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Research Paper

Ion exchange chromatography of spirulinaextract forlectin protein purification and its antibacterial activity

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Abstract
Use of Spiriluna plantesis(blue-green algae) became more prominent after its uptake by the NASA for their astronauts as a dietary supplement during their space mission. The species has a great ability to perform immunological functions and anti-cancerous activities.This paper discusses water and salt extraction of a coagulant protein from the species, purification using ammonium sulphate and ion exchange chromatography and antimicrobialactivity. The protein extract is a cationic protein with PI greater than 9 and molecular mass of 31kDa. The crude extract is thermoresistant and found active after 6 hours treatment with heat from 60-90 ^{o}C. Simple methods for both the purification and assay of SP protein are presented, which are required for large scale production of protein having such miraculous properties.

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I. Introduction

Spirulina had long history since 9th century Kanem Empire as food supplement. In 1961, Leo Szilard postulated the food supplements based on algae and named it "Amruss" and after that first commercially production plant of Amruss was established by Sosa Texcoco in 1970 [1,2].In 1965 the botanist Jean Leonard confirmed that the Amruss is made up of spirulina which brings a bloom in sodium hydroxide production in 1970 [3].

The name spirulina has been coined because of the spiral nature of filaments of cyanobacterium [4]. It is a dried biomass of *Arthrospira plantesis* which is an aerobic photosynthetic bacterium found abundantly in fresh as well as in marine water [5,6]. The lectin protein content of spirulina is very high and therefore it is a potential source to extract and purify its protein content [7]. Other than protein significantamount of other supplements are also found in it, such as vitamin B12, provitamin A(β-carotenes), and minerals like iron [8].

The motivation behind using Spirulinain this work is its application in development of drugs which is beneficial in modulating the immunological functions in human body and performing certain antiviral activities [9,10].

Spirulina extracts are found to be particularly promising in resembling anti-bacterial activities [11]. Lectin protein extracted from spirulina can have strong tendency to bind to the monosaccharides and glycoproteins [12]. The micro-organism surfacesare composed of carbohydrates which are specific for the binding to the lectin protein.Majority of micro-organisms,are encapsulated with carbohydrate cell and are gram-negative/gram-positivebacteria composed of variety of carbohydrates such as teichoic acid, peptidoglycan and lipopolysaccharides. The binding between lectin and microbial surface content (carbohydrates) shows agglutination activity. In this work colony of Pseudomonas aeruginosa a gram-negative bacteriumhas been used to study antibacterial activity [13-15].

The first extraction and purification of protein was introduced by Edwin Joseph Cohn in 1943 [16]. The solubility properties, isoelectric point, precipitation, and crystallization plays a significant role in selection of a particular chromatography technique for purification of protein. Among various protein purification methods chromatography is certainly a commonly used technique for higher resolution efficiencies for protein purification. It is an ideal technique for capturing molecules from dilute solutions which often encountered in bioprocessing. Ion exchange chromatography has been chosen for this work based on pre-evaluated properties of lectin protein [17].

Ion-exchange chromatography, as the name suggests, is the exchange of ions which are oppositely attracted to the corresponding ion. Protein with known isoelectric point or pH of the environment corresponds to some charge on their surface. While working on pH lesser or more than isoelectric point charge on a protein can be determined. On the basis of charge, an appropriate exchanger is selected either cation or anion exchangers.

The exchangers are nothing but the matrix of beads which bear a charge (positive or negative). The protein with opposite charge to the matrix binds with it and further elution of the protein takes place by passing it through a salt gradient to obtain high-resolution purification of the protein. In this work cation-exchange chromatography is performed as the pH of the environment is lower than its isoelectric point [18,19].

In this study lectin protein has been extracted and purified from crude extract of spirulina using ion exchange chromatography. Various parameters of purification have been optimized by repeatedly performing experiments starting from some random unknown values after pre-examination of impure protein. The purified lectin has-been used to study antibacterial activity using Kirby-Bauer method. Pure lectin shows good antibacterial activities.

II. Materials and Methods

Materials and Equipments

Spirulina Powder Arthrospira Platensis was purchased from Parry Nutraceutical, Chennai, India. Tris-base and HCl were purchased from hi-media and CHD. Mini-PROTEAN Bio-Rad vertical electrophoresis unit have been used for SDS page analysis Running buffer (tris-glycine) was prepared in laboratory for SDS page. SP Sepharose resin were purchased from Sigma Aldrich and Column was packed in lab for ion exchange chromatography. GE Acta prime plus was used to perform final stage of purification. Nanodrop spectrophotometer were used for optical density measurements. MicroKwik culture purchased from Carolina, Burlington for antibacterial activity.

Extraction

The spirulina powder 80 gram were homogenized in 240ml, 50mM tris-HCl(pH 8.2) for about 15 minutes to obtain the crude extract. Further the sample has been centrifuged for 30 min at 10,000rpm and temperature was fixed at 4℃. Supernatant has been collected and used for further processing.

Purification Step I

In order to separate the enzyme from the pool of proteins partial purification was done using solid ammonium sulphate salt. Protein was precipitated using two-fold (30%-80%) saturation of the sample. After saturation (30% & 80 %) the sample were left for overnight. Next day the sample were centrifuged at 10.000 rpm by keeping temperature fixed at -4^{0}C and the pellets were collected and stored at -20℃.

Purification Step II

The obtained pellet after step I of purification were homogenised in running buffer. Running buffer solution with protein has been loaded in High-trap GE Acta primeplus. The column was equilibrated with 50 mM tris-HCl (pH 8.2) and 1mL/min flow rate was optimized and fractions of 1mL were collected in an automatic fraction collector. further, elution was performed using different concentrations of saline (NaCl) tris buffer for 0.1M, 0.2M, 0.3M, 0.4M and 0.5M.

Characterization

SDS PAGE was performed using Mini-PROTEAN apparatus (Bio-Rad). After electrophoresis, the purified sharp bands were observed above 2.5cm to the electrophoretic front with respect to the low range markers. Molecular mass has been determined using SDS page 12% gel. Using Bradford method with bovine serum albumin as a standard, protein was quantified.Protein has been tested for its thermal resistivity by incubating the crude extract at different range of temperatures from 60^{o}C to 90 ℃. The optical density of the protein was measured using nanodrop for all the fraction of solution collected from Acta prime.

Antimicrobial activity

Pseudomonas aeruginosa(gram negative), Escherichia coli(gram negative), Micrococcus luteus(gram positive) MicroKwik culture purchased from Carolina, Burlington, was used to check the antimicrobial activity. Cultures were grown overnight in 0.3M phosphate buffered LB media at 37℃ in shaker. After the centrifugation of culture, the pellet was resuspended in 50mM phosphate buffer. the suspension culture was introduced with the protein and incubated at 37℃ for 3 hour. Cellular moieties were viewed under microscope. The buffer(50mM tris-HCl pH 8) used to dilute the protein was used as control.150µl of the seed protein was added to agar plates of the bacterial colonies.

III. Results and discussion

Salting-in and salting-out

The initial extraction from crude extract of spirulina has been done using Tris-HCl of pH8. No organic contaminations were found in initial stages and therefore the adapted protocol for protein extraction was not challenging. In some cases, organic compoundscreate difficulty in the extraction process and modified protocols are need to be followed. Previous researches have very less to talk about the nature of the protein from spirulina

but our work is able to provide valuable and important results which are helpful in proving its significance and potential applications in drug designing.

Initially, a lower concentration of salt has been used to solubilize the protein and further the precipitation of protein with ammonium sulphate followed by its dialysis presents the turbid nature of the protein. Tris-buffer has been used to solubilize the formed precipitate in salting-out process. The used buffer shows its excellent solubility and compatibility with thecrude protein.

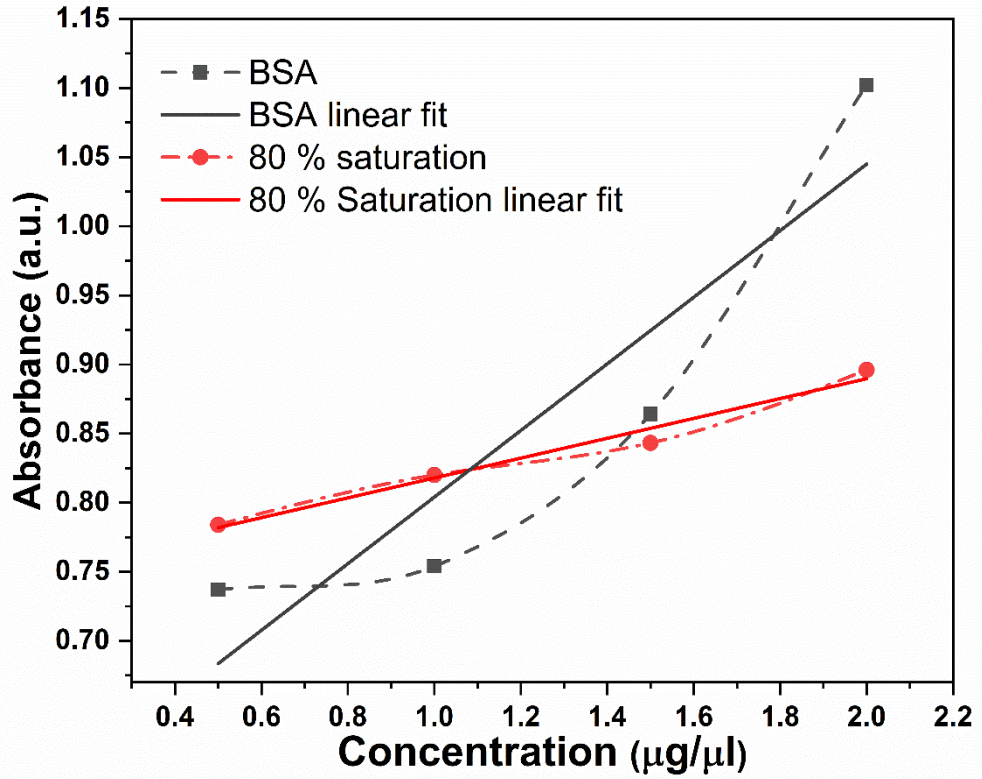

Figure 1 Bradford reagent test using BSA as standard for protein quantification for partially purified protein

Protein Quantification

After the extraction protein was quantified from partially purified extract.The sensitivity of dye in laemmli SDS protein loading buffer and column matrix depends on concentration of protein in sample. The quantification of protein has beendone using Bradford reagent with bovine serum albumin as standard. 1mg/mL BSA was used to prepare four 100µL samplesof different concentrations of BSA and the protein extract each. The obtained results are shown in figure 1. The data for both BSA and protein sample has been plotted and linearly fitted using Origin-lab tool. The linear fitted solid line in black and red represents concentration vs absorbance for BSA and partially purified protein respectively. After comparison with BSA the protein concentration in sample obtained for salting-in sample was 3µg/µL.

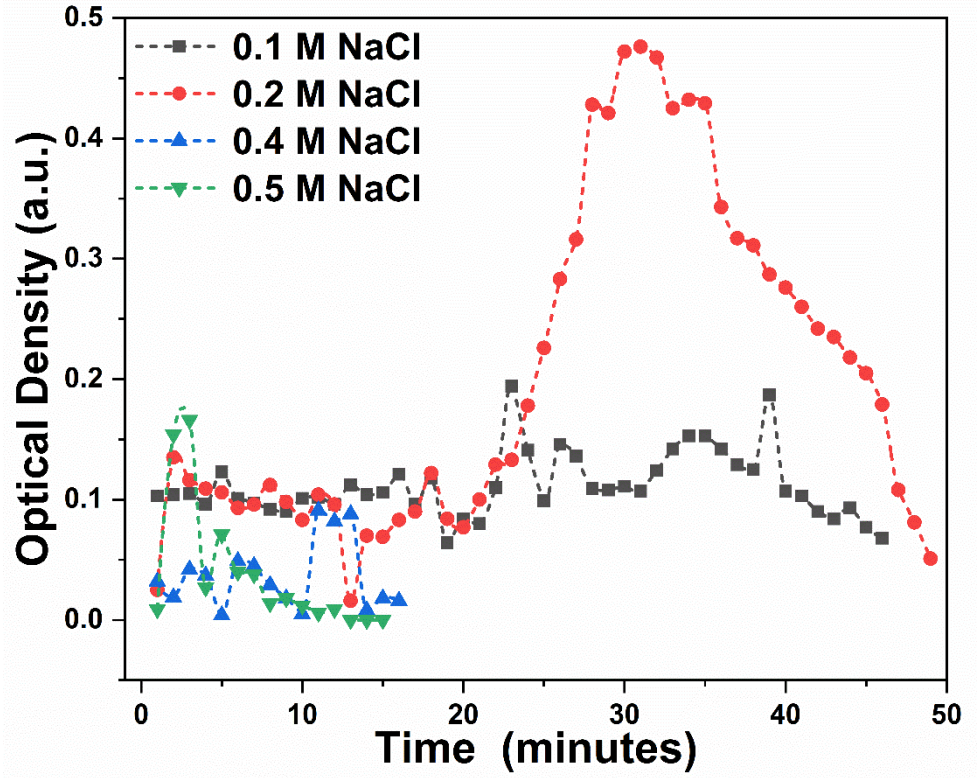

Figure 2 Chromatogram for purified protein based on optical density measured using nanodrop for different NaCl salt concentrations.

Purification

The high value of isoelectric point of spirulina extract proved to be helpful to go for a simple purification using cation exchange chromatography. The absorbance at 280nm of the bound protein is shown in figure 2. On applying salt gradient to the column with different concentrations of saltthe characteristic result was observed at 0.2M NaCl after collecting 33 mL of fractions. 0.437 is the highest peak value observed in the figure which shows the highest concentration of protein. Other peaks also show protein content at 0.1M, 0.2M and 0.5M NaCl concentrations. No characteristic peak was observed with 0.3M NaCl as the optical density and concentration of protein were recorded as 0.00 using nanodrop spectrophotometer.

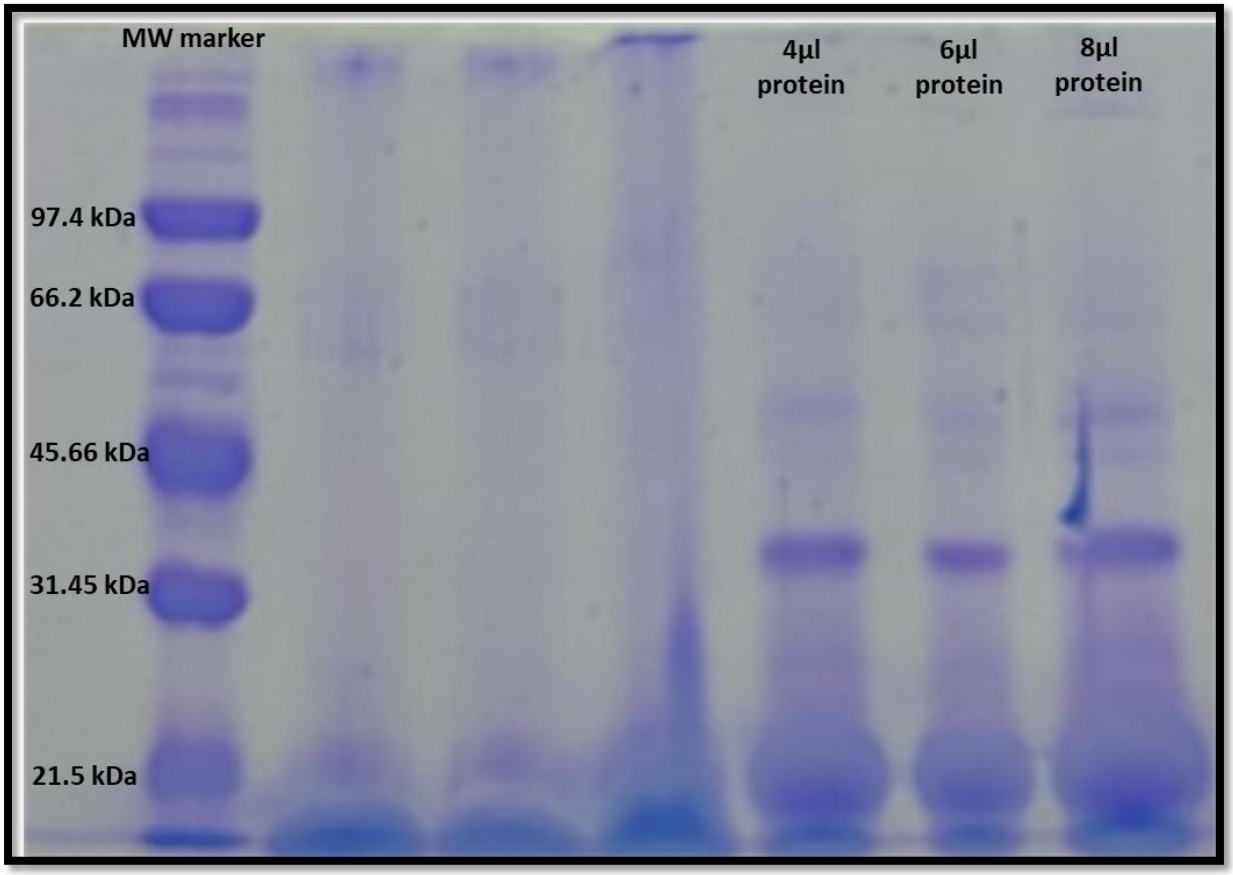

Figure 3 Analysis of final purified protein on SDS PAGE for protein purity and molecular weight estimation

Characterization

The purity of the protein was checked on SDS PAGE. The bands were observed at 31 KDaon the gel. The samples were run on 12% resolving gel to obtain sharp bands of protein which confirms its purity. It is clear from SDS-PAGE results that each fraction consists of a single homogenous protein.

The protein is thermostable and shows activity after 5h heat treatment at 90℃. Antibacterial response of the sample was found to be the same as without heat treated sample.

Antibacterial activity

Table 1

Seeds	**Lectin Fraction (%)**	**Inhibition zone diameter of the tested microorganism (mm)**		
		Pseudomonas aeruginosa (-)	**Escherichia coli (-)**	**Micrococcus luteus (+)**
Spirulina	30	**12±0.5**	**9±0.5**	**Nil**
Spirulina	95	**18±0.5**	**13±0.5**	**Nil**

Table 1 shows antibacterial activity of Pseudomonas aeruginosa and Escherichia coli which are gram negative bacteria shows inhibition halos of characteristic diameter as shown. 30% pure protein has been comparatively less effective with respect to 95% pure protein in inhibiting the bacterial colonies on the media plate. Gram positive bacteria was not able to show a specific inhibition using this protein.

IV. Conclusion

High purity lectin protein was obtained from Spirulina using ion cation exchange chromatography technique. Highest concentration of protein was observed in 0.2 molar NaCl salt gradient. The purity of protein was found to be very high based on bands in SDS PAGE after purifications. 31 Keda weight was observed for purified protein.Purified protein shows very good antibacterial activity whichmay provide a unique possibility of using lectin from spirulina as novel material for drug development of antimicrobial compounds. As the chances of biowarfare increases in competitive world there will be high demand for novel targets for futuristic

modern drug designing. In this course,lectin from spirulina which is highly abundantmay prove to be a crucial component against designing drugs for infections caused by Pseudomonas and other similar bacteria.

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