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Characterization of chitinase enzyme from the gut content of mosquitofish, *Gambusia affinis*

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ABSTRACT

Identified the activity of chitinases enzyme from the gut content of mosquito fish, *Gambusia affinis*, is a novel study. Fish was collected from the Nilgiris district of Western Ghats, India was acclimatized and reared in the laboratory. Dissected guts were homogenized and centrifuged under ice cold condition. Enzyme activity was estimated by adopting calorimetric method. Gut extract chitinases showed positive results of bio-physical and bio-chemical properties and its optimum activity were observed at pH 6.3, temperature around 32°C and substrate concentration was 0.6 U/g. Chitinase is more active in the near acidic than alkaline pH and suggestive of enzyme secrets in the foregut of the fish and it can be assumed as fish origin. Mosquito fish introduction is being a major cause of bio-diversity decline in any fresh water ecosystem, can be used as chitinase enzyme source instead of using in mosquito control. Further studies on mosquito fish chitinase enzyme purification may open the possibility of industrial uses.

Introduction

The study of digestive enzymes of fish relates its food habits to the enzymes found in the gut and is widely used in nutritional physiology as important means of investigating digestive abilities of fish. According to Herve` Moreau *et al*., (1988) the presence of appropriate enzymes determines the ability of an organism to digest a given food item. No information is available on the quantitative and qualitative assays of digestive enzymes in the gut of mosquito fish, *Gambusia affinis*. Chitinases are associated with chitin eating habit and have been detected in the stomachs of Atlantic cod, Japanese sea bass and trout (Okutani 1966; Danulat & Kausch 1984; Danulat 1986).

Chitinolytic enzyme activities vary greatly between fish species and with the various methodologies used to examine them (Fange *et al*., 1979; Lindsay 1984). The primary function of chitinolytic enzymes is still debatable and likely varies between the species. Along the alimentary tract of fishes, chitinolytic enzymes are believed to have various roles (Clark *et al*., 1988; Jeuniaux 1993). Chitinases are primarily associated with the stomach where they disrupt exoskeletons allowing other digestive enzymes access to nutrient-rich inner tissues. Chitinases have also been found in the intestines where they may aid in removal of fragment blockage (Lindsay 1984). Chitobiases are mostly associated with the intestine, and pyloric caeca, where they further break down chitin into single units of N-acetyl- glucosamine (NAG) and may serve a nutritional function (Clark *et al*., 1988; Jeuniaux 1993). The enzymatic hydrolysis of chitin has the potential to result in additional energy gain from a meal.

The introduction and spread of exotic species is regarded by many as a major threat to global biodiversity (Vitousek *et al*., 1997; Kolar & Lodge 2001; Sakai *et al*., 2001; Lee 2002; Dudgeon *et al*., 2006). In particular, studies of fish introductions to freshwater ecosystems have shown that some species can reduce native fish populations, degrade aquatic habitats, compromise gene pools, and increase the risk and spread of alien diseases and parasites. As a consequence, the introduction of alien fishes is a major cause of biodiversity decline in freshwater ecosystems (Courtenay & Stauffer 1990; Courtenay & Moyle 1992; Fuller *et al*., 1999; Canonico *et al*., 2005) and, on a global basis, fish introductions are a prime cause of the extinction of many indigenous fish populations (Reid *et al*., 2005).

Inter-specific competition for resources may extend to predation, by gambusia, of eggs and larvae of endemic fishes and amphibians. In Australia, gambusia was suggested to be an imminent threat to red finned blue eye (*Scaturiginichthys vermeilipinnis*, Pseudomugilidae) and Edgbaston goby (*Chlamydogobius squamigenus*, Gobiidae) (Unmack & Brumley 1991; Unmack 1992; Wager 1994, 1995). They also negatively affect southern blue eye (*Pseudomugil signifer*) populations (Howe *et al*., 1997) and tadpoles (Morgan & Buttemer 1997; Webb & Joss 1997). Glover (1989) reported gambusia caused a decrease in desert goby (*Chlamydogobius eremius*) and spangled perch (*Leiopotherapon unicolor*, Terapontidae) populations inhabiting Clayton Bore in South Australia. Speculation that gambusia preyed on the eggs and larvae of rainbow fish (Melanotaeniidae) in the wild (Arthington & Lloyd 1989) was confirmed over summer 1997/98 in a field study in the upper Orara River, near Karangi, New South Wales (Ivantsoff & Aarn 1999). In New Zealand, Barrier & Hicks (1994) showed that although gambusia was harassed by the larger black mudfish (*Neochanna diversus*, Galaxiidae), gambusia ate their larvae. Predation of *G.affinis* has eliminated Gila topminnow (*Poecilliopsis o. occidentalis*) from almost its entire range of North America. *G. affinis* is commonly distributed in almost all freshwater bodies where crusteceans and insect larvae are

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available as food source and has the possibility of being a good source of chitinase. Hence, the current study was undertaken to explore the possibility of identifying the chitinase from the *G. affinis* gut and no studies have yet been conducted in this aspect.

Materials and methods

Healthy and disease free advanced fingerlings were collected from Avalanche and T.R. Bazaar areas of the Nilgiris, Western Ghats, India with the help of local fishermen. Fish stock was acclimatized for a period of two weeks in a metal drum (cemented inside) containing same source of water and the fish was fed with artificial diet enriched with chitin. Fish was dissected in an ice cold condition and the whole gut was separated and the inner content of the fish was washed and removed. Gut of fifty fishes was collected together and grinded using the mortar and pestle in physiological saline. In order to achieve complete extraction the gut extract was sonicated at 20 Hz for 15 minutes using Probe Sonicator, PCI, Mumbai. The extract was centrifuged at 10,000 x g at 4^{o}C for 10 minutes and the precipitated cell debris was discarded. The supernatant was tested for the activity of chitinase according to the procedure described by Monreal & Reese (1969) using N-Acetyl-D-Glucosamine as standard. The enzyme activity was estimated as amount of reducing sugars released using calorimetric method at 540 nm. The enzyme activity was measured in Units per gram of protein (Hartree 1972) in the extract.

To estimate the activity of the enzyme at various pH, phosphate buffered saline ranging from pH 6.0 to 8.0 were prepared. The enzyme was fractionated into two, based on the pH using ammonium sulphate precipitation method. For this the pH of the extract was adjusted to 7.2 and centrifuged to precipitate the protein fraction. Using chilled acetic acid the protein was precipitated at pH 3.0. The precipitate was re-dissolved and assayed for enzyme at various acidic pH. Similarly the chitinase in the extract was precipitated in crude extract using 0.1M ammonium hydroxide at pH 7.0 and removed. Further precipitation was done at pH so to get the basic proteins and assayed for chitinase. The fractionated enzyme was dialyzed in a semi-permeable membrane bag against flowing double distilled water for two days. This method is done to remove the excess ammonium ions or hydroxyl ions, which may be intercalated during ammonium fractionation.

Various enzyme fractions designated as 1 and 2 were scanned for enzyme activity at different temperature. The extract was incubated in a water bath at concerned temperature for two hours and activity was studied. Initially the activity was calculated from 15 to 50^{o}C with 5^{o}C gap. A close scan of the enzyme activity was done in 10^{o}C where the optimum activity is found. In this range the chitinase enzyme activity was estimated at every 1^{o}C gap.

The chitinase enzymes fractions were assayed at various concentration of starch as the substrate. For this various substrate concentration ranging from 0.1 to 0.9 g/ml was used at the optimum pH and temperature and observed as per the methodologies described. The values were plotted in a standard graph and half the maximal velocity (Km) is estimated.

Results

The activity of chitinase enzyme in the gut extract at various pH is illustrated in the figure-1. The data shows the enzyme have maximum activity in between 6 to 7 pH. On either side of this pH the enzyme activity decreases. The activity of chitinase enzyme in the gut extract at various temperatures is illustrated in the figure-2. The maximum activity was recorded in between 30 to 35^{o}C. There was a steep fall in the activity of enzyme after 45^{o}C. The activity also found to be ceases after 55^{o}C. The activity of chitinase enzyme in the gut extract at various substrate concentrations is illustrated in the figure-3. This shows that the maximum substrate saturation is the 0.5 only and the activity was not increasing after this concentration.

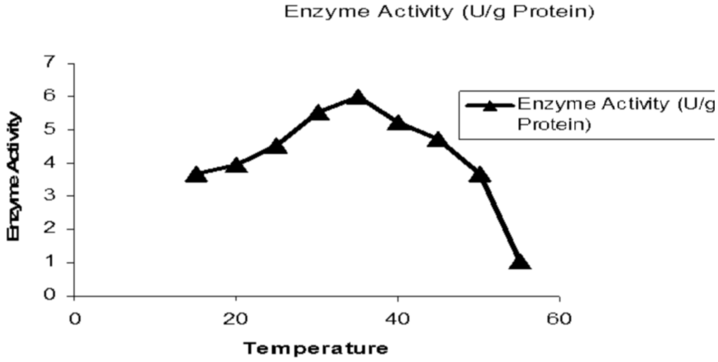

Fig. 1. Activity of Chitinase in *G.affinis* guts content at various pH

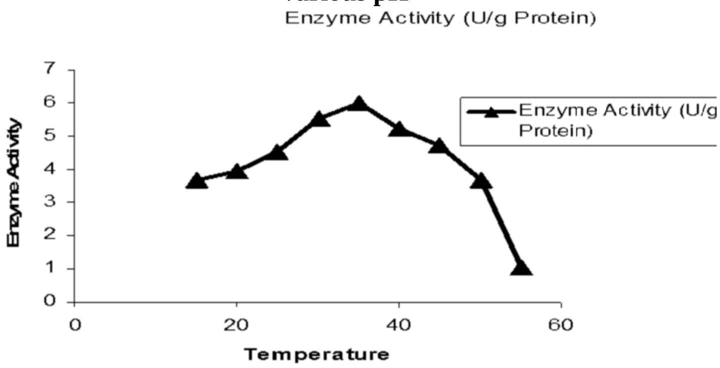

Fig. 2. Activity of Chitinase in *G.affinis* gut content at various Temperatures

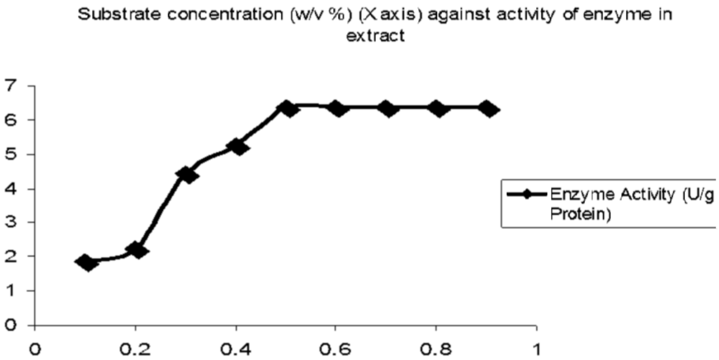

Fig. 3. Substrate concentrations and its half maximal velocity of *G.affinis* gut content

Discussion

Over five decades, the presence of chitinase enzymes in several fish species guts content has been demonstrated and gave positive results, the exceptions being the cyprinid *Abramis brama* and the African lung fish *Protopterus aethiopicus* (Danulat 1987). Some authors have claimed that chitinase activities measured in the gut contents of the fish species under study were of bacterial origin only (Goodrich & Morita 1977[b]). Most searchers working on "fish gut chitinases" found indications of both chitinase originating from fish tissues and bacterial chitinase. The enzyme produced by chitinolytic bacteria is characteristically different from "fish own" chitinase, for example with regard to the optimum pH-range for its activity (Okutani 1966; Lindsay *et al*., 1984; Danulat 1986). The study on optimum pH-range of chitinase showed that the enzyme is active under physiological conditions in the cod: its optimum was determined at pH 5.1. The enzyme in the stomach extracts was very active also at pH values lower than 5.1 while the one in extracts of the intestine showed high activity at pH 7.5 but rapid decrease of activity at pH values below the optimum (Danulat & Kausch 1984). Current study data shows the chitinolytic enzyme is more active in the near acidic pH than the alkaline pH. This is suggestive that it may be secreted in the foregut of the fish, G. *affinis* and so chitinase enzyme can be assumed as a fish origin.

In view of being a threat to the local ecosystem by its prolific breeding nature (Unmack & Brumley 1991; Unmack 1992; Wager 1994, 1995), competitive advantage over other endemic faunas (Barrier & Hicks 1994) and on a global basis it is cause of extinction of many indigenous fish species so the fish *G. affinis* can be used as chitinase source instead of mosquito control. A great deal of interest has been generated on chitinase because of its applications in the bio-control of plant pathogenic fungi (Ordentlich *et al*., 1988), molting process of insects, production of chito oligosaccharide (Terayama *et al*., 1989), single cell protein (Vyas & Deshpande 1991) and mycolytic enzyme preparation (Vyas & Deshpande 1989). Further studies on this fish chitinase purification may open the possibility of industrial usage.

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