Purification and characterization of esterase from marine Vibrio fischeri isolated from squid

A Mohankumar* & P Ranjitha

* Department of Zoology, Chikkanna Govt. Arts College, Tirupur 641 602, Tamilnadu, India

[E-mail: moniver@satyam.net.in]

Research Department of Microbiology, Sengunthar Arts and Science College, Tiruchengode

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Bioluminescence bacterium Vibrio fischeri was produces esterase enzyme when the medium contained specific substrate. Esterase was purified from the culture supernatant. Most active fractions were obtained using the technique of precipitation with 1N HCl. Precipitated fraction was purified by ion exchange chromatography (DEAE-Cellulose) and gel filtration chromatography (Sephadex G200). Enzyme purity was determined by RP-HPLC. Purified active fraction exhibiting final specific activity of 300U/mg and characterized; the optimum pH was 7.5, the optimum temperature was 30°C. Enzyme was very stable at the temperature 30°C and at wide range of pH. Enzyme was monomeric protein having molecular mass of 37 kDa estimated by native PAGE assay.

[Keywords: Vibrio fischeri, Extracellular enzymes, Esterase, RP-HPLC]

Introduction

Marine microorganisms which are salt tolerant, provide an interesting alternative for therapeutic purposes. Marine microorganisms have a diverse range of enzymatic activity and are capable of catalyzing various biochemical reactions with novel enzymes. Especially, halophilic microorganisms possess many hydrolytic enzymes and are capable of functioning under conditions that lead to precipitation of denaturation of most proteins. Further it is believed that sea water, which is saline in nature and chemically closer to the human blood plasma, could provide microbial products, in particular the enzymes, that could be safer having no or less toxicity or side effects when used for therapeutic applications to humans.

The Photobacterium (Vibrio) fischeri group consists of rod-shaped cells with a light yellow, cell-associated pigment and a tuft of sheathed flagella. Species is restricted to the marine environment and has a specific requirement for sodium ion for growth. It occurs both free living in sea water and as the specific luminous symbiont of the monocentrid fish and squid.

Esterases are distinguished from lipases in that their action is generally restricted to short-chain fatty acids. Esterases catalysis of a large number of aliphatic and aromatic esters. Although the molecular and catalytic properties of this protein from mammalian sources have been well studied, only limited investigations have been made into properties of microbial esterases. Because of the potential food applications, the general economic attractiveness of extracellular enzyme is higher than the intracellular microbial industrial enzymes.

Exterases have a wide range of industrial applications. Global market for industrial enzymes is constantly growing with a rate of 5-10% per year. Numerous advantages of the new set of esterases compared to commonly used esterases present a great economical potential to a suitable industrial partner in each of the application sectors. Information of lipolytic enzymes produced by marine Vibrio spp is particularly limited. Present study consists on the esterase production of V. fischeri isolated from squid and on the purification and partial characterization of its esterase.

Materials and Methods

Squids (Sepia sp.) were collected from Poombukar, East coast of Tamil Nadu and cooled to about 8°C before opening the mantle cavity along the ventrum.
Light organ fluid containing bacteria was obtained from pore that leads into channels with in the organ tissue. Whole light organs were removed by dissection and homogenized in 700 µl of sterile seawater. Material obtained in either of these ways was serially diluted in seawater complete (SWC) broth and the samples were spreaded on SWC (0.38M NaCl, 0.02M MgCl₂, 0.5% peptone, 0.3% yeast extract, 2% agar and 0.3% glycerol) agar plates and identified the luminous bacteria based on the work of Reichelt and Baumann and VHA (Vibrio Hareyi Agar) differential media. All strains were precultivated on the solid maintenance SWC medium. For detection of esterase activity the following basal medium (Zobell 2216E, slightly modified) was used. It contains 10 g of peptone, 1 g of yeast extract, 0.05 g of CaCl₂, 15 g of bacto agar made in L with aged seawater, pH was adjusted to 7.6. The media supplements with either of 1% tween-20 or 0.25% Triacylglycerol (trubutyrin) were then poured into petri dishes, after solidification of these media inoculated and incubated at 30°C for 10 days. The total diameter, minus the diameter of the colony was considered to be proportional to the esterase activity rate. After 1-10 day incubation the halos, clear (on tributyrin) or turbid (on all other substrates) were measured.

Broth culture was streaked on the spirit blue agar plates with substrate (Tween-20 or Triacylglycerol). Then the plates were incubated at different temperature 6°C, 17°C and 30°C for up to 15 days. The plates were observed after 6 hrs and every 12 hr for the clearing of the blue or deep blue color around each streak. Esterase activities at different hours were compared by measuring the width (millimeter) of areas of cleaning or area of deep blue color around the colonies. 150 µl of culture was inoculated into 200 ml of sea water medium (pH 7.0) with substrate and incubated at 30°C for 3 days. The portion was centrifuged at 20,000 x g at 4°C for 30 min. Supernatant was filtered using 0.45 µm cellulose acetate filter units. Cell free filtrate was used as the crude enzyme for the purification experiments. Crude extract was precipitated by slowly adding 1 N HCl at 4°C with stirring until pH 4.3 was attained. Precipitate containing the esterase activity was collected by centrifugation 10,000 rpm for 30 min. Pellet was dissolved in 10 mM phosphate buffer and make up the pH 7.5 by the addition of 1 N NaOH. Dissolved pellet was first applied to a column of DEAE-Cellulose, equilibrated with 20 mM phosphate buffer, pH 7.5. Proteins were eluted at a flow rate of 18 ml/h with a linear NaCl gradient (0.1 to 0.6 M). Fractions containing strong esterase activity were pooled (fraction size of 3 ml) and concentrated.

Esterolytic fractions collected from DEAE cellulose chromatography were redissolved in a small volume of 50 mM phosphate buffer, pH 7.0, and further purified by gel filtration on Sephadex G200, equilibrated with 50 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl. Elution with the same buffer was at the flow rate of 12 ml/hr, 3 ml of fractions were collected. Elution containing esterase activity was pooled and used for further characterization.

Enzyme assay used for the determination of esterase activity upon emulsified substrate and Tween solution. Final concentration of Triacylglycerol (TAG) was 10-50 mM, Tween 0.8-20% (v/v). Mixture of the substrate and buffer (final volume 8 ml) was adjusted to pH 8.0 by 1 N NaOH and the pH was maintained for 3 min by titration with 50 mM NaOH solution (blank). Then 1 ml of the esterase solution in the buffer was added and the esterolytic reaction was observed for 40 min by titration as mentioned above. Enzyme activity is expressed as U/ml and one unit (U) of activity is defined as µmols of free fatty acids liberated/min/ml by the enzyme solution under assay conditions.

In order to study the undenatured protein profile of esterase enzyme from V. fischeri in cell free broth, electrophoresis performed by the method described by Lammeli with some modifications. Gel was casted by using discontinuous buffer system having 10% resolving gel and 5% stacking gel. For measurement of molecular mass of protein, commercial broad range molecular mass standard proteins were used. Protein bands were located by coomassie blue staining. Purified enzyme were resuspended in 100 µl of water plus 0.1% trifluoroacetic acid (HPLC grade) and loaded on to a C 18 reverse phase column (reversed phase C 18 column, 10- µm particle size, 22 mm i.d., 25 cm length). Prior to loading, the column was pre-equilibrated with water plus 0.1% (V/V) trifluoroacetic acid. Protein was eluted from the column using a linear gradient of 0% to 70% (V/V) acetonitrile (HPLC grade) plus 0.1% (V/V) trifluoroacetic acid and a flow rate of 1.0 ml min⁻¹. The elution profile was monitored by A₂₈₀. Fractions were collected in 1 min intervals and each fraction
was evaluated for the presence of enzyme activity after dialyzing the samples extensively.

**Characterization of Lipase Enzyme**

Enzyme solutions at a concentration of 70 mg/ml were adjusted to various pH ranging from pH 2 to 11 with either 0.1 N NaOH or 0.1 N HCl and aliquots were incubated at 30°C for 4 hr. Then aliquot was removed and assayed for activity. Enzyme assays were conducted at various pH in an emulsified reaction mixture containing 0.4 ml of Tween or TAG, 4 ml 50 mM Tris, 0.1 ml enzyme. The pH was maintained by pH stat with 0.02 NaOH. After incubation of the reaction mixture for 40 min at 30°C, it was titrated to pH 9. Quantity of free fatty acids released was calculated from the total quantity of base used. Control reaction mixture contained heat inactivated enzyme.

The solution of enzyme at the concentration of 45 mg/ml was adjusted to pH 7.0 with 0.02 NaOH and aliquots were incubated at temperatures ranging from 5°C to 65°C for 4 hr. Then the aliquots were assayed for activity. Enzyme activity was determined at various temperatures ranging from 5°C to 65°C. Reaction mixtures (except enzyme) were held at the respective temperature for 5 min before the addition of the enzyme. The esterase enzyme was incubated with various concentrations of substrate and the final substrate concentration ranged from 0.5 to 10% of tween 20. Different concentrations of enzyme also studied in the concentration of 5, 10, 15 and 20 microlitres.

Enzymes were preincubated for 1 h at 30°C (pH 7.0) and in 0.1 M Tris-HCl buffer with various ions and other chemicals (one at a time). Assay was performed with the mixture, which did not contain CaCl₂ (except in test sample). The ions used were included NaCl (10 mM), BaCl₂ (0.001 M), MgCl₂ (0.001 M), KCl (2 mM), FeSO₄ (0.001 M), CaCl₂ (0.001 M), SrCl₂ (0.001 M), NaF (2 mM), MnCl₂ (2 mM), CuO₂ (2 mM). Other chemicals tested were ethylene diamine tetra acetic acid (0.5% EDTA), the ammonic detergent, sodium dodecyl sulphate (0.5% SDS).

**Results and Discussion**

Total viable luminous count range was varied from 4 to 18 CFU/ml. *Vibrio fischeri* encountered 100% of luminous bacteria. Habitats of this squid species must receive a significant input of cells of symbiotic *V. fischeri*. Luminous *V. fischeri* isolate was motile Gram negative rods. They produced yellow colonies on SWC agar plates. They were halophiles, unable to grow in the absence of NaCl. The colony morphology of the luminous *V. fischeri* strains tested on VHA was small (2 to 5 mm) dark blue green colonies. Harris *et al.*, reported that the VHA media displays great potential as primary isolation medium and offers significant advantages over thiosulfate-citrate- bile salts-sucrose agar.

In ZoBell modified media, the *V. fischeri* was showed 36 mm of halos after 10 days incubation indicates that the strain *V. fischeri* showed significant esterase activity. In fact it actively splits tween 20 than tributylin as good substrate. Bruni *et al.*, reported that the most strains of *Pseudomonas* sp. NCMB 1082 was split all tweens, tributyrl, but not triolein, 9 strains showed good activity on water soluble tweens, 4 on tween 85. In spirit blue agar, the width of hydrolysis areas were measured to observe the esterase activity of luminous *V. fischeri* bacteria at different temperatures Fig. 1. At 30°C, the esterase activity of strain was appeared after 7 hr with a large discoloration area or dark blue halos and the widest area observed after 1 day. At 17°C, the activity began after 12 hr, the size of halos increase after 3 days but the area of hydrolysis was smaller than that at 30°C. Apparently low activity was observed at 6°C. More or less similar mm of halos as that in ZoBell modified agar was observed in spirit blue agar with the substrate (tween, tributylin) at 30°C. Higher activity at 30°C may be attributed to maximum growth of the organism and subsequently increased esterase secretion.

1 N HCl precipitate of crude extract eluted in DEAE-Cellulose chromatography. Esterolytic fractions were assayed in chromogenic agar plate with the substrate of tween 20 showed yellow zone around the active fractions. Pooled material was eluted in gel filtration (Sephadox G200) chromatography. Esterase activity was represented by fractions between 28-34. Figure 2 shows gel filtration chromatography. This esterase had substrate specificity on tween 20, tributylin and exhibited high activity only on the compound of sorbitan monoester. Purified esterase from gel filtration chromatography showed single peak corresponding to protein peak and exhibiting final specific activity of 300 U/mg. In native PAGE electrophoresis it showed single band indicates esterase from *V. fischeri* was monomeric protein.
An esterase hydrolyzing tween 80 (polyoxyethylene sorbitan monooleate) was purified from sonicated cell lysates of *Mycobacterium smegmatis* ATCC 14468 by DEAE-Cellulose, Sephadex G150, phenyl sepharose and diethyl – (2-hydroxypropyl) aminoethyl column chromatography and by subsequent preparative polyacrylamide gel electrophoresis. The esterase had narrow substrate specificity; it exhibited a high activity only on compounds having both polyoxyethylene and fatty acyl moieties such as tweens. Purification of the esterase was performed in three steps of anion exchange chromatography and finally by gel filtration chromatography. Separation on Sephacryl S-300 gave a symmetrical single peak. Smacchi *et al.*, reported that the chromatography in both DEAE-cellulose and on Sepharose 6B resolved the esterase activity into one peak corresponding to the major protein peak.

The esterase from *V. fischeri* was showed molecular mass of 37 KDa. Tomioka estimated that the molecular weight of esterase was to be 36,000 by sodium dodecyl sulfate polyacrylamide. The purified extracellular *Arthrobacter nicotianae* esterase showed a single band on SDS-PAGE corresponding to a molecular mass of about 32 kg mol⁻¹, suggesting that the enzyme is a monomer. Kakariari *et al.*, obtained only one band with the crude cell free extract.
in SDS-PAGE, indicating a simple enzyme system, significantly different from the complex esterase system of the *P. freudenreichii* ssp *freudenreichii* strains described by Dupuis and Boyaval.

Purity of the preparation of enzyme was confirmed by RP-HPLC. Figure 6 shows a reverse-phase HPLC chromatogram recorded for the purified and nonpurified lipolytic enzymes. Active pure fraction of esterase showed single peak visualized at the retention time between 5.0 and 10.0 min. Unpurified (curde) enzyme have several peaks indicates the presence of proteins impurities. Sekeroglu *et al*., used HPLC system of an HP series to identify the enzyme product. Tojo *et al*., estimated purified enzymes by microbore reverse phase HPLC based on protein concentrations.

**Characterization of Esterase**

*Vibrio fischeri* esterase had an optimum pH of 7.5 on tween 20 substrate (Fig. 3). In this pH the esterase enzyme was retained 100% of the optimum activity. As indicated in Fig. 4, the enzyme had a temperature optimum of 30°C. At the temperatures below 25°C, activity slowly decreased. Sixty percentage of the activity available at 37°C. Smacchi *et al*., stated that the extracellular esterase of *A. nicotianae* 9458 had pH and temperature optima of 7.0 and 30°C, respectively. Similarly crude preparations of esterases from lactic acid bacteria and purified esterase from *L. plantarum* had pH and temperature 6.5-7.0 and 30-40°C respectively. Rhee *et al*., determined that the optimal pH for Est E1 esterase protein at various pH values of pH 3.0 to 9.5. The temperature range was 30 to 90°C, at lower temperature the enzyme still showed activity.

The enzyme was stable over a wide pH range. In pH 6 retained 60% and pH 9 retained 70% activity. At pH below 4 or above 10 the loss of enzyme activity was appreciable. Gradual loss of activity with increased temperature up to 65°C. The temperature above 30°C, 30% actively retained at 50°C, 70% of the activity retained at 40 and 45°C, 90% activity at 35°C. Tomioka reported that tween-hydrolyzing esterase of *Mycobacterium smegmatis* was stable to heat treatment at 100°C and to a wide range of pH. Esterase activity was reached at maximum in between the concentration of 0.5 to 8% of tween. Concentration above 8% reduced 50% of the enzyme activity. Macarie *et al*., assayed the esterase activity in the range of substrate concentration from 0.3 to 2.7 mM using pNPC6. More than 2 mM inhibit the esterase activity. Bendikiene *et al*., found the tween 85 was the best substrate among all the detergents studied and 10% concentration was optimal for the hydrolysis by lipolytic enzyme from *Pseudomonas mendocina* 3121-1. A linear relationship was also evident when enzyme activity was measured as a function of protein concentration. Same direct
Fig. 4—Effect of Temperature on the Esterase from *V. fischeri*

Fig. 5—Effect of inhibitors on Esterase enzyme
proportionality was found when crude extract were used as the source of enzyme\textsuperscript{20}.

Effect of Metal Ions, Chelators and Other Chemicals on Esterase

Figure 5 showed the results on the effect of various inhibitors on the activity of the esterase. Except NaCl, CaCl\textsubscript{2}, which were showed no effect on enzyme activity. All other ions tested in the present study showed inhibition with relative degree of variation. Evidently even the low concentration of NAF and CuO\textsubscript{2} were highly retarded the easterase activity. Inhibitory action of former was stronger than that of latter. Action of these two compounds could be attributed to their effect in creating the imbalance of ions in the reaction mixture by absorption or release, respectively. In this study the SDS was highly favored the activity of esterase (133\%). Esterase enzyme activity was not inhibited by the EDTA. It produced 67\% of the activity in the presence of EDTA. Similarly Moskowitz \textit{et al.}, reported that the esterase of \textit{Mucor miehei} was relatively unaffected by the high concentration of various salts, ethylene diamine tetraacetate (EDTA) or sulphydryl inhibitors\textsuperscript{21}. \textsuperscript{21}Kakariari \textit{et al.}, showed that the esterase from \textit{P. freudenreichii} ssp \textit{freudenreichii} did not affected by EDTA, other metal ions except Cu\textsuperscript{2+}, Hg\textsuperscript{+}, Fe\textsuperscript{2+}. Reverse-phase HPLC chromatogram is shown in Fig. 6.

References
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