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Extraction and identification of substances present in the green thalloid alga *Ulva lactuca* Linnaeus, 1753 (Chlorophyta: Ulvales) from the Rocky shore of Visakhapatnam

K. Umadevi[1*], R. Prasanthi[2], S. Syamala Devi[1], S. Rupa[1]

[1] Department of Marine Living Resources, Andhra University, Visakhapatnam 530003, India
[2] Department of Biochemistry, Andhra University, Visakhapatnam 530003, India
*Corresponding author.

Abstract

A commonly occurring marine seaweed in the intertidal rocky shore in Visakhapatnam coast *Ulva lactuca* is studied for the occurrence of bio-active substances of economic importance adopting chromatography techniques. The crude extract of Ulva lactuca is prepared using 10% Trichloro acetic acid. Then the crude extract is separated using a silica gel 60 column in Dichloromethane. The bands appeared in the column chromatography were eluted into a single test tube. The eluted extract was subjected to paper chromatography and thin layer chromatography.The obtained R_f values revealed that, α-amylase was present in the extract of *Ulva lactuca*. This study opens new opportunities for considering *Ulva lactuca* as a source of α-amylase for industrial production as this enzyme is having industrial applications.

Key words: Alpha amylase; Column Chromatography; Paper Chromatography; Thin Layer Chromatography; *Ulva lactuca.*

INTRODUCTION

The sea weeds are used as food source, fodder, manure and as medicine since centuries ago. They contain proteins, halogens such as iodine, bromine, vitamins and more than 60 trace elements, substances such as growth stimulants and antibodies. Substances of plant origin would function as antioxidants, anti-coagulants, anti-hypertensive, anti-tumour and anti-bacterial. The marine micro and macro algae have been considered as exploitable plant resources, there by attracted the attention of scientists and industrialists for extraction of bioactive substances to develop nutritious foods and novel drugs (Sun et al, 2010; Liu et al., 2011).

Sea weeds are the only source of substances such as agar, alginates and carrageenan. Agar extracted from marine algae had been used very widely in microbiology to help grow bacterial cultures. Apart from these phytochemicals, products such as mannitols, laminarin and fucoidins are also obtained from marine thalloid algae (Khan and Satam, 2003; Vauchel et al, 2008; Gates, 2010: Abraham et al, 2018).

Marine thalloid algae are rich sources of enzymes. Enzymes are biological catalysts which initiate or accelerate biochemical reactions in the living cells, process reactions which are difficult to continue under normal conditions found in the cell. Haloperoxidases are known to be used in oxidative destruction of microorganisms. They have been detected in bacteria, fungi, micro algae, thalloid algae and also in mammals. The green marine thallaiod algae *Ulva lactuca*, commonly called as sea lettuce in the family Ulvaceae, contains commercially valuable bioactive substances. (Patil and Rebecca, 2014; Rebecca et al., 2016).

Amylases are widely distributed in the marine green thalloid algae. They degrade starch and related polymers into substances related to amylolytic enzymes (Saini et al. 2016). There are several types of amylases, namely endoamylases and exoamylases, α – amylases, β – amyalses, γ - amylases which are functionally different. All

types of amylases are used in the pharmaceutical industry in manufacturing digestive antacids and tonics. For conversion of starch into glucose and fructose syrups by liquefaction process, alpha amylases are used (Nielsen and Borchert, 2000).

The green marine seaweed *Ulva lactuca* is abundant in the rocky shore of Visakhapatnam. Because of its abundance and feasibility of collection, knowing the fact that, green marine thalloid algae are rich sources of enzymes, *Ulva lactuca* has been selected to study the various substances present in this species of marine thalloid algae. The aim of this study is to identify presence of enzymes present in it, to explore the possibility of using *Ulva lactuca* as an alternate natural source of enzymes.

Materials and methods

Sample collection

Samples of *ulva* were collected from the rocky shore- Tenneti park area of Visakhapatnam (Latitude: 17º 41' 12.5" N and Longitude: 83º 13'6.5" E) on 25 November 2018 during low tide. The sample was sent to experts for scientific identification of species.

Cleaning and drying of the sample

The sample was washed three times with fresh water to remove sand and unwanted matter. The holdfasts were removed, the leaves were selected and then sun dried on a clean surface (Fig.1A). The biomass was dried daily 2 hours in the mid-day12 pm-2 pm (temperature 28ºC-30ºC) until the leaves broken to coarse powder when squeezed (Fig.1B). The dried sample was made to smooth powder using laboratory motor and pestle.

Sample preparation

Ten grams of dry powdered sample was introduced into the beaker containing 100 ml of 10% TCA solution and soaked overnight (Fig.1C). In the next morning the soaked sample was made into a paste with the help of a motor and pestle initially and then homogenized (Omni, Model- Micro ES). The mixture was centrifuged (Eppendorf Model-5810R), transferring the sample into Eppendorf tubes; at 10,000 rpm for 10 minutes at 4^0C (Fig.1D). The supernatant obtained was used in the analysis, debris was discarded.

Column chromatography

Preparation of the column

A cylindrical glass column containing a porous disc at the bottom was taken to load the sample. The column was washed with tap water and then with distilled water before use. The column is loaded (20 cm length) with silica gel 60 slurry prepared with dichloro methane (DM) adopting standard procedure (Srivastava, N., et al., 2021). Care was taken in loading the gel into the column to prevent the formation of air bubbles. Excess Dichloromethane is drained out into the breaker above the gel surface. With the help of a pipette, the sample was loaded up to 7 cm length above the silica surface of the column. Now mobile phase solvent petroleum ether was poured on the sample solution. The mobile phase solvent is then allowed to flow down through the column. The sample was allowed to run.

Clear bands appear in the column due to the adsorption of substances/compounds present in the extract with different polarities. The bands formed at the top of the column indicate substances with greater adsorption. The compounds present in the extract with low adsorption will run down to the column and form bands at the bottom. Substances formed individual bands in the column were collected as a single fraction in a 10 mL test tube, which was used for paper chromatography and thin layer chromatography to identify the substances (Fig. 2). The results were presented in the results' section.

Paper chromatography

Paper chromatography was performed using *Ulva lactuca* extracts eluted from the column adopting standard methods (Gupta et al., 2018). Whatmann filter paper (12 ×5 cm) containing 90% α- cellulose was used as adsorbent paper. In this study the mobile phase was aqueous acetone (50% v/v). With a micro pipette 0.2 µL of sample was loaded at four spots, at a distance of 1.5 cm to each other, on the base line of chromatography paper. The spots are allowed to dry at room temperature for 2 minutes and then clipped to the roof of the chromatography chamber keeping the free end in the solvent system. Care was taken to confine the spot to a minimum area of 0.5 cm dia. also the solvent do not touch the base line on which the sample spots were present.

After the solvent run to a maximum distance on the paper, the paper strip was removed from the chamber, then dried at room temperature followed by air drying for 5 minutes. Then Ninhydrin was sprayed uniformly on the paper to locate the coloured spots. The limit of solvent exposed was marked with a pencil and measured from the base line. The paper was dried to room temperature after spraying Ninhydrin. The ratio of the distance between the base line and the coloured spot formed of the substance (a) to that of distance travelled by solvent (b) was expressed as Retention factor (R_f).

$$\text{Retention factor } (R_f) = \frac{\text{distance travelled by solute (cm.)}(a)}{\text{distance travelled by the solvent (cm.)(b)}}$$

The results were presented in the results' section.

Thin layer chromatography

Thin layer chromatography was performed using the fraction of the eluted extract of *Ulva lactuca* adopting standard methods (Santiago, M., Strobel, S., 2013). In this study 96% ethanol and water in 70:30 ratio was used as mobile phase taken in 1000 mL beaker, covered with aluminium foil, until the silica gel plate loaded with sample was placed in. Readymade (Merck life sciences Pvt Ltd) silica gel 60 plates (12cm length, 12cm breadth, 0.25 mm thickness) were taken. A base line was drawn 2 cm above the lower edge of the plate. Using a micro pipette 0.2 µL of sample was released at four spots at an intervals of 1.5cm distance to each other on the silica gel plate on the base line. Each spot occupied a minimum area of 0.5cm diameter on the silica gel plate. After air drying, the silica gel plate was placed in 1000 mL breaker containing solvent. Precaution was taken that the solvent shall not touch the sample spots on the base line of the silica gel plate. The solvent was allowed to run up to ¾ distance of the plate from the base line. The plat was then removed from the solvent and the limit that the solvent run was marked. After air drying the silica gel plate, Ninhydrin was sprayed on the plate uniformly with the help of sprayer. After spraying Ninhydrin, the silica gel plate was air dried and kept in hot air oven for 10 minutes at 100°C. The upper limit of the distance that the sample run on the plate from its base line was marked with a pencil and measured (a). In the same way the distance of the solvent front was also measured (b). The Retention factor (R_f) was calculated using the ratio of distance moved of solute to that of the distance moved of solvent from the base line.

$$\text{Retention factor } (R_f) = \frac{\text{distance travelled by the solute (cm.)(a)}}{\text{distance travelled by the solvent front (cm.)(b)}}$$

The results were given in the results' section. The R_f value obtained in this study was compared with the results of Suribabu and Hemalatha (2014).

Results

Identification of the sample

The green thalloid algal species sellected to study is identified as *Ulva lactuca* Linnaeus, 1753 (Desikachari, 1957; Umamaheswararao and Sriramulu, 1970). The complete taxonomic status is as follows. Kingdom:Plantae; Phylum: Chlorophyta; Class: Ulvophyceae; Order: Ulvales;
Family: Ulvaceae; Genus: Ulva; Species: U. lactuca.

Column chromatography

Figure 2A shows the silica gel (Dichloromethane) column loaded with sample extract. Although 8 bands were formed in the column visible to the naked eye, all were collected into a single test tube and treated as a single sample while conducting the paper chromatography and thin layer chromatography for identification of substances.

Paper chromatography

A single purple colour spot was developed on the paper chromatogram (Fig. 2B). The R_f value is 0.75, which is identified as α-amylase (Consden, R., 1944).
In this study, it was observed that within 2 hours the solvent travelled to a maximum distance of 5.7 cm in two hours.

Thin layer chromatography

In the thin layer chromatography the coloured spot developed for the eluted fraction from the base line after spraying Ninhydrin is depicted in the Figure 2C. The R_f value is 3.8. The obtained R_f value for the extracts of *Ulva lactuca* is compared with the results of Suribabu and Hemalatha (2014) and identified as α- Amylase.

Discussion

Chromatography is an analytical technique used for the authentication and identification of bioactive compounds available in plants. In the pharmaceutical industries' high performance liquid chromatography is used for the isolation and purification of bioactive compounds available in plants (Annadurai, 2021). In the pharmaceutical industries large steel columns packed with Silica will be used as stationary phase, which not only isolate but also gives information about the new substances present in the sample. Non polar to high polar solvents such as Toluene, Dichloromethane, Acetone, Ethanol, Methanol and their aqueous solutions will be used as a gradient solvent system for separation and elution of various organic compounds from the plant based organic extracts. The volume of solvent used will be changed with the amount of sample released into the column for purification. Since this study was undertaken in the laboratory to isolate and identify the substances present in the *Ulva lactuca* extract, dichloromethane was used as mobile phase and Silica gel 60 as stationary phase in the column chromatography. For paper chromatography 50% aqueous acetone was used as mobile phase whereas 96% ethanol and water in 70:30 ratio used as mobile phase in thin layer chromatography.

In this study alpha amylase was obtained in paper chromatography and also in the thin layer chromatography. Alpha amylase is having industrial applications. Amylases are important industrial enzymes, which are having wide applications. These are used in the conversion of polysaccharides to glucose, maltose and maltotriose units. Amylases are also used in the bread baking process, as the addition of amylases increases the softness and volume of dough. In addition, the small oligosaccharides, glucose and maltose produced by α- amylase enzymes enhance the browning of the crust and gives mouth-watering baking flavor (Lundkvist et al., 2007).

Amylases play a very vital role in clinical, medical and analytical processes and also in starch saccharification in the textile, food, distilling and brewing industries (Atkinson & Movituna, 1991; Lundkvist*et al*., 2007). Thirty percent of the world's enzyme production comprises of α- amylases. These enzymes are widely used in the production of cyclodextrins (Svensson, 1994). Alpha and β amylases and 1-4 & 1-9 glucosidases play an important role in the degradation of starch in fruits ripening process (Lajalo, 2001). In the fruit ripening process many farmers are exposing fruits to Ethylene gas, calcium carbide (Madhuwanti and Marapana, 2019). The plant based α amylases can be used for replacing the above chemicals in the fruit ripening process (Brummel et al., 2004). *U. lactuca* can be considered as an alternate source of alpha amylases to be used in the fruit ripening process. In the starch hydrolysis, α amylases are widely used to convert starch into fructose and glucose syrups (Nielsen and Borchert, 2000).

Alpha amylases are used in the digestion of corn starch to produce malto-oligomer mix a new commercial product having low glucose content. It is less sweet than sucrose, so used as a substitute for sucrose and other saccharides (Takata et al., 1992, Vander et al., 2002). In the textile industry Alpha amylases are applied to cloth, to remove the starch and also as desizing agent (Iqbal et al., 1997). In the industry while liquefaction, starch becomes highly viscous after few hours of its use. Therefore, thermo stable alpha amylase is used as a thinning agent, which reduce viscosity, brings partial hydrolysis of starch to avoid the subsequent retrogradation of starch during cooling (Sang-Lang et al., 2011). The degradation of starch with plant based α amylases also reduce pollution in the ambient waters into which the sewage water of the industry is released. Bacterial α amylases partly replaced malt in the brewing industry there by caused the process cost effective.

The use of α amylases extracted from *Ulva lactuca* may be tested for their efficiency in replacing malt in the brewing industry and also its cost effectiveness (Santamaria et al., 1999). Starch is used in the paper industry as a mounting adhesive later on removed using alum or protein glue which damages paper causing embrittlement. The starch used as mounting adhesive will be hydrolyzed and removed after applying α amylases in immersion or as a gel, without damaging the texture of the paper (Okolo et al., 1996).

In the food and feed industry, in the manufacturing of detergents and in the building construction also α amylases are widely used. The addition of α amylases to food and feed will improve the nutritional value reducing using the raw starch. *Ulva lactuca* α-amylases can be a better replacement, in the place of synthetic/bacterial α-amylases. Adding α-amylases to detergents improve the removal organic dirt from cloth, protects the colour and gives stability to the detergent bar composition. Starch modified by the α-amylases is used in the gypsum board manufacturing used in the construction of dry wall (Borchert et al., 1995; Mirasol et al., 1997: Atsushi and Eiichi, 1998). Alpha amylases are also used in the ethanol production reducing the usage of synthetic enzymes and bacterial enzymes. Ethanol can be used as bio fuel without generating pollution unlike fossil fuels (Kirk et al., 2002).

There are manifold uses for α-amylases in the industry. They are used in textile industry, food industry, feed industry, detergent manufacturing, building construction, pharmaceutical industry and paper industry. So far synthetic, bacterial amylases are widely used in all the above mentioned industries which demand more investment and also pollution through the release of sewage.

Ulva lactuca is native seaweed, grows luxuriantly in the rocky shores all along the Indian coast. It is already cultured in the open sea farms in Tamilnadu. Further studies on quantification and purification of α-amylases and cost effectiveness of production from *Ulva lactuca* will provide insight to the industrial production of this enzyme from this species of seaweed.

Ulva lactuca extracts in the paper chromatography and thin layer chromatography studies indicated the presence of α-amylase enzyme. Alpha amylases are extensively used in food, feed, pharmaceutical, textile, detergent, paper industries and also in the bio fuel production, where synthetic or bacterial amylases are playing a vital role. The use of synthetic and bacterial amylases may cause pollution and contamination. The use of α-amylases extracted from *Ulva lactuca* may be a better replacement for synthetic and bacterial amylases used in the above industries, in terms of performance, cost-effectiveness and to reduce pollution.

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Conflicts of interest

The authors declare that they have no potential conflicts of interest.

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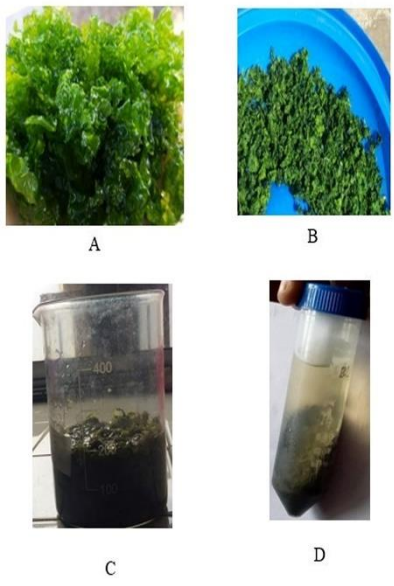

Figure 1. *Ulva lactuca* thallus preparation for chromatograpy

A. Fresh thallus; B. Sun dried thallus; C. Digestion of dried thallus
D. Extract after centrifugation

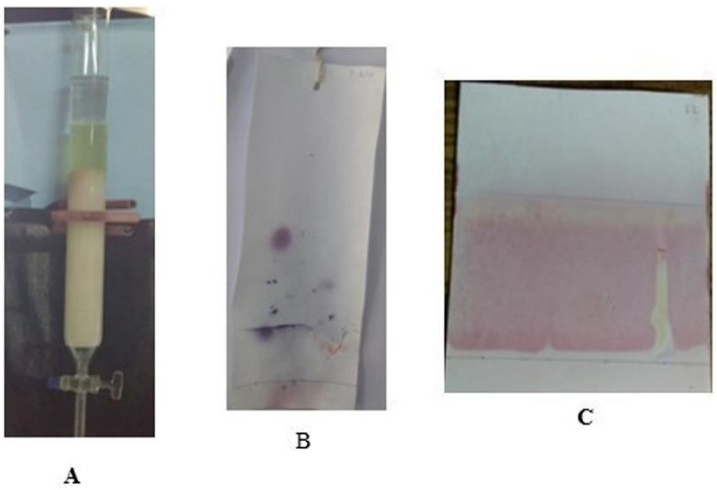

Figure 2. *Ulva lactuca* chromatograms
A. Separation of extract in a column; B. Paper chromatogram; C. Thin layer chromatogram