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Isolation of Oxylipin from Rice Bran as Antibacterial Principle against Pseudomonas Aeruginosa

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Abstract: *Lipoxygenase (linolenate: oxygen oxidoreductase (E.C.1.13.1.13) is found in a wide variety of plants, particularly the legumes. It is present in groundnut, soyabeans, peas, potato and wheat. Under appropriate conditions, this enzyme leads to the deterioration of fat-soluble vitamins and essential fatty acids of oils and fats. Since little is known about this enzyme in rice bran (which is a good source for edible grade oil), work on this aspect is undertaken. This enzyme when reacted with substrate (rice bran oil) yields oxylipins. Oxylipins are saturated and unsaturated oxidized fatty acids. These compounds include the eicosanoids (e.g. prostaglandins, thromboxanes, leukotrienes, lipoxygenase products), many of which are pharmacologically important compounds with biological activities. These compounds are highly toxic towards bacteria and fungi. This study aims at isolating lipoxygenase from rice bran and using the oxylipin produced by the enzyme reaction which is used to inhibit Pseudomonas aeruginosa, an opportunistic human pathogen. Results of partially purified enzyme activity are found to be 0.65/ml/min. Whereas inhibition zone of 2.2 cm is observed with the produced oxylipins which are lower than that of the used standard antibiotic Streptomycin i.e., 2.6 cm. In conclusion partially purified enzyme has provided an oxylipin showing low inhibition compared with standard which is not surprising as it is a pure antibiotic. Further the study is suggestive to use the pure compound as antibiotic.*

Keywords: Rice bran, Lipoxygenase, Oxylipin, Pseudomonas aeruginosa.

1. INTRODUCTION

Rice bran is a waste found in rice mills, Cycloxygenases & lipoxygenases are the main pro-inflammatory enzymes in Rice bran [1]. Lipoxygenases (LOXs; EC 1.13.11.12) are widely distributed in plants and animals. These are nonheme iron-containing dioxygenases which initiate the synthesis of oxylipins. Plant oxylipins are a large family of metabolites derived from polyunsaturated fatty acids [2]. Enzyme extracted from rice bran is used to produce oxylipins using rice bran oil as substrate. To get a better insight into the biological activities of oxylipins, in vitro growth inhibition assays are used to investigate the direct antimicrobial activities on pathogenic microorganisms including bacteria, oomycetes, and fungi [3]. The reaction of crude enzyme extract with substrate (rice bran oil) produces oxylipins which have shown inhibitory activities against disparate microorganisms. The extraction and purification of the enzyme is carried out to accomplish the desired objective. The samples collected from different rice mills are tested for their enzyme activities and the enzyme with highest activity is selected for partial purification using ammonium sulphate precipitation. Partially purified enzyme is used for oxylipin production; produced oxylipins are used as inhibitor for further study with test organism.

2. MATERIALS AND METHODS

2.1 Sample collection

Three samples of Rice bran are collected from the rice mills situated at different locations of Raichur in the Industrial Area (Karnataka), India. Out of three samples collected two are unsteamed (without finishing operation) i.e., sample 1 and sample 2 and the other is steamed i.e., sample 3.

2.2 Culture collection & maintenance

Culture of Pseudomonas aeruginosa is collected from the Microbiology lab, Ballari Institute of Technology and Management, Bellary, Karnataka, India. The culture is maintained by sub-culturing in nutrient broth at 37 °C for 24 hours and used for further analysis.

2.3 Extraction of crude enzyme

50 g of rice bran is soaked in 100 ml of distilled water and kept overnight at Room Temperature. The soaked sample is filtered using Wattmann filter paper, filtrate is collected & subjected to centrifugation at 6000 rpm for 30 min, and the supernatant is re-centrifuged at the same rpm for 30 min, the pellet obtained is dissolved in 1 ml phosphate buffer of pH-7 and is used as crude enzyme for further analysis [4].

2.4 Protein assay

In order to find the concentration of the protein, Standard Bovine Serum Albumin (BSA) graph is prepared using Lowry's method. Serially arranged test tubes containing reaction mixture with varying concentration of BSA from 0.1, 0.2, 0.3….. 1.0 ml (1mg/ml) made up to 1 ml with phosphate buffer added with 2 ml of Alkaline Copper reagent and incubated for 20 min. The reaction is terminated by adding 0.5 ml of Folin & Ciocalteu's Phenol reagent. Optical density (OD) is measured at 660 nm after 5 min of incubation. The graph is plotted taking OD Vs Protein concentration.

2.5 Activity of crude enzyme

The assay is carried out as described by B S Shastry and Rao (1975), with few modifications. The reaction mixture consist of 3 ml enzyme, 0.2 ml 10 % KOH, 0.5 ml substrate (dissolve 0.1 ml of rice bran oil in 60 ml of absolute alcohol) and 0.3 ml water are mixed together and incubated for 20 minutes at room temperature (RT). This mixture is titrated against 0.1N HCl. The procedure is repeated for all the three samples [5].

2.6 Partial purification of enzyme

50 ml of crude enzyme sample is used for partial purification by adding 8.2 g of ammonium sulphate to give 30 % saturation, 2.8 g for 40 % saturation and 2.64 g to obtain 50 % saturation. Each time the samples are centrifuged and pellet is dissolved in phosphate buffer and the supernatant is used for further saturation till 50 %. The enzyme activity at each saturation is calculated using the previously mentioned procedure, and stored at 4 °C for further use. These three saturated pellets are used as the enzyme for Oxylipin production [6].

2.7 Oxylipin production

The reaction of enzyme (0.5 ml) with already prepared substrate (2 ml) followed by addition of 1% Manganese sulphate, 1% KOH and incubated for 15 minutes is carried out. Two different layers are obtained using dilute sulphuric acid. Top layer containing desired oxylipins was separated and stored at 4°C for further analysis [7].

2.8 Gas Chromatography mass spectroscopy (GC-MS)

Gas chromatography mass spectral (GC-MS) is performed with SHIMADZU QP2010S using facility at (Karnataka University Dharwad). The mass spectrometer is auto tuned with decafluorotriphenylphosphine with a70 x 106 eV fragmentation energy. Spectra are recorded at a scan speed of 380 atomic mass units (AMU)/s (4 samples per 0.1 AMU) with a 0.5-s delay between scans of 50 to 500 AMU. The spectrometer is operated in the peak finder mode at an electron multiplier voltage of 1,800 V. The threshold of detectability was 200 linear counts [8].

2.9 Temperature

In order to check the optimum temperature of the test organism 50 ml of nutrient broth was inoculated with 1 ml(106 cells/ml) of the culture and the culture incubated at 10 ºC, 30 ºC(RT), 40 ºC, 50 ºC, 60 ºC and 70 ºC temperatures respectively. OD at 600 nm was measured and plotted the graph.

2.10 pH

In order to check the optimum pH of the test organism 50 ml of nutrient broth prepared in buffers of pH 4, 6, 8, 10 and 12 inoculated with 1 ml (106 cells/ml) and the culture incubated at 40 °C OD at 600nm is measured and plotted the graph.

2.11 Inhibitory effect

Antibacterial activity of the oxylipins determined by agar well diffusion method which is a modified method of Kirby Bauer technique [9, 10]. The inoculums culture of Pseudomonas aeruginosa is spread evenly on the surface of gelled agar plates. Wells of (4 mm) are aseptically punched on the agar using a sterile borer. 10, 15 and 20 µl of oxylipin, 20 µl of standard Streptomycin (positive control) and 20 µl of DMSO (Vehicle control) are loaded into the wells. The plates are refrigerated at 4 ºC for 15 min and incubated at 37 ºC for 24 hrs. Further, the same procedure is followed for varying concentrations of oxylipins in increasing order.

3. RESULTS AND DISCUSSIONS

3.1 Protein Assay

Standard Bovine Serum Albumin graph is obtained using Lowry's method. The concentration of protein in the enzyme sample is found using this standard graph.

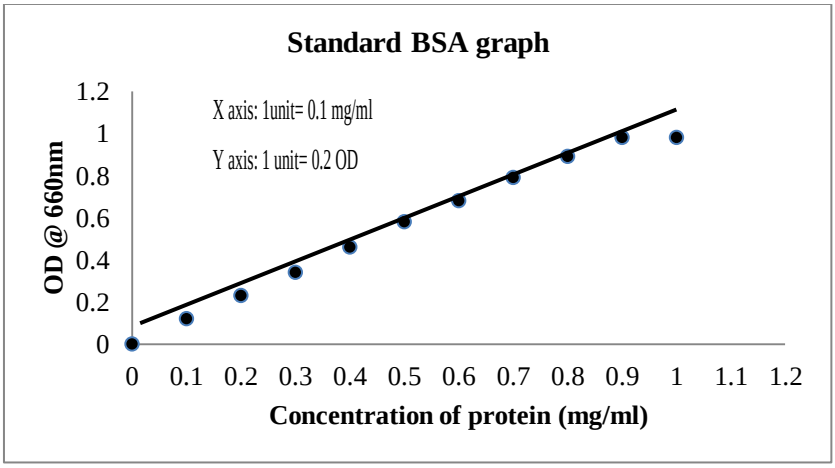

Fig 1. *Standard BSA graph*

3.2 Activity of enzyme

The enzyme activity is defined as the amount of isolated lipoxygenase required to give maximum product oxylipin per ml/min and the specific activity is the ratio of enzyme activity per ml/min with total amount of protein present. The enzyme activity is estimated using the procedure mentioned previously and the specific activity is estimated using the formula:

Specific Activity= Enzyme Activity/ Total protein Concentration (Table-01)

Table 1. *Activity & Specific activity of crude lipoxygenase enzyme.*

Sample	Activity of enzyme (ml/min)	Specific activity
Steamed	0.0275	0.0315
Unsteamed 1	0.0425	0.0561
Unsteamed 2	0.09	0.0863

Reaction mixture containing 3 ml of enzyme, 0.2 ml of 10% KOH, and 0.5 ml of substrate (dissolve 0.1 ml of rice bran oil in 60 ml of absolute alcohol) with the total volume of 4ml with

distilled water is incubated for 20 min at room temperature and titrated against 0.1N HCl. The activity of lipoxygenase enzyme in unsteamed sample 2 is found to be high i.e., 0.09 followed by unsteamed sample 1(0.0425) and steamed sample (0.02) (table1). However, previous studies on the activity of lipoxygenase enzyme is found using Warburg Respirometer by B S Shastry and M R Raghavendra Rao [5].

3.3 Ammonium sulphate purification

Partial purification using different concentrations of ammonium sulphate is carried out to obtain saturations of 30 %, 40 % and 50 %. After each saturation the activity of the enzyme is estimated and tabulated.

Table 2. *Ammonium sulphate purification.*

Saturation %	Ammonium sulphate (gm)	Activity (ml/min)
30	8.2	0.1
40	2.8	0.06
50	2.64	0.1325

8.2, 2.8 and 2.64g of NH4(SO4)2 used to obtain 30, 40 and 50 % of saturation respectively. Saturated protein solution is kept for overnight at 4oC, centrifuged and the pellet is dissolved in 6 ml, 0.9 ml, 0.5 ml of phosphate buffer (pH-7.2) respectively and stored at 4° C. The saturated enzyme with highest activity is used for oxylipin production.

By this procedure, a 10-fold purification with 8 % recovery of total activity is achieved in the work of B S Shastry and M R Raghavendra Rao.

3.4 Oxylipin production

Oxylipin is produced using 50 % saturated pellet dissolved in phosphate buffer as per the method cited earlier.

3.5 Gas Chromatography- Mass Spectrometry (GC-MS)

The obtained oxylipin is subjected for GC-MS in this study. Kenji Matsui et al., (2009) reported that the peaks appeared at retention time 1, 11 and 12 min are acetone, butyl-1-isothiocyanate and 2,4-hexadienal respectively. Further this study also observes the GC-MS peaks at the retention time 1, 11 and 12 representing tentatively the compounds as acetone, butyl-1-isothiocyanate and 2,4-hexadienal respectively.

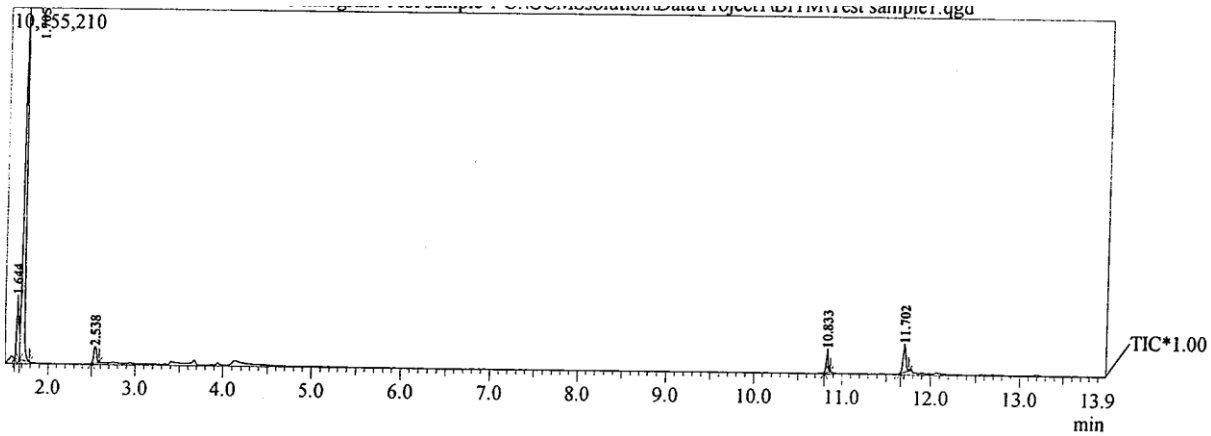

Fig 2. *GC-MS of oxylipin produced using rice bran*

Peak Report TIC

Peak#	R.Time	I.Time	Area	Area%	Height	Height%
1	1.644	1.617	3298592	12.47	1829308	13.21

3.6 Temperature study

In order to check the optimum temperature of test organism, Pseudomonas aeruginosa, the culture is grown in nutrient media at different temperatures ranging from 10o C to 70o C. The activity at each temperature is measured using the procedure mentioned previously and graph of activity Vs temperature is plotted.

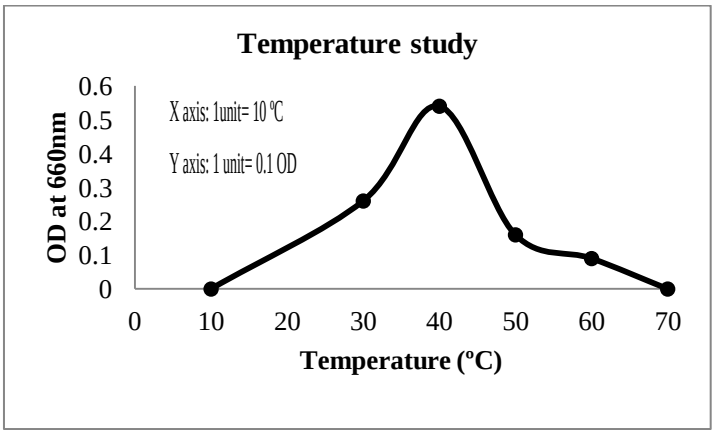

Fig 3. *Temperature study*

The graph illustrates that the increase in temperature has increased the activity (optical density) from 0 to 0.54, and showed the decrease at further increase in temperature indicating the optimum temperature as 40 °C (fig 2).

3.7 pH study

In order to check the optimum pH of test organism, Pseudomonas aeruginosa, the culture is grown in nutrient media dissolved in different buffer solutions of varying pH ranging from 4 to 12 pH. The activity at each pH is measured using the procedure mentioned previously and graph of activity Vs pH is plotted.

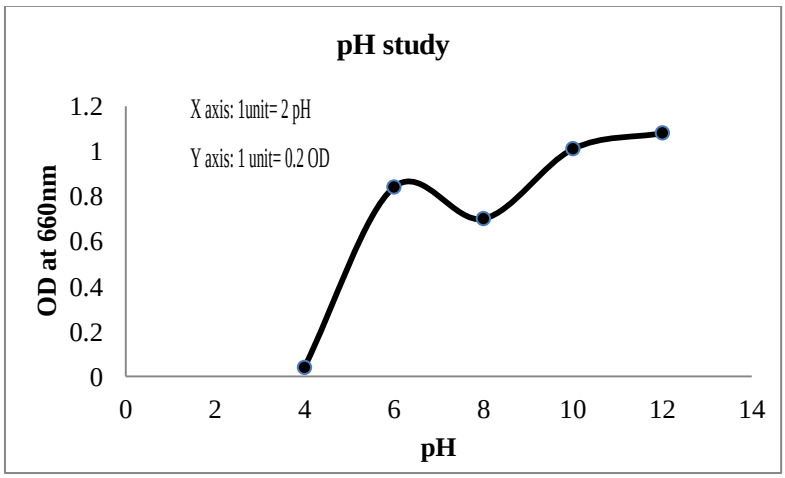

Fig 4. *pH study*

The graph illustrates that the increase in pH has increased the activity (optical density) from 0.04 to 1.08, except that at pH 8 there is a slight decrease in activity indicating the optimum pH as 12 (fig 3).

3.8 Inhibitory Effect of Oxylipin

Inhibitory effect of oxylipin on Pseudomonas aeruginosa is determined using agar well diffusion method.

Table 3. *Inhibitory effect*.

Conc of oxylipin (mg/ml)	Zone of inhibition (cm)
1	1.2
2	1.4
3	3.4
4	4.0
5	4.4

Fig 5. *Inhibitory effect of oxylipin*

Varying concentrations of oxylipin is prepared in increasing order by diluting with DMSO and the corresponding zone of inhibition for each concentration of oxylipin is noted.The zones in the above picture are represented as B-blank, O-Oxylipin, S-Streptomycin, D-DMSO.

4. CONCLUSION

Lipoxygenase enzyme is isolated from rice bran which is a waste product of rice milling used for the production of Oxylipin. Various assays are carried out in order to obtain oxylipin from partially purified enzyme. Inhibitory effect of the oxylipin is studied against Pseudomonas aeruginosa with varying concentrations in the increasing order. In addition, the inhibitory effect of oxylipin is compared with that of a commercially available antibiotic Streptomycin. Since, oxylipin is not used in the pure form the inhibition was comparatively less but yet with some more study on purified form of oxylipin is required which will help proving oxylipin as an alternative safer antibacterial agent.

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