction III showed 7.99% , 20.67% and29.30% of inhibition at the same concentration respectively against HT-15 and

the purified crude of fraction I, fraction II and Fraction III showed 8.0% , 9.12% and13.02% of inhibition at the same concentration respectively against HT-29. The purified sample of G1, G2 and G3showed 18.63 , 15.80% and76.31% of inhibition at the same concentration respectively against HT-15 and the purified sample of G1, G2 and G3showed 12.90% , 12.53% and69.32% of inhibition at the same concentration respectively against HT-29. Estimation of amino acids was done using an automatic amino acid analyzer for the two fractions of FIII and G3.

**Introduction**

**Marine environment**

Over 70% of the earth's surface is made up of oceans, making them a vast resource for finding possible chemotherapeutic drugs. As a measure of biodiversity, greater taxonomic levels indicate that there are more phyla in the waters than on land. Twenty-one phyla are exclusive of the sea, but just one of the thirty-three recognised phyla of extant animals is exclusive of the land.

**Bioactive compounds**

Using marine creatures, either fully or partially, to create or alter products, enhance plants or animals, or create microbes for particular use is known as marine biotechnology. Many biological processes that apply to both aquatic and terrestrial creatures have been clarified by humans with the use of various molecular and biotechnological tools. According to Harvey (2000), just 10% of the more than 25,000 plants have had their biological activity examined.

Bacteria and fungi are significant producers of antagonistic substances in terrestrial environments. A comparable function in the oceans is anticipated for these organisms, and this expectation has indeed been confirmed. Reports have documented the antibiotic, antiviral, antifungal, and antiyeast properties of these microorganisms. Additionally, some growth-

stimulating characteristics have been identified, which could be beneficial for research on wound healing, carcinogenic effects, and cancer studies. Among the numerous bacteria that demonstrate antimicrobial properties, a variant of the ichthyotoxic Pseudomonas piscicida has shown considerable antagonism towards various microorganisms (Buck, J. D. et al., 1962).

Given that researching marine bacteria and their possible contributions to the development of pharmacologically active substances is an intriguing new area, this study involved the isolation and characterization of bacteria exhibiting antimicrobial properties from clinical pathogens.

**Materials and methods**

**Collection of Sample**

The marine water samples were collected from Kilakarai the deep sea

**Isolation of Bacterial Strains from the Samples by serial dilution Method**

The bacterial colony were isolated by serial dilution method and identified by standard method biochemical characterization followed by R.C Dubey.

**Extraction of total proteins**

Marine bacteria isolated as above were cultured in 300 ml Marine FePO4 0.1 g, dissolved in 1 l seawater, pH 7.2–7.6) for the production of bioactive compounds in 500 ml Erlenmeyer flasks. Flasks were incubated on a rotatory shaker at 220 rev/min at 25℃. After 7 days of cultivation. The culture was centrifuged. After centrifugation (16,000 g, 20 min) at 4 °C, the supernatant was collected and the crude extract obtained. The crude extract of bacteria was fractionated by salting out with increasing concentrations of ammonium sulfate. Solid ammonium sulfate was slowly added to the above crude extract with gentle stirring, up to 35% saturation in 20 min. After the crude extract was left at 4 °C with the ammonium sulfate under vortexing for another 40 min , the protein precipitate was collected by centrifugation (16,000 g, 20 min) at 4°C. The supernatant was transferred to another beaker, and solid ammonium sulfate was added to it up to 70% saturation. The mixture was treated as above. Likewise, another protein precipitate was obtained at 70%−100% saturation of ammonium sulfate. Each of the three protein pellets was suspended in 10 ml of ice-cold PBS (10 mM, pH 8.0), and dialyzed against a large volume (3 L) of distilled water for 24 h at 4 °C. Dialysis bags were employed. During this process, the dialysate was changed three times to completely remove any residual ammonium sulfate. (Murphy, et.al.1990)

**Antimicrobial Activity by Agar Well Diffusion Assay** (Perez *et al.,* 1990)

The agar well diffusion method was used for the inhibitory effects of crude extract of marine Bacillus against the clinical pathogens such as *Staphylococcus aureus*, *pseudomonas aurigenosa,* extract was loaded on the Muller Hinton agar plates. This was swabbed with clinical pathogen such as *Staphylococcus aureus, pseudomonas aurigenosa,* Five wells (6 mm in diameter) were made equidistance in each of the plates using a sterile cork borer. Up to 25µl to 100 µl of each concentration of the extract were respectively introduced into the wells using sterile automatic pipettes, with the stock in one well. It was allowed to diffuse at room temperature for 2 hrs and the plates were incubated to 37°C for 24 hrs. Diameters of the inhibition zones were measured. The antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by marine microbes.

**Anticancer activity – cytotoxicity study**

**Human cell lines:**

HT-29 and HT-15 human cancer cell lines of colon grown in RPMI medium were obtained from CMC hospital vellore.

**In-vitro cytotoxicity studies (Sini, *et, al.,*2012)**

The crude extract of marine Bacillus was studied for short term in vitro cytotoxicity using HT-15 HT-29.10mg of the extract was taken in an Eppeandorf vial of capacity 1ml and dilute to four different concentrations with its duplicate and control (50%) using solvent and mixed with the help of a vortexing machine. The cell viability was checked by trypan blue dye (1%). The cell suspension (1x 106 cells in 0.1ml) was added to tubes containing various Concentrations of the test compounds and the volume was made up to 1ml using phosphate buffered saline (PBS). Control tube contained only cell suspension. These assay mixtures were incubated for 3 hour at 37ºC. After incubation 0.1 ml trypan blue was added and number of dead cells determined by using haemocytometer. The percent viability was calculated by using formula: % viability = (live cell count/total cell count)*100.

**Micro culture tetrazolium (MTT) assay**

Cell viability was assessed by MTT assay (Micro culture tetrazolium/formazan assay) in the presence and absence of different concentrations of the plants extract. The cells were

seeded in 96-well plates. Four wells for each concentration were seeded and triplicate plates were used the cell line. Then, the cells were incubated at 37°C. After 24 h the medium was replaced by fresh medium containing different concentrations of the plants extract. Then, the medium was changed by fresh medium containing MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 4-diphenyltetrazolium bromide] with a final concentration of 0.5 mg/ml (after 24 h). The cells were incubated for another 4 h in a humidified atmosphere at 37°C and after that the medium containing MTT was removed and remaining MTT formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm immediately using an ELISA reader. $IC_{50}$ was defined as the concentration of the extract that produced a 50% decrease in cell viability relative to the negative control which was wells exposed to the solvent without any extract.

**Purification of proteins**

**Anion exchange chromatography** (DEAE Sepharose Fast Flow):

The Fraction-I, Fraction-II, and Fraction-III were obtained at the ammonium sulfate saturation of 0-35%, 35-70% and70-100%, Fraction III were active in the antimicrobial activity. Fraction III were obtained from the crude extract at 70%−100% saturation of ammonium sulfate was dialyzed against 10 mM Tris-HCl, pH 7.46 for 5 h and the dialyzed solution was subsequently injected into a DEAE Sepharose Fast Flow column, which was pre-equilibrated with the aforementioned Tris-HCl buffer. The column was washed with the same buffer until the baseline returned to zero and remained stable. The column was then eluted with increasing concentration of NaCl prepared in 10 mM Tris-HCl buffer, pH 7.46 at 4 °C. Aliquots of 5 ml/tube were collected at a flow rate of 1.2 ml/min and the absorbance was measured at 280 nm. Seven A280 nm peak fractions, named G-1, G-2, and G-3 were collected respectively.

**Protein assay**

The protein content of total protein extract fraction-III, G-1, G-2 and G-3 was determined by lowry's method

**Requirements**

- Standard solution
- Protein sample
- Pipette
- Test tubes
- Spectrophotometer

**Procedure**

- Pipetted out 0.2 - 1 ml of the working standard solution into a series of test tubes.

- 1ml of the samples were taken in other test tubes and the volumes of all the test tubes were made upto 1ml with distilled water.
- 5ml of alkaline copper reagent was added into all the tubes, mixed well and allowed to stand for 10 minutes.
- Then 0.5ml of Folin's reagent was added and incubated at room temperature in dark for 30minutes.
- The color developed and it was read at 660nm.
- A standard graph was drawn and the amounts of protein in the samples were calculated.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

The Sodium dodecyl sulfate-polyacrylamide gel electrophoriesis (SDS-PAGE) analysis was Performed.(fraction III and G1)

**Analysis of amino acid component**

The qualitative and quantitative estimation of amino acids was done using an automatic amino acid analyzer (Shimatzu-High performance liquid chromatography LC 4A) 20 µl of the purified sample was injected into single column and analyzed using sodium buffer system (Yamamoto *et al.,* 1994).

## Results and discussion

**Collection and transport of sample**

The marine water samples were collected from the kanyakumari deep sea, and the sample was transferred to the lab aseptically, the collected sample was stored in the air tight container.

**Isolation of marine organism**

Isolation using differential media. The water sample was serially diluted and spreaded into the marine agar medium. After incubation period the colonies were counted and the dominated colonies were picked out and their morphology morphological characterization was studied. The dominated colony were streaked and the pure culture were stored for further studies .The isolated colony results as *Pseudomonas sp*

**Crude extraction**

The Fraction-I, Fraction-II, and Fraction-III were obtained at the ammonium sulfate saturation of 0-35%, 35-70% and70-100%,

**Antimicrobial Activity by Agar Well Diffusion Assay** (Perez *et al.,* 1990)

The antimicrobial potential of the marine microbes the Fraction I, Fraction II, Fraction III shows the greater effect when against with the clinical pathogens. *Staphylococcus aureus,*

*Pseudomonas sp,* Fraction III shows greater zone of inhibition in various concentration of (25 µl, 50 µl, 75 µl, 100 µl). The higher concentration of 100 µl of sample shows the greater results. The high concentration of the 100 µl of the crude sample exhibit higher zone of inhibition against the *Staphylococcus sp* (14mm), *Pseudomonas* (13mm), which shown in the table 3. The results suggested that the fraction-III warranted further purification in order to unveil the active components of Pseudomonas sp.

**The curve of elution in DEAE Sepharose Fast Flow chromatography.**

Column specification: 1.6 × 30 cm; Equilibrate liquid: buffer C (Tris-HCl, pH 7.46, 10mM); Sample: Fraction-III; Detection wavelength: UV 280 nm; Flow rate: 1.2 mL/min; Collection rate: 5 mL/tube. Aliquots of 5 ml/tube were collected at a flow rate of 1.2 ml/min and the absorbance was measured at 280 nm. Three A280 nm peak fractions, named G-1, G-2, G-3 were collected respectively.

**Anticancer activity on HT-15and HT-29cell line**

The present study crude extract of marine bacillus showed anticancer activity against HT-15and HT-29 cell line in a dose dependant manner. The crude extract of of marine Bacillus sp showed activity of 7.88 at 400µg/ml. Whereas, the purified crude of fraction I, fraction II and Fraction III showed 7.99% , 20.67% and29.30% of inhibition at the same concentration respectively against HT-15 and the purified crude of fraction I, fraction II and Fraction III showed 8.0% , 9.12% and13.02% of inhibition at the same concentration respectively against HT-29. the purified sample of G1, G2 and G3showed 18.63% , 15.80% and76.31% of inhibition at the same concentration respectively against HT-15 and the purified sample of G1, G2 and G3showed 12.90% , 12.53% and69.32% of inhibition at the same concentration respectively against HT-29.

**Characterization of purified proteins**

To estimate the molecular weight of G-3, Molecular weight marker (range from 14.4kDa to 97.4 kDa). Three bands appear, the molecular weight of band 46kDa, band 35kDaand band 32kDa.

**Estimation of amino acids**

Estimation of amino acids was done using an automatic amino acid analyzer for the two fractions of FIII and G3. The amino acids present in these two sample were shown in the table

**Discussion**

Marine bacteria have been recognized as important and untapped resource for novel bioactive compounds (Anand, T.P,et.,al.2006; Uzair, B,et.,al.2006). Development of marine

biotechnology is expected to produce novel compounds that may contribute significantly towards drug development over the next decade. In this study, we isolated several marine bacteria for extraction of some possible types of antimicrobial compounds active against drug resistant organisms. To demonstrate and characterize this possible antagonistic ability, antibiotic resistant clinical isolates and indigenous marine bacteria were used as revelator.

In present study the antimicrobial potential of the marine microbes the Fraction I, Fraction II, Fraction III shows the greater effect when against with the clinical pathogens. *Staphylococcus aureus, Pseudomonas sp,* Fraction III shows greater zone of inhibition. The results suggested that the fraction-III warranted further purification in order to unveil the active components of Pseudomonas sp. From the curve of elution in DEAE Sepharose Fast Flow chromatography Three A280 nm peak fractions, named G-1, G-2, G-3 were collected respectively. In the previous studies of the Liyan Song *et.al* 2008 isolated seven fraction from the protein of invertebrates.

The antimicrobial potential of the marine microbes the G-1, G-2, G-3 shows the greater effect when against with the clinical pathogens. *Staphylococcus aureus, Pseudomonas sp*. Among that G-3 shows the maximum results of 18.2 mm and 15.4mm against *Staphylococcus aureus, Pseudomonas sp* respectively.

Estimation of amino acids was done using an automatic amino acid analyzer for the two fractions of FIII and G3. In the previous (Yamamoto *et al.,* 1994 study the aminoacids were studied and results were similar to the present studies).

In the present studies the crude extract were partially purified by the ion exchange chromatography and the antimicrobial activity was analysed and the compound was considered as the bioactive compound because of the biopotentiaal of the compound against the clinical pathogen. Kondratieva L.M.,and Vakhusheva E.V 1991).

**Cytotoxicity**

The crude extract from marine bacillus was initially evaluated for their effects on cell viability through cytotoxic test. Cytotoxic effect of crude extract from marine bacillus was observed for 72 h of incubation period and the $CC_{50}$ value was calculated as 500µg/ml.

**Anticancer activity on HT-15and HT-29cell line**

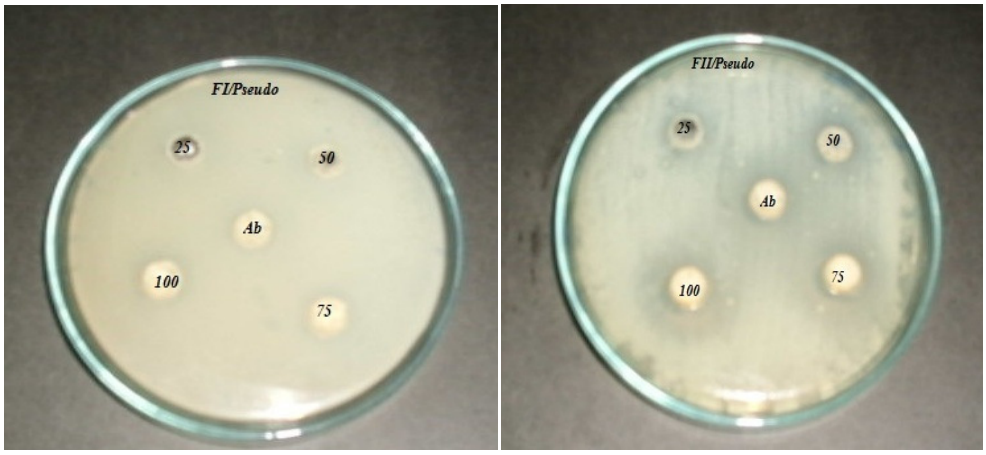
The purified crude of fraction I, fraction II and Fraction III showed 7.99% , 20.67% and29.30% of inhibition at the same concentration respectively against HT-15 and the purified crude of fraction I, fraction II and Fraction III showed 8.0% , 9.12% and13.02% of inhibition

at the same concentration respectively against HT-29. the purified sample of G1, G2 and G3showed 18.63% , 15.80% and76.31% of inhibition at the same concentration respectively against HT-15 and the purified sample of G1, G2 and G3showed 12.90% , 12.53% and69.32% of inhibition at the same concentration respectively against HT-29. The IC50 value shows the inhibition concentration at which only 50% of the cells are viable. It was reported that crude materials that require a drug concentration greater than 30 µg ml-1 to exhibit cytotoxicity were not considered as cytotoxic agents (Quah,1995).

Molecular weight of the G-3 were analysed with Molecular weight marker (range from 14.4kDa to 97.4 kDa). Three bands appear, the molecular weight of band 46kDa, band 35kDaand band 32kDa (Liyan Song *et.al* 2008).

Based upon the results of the *in vitro* antimicrobial assay, two pratien samples Fraction III and G3 from *Pseudomonas sp* shows the bioactivity-guided fractionation and purification. Important characteristics of proteins G3- were identified, showing three bands and the molecular weights to be 46kDa, band 35kDaand band 32kDa. Additionally, our present study reveals that the studies of amino acids pharmaceutical agents were discovered by screening natural products from marine microorganisms

**Figure 5: Antimicrobial activity of the crude extracts (Fraction I, Fraction II. Fraction III) against *Pseudomonas aeruginosa***



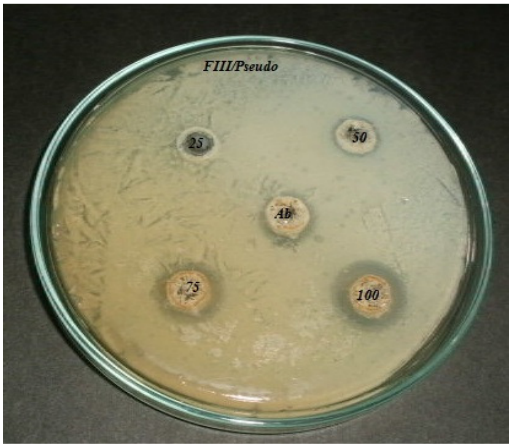



**Figure 6: Antimicrobial activity of the crude extracts (Fraction I, Fraction II. Fraction III) against *Staphylococcus aureus***

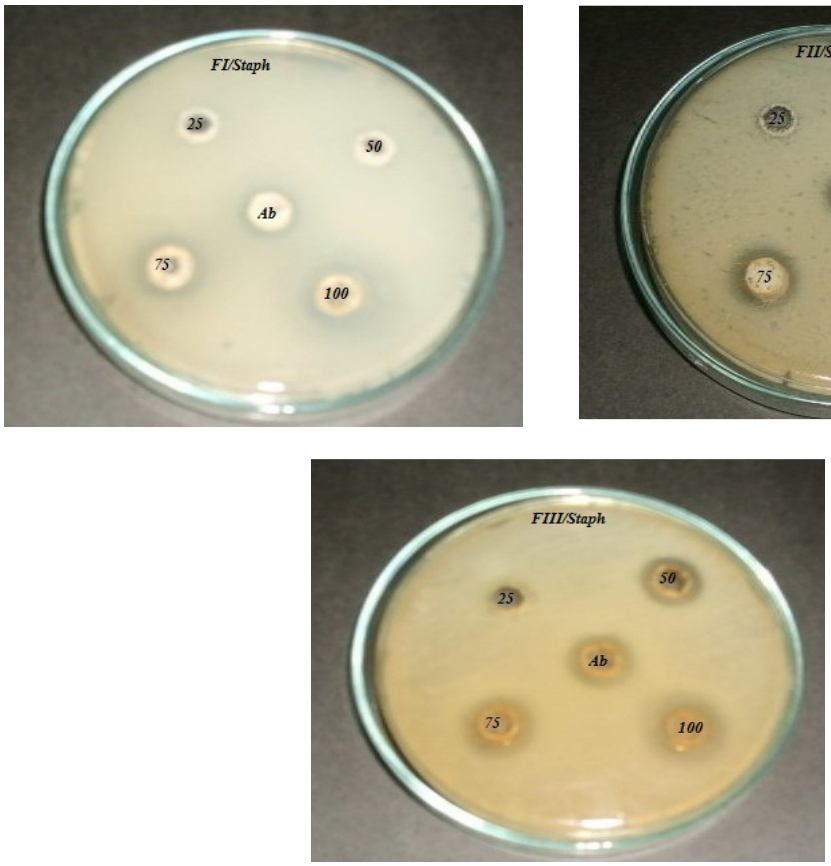



**Figure 7: Antimicrobial activity of the purified sample (G1, G2, and G3) against *Pseudomonas aeruginosa***

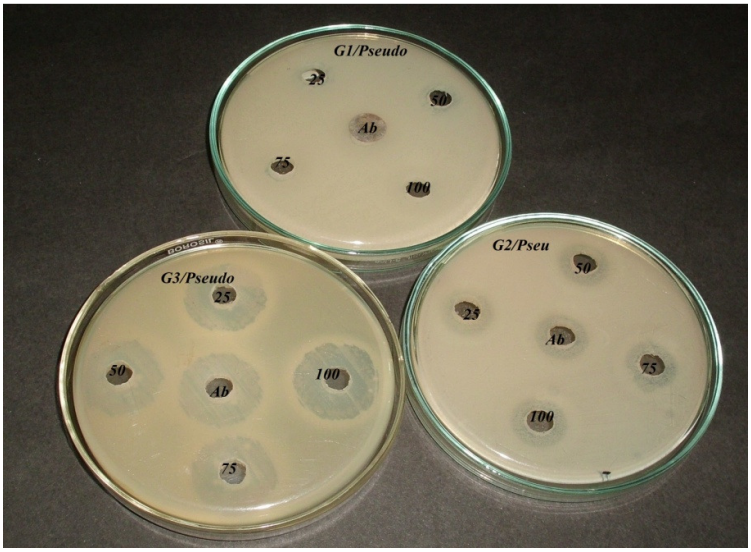



**Figure 8: Antimicrobial activity of the purified sample (G1, G2, and G3) against *Staphylococcus aureus***

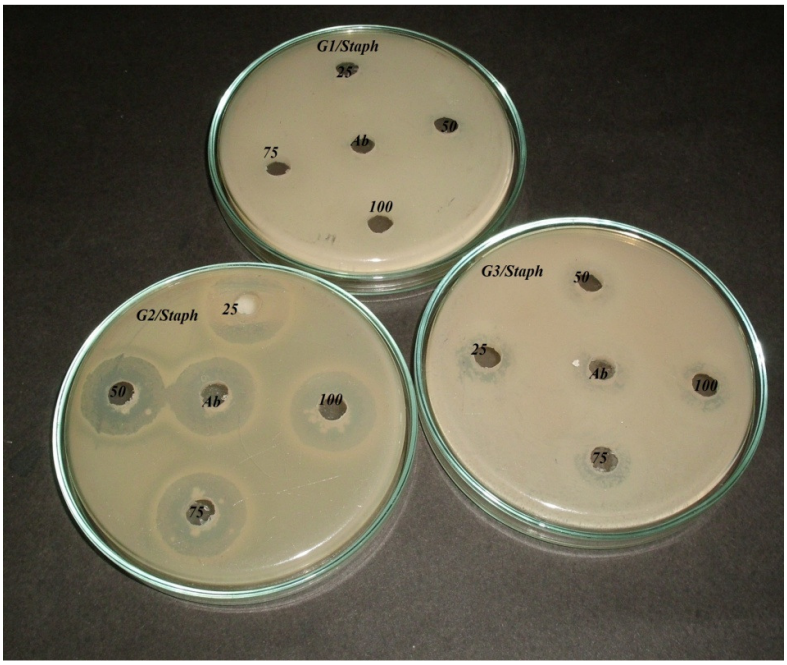
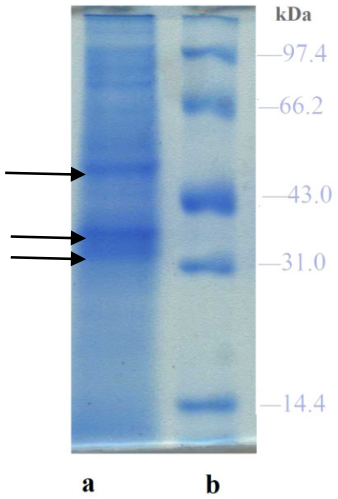


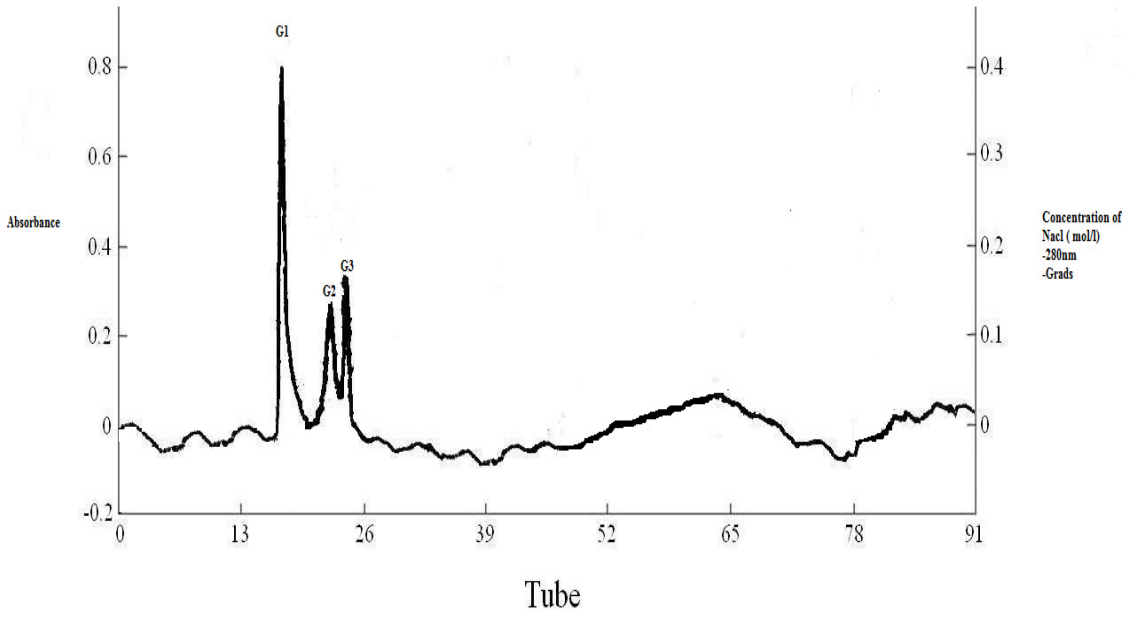

**Figure 9: The SDS-PAGE of G3**

Molecular weight marker (range from 14.4kDa to 97.4 kDa). Three bands appear, the molecular weight of band 46kDa, band 35kDaand band 32kDa




**The curve of elution in DEAE Sepharose Fast Flow chromatography.**

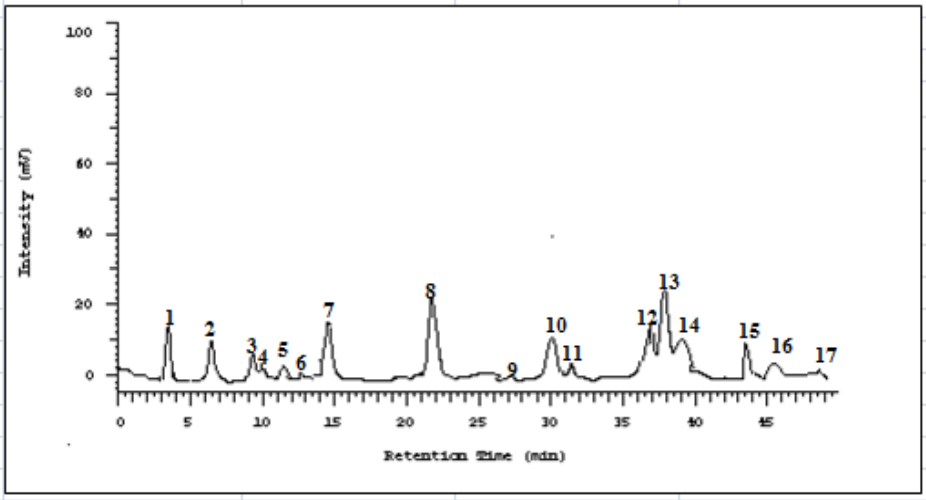
Column specification: 1.6 × 30 cm; Equilibrate liquid: buffer C (Tris-HCl, pH 7.46, 10 mM); Sample: Fraction-III; Detection wavelength: UV 280 nm; Flow rate: 1.2 mL/min; Collection rate: 5 mL/tube.




**Analysis of Aminoacids**

# D-7000 HPLC System Manager Report

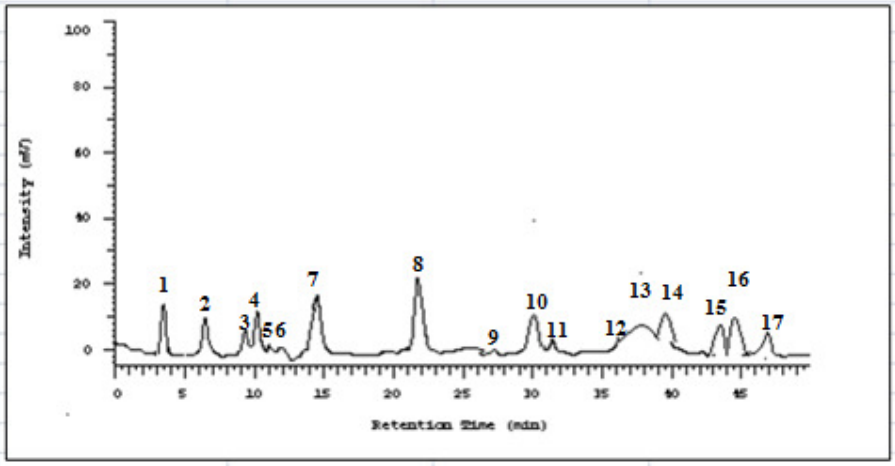
| | | | |
|---|---|---|---|
| **System Name:** | **LACHROM L-7000** | **Series:** | **AMINO ACIDS** |
| **Proc. Method:** | **Atoz** | **Sample Name:** | **FIII** |




| No. | COMPONENT NAME | R.T. | AREA | AREA % |
|---|---|---|---|---|
| 1 | ASPARTIC ACID | 4.06 | 10123 | 0.113 |
| 2 | GLUTAMIC ACID | 6.49 | 454 | 0.0343 |
| 3 | ASPARAGINE | 9.21 | 116 | 0.0011 |
| 4 | GLYCINE | 13.43 | 147 | 0.0054 |
| 5 | THREONINE | 14.9 | 3465 | 0.201 |
| 6 | ARGININE | 18.09 | 2604 | 0.109 |
| 7 | ALANINE | 19.55 | 134 | 0.005 |
| 8 | CYSTINE | 21.66 | 3423 | 0.2007 |
| 9 | TYROSINE | 24.78 | 121 | 0.008 |
| 10 | HISTIDINE | 27.44 | 324 | 0.0456 |
| 11 | VALINE | 29.91 | 1343 | 0.073 |
| 12 | ISO-LEUCINE | 36.65 | 343 | 0.024 |
| 13 | PHENYL ALANINE | 37.95 | 6545 | 0.298 |
| 14 | LEUCINE | 39.36 | 4067 | 0.189 |
| 15 | LYSINE | 43.41 | 6232 | 0.785 |
| 16 | PROLINE | 45.12 | 121 | 0.005 |
| 17 | TRYPTOPHAN | 46.13 | 878 | 0.078 |
| | | | | **2.1850** |

# D-7000 HPLC System Manager Report

| | | | |
|---|---|---|---|
| System Name: | LACHROM L-7000 | Series: | AMINO ACIDS |
| Proc. Method: | Atoz | Sample Name: | G3 |




| No. | COMPONENT NAME | R.T. | AREA | AREA % |
|---|---|---|---|---|
| 1 | ASPARTIC ACID | 4.03 | 129124 | 9.972 |
| 1 | GLUTAMIC ACID | 6.49 | 90531 | 8.35 |
| 2 | ASPARAGINE | 9.24 | 67525 | 1.549 |
| 3 | SERINE | 10.22 | 1301 | 0.072 |
| 4 | GLYCINE | 13.45 | 2012 | 0.201 |
| 5 | THREONINE | 14.89 | 121210 | 7.543 |
| 6 | ARGININE | 18.08 | 119 | 0.063 |
| 7 | ALANINE | 19.53 | 115 | 0.004 |
| 8 | CYSTINE | 21.67 | 305237 | 22.100 |
| 9 | TYROSINE | 24.76 | 115 | 0.004 |
| 10 | HISTIDINE | 27.25 | 10741 | 0.537 |
| 11 | VALINE | 29.90 | 10521 | 0.425 |
| 12 | ISO-LEUCINE | 36.62 | 12012 | 3.874 |
| 13 | PHENYL ALANINE | 37.93 | 16530 | 5.325 |
| 14 | LEUCINE | 39.34 | 18491 | 1.370 |
| 15 | LYSINE | 43.39 | 138124 | 13.771 |
| 16 | PROLINE | 45.11 | 209671 | 20.652 |
| 17 | TRYPTOPHAN | 46.12 | 475 | 0.321 |
| | | | 1133866 | 96.133 |

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