STUDIES ON THE DIGESTIVE ENZYMES OF THE CULTIVABLE GREY MULLET *LIZA PARSIA* (HAMILTON BUCHANAN, 1822)

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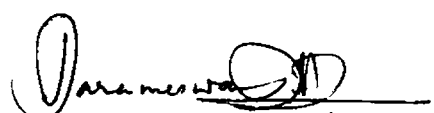
MAY 1989

TO MY PARENTS

C E R T I F I C A T E

This is to certify that the thesis entitled **"STUDIES ON THE DIGESTIVE ENZYMES OF THE CULTIVABLE GREY MULLET *LIZA PARSIA* (HAMILTON BUCHANAN, 1822)"** is the bonafide record of the research work carried out by Shri.**K.PALANISAMY** under my guidance and supervision in the Centre of Advanced Studies in Mariculture, Central Marine Fisheries Research Institute, and that no part thereof has been presented for the award of any other Degree.



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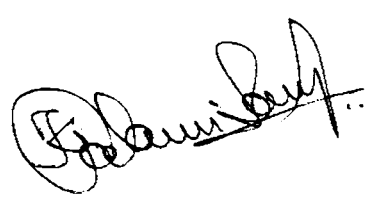
D E C L A R A T I O N

I hereby declare that this thesis entitled "STUDIES ON THE DIGESTIVE ENZYMES OF THE CULTIVABLE GREY MULLET *LIZA PARSIA* (HAMILTON BUCHANAN, 1822)" has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.



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CONTENTS

.....2

PREFACE

PREFACE

The fishes belonging to the family Mugilidae, commonly known as "Mullets" are widely distributed in coastal waters and estuaries of the tropical and subtropical belt. A few species occupy the warm temperate and cool temperate waters and they are also known to ascend the freshwater regimes of these areas. Historically, mullets are described in the records of the fifteenth and sixteenth centuries. Their fisheries appear to date back to the times of the ancient Greeks and Romans. Presently, they contribute to fisheries of varying magnitude in several regions of the coastal areas of world oceans and contiguous seas, especially in the South-West Asia, India, Mediterranean countries, East European countries, Central and South America and the Pacific basin (Nash and Shehadeh, 1980). In 1985, the total landings of Mugilidae from these areas were estimated at 2 10 261 metric tons constituting 0.27% of the total world production which was estimated at 84 million metric tons (FAO, 1985).

In India, mullets are caught all along the coast in lagoons and creeks and in the adjacent estuaries and brackish water bodies. The important fishing areas in the country are the estuaries of the rivers Ganga, Mahanadi, Godavari, Krishna and Cauvery and the brackish water lakes of Chilka and Pulicat on the east coast; the estuaries of Narmada, Tapti, the Gulf of Kutch and the backwaters of Kerala on the west coast.

In 1985, the estimated landing of mullets in India was 5092 tonnes, forming 3.23% of the total marine fish production of the country (CMFRI, 1989).

Besides contributing to the capture fisheries, mullets form one of the most extensively cultured group of fishes. The significance of the resources of mullet is more in their potential as cultivable fishes for extensive and intensive fish farming than in the existing capture fisheries. It is presently employed in the fish cultivation in about fifteen countries in the world, and has great potential for augmenting fish production through aquaculture and technology transfer in many more countries.

Characteristics of mullets such as feeding low in the food chain, capacity to tolerate wide fluctuations in environmental conditions, fast rate of growth, limited breeding problems, periodic abundance of fry and fingerlings and the great demand as a delicious table fish, make them ideal for culture in different ecosystems of coastal sea water, estuaries, brackish water bodies and even in fresh water environment.

Mullets form an important constitutent in the catches of traditional brackishwater fish culture operations praciced in West Bengal, Kerala, Karnataka and Goa. They are cultivated in low lying fields near estuaries and deltaic areas, as well as in paddy fields. Although, the production in these culture systems are relatively low due to poor management, unsatisfactory water supply, unscientific stocking, lack of proper feeding and protracted periods of culture, recent efforts on culture of this species in specially prepared farms undertaken at Cochin, has given encouraging results.

Liza parsia, the species selected for the present study is a medium-sized fish, which grows up to a maximum size of 330 mm. It supports the local fisheries in Hooghly-Matlah estuary, Mahanadi estuary, Pulicat Lake and in the South-West coast of the country.

The fishery and biology of mullets such as the catch rates, breeding aspects, larval rearing, seed production, field culture and ecophysiology have been extensively studied. However, indepth information on aspects such as nutritional requirement, digestion and digestive processes in these euryhaline fishes are limited.

Culturing of fish in captivity demands a detailed knowledge on well balanced diet and adequate feeding. Formulation and production of nutritionally balanced diets for fish require research, quality control and biological evaluation. It is often assuemed that what is ingested is also digested, but this is not always be the case. Digestion depends upon both the physical state of the food and the kind and quantity of enzymes in the digestive tract.

The ability of fish to digest a particular component of diet can be ascertained by investigating the complement of digestive enzymes present along the digestive tract. Investigations on the basic digestive physiology will not only enhance our present knowledge on nutrition and feed development, but will also contribute in understanding the digestive functions of lower vertebrates.

It is against this background that the present topic of investigation "Studies on the digestive enzymes of the cultivable grey mullet Liza parsia Hamilton - Buchanan, 1822" has been selected.

The thesis is arranged and presented in eight chapters. The first chapter deals with an introduction to the topic of study and a review of relevant works done in the same field highlighting the present status of our knowledge on the subject, and relevance of such study in coastal aquaculture operations. In the second chapter, the general material and methods employed for the

experimental work are described. The third chapter presents distribution pattern of different digestive enzymes along the digestive tract. The fourth chapter includes the results of studies on the characterisation of digestive enzymes. Changes in digestive enzyme profile in different size groups investigated are dealt with in the fifth chapter. The sixth chapter includes results of studies on the relationship between feeding periodicity and the activity of digestive enzymes. The sequential changes in the digestive enzyme activities with time after feeding is presented in chapter seven. The influence of dietary composition on the relative contribution of digestive enzymes forms the content of the eighth chapter. Relevant discussions on the results obtained are presented in different chapters.

One of the options for increasing the food production in India is through aquaculture, utilising the large areas of coastal water bodies already available. Scientific farming practices would ensure desired production rates in the culture of mullets, and it is hoped that the results of investigation embodied in this thesis would enhance our knowledge on the digestive physiology of mullets which in turn would aid in implementing planned aquaculture of these fishes.

CHAPTER 1
INTRODUCTION

1. INTRODUCTION

1.1 General Introduction

There has been a spectacular increase in the exploitation of fishery resources from the world oceans and contiguous waters in recent years. However, since the wild stocks of aquatic organisms are limited, we must eventually exercise a ceiling on the harvest of aquatic organisms from the wild in order to maintain a balanced ecosystem. Further, this natural resource can no longer meet the demand of the ever increasing population. Thus, aquaculture, an ancient practice in some countries, has received considerable attention throughout the world in recent years as an alternative source to meet the nutritional demand of people. Although much prominence is given to the aquaculture of shellfishes, especially shrimp culture, fishes still contribute a major share to world aquaculture production , and there is an ever increasing demand to cultivate the finfishes in brackish and marine waters.

Fish farming is an age old practise as exemplified by the pond culture of carp which was stated to be conducted in a primitive form 2000 years ago. Although improvements were brought in the culture methods over the years continuously, the advances which have transformed fish forming from an art to science gathered momentum during the past three decades only. In fact rapid increase in the number of fish hatcheries and progress in the commercial aquaculture in recent years are the fruits of industrious research. With the adoption of composite fish culture of carp, India has achieved significant production levels per unit area in fresh water

fish culture. However, brackishwater aquaculture is relatively a new area and the important groups of cultivable finfishes are the mullets, milkfish and Cichilds.

Members of the fish family Mugilidae possess vast potential as important protein source for mankind and hence have the brightest future of all the marine and brackishwater finfishes in the developing technology of aquaculture. They are widely distributed geographically, and have the capacity for tolerating extreme conditions of temperature, salinity and dissolved oxygen. They are hardy animals in nature which thrive on good husbandry and are capable of withstanding poor farming practices.

Like most fish, mullet is a highly rated food fish. Ghosh and Guha (1934) listed Liza parsia as one of the best sources of vitamin A in the common Indian food stuffs. Higashi (1961) classified mullet among the active fishes because of its high content of both pantothenic and folic acids. According to Pottinger and Baldwin (1940), the aminoacids of mullet include large proportions of lysine and arginine with lesser quantities of histidine and tryptophan. The protein content of a whole mullet is about 20% and of dried flesh 96%. Thus, among mullets, Liza parsia cultivated in traditional filtration ponds in West Bengal and Kerala hold immense potential as a candidate species for further intensification of brackishwater aquaculture by virtue of their nutritional value.

In culturing fish in captivity, nothing is more important than well balanced fish feed and adequate feeding. If there is no utilizable feed intake by the fish, then there will be no growth and death

eventually results. The production of nutritionally balanced diet for fish requires research, quality control and biological evaluation. The nutritional value of a diet is ultimately determined by the ability of the animal to digest and absorb it. It is often assumed that what is ingested is also digested, but that is not always the case. Digestion depends upon both the physical state of the food and the type and quantity of enzymes in the digestive tract. There are specific differences in the kind and amount of enzymes present in the digestive system. Some fishes are more efficient than others in digesting the different food components. Since food is not useful until absorbed and made available for metabolism, the decision for the inclusion of an ingredient in a fish diet for purposes other than bulk is partially determined by the ability of the fish to digest it. The efficiency of fish to digest a particular component of diet can be ascertained by investigating the complement of digestive enzymes present along the digestive tract.

Studies concerning the digestive physiology of the fishes would provide a more logical and efficient approach to determine the dietary requirements of the fishes for mariculture. Extensive studies were conducted on the digestive processes of terrestrial vertebrates, particularly the homoiotherms and the outcome of such studies have gained application in agricultural research and led to improvements of feed and husbandry technology. To date, fish have received less attention. Nevertheless, the limited reports have revealed the fact that digestive enzyme complements of fish are qualitatively similar to those of other vertebrates and they are

influenced by dietary changes, age and other factors.

1.2 Review of Literature

Fishes of the family Mugilidae belong to the order Mugiliforms which are Actinopterygian teleosts. The majority of mugilid species inhabit the tropics and the substropics with some species distributed in warm temperate zones and a few penetrating cool temperature waters. Besides, mullets have long been regarded as typically estuarine fishes (Thomson, 1966).

A wealth of information is available on the various aspects such as taxonomy, habitat, morphology, anatomy, reproduction, age and growth, population structure, behaviour, migration and fisheries. Thomson (1966) have presented a global review of the greymullets and updated the information available till late sixties. Later reports on mullets during seventies and eighties mainly centered around studies concerning aquaculture aspects. Oren (1981) have presented the knowledge accumulated on different aspects such as taxonomy, reproduction, age and growth, food and feeding, energy metabolism, artificial propagation, parasites and diseases and aquaculture methods of grey mullets in the compendium "Aquaculture of grey mullets" edited by him.

In India, with its extensive estuarine waters in Kerala, West Bengal and Tamilnadu, mullet is farmed from ancient times. Research on intensive culture began in the 1920's when rearing experiments with young mullet were conducted in Tamilnadu at the Fisheries Department farm at Ippur (Campbell, 1921; Hornell, 1922) and at the

Chingleput Fort moat fish farm (Gravely, 1929). In the 1940's, two further aspects were emphasized, viz. feasibility of acclimating mullet juveniles to freshwater and development in polyculture practise. The first acclimation experiments were undertaken in Tamilnadu with Mugil troschelli and Mugil vigiensis (Devanesan and Chacko, 1943; Job and Chacko, 1947). This was followed by acclimatization studies on Mugil (= Liza) parsia in West Bengal (Mookerjee, et al., 1946) and on Mugil cephalus and Mugil seheli in Tamilnadu (Ganapathi and Alikunhi, 1949).

Hora and Nair (1944) suggested improvements for the brackishwater farming of mullet in the Ganges delta. Basu (1946) advanced the adoptation of Chinese and Philippine practices for West Bengal farms. Later, Pillay (1947) reported on his extensive examination of culture in West Bengal, Tamilnadu and Kerala. Devasundaram (1951), Pillay (1953) and Sarojini (1956, 1958) have advocated polyculture of mullets in coastal and brackish water areas.

The acclimation experiments led Panikkar (1951) to suggest that temperature and salinity tolerances of the mullet species be studied in detail.

Based on his experiments, Pillay, (1954) suggested Mugil parsia (Ham) as an important cultivable brackishwater mullet in deltaic West Bengal. Sarojini (1954) observed the food contents of Mugil parsia in relation to feedubg habits. Later, Luther (1967) has reviewed the work done on grey mullets in India.

Ghosh (1967) reported on acclimatisation of Mugil parsia fry in

fresh water. Mohanty (1973) recorded that juveniles of M. cephalus acclimated to freshwater were more tolerant to changes in conditions and that tolerance varied with length. Successful spawning and larval rearing of Mugil macrolepis was achieved by Sebastian and Nair (1974).

Ghosh et al. (1975) studied in detail the food requirement and supplementary feeding as a tool for enhanced production in the culture of Mugil parsia (Ham). Ghosh (1975) observed the effect of cobalt on the survival and growth of Mugil parsia. Later, Das (1976, 1977) conducted feeding experiments and analysed food constituants of Mugil cephalus.

Works carried out by the Indian workers in the eighties included mainly studies on pituitary gland of selected cultivable mullets (Asha, 1983); spawning biology of L. Parsia (Kurup and Samuel, 1983); genetic variation (Karia, 1984); effect of environmental factors on developing eggs and early larvae of L. parsia. (pillay, 1984); electrophoretic studies on Mugil cephauls and L. parsia (Mary Mathews, 1985); lactate dehydrogenase isozymes in Mugil cephauls and L. parsia (Ravi, 1986); seed identification of four species of mullets off Ratnagiri, (Barve and Jalihal, 1987); histological and biochemical changes during spermatogenesis in Mugil cephalus and L. parsia (Elezabeth, 1987); morphohistology of the digestive tract of L. parsia in relation to its food habits (Gosh et al., 1987); food ration for rearing the fry of the L. parsia (Kiron and Paulraj, 1987); biochemical aspects of ovarian maturation in L. parsia (Muthukaruppan, 1987); influence of

salinity on the growth and feed utilisation in L. parsia fry (Paulraj and Kiron, 1987) and low ambient oxygen tolerance in fry and fingerlings of Chanos chanos and Mugil cephalus (Usha Devi, 1987).

A thorough survey of literature suggest that reports on digestion and digestive enzymes of mullets are rather scanty. However, as early as in 1935, Ishida reported on the digestive enzyme of Mugil cephalus without emphasizing significance to any specific enzyme. Similarly, after a long period, Richard et al. (1982) followed the modifications in the digestive enzyme profile during the first year of life of the mullet M. capito in French water.

Studies pertaining to specific digestivse enzymes are very limited. Leucine aminopeptidase was assayed, using leucinamide, in Mugil auratus (Kleine, 1963). Chymotrypsin activity was assayed in Mugil auratus (Kleine, 1963; Albertini - Berhaut and Alliot, 1978) and M. capito (Albertini - Berhaut and Alliot, 1978). Trypsin, carboxypeptidase A and B and elastase were reported only in Mugil auratus and M. capito (Albertini - Berhaut and Alliot (1978). Proteases in general were assayed in the alimentary canal of Mugil sp. (Volya, 1966), in the stomach, pyloric caeca and intestine of M. auratus, M. capito and M. saliens (Albertini - Berhaut and Alliot, 1979) and in Liza aurata and L. ramada (Albertini - Berhaut, 1986). Studies on amylase is limited to the report made by Volya (1966) on Mugil sp. Similarly, only one report is made on the incidence of glycosidase activity in L. aurata and L. ramada (Albertini - Berhaut, 1986).

Reports on the digestive enzymes of mullets from Indian waters are rather limited to the works by Seshadri (1957, 1960). He studied the amylases of liver and intestine of M. speigleri, M. dussumieri and M. cephalus (Seshadri, 1957). Later in 1960, he studied the distribution of pancreatic tissue and reported amylase activity in mullets, viz. M. speigleri, M. cephalus and M. dussumieri, especially in liver, stomach and intestine of these fishes. Besides, he also investigated the temperature and pH optima for amylase activity. Seshadri (1967) also compared the amylase acativity in the above said mullets and a murrel Ophiocephalus punctatus.

The other reports on mullets, related to physiology of alimentary canal are the reports made by Agrawal and Bala (1967) on M. corsula, and Kutty and Mohamed (1975) on the metabolic adaptations of mullet Rhinomugil corsula with special reference to energy utilisation. Further, Kutty (1981) has also reviewed the energy metabolism of mullets.

Hence it is obvious that there is no work on the digestive enzymes of Liza parsia which is one of the potential candidate speices for aquaculture.

1.2.1 Carbohydrases

Studies on the carbohydrases were largely confined to the identification of amylase in different groups of fishes such as selachians (Weinland 1901; Herwerden, 1908; Beauvalet, 1933); Scyllium (Sullivan, 1907); bluegill and pickerel (Kenyon, 1925; McGeachin and Debnam, 1960); carps (Kenyon, 1925; Vonk, 1927, 1941;

Morishita et al., 1964; Ushiyama et al., 1965; Onishi et al., 1974); Anguilla japonica (Oya et al., 1927; Morishita et al., 1964); Fundulus (Babkin and Bowie, 1929); Raja sp. (Babkin, 1929); Zoarces (Mackay, 1929); Brevoortia sp. (Chesley, 1934; Pleuronectes (Bayliss, 1935; Volya, 1966); Calotomus and Thalassoma (Ishida, 1936; Turpayev, 1941; Al-Hussaini, 1949; Kawai and Ikeda, 1972, 1973); brook trout (Philips et al., 1948); Gobio and Rutilus (Al-Hussaini, 1949); Tilapia nilotica (Al-Hussanini, 1953; Moriarty, 1973); scarid and labrid fishes (Gohar and Latiff, 1957); Tilapia sp. (Fish, 1960; Nagase, 1964); mackerel (Kandyuk, 1967); Pagrus major and Plecoglossus altivelis (Kawai and Ikeda, 1971); Tilapia shirma (Cockson and Bourne, 1972); Gadus morhua (Overnell, 1973); Salmo gairdneri (Kitamikado and Tachino, 1960a; Kawai and Ikeda, 1973a); white sturgeon (Budding ton and Doroshov, 1986b) and Clarius gariepinus (Uys and Hect, 1987).

Amylase was also studied in salmonoid fishes and in bream Abramis brama with reference to various growth stages and season (Onishi and Murayama, 1970; Kuzmina, 1980). Development of amylase and maltase activity after hatching was monitored in Salmo gairdneri (Kawai and Ikeda, 1973a), Cyprinus carpio and Acanthopagrus schlegelii (Kawai and Ikeda 1973b) and lake sturgeon Acipenser fluvescens (Buddington, 1985). Onishi et al. (1973 a,b, 1976) studied the sequence of amylase after feeding, interaction among the secretion, inactivation and synthesis of digestive enzymes including amylase after feeding and response of amylase to twice a day feeding in carp. They also studied the activity ratio of

amylase to protease in cultivated salmonoid fishes during their maturation process (Onishi et al., 1974). Amylase activity was investigated in relation to pH in Hypophthalmichthys molitrix and Aristichthys nobilis (Bitterlich, 1985). Later, Uys et al. (1987) studied the changes in the amylase activity after different hours of feeding in Clarias gariepinus.

Other carbohydrases such as maltase, sucrase, lactase, melibiase, cellobiase and methyl alpha-D-glucosidase were detected in carp (Kawai and Ikeda, 1971). Olatunde and Ogunbiyi (1977) recorded carbohydrases in Physailia pellucida, Eutropius niloticus and Schilbe mystus. Neiderholzer and Hofer (1979) detected cellulase activity in Rutilus rutilus and Scardinus erythrophthalmus. Further, carbohydrases were also recorded in milk fish Chanos chanos (Chiu and Benitez, 1981) dover sole Solea solea (Clark et al., 1984) and in Atlantic halibut Hippoglossus hippoglossus (Glass et al., 1987).

1.2.2 Proteases

Reports available in the literature pertaining to proteases mainly centres around acid proteases and alkaline proteases. Pepsin, chymotrypsin and trypsin have drawn the attention of several investigators over the long years.

Acid Protease

Pepsin has been the centre of attraction for several workers who attempted studies on digestive enzymes of fishes. Peptic activity was demonstrated in several fishes such as pike (Fick and Murisier,

1874); bluegill, carp, crappie, pickeral and white bass (Kenyon, 1925); Esox lucius (Kenyon, 1925 Fish 1960; Nagase, 1964); herring (Almy 1926; Mackay, 1929); perch (Hykes et al., 1934); Pleuronectes (Bayliss 1935; Kandyuk, 1967); Oncorhyncus (Norris and Elam, 1940); Thunnus (Norris and Mathies, 1953); Trutta fario (Buchs, 1954); Salmo gairdneri (Kitamikado and Tachino, 1960b; Morishita et al., 1964); Tilapia mossambica (Fish, 1960; Nagase, 1964; Morishita et al., 1964; Hsu and Wu, 1979); Anguilla japonica (Morishita et al., 1964; Hsu and Wu 1979); Seriola quinqueradiata (Morishita et al., 1964); Ictalurus (Nordlie, 1966; Smit 1967); Trachurus (Kandyuk, 1967); dogfish (Merret et al., 1969); bonito (Kubota and Ohnuma, 1970); Limanada yokohamae, Kareius bicoloratus and Paralichthys olivaceus (Yasunaga, 1972); Tilapia nilotica (Moriarty, 1973); Dicentrarchus labrax (Alliot et al., 1974); Eutropius niloticus, Physailia pellucida and Schilbe mystus (Olatunde and Ogunbiyi, 1977); Engraulis encrasicholus (Estblier and Gutierrez, 1978); Carassius auratus, Channa maculatus, Clarias fuscus and Ctenopharyndodon idella (Hsu and Wu, 1979); Cyprinus carpio (Hsu and Wu, 1979; Jonas et al., 1983); sardines (Noda and Murakami, 1981); Boreogadus saida (Arunchalam and Haard, 1985); Solea solea (Clark et al., 1985a); Clarias gariepinus (Uys and Hecht, 1987) and Hippoglossus hippoglossus (Glass et al., 1987).

Fick and Murisier (1874) demonstrated that pike pepsin adapted to functioning at low temperature. Pepsin was mostly identified employing substrates such as gelatin (Bodansky and Rose, 1922), fibrin (Vonk, 1927) and casein (Bayliss, 1935). Optimal pH for pepsin was reported to be 2.5 - 2.8 for herring (Almy, 1926), 1.65

- 1.8 for the perch (Hykes, et al.,1934) and 1.5 - 2.5 for Pleuronectes sp. (Bayliss, 1935). Vonk (1927, 1929, 1941) studied the pH optima of the pepsins of Acanthias, Esox, frog, Testudo and pig and obtained values which were all near to pH 2.0. He regarded this pH 2.0 as the one that indicate the essential identity of the enzyme throughout the vertebrate series. Other specific studies on pepsin included development of pepsin in rainbow trout, carp and black sea bream (Kawai and Ikeda, 1973 a,b); relation of peptic activity to feeding habits of fishes (Hsu and Wu, 1979); utilisation of pepsin in food processing industry (Haard et al., 1982); influence of diet on peptic activity in Brycon (Reimer, 1982); adaptation of peptic activity to function at low temperatures in rainbow trout (Twining et al., 1983); pepsin as rennet substitute (Brewer et al., 1984); development of peptic activity after hatch in lake sturgeon (Buddington, 1985) and in whtie sturgeon (Buddington and Doroshov, 1986a); annual cycle in peptic activity (Reimer, 1986), and changes in peptic activity after feeding (Uys et al., 1987).

Alkaline protease

Several alakaline proteases have drawn the attention of investigators in the past. They mainly included trypsin chymotrypsin, elastase, leucine aminopeptidase, carboxypeptidases endopeptidases, aminopeptidase, and collagenase. These enzymes were reported to be detected in fishes right from the dawn of 20th century. However, comparatively the literature accumulated in these years on these lines are rather limited.

Protease in general

Protease activity, without any specific reference to a particular enzyme, was reported in tuna (Kashiwada, 1952); Sebastes (Stern and Lockhart, 1953); perch and Tilapia sp. (Fish, 1960); rainbow trout (Kitamikado and Tachino, 1960b); carp (Chepik, 1966; Trofimova, 1973; Shcherbina et al., 1976 Jonas; et al., 1983); white amur (Hickling, 1966; Stroganov and Buzinova, 1969); mackerel (Ooshiro, 1968, 1971 a,b,c); Scomber scombrus (Kala,c 1975, 1976); Seriola quinqueradiata (Kamoi et al., 1975); Clupea harengus and Mallotus villosus (kalac, 1978a); Chanos chanos (Benitez and Tiro, 1982) and Hypophthalmichthys molitrax and Silurus glanis (Ragyanski et al., 1977, Jonas et al., 1983); Dicentrarchus labrax (Tue, 1983).

Further studies on proteases mainly concentrated on the response of these digestive enzymes to dietary changes in carp (Kawai and Ikeda, 1972); development of the protease in both C. carpio and black sea bream (Acanthopagrus schlegelii) (Kawai and Ikeda 1973a); protease activity in relation to various growth stages of Oncorhyncus nerka, O. masou, Salmo gairdneri irideus and Salvelinus fontinalis (Onishi and Murayama, 1970); response of protease to twice a day feeding in carp (Onishi et al., 1973a, 1976); possibility of incorporating proteolytic enzymes in fish food (Dabrowski and Glogowski, 1977); relationship between feeding habits and proteases of fresh water cultured fishes (Hsu and Wu, 1979); seasonal changes of protease activity in Rutilus rutilus and Scardinius erythrophthalmus (Hofer 1979b), and effect of feeding

acidified feeds on protease activity in rainbow trout (Rungruangsak and Utne, 1981).

Alkaline protease - in general

Alkaline protease as a component of digestive enzyme was reported in carp, crappie and pickerel (Kenyon, 1925); herring (Almy, 1925); Anguilla sp. (Oya et al., 1927); Fundulus sp. (Babkin and Bowie, 1928); Zoarces anguillaris (Mackay, 1929); Ameiurus sp. Micropterus sp. and Tinca sp. (Beauvalet, 1933, Schlottoke, 1939; Al-Hussaini, 1949); Parasilurus sp. (Oya and Yokota,1933); Carassius auratus and Micropterus salmoides (Chesley, 1934); Pleuronectes platesa (Bayliss, 1935); Calotomus sp. Salarias sp., Spheroides sp. and Thalassoma sp. (Ishida, 1936); Lota vulgaris, Perca fluviatilis and Trutta iridiea (Schlottke, 1939); cod and mackerel (Johnston 1941); Gobio sp. and Rutilus sp. (Al-Hussaini, 1949); Lepomis marchrochirus (Lawrence, 1950) and Sardinops melanosticta (Murakami and Noda, 1981).

Trypsin

Trypsin is the major enzyme among the alkaline proteases to be studied largely in several fishes. It was detected in carp (Knauthe, 1898; Beauvalet, 1933; Bondi and Spandorf, 1954; Cohen et al., 1981a; Khablyuk and Proskuryakov, 1983 a,b); Raja sp. (Weinland, 1901); Torpedo sp. (Weinland, 1901; Babkin, 1929); Carcharias littoralis, Mustelus sp. (Sullivan, 1907); Squalus sp. (Sullivan 1907; Prahl and Neurath, 1966; Lacko and Neurath, 1970); labrid and scarid fishes (Gohar and Latiff, 1963); Tilapia sp.

(Morishita et al., 1964; Nagase, 1964); salmon (Croston, 1960, 1965; Uchida et al., 1984); Ginglymostoma cirratum and Squalus suckleyi (Zendzian and Barnard, 1967); Chimaera monstrosa (Nilsson and Fange, 1969); Myxine glutinosa (NIlsson and Fange, 1970); Protopterus aethiopicus (Reeck et al., 1970); Gadus morhua (Overnell, 1973); Tilapia nilotica (Moriarty, 1973); Dicentrarchus labrax (Alliot et al., 1974); Carassius auratus (Jany., 1976); Mallotus villosus (Hjelmeland and Raa, 1982); Parasilurus asotus (Yashinaka et al., 1983 c, 1984b); Anguilla japonica (Yashinaka et al., 1984 e, 1985 b,e); Gadus ogac (Simpson and Haard, 1984a); Solea solea (Clark et al., 1985a); Hypophthalmichthys molitrix and Aristichthys nobilis (Bitterlich, 1985). Acipenser transmontanus (Buddington and Doroshov, 1986b); Hippoglossus hippoglossus (Glass et al., 1987); Clarias gariepinus (Uys and Hecht, 1987), and in rainbow trout (Stevens and Mc Leese, 1988).

Chymotrypsin

Chymotrypsin was detected in the digestive enzyme components of Engraulus encrascicholus (Kleine, 1963); salmon (Croston, 1965); Squalus sp. (Prahl and Neurath, 1966; Lacko and Neurath, 1970); Chimaera monstrosa (Nilsson and Fange, 1969); Myxine glutinosa (Nilsson and Fange, 1970); Gadus morhua (Overnell, 1973); Tilapia nilotica (Moriarty, 1973); Dicentrarchus labrax (Alliot et al., 1974); carp (Cochen et al., 1981a; Kablyuk and Proskuryakov, 1983a,b); Solea solea (Clark et al., 1985a). Acipenser transmontanus (Buddington and Doroshov, 1986b); Clarias gariepinus (Uys and Hecht, 1987) and Hippoglossus hippoglossus (Glass et al., 1987).

Carboxypeptidases

Carboxypeptidases A and B were recognised by earlier investigators. In general, this group of enzyme is reported to be present in labrid and scarid fishes (Gohar and Latiff, 1963); Thynnus secundodorsalis (Zendzian and Barnard, 1967); Chimaera monstrosa (Nilson and Fange, 1969); Protopterus aethiopicus (Reeck and Neurath, 1972); Gadus morhua (Overnell, 1973); Tilapia nilotica (Moritary, 1973); Dicentranchus labrax (Alliot et al., 1974); carp (Cohen et al., 1981; Khablyuk and Proskuryakov, 1983 a,b); Parasilurus asotus (Yoshinaka et al., 1984 a,c,d; 1985 c,d); Acipenser transmonatanus (Buddington and Doroshov, 1986b), and Hippoglossus hippoglossus (Glass et al., 1987).

Elastase

Elastase enzyme was detected in Dasyatis americana and Thynnus secundodorsalis (Zendzian and Barnad, 1967); Chimaera monstrosa (Nilsson and Fange, 1969); Protopterus aethiopicus (de Haen and Gertler, 1974); carp (Cohen et al., 1981a; Khablyuk and Proskuryakov, 1983a, b); Parasilurus asotus (Yoshinaka et al., 1982, 1983a, 1984a, 1985a,b); Solea solea (Clark et al., 1985b); Anguilla japonica, Cyprinus carpio, Dasyatis akajei, Lateolabrax japonicus, Lophiomus setigerus, Musteus manazo, Salmo gairdneri, Seriola quinqueradiata and Thunnus thynnus (Yoshinaka et al., 1985b) and Acipsenser transmontanus (Buddington and Doroshov, 1986b).

Leucine aminopeptidase

Leucine aminopeptidase was reported in Engraulus encrascicholus and Mullus barbatus (Kleine, 1963); Myxine glutinosa (Nilsson and Fange, 1970); Gadus morhua (Overnell, 1973); carp (Khablyuk and Proskuryakov, 1983 a,b); Solea solea (Clark et al., 1985a); Atherser transmontanus (Buddington and Doroshov, 1986b), and Hippoglossus hippoglossus (Glass et al., 1987).

Other alkaline proteases detected in the fishes were dipeptidase and aminopeptidases in labrid and scarid fishes (Gohar and Latiff, 1963), Chimaera monstrosa (Nilsson and Fange, 1969) and in Myxine glutinosa (Nilssson and Fange, 1970) and collagenase in Solea solea (Clark et al., 1985a).

Further studies on alkaline proteases, besides identification of the same in fish digestive system were made by few investigators. They include investigations on trypsin, chymotrypsin, elastase and carboxypeptidase B from carp pancreas with respect to their physical and kinetic properties (Cohen et al., 1981a,b); activation of trypsinogen and chymotrypsinogen and stability of the activated enzymes and distribution of these activities in the pancreatic extract of Parasilurus asotus (Yoshinaka et al., 1981 a,b); Production of fish sauce from sardine by utilization of its proteolytic enzymes (Yoshinaka et al., 1983b); feasibility of utilisation of trypsin isolated from the pyloric caeca of Gadus ogac as a food processing aid (Simpson and Haard, 1984 b,c); development of trypsin, chymotrypsin and aminopeptidase in Corgonus, Salmo gairdneri and Rutilus rutilus (Lauff and Hofer,

1984); development of radioimmunoassay for trypsin and chymtrypsin (Hjelmeland and Jorgensen, 1985); changes in the activities of trypsin and chymotrypsin after feeding in Clarias gariepinus (Uys et al., 1987), and influence of feeding level on the kinetics of trypsin from rainbow trout (Steevens and McLeese, 1988).

Work on digestive enzymes of fishes other than mullets in Indian waters is rather limited. Digestive enzymes, in general, have been investigated in Ophiocephalus punctatus (Mahalanabis and Riochaudhri, 1950); Carassius auratus and Micropterus salmonoides (Sarabahi, 1951); Tilapia mossambica (Moitra and Das, 1967), Catla catla, Cirrhinus mrigala and Labeo rohita (Dhage, 1968); Epinephalus tauvina (Dhage, 1969); Labeo calbasu and Mystus seenghala (Sehgal, 1969 a,b); Periophthalmus koelretueri (Dhage and Mohamed, 1977), and in some fresh water teleost fishes (Agrawal et al., 1975).

Ghanekar et al., (1956) observed alpha and beta amylases in the extracts of various organs of elasmobranchs. Sundaram and Sarma (1960 a,b) purified and studied the properties of a protease isolated from the guts of Rastrelliger kanagurta and Etroplus suratensis. Sastry (1974) reported lipolytic activity in Clarias batrachus and Ophiocephalus punctatus and Ghosh (1976) correlated the digestive enzymes with the fond habits of clarias batrachus. Saigal et al (1974) and Ghosh et al., (1977) reported on the carbohydrases , protienase and lipase in Heteropneustes fossilis. Dhaliwal (1975, 1977) determined the optimum pH for the activity of certain carbohydrases in Cyprinus carpio and Mystus vittatus, and

detected protease and lipase in Mystuss vittatus. Mukhopadhyay (1977) reported on the specific activities of amylase, cellulase, protease and lipase in Clarias batrachus and changes in their activities with the changes in the nutritional status of the diet. Sinha (1978) investigated the changes in amylase, protease and lipase activities in the alimentary tract of Cirrhinus mrigala during different life history stages.

Goel and Sastry (1973) investigated alkaline phosphatases in Clarias batrachus, Ophiocephalus punctatus, Ophiocephalus gachua and Barbus sophore. Later, Shafi (1978) also studied localisation and distribution of alkaline phosphatase in Clarias batrachus. Dalela et al. (1976) surveyed the acid phosphatase in Colisa fasciatus, Macrognathus aculeatus, Notopterus notopterus and Nandus nandus. Sinha (1979) localised the distribution of acid and alkaline phosphatases in Cirrhinus mrigala.

Sastry (1977) investigated on peptidase activity in Clarias batrachus and Ophiocephalus punctatus. Mukhopadhyay et al. (1978) purified an alkaline protease from Clarias batrachus and studied the effect of dietary protein levels on the proteolytic activity. Desai (1978) studied distribution of cholinesterase in migratory Hilsa ilisha non migratory Hilsa foli.

The physiology of digestion was studied in Mastacembelus pancalus (Agrawal and Tyagi, 1963), Colisa fasciata (Agrawal and Singh, 1963) and Mystus vittatus (Tyagi, 1971). Sastry and Malik (1979) studied the effect of Dimecron on the digestive system of fresh water fish Channa punctatus. Further, Sastry et al. (1979) and

Sastry and Gupta (1979a,b, 1980) investigated alteration in the activities of different digestive enzymes of C. punctatus exposed to different heavy metals such as lead nitrate and mercuric chloride. Mukundan et al. (1985) purified a lipase from the hepatopancreas of oil sardine Sardinella longiceps and studied its characteristics and properties. Bahuguna and Bahuguna (1987) investigated the digestive physiology of hillstream fishes with reference to amylase, glycogenase, maltase, lipase and proteinase in relation to their diet. Patra and Ray (1987) studied the influence of dietary protein source on protease activity, protein synthesis and biochemical composition in Anabas testudineus. Phadate and Srikar (1987) investigated the effect of formulated feeds on protease activity and growth of Cyprinus carpio, Catla catla and Hypopththalmichthys molitrix.

1.3 Research Approach

A detailed analysis of the review of literature presented in the foregoing section leads to arrive at the following inferences:- Studies on mullets in the past years, in abroad, mainly centered around detection of various digestive enzymes in the different species of mullets. Except for the sporadic reports on the digestive enzyme profile during development, hardly any detailed report is available on any aspect of digestion or digestive enzymes.

In India, mullets have been the subject of study for several workers in areas other than nutrition, digestion and digestive enzymes. Thus, extensive studies have been conducted on various

aspects including acclimatisation of mullets to varying environments, food and feeding, energy metabolism, reproductive physiology, spawning and larval rearing and development of polyculture practise.

Commercial fishculture practise demands detailed information on nutrition and digestion capabilities of the candidate species. There exists a dearth of knowledge on nutrition, digestion and digestive enzymes of mullets in general and particularly on Liza parsia, which is one of the potential candidate species for aquculture in India.

The aquaculture practise of grey mullet is posed with several questions when one tries to understand different aspects of nutrition and digestive ability as follows:

1. What kind of qualitative and quantitative distribution profile the digestive enzymes evince in the grey mullet Liza parsia?

2. How does the digestive enzyme respond to the changes in the physico-chemical factors prevailing in their habitats?

3. Whether the digestive enzymes are influenced by the size, type of food and feeding habits of the fish?

4. Is there any change in relative contribution of various digestive enzymes in response to changes in the dietary composition?

The present study is an attempt to throw light in solving the

problems faced by this industry in economising the operations of the aquaculture of mullets, which in turn would popularise fish culture as profitable as shelfish culture. The major focal themes of the present investigations are:

1. Collection of fishes from natural environment and analysis of their digestives enzymes present along the different regions of the digestive tract, both qualitatively and quantitatively;

2) Effect of physico chemical factors on the activity of the various digestive enzymes present in the diffeerent regions of the digestive tract;

3. Effect of inhabitors and metal ions on the activity of various digestive enzymes;

4. Relationship between size of fish and the activities of enzymes in the digestive tract;

5. Relationship between feeding habits and the digestive enzymes;

6. Temporal changes in digestive enzymes activity; and

7. Influence of dietary composition on the digestive enzymes.

CHAPTER 2

MATERIAL AND METHODS

2. MATERIAL AND METHODS

2.1 Animals

The fishes belonging to the family Mugilidae, commonly known as 'mullets', are widely distributed in the coastal waters and estuaries of the tropical and subtropical zones of all seas. The species taken for the present investigation is the gold spot mullet Liza parsia (plate-1), occurring commonly along the coasts of Pakistan, India and Sri Lanka. Contemporary species of fishes belonging to the family Mugilidae are assigned to fourteen genera with sixty four species (Thomson, 1981). Liza parsia is a schooling species occurring in shallow coastal waters, estuaries, lagoons, sometimes entering tidal rivers. They are found to survive in ponds with 87 ppt salinity. Spawning takes place in the sea. They feed on small algae, diatoms and other organic matter both living and detrital, taken in with sand and mud. Fries of mullets feed on copepods and floating algae and usually occur in shallow inundated areas along the sea coast. It is one of the common mullets in the Indian seas and an important cultivable brackish water fish along the coasts of India.

2.2 Collection and Transportation of animals

Fishes used in the present investigation were collected from Cochinbackwater nearby Vypeen island (Fig.1). Adults of Liza parsia of size range 160 to 180mm in total length caught in 'Chinese dipnet' were transferred to sterile polythene bags and transported to the laboratory in ice box (0-2°C) and kept in deep freezer for

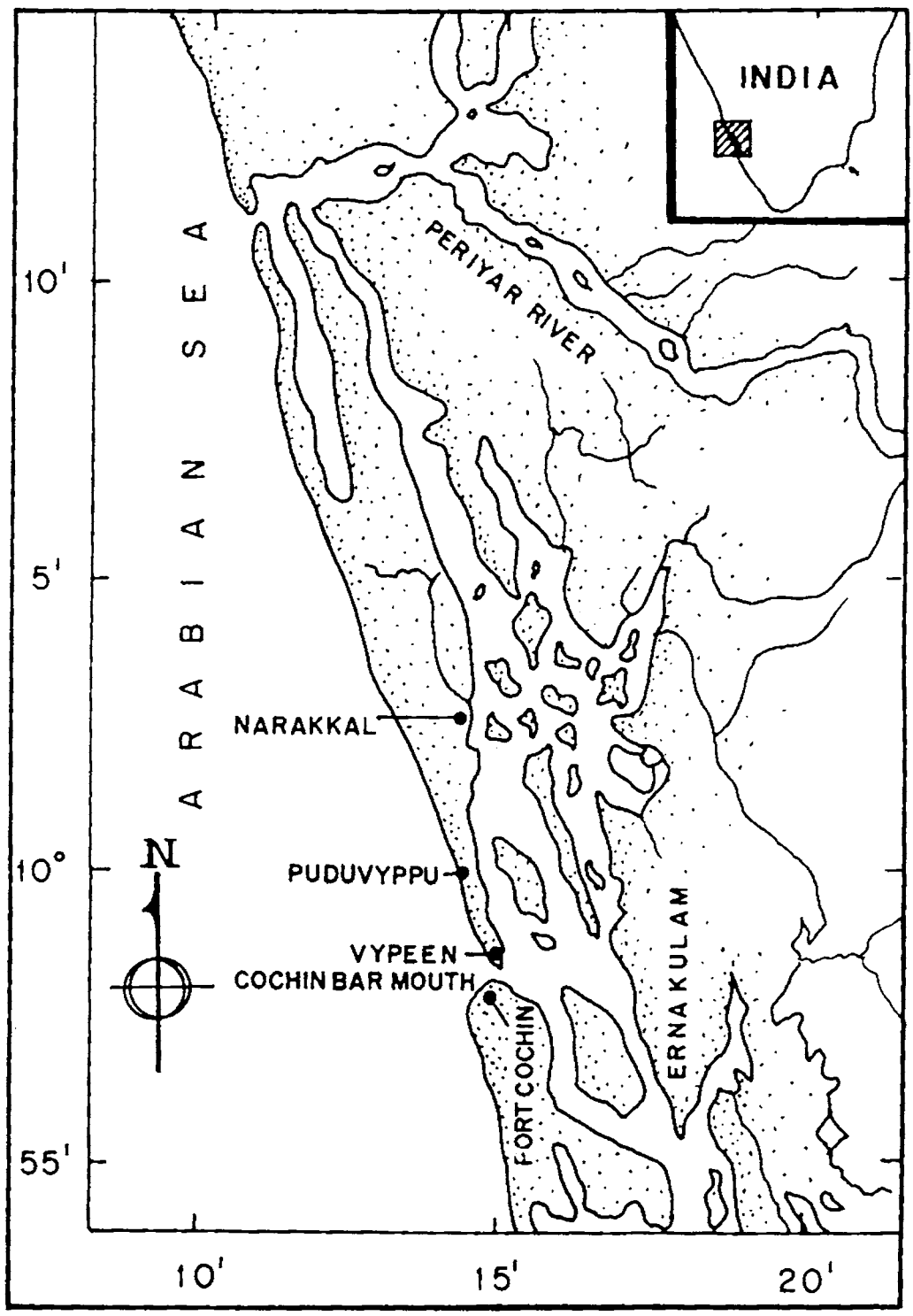

Fig.1 Map showing the collection sites in the Cochin Backwater, Southwest coast of India.

Plate - 1. The Gold Spot mullet - Liza parsia

plate-1

subsequent analysis.

When required the animals were transported alive to the laboratory in polythene transportation bags of 15 l capacity, half filled with the water collected from the same site where the fishes were caught. When the water contained sediment and silt it was used after filtering through bolting silk. Each bag contained not more than two specimens at a time. The whole operation was completed in about three hours during which no mortality was recorded.

2.3 Maintenance of animals in the laboratory

In the laboratory, fishes were maintained at room temperature (28° ± 2°C) in 1000 l fibre reinforceed plastic tanks, filled with filtered sea water of appropriate salinity (15 to 20 ppt) provided with a biological filter and under continuous aeration.

2.4 Substrates and chemicals

The enzyme substrates such as : glycogen, raffinose, trehalose, maltose, lactose, soluble starch, dextrin, salicin, sucrose, melibiose, cellobiose, microcrystalline cellulose, carboxymethyl cellulose, p-nitrophenyl-alpha-D-glucopyranoside, p-nitrophenyl-beta-D-glucopyranoside, p-nitrophenyl-beta-D-galactopyranoside, for estimating carbohydrases; casein, haemoglobin, N-alpha-benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA), N-alpha-p-tosyl L-arginine methyl ester hydrochloride (TAME), N-benzoyl-L-tyrosine p-nitroanilidae (BTNA), hippuryl-L-phenylalaninne, hippuryl-L-arginine, elastin-orcein, L-leucine-p-nitroanilide for assaying different proteases; alpha-napthyl acetate for estimating esterase;

p-nitrophenylphosphate for estimating phosphatases and inhibitors N-tosyl-L-phenylalanine chloromethylketone (TPCK), N-alpha-p-tosyl-L-lysine chloromethyl ketone (TLCK), phenyl methyl sulfonyl fluoride (PMSF) were all purchased from Sigma chemical company, USA. All other chemicals used in this investigation were of analytical reagent grade.

2.5 Preparation of enzyme extract

All procedures in the preparation of crude enzyme extracts were carried out at temperatures 4° to 8°C. Length and weight of the fishes were recorded before dissection. The live fishes were made unconscious by a strong sharp blow on the head or sacrificed by cervical dislocation. The digetive tract was dissected out by making an incision ventrally. The whole digestive tract was separated into the following distinct regions viz. oesophagus, cardiac stomach, pyloric stomach, pyloric caeca, anterior intestine, posterior intestine, liver with gall bladder and spleen or as required in different experiments. Care was taken to avoid puncturing the gall bladder and hence liver with gall bladder and its contents were used. Unless otherwise stated, all the regions were washed with chilled double distilled water to free the ingested materials. Adhreing mesenteric and adipose tissues were stripped from all organs. The intestine of the mullet, which had an almost uniform size throughout its length range was divided into two equal segments and designated as anterior and posterior intestines. These excised organs were quickly frozen and stored in deep freezer until used for enzyme assay. Each digestive organ

excised from the fish was extracted separately for these enzyme assays.

After slow thawing in a cold room at 4°C, the digestive tissues were blot dried and weighed individually. The tissue was cut into small pieces and homogenized in a Potter-Elvehjem type homogenizer (Remi model, type LT 56-3, provided with speed regulator) in chilled double distilled water to give a 1:10 dilution. While homogenization, the homogenizing tube was kept immersed in a cooling jacket to prevent over-heating (Hess and Brand, 1972). The homogenate thus obtained was spun in a sorval model 5B refrigerated centrifuge at 25,000 g for 20 minutes at 0°C. The clear supernatant, which contained the enzymes, designated as crude enzyme extract, was decanted and stored in small vials in a deep freezer. When required the frozen crude extract was brought to room temperature by thawing slowly at 4°C and used to estimate enzyme activity. Any remaining portion of the enzyme extract was not stored further as repeated freezing and thawing denature the enzymes (K.D.Jany, Personal communication). The crude extracts were not stored for more than a weak unless otherwise stated.

2.6 Enzyme Assays

2.6.1 Carbohydrases:

The different carbohydrases, maltase, dextrinase, sucrase, trehalase, alpha-glucosidase, raffinase, melibiase, cellobiase, salicinase, beta-glucosidase, beta-galactosidase, amylase and cellulase were assayed along the digestive tract of L. parsia.

The crude enzyme extract was allowed to react with an appropriate carbohydrate substrate and the rate of hydrolytic products formed upon the reaction was taken as a measure of enzyme activity. The concentration of different carbohydrate substrates employed were 1.0% for starch, glycogen, dextrin, cellobiose, maltose, lactose, carboxymethyl cellulose and microcrystalline cellulose; 5.0% for salcin and melibiose and 10.0% for raffinose, trehalose and sucrose. The substrates were dissolved in 0.1 M Tris-0.01 M EDTA buffer, pH 7.0 and stored in a refrigerator. A drop of toluene was added to the substrate solution as a preservative. The incubation mixture contained 0.50 ml crude enzyme extract and 1.0 ml of pre-incubated substrate solution. The incubations were carried out at 37°C (±0.5°C) for 3 hours. A blank containing no substrate and a control containing no crude enzyme extract were run simultaneously with the reaction mixture. The concentration of reducing sugars at the end of 3 hour and incubation period was measured by the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952). The enzyme activity was expressed as micro gram glucose liberated/mg protein/hour. For the synthetic substrates, p-nitrophenyl-alpha-D-glucopyranoside, p-nitrophenyl-beta-D-glucopyranoside and p-nitrophenyl-beta-D-galactopyranoside, 0.20 ml of crude enzyme extract was incubated with 1.00 ml of 0.01 M substrate solution and 1.80 ml of 0.1 M Tris-0.01 M EDTA buffer, pH 7.0. These substrates were dissolved in the same buffer used in the reaction mixture. The hydrolysis of the substrate was determined by the increase in the absorbance at 405 nm, due to the formation of p-nitrophenol, in a spectrophotometer (ECIL, Spectrophotometer model GS 865 D). The

enzyme activity, calculated by employing a standard curve with p-nitrophenol, is expressed as micro gram p-nitrophenol liberated/mg protein/hour.

2.6.2 Acid protease

Pepsin, an endoprotease, is the major acid protease in the gut of fishes acting in an acidic pH range. Pepsin like activity was determined by the method of Anson (1938) utilising acid denatured haemoglobin as substrate. A 2% solution of haemoglobin was prepared in 0.06 N HCl, which produces an acidity of pH 1.8 that denatures the haemoglobin. The haemoglobin substrate solution was stored at 4°C until used in the assay. Before addition to assay mixture, the substrate solution was prewarmed to 37°C for 15 minutes.

The assay system consisted of 50 ml of the substrate solution in a test tube equilibrated at 37°C, to which 1.0 ml of the crude enzyme extract was added. After 30 minutes incubation period, 10.0 ml of 5% chilled TCA was added and allowed to stand at room temperature for 10 minutes. The contents were then centrifuged at 5000 g for 10 minutes and the supernatant was used for further analysis. To 5.0 ml of the supernatant in a clean test tube 10.0 ml of 0.5 N NaOH was added and mixed well. To this 3.0 ml of diluted phenol reagent (Phenol reagent and distilled water mixed in 1:2 proportion) was added with constant shaking with the help of a cyclomixer (Remi Model). The blue colour that developed was read in a spectrophotometer at 650 nm. Blanks were prepared for every assay following the same proceedure adopted for sample, preparation, except for the addition of TCA to the substrate just prior to

addition of enzyme extract. The colour value is expressed in equivalents of tyrosine with the help of a standard curve prepared with tyrosine. The enzyme activity is expressed as micro mole tyrosine liberated/mg protein/hour.

2.6.3 Alkaline Protease

The protease activity in the alkaline pH range, tested in the post gastric region of the gut, included variety of enzymes such as trypsin, chymotrypsin and peptidases which were assayed using specific synthetic substrates.

The total alkaline proteolytic activity was determined by the casein digestion method presented by Kunitz (1947). The substrate solution contained 1% casein in 0.1M phospate buffer, pH 7.6. The suspension was heated for 15 minutes in a boiling water bath to obtain a homogenous solution. A drop of toluene was added as preservative. The casein solution was stored at 4°C until used in the assays and it was discarded after a week. Before addition to assay mixture, the substrate solution was prewarmed to 37°C for 15 minutes.

The assay system consisted of 0.125 ml of the crude enzyme extract and 0.875 ml of 0.1 M phosphate buffer, pH 7.6 and 1.0 ml of prewarmed casein solution. The mixture was incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 3.0 ml of ice cold 5% TCA. The mixture was then allowed to stand at room temperature for one hour and then centrifuged for 20 minutes at 3000 g. The absorbance of the supernatant solution was determined

at 280 nm in a UV/VIS spectrophotometer (ECIL GS 865 D). All assays were corrected for by a blank determination which was conducted similar to the regular assay, except that the crude enzyme extract was added to the blank tube after the TCA precipitation step.

The absorbance values were converted into enzyme units by employing a standard curve made with tyrosine. The specific activity is expressed as micro mole-tyrosine liberated/mg protein/hour under the assay conditions stated here.

2.6.4 Trypsin (EC 3.4. 4.4)

Tryptic activity was determined according to the method of Erlanger et al. (1961) with BAPNA as substrate. A 1.0 mM substrate solution was prepared by dissolving the BAPNA in 1.0 ml dimethyl sulfoxide. The solution was then adjusted to a final volume of 100 ml with 0.04 M Tris buffer, pH 8.2 containing 0.02 M CaCl2. The assay mixture consisted of 2.9 ml of the substrate solution and 0.1 ml of the crude enzyme extract in a cuvette. The increase in absorbance at 410 nm was monitored for 10 minutes at room temperature. The reference cuvette contained 2.9 ml of the substrate solution and 0.1 ml distilled water. A standard curve made up of P-nitroaniline was used to convert absorbance into enzyme units. The specific actiivty of trypsin is expressed as hano moles of P-nitroaniline liberated/mg protein/minute.

Trypsin-like activity was also measured by recording enzymic hydrolysis of 10^{2}M TAME at 247 nm following the method of Hummel (1959). The activity is expressed as enzyme units per mg protein,

one enzyme unit being equivalent to a difference of 0.001 in optical density per minute under the experimental conditions.

2.6.5 Chymotrypsin (EC 3.4.4.5)

Chymotrypsin was assayed by using BTNA as substrate. A 1.0 m mM substrate solution was prepared by dissolving BTNA in 30 ml of 95% ethanol. The solution was then adjusted to a final volume of 50ml with distilled water. The assay mixture consisted of 1.50ml of substrate solution and 1.4 ml of 0.1M Tris-HCl buffer, pH 7.8 containing 0.1 M CaCl2 in a spectrophotometer cuvette. After adjusting the absorbance at 410 nm to zero, the reaction was initiated by the addition of 0.10 ml of the crude extract. The increase in absorbance at 410 nm was monitored for 10 minutes at room temperature. Assays were corrected for by a reference mixture which contained all components of the assay exccept the crude enzyme extract, instead of which 0.10 ml of distilled water was added. The absorbance values were converted into equivalents of p-nitroaniline with the help of a standard curve using p-nitroaniline. The specific activity of chymotrypsin is expressed as nanomoles p-nitroaniline liberated /mg protein/minute.

2.6.6 Elastase (EC 3.4. 21.11)

The elastase activity was estimated by employing elastin-orcein (Sacher et al., 1955) according the method described by Appel (1974). The mucoprotein fraction of insoluble orcein-elastin is degraded by the enzyme to soluble hydrolysis products, the amount of dye liberated into solution per unit time is a measure of the

enzyme activity. The assay system which consisted of orcein-elastin 20 mg, 0.1 M Tris buffer pH 8.8 2.00 ml, and the crude enzyme extract 1.00 ml was incubated in a water bath provided with shaker at 37°C for 1 hour. At the end of the incubation period, 2.00 ml of phosphate buffer (0.7 M; pH 6.0) was added to stop the reaction. Then the mixture was centrifuged and the absorbance of the supernatant fluid was determined in a spectrophotometer at 578 nm. A zero time blank was prepared with all the reagents mentioned as above except that the phosphate buffer was first added to the assay mixture followed by the crude enzyme extract. Control was maintained to determine the spontaneous hydrolysis, if any, of the substrate by employing the same method desccribed above for the sample and instead of tissue homogenate 1.0 ml of physiological saline was added. The absorbance values were converted into enzyme units by comparing with a standard graph prepared with the dye, orcein. The enzyme activity is expressed as micro mole of orcein liberated/mg protein/minute.

2.6.7 Carboxypeptidase A (EC 3.4 12.2)

Carboxypeptidase A was measured by employing hippuryl-L-arginine as substrate according to the method of Folk and Schirmer (1963). The rate of hydrolysis of hippuryl-L-phenylalanine was determined by measuring the increase in absorbancy at 254 nm. The assay system consisted of 0.025 M Tris-HCl buffer containing 0.5 M sodium chloride, pH 7.5; 0.001 M Hippuryl - L-phenylalanine in 0.025 M Tris-HCl, pH 7.5, with 0.5 M sodium chloride. The assays were run at room temperature. 2.9 ml of substrate solution was pipetted into

each cuvette and adjusted to zero optical density at 254 nm. Appropriate amount of crude enzyme extract was added and the increase in absorbance was monitored for 10 to 15 minutes.

The absorbance values were plotted against time in a graph. The change in absorbance per minute was calculated from the linear portion of the curve. The enzyme activity was calculateed based upon the extinction coefficient of hippuric acid (0.36) formed during the reaction. The unit of enzyme activity is defined as the hydrolysis of 1 milli mole of substrate/gm protein/minute under the assay conditions.

2.6.8 Carboxypeptidase B (EC 3.4.12.3)

Carboxypeptidase B activity in the crude enzyme extracts was measured by the spectrophotometric method of Folk et al. (1960) employing hippuryl-L-arginine as substrate. The reaction velocity was determined by an increase in absorbancy at 254 nm resulting from the hydrolysis of hippuryl-L-arginine. The reagents included 0.025 M Tris - HCl Buffer, pH 760 containing 0.1 M sodium chloride; 0.001 M Hippuryl-L-arginine in 0.025 M Tris-HCl buffer pH 7.6, containing 0.1 M NaCl. The assay was conducted in the spectrophotometer cuvettes at room temperature. The assay system contained 2.90 ml of the substrate solution in a cuvette which was adjusted to zero optical density at 254 nm. Appropriate amount of enzyme extract was added to the above solution and the increease in absorbance was monitored. The optical density values were plotted against time. Change in absorbance per minute was determined from the initial linear portion of the curve. The enzyme activity was

calculated based upon extinction coefficient of hippuric acid formed (0.349) during the reaction. The unit of enzyme activity is defined as the hydrolysis of one micromole of hippuryl-L-arginine±gm protein/minute under the assey conditions.

2.6.9 Leucine aminopeptidase (EC 3.4.11.1.)

Leucine aminopeptidase activity was assayed with L-Leucine-p-nitroanilide as substrate as described earlier by Appel (1974). The p-nitroaniline liberated on enzymatic hydrolysis was determined by measurement of the increase in absorbance at 410 nm with a spectrophotometer. The assay system consisted of 2.0 ml of 1.2 mM Leucine-p-nitroanilide solution in 50mM Tris buffer pH 7.2 and 1.0 ml of enzyme extract. The assay was conducted in spectrophotometer cuvettes at room temperature and the increase in absorbance was monitored for 30 minutes. At the end of 30 minutes incubation period, the increase in absorbance was noted. A standard curve, prepared with p-nitroaniline, was used to convert the absorbance into amount of p-nitroaniline released. The enzyme activity is expressed as nano mole p-nitroaniline liberated/mg protein/minute under the specified conditions.

2.6.10 Esterase

Exterase acctivity was measured by employing alpha-napthyl acetate as substrate according to Nachlas and Seligman (1949) with slight modifications. The alpha-napthol liberated from alpha-napthyl acetate by the enzymatic activity of the sample combines with fast blue RR to produce a coloured compound and this was measured at

590 nm in a spectrophotometer to quantify the esterase activity. A 0.003 M alpha-napthyl acetate was prepared by initially dissolving it in 1.0 ml of acetone and then making up the volume with double distilled water. Arresting reagent was prepared by mixing two parts of 1% (W/V) fast blue RR with five parts of 1% (W/V) sodium lauryl sulphate, just before adding to the assay system. The assay system consisted of 1.0 ml of the 0.003 M alpha-napthyl acetate solution, 1.950 ml of 0.1 M phosphate buffer pH. 7.0, 0.050 ml of the appropriately diluted crude enzyme extract. The assay mixture in the test tubes was incubated at 37°C for 30 minutes. At the end of the incubation period, 2.0 ml of the freshly prepared arresting reagent was added to stop the reaction. The blue colour developed was measured at 590 nm and a standard graph prepared with alpha napthol was used to quantify the enzyme activity. The specific activity is expressed as micro gram alpha napthol liberated/mg protein/minute under the specified conditions.

2.6.11 Acid phosphatase (EC 3.1.3.2)

Acid phosphatase activity was assayed by employing p-nitrophenyl phosphate as substrate according to the method described by Walter and Schutt (1974). The amount of p-nitrophenol liberated per unit time, as determined in alkaline solution at 405 nm is a measure of the phosphatase activity. The acid buffer substrate solution (5.5 mM p-nitrophenyl phosphate) was prepared by dissolving 0.41 gm citric acid + 1.125 gm sodium citrate + 0.203 gm of p-nitrophenyl phosphate in double distilled water. The pH was adjusted to 4.8 and then the volume was made upto 100 ml. The assay mixture consisted

of 1.90 ml acid buffer substrate solution and 0.10 ml of crude extract in test tubes. The incubation was carried out at 37°C for 30 minutes. At the end of the incubation period, the reaction was terminated by the addition of 2.00 ml of 0.1 N NaOH. The absorbance of the mixture was measured at 405 nm. A sample blank was prepared by the same procedure mentioned above except that the enzyme extract was added to the assay mixture after the addition of 2.0 ml of 0.1 N NaOH. A standard graph prepared with p-nitrophenol was used to convert the absorbance into amount of p-nitrophenol liberated and the enzyme activity is expressed as nano mole p-nitrophenol liberated/mg protein/minute under the assay conditions stated.

2.6.12 Alkaline phosphatase (EC 3.1.3.1)

Alkaline phosphatase activity was determined by employing p-nitrophenylphosphate as substrate according to the procedure described by Walter and Schutt (1974). The amount of P-nitrophenol liberated per unit time, as determined in alkaline solution at 405 nm has been taken as a measure of the phosphatase activity. The alkaline buffer substrate solution (1.25 mN p-nitrophenylphosphate) was prepared by dissolving 1.052 gm diethanolamine + 8.0 ml 0.1 N HCl + 46.40 mg P-nitrophenylphosphate in 85 ml of double distilled water and the pH adjusted to 9.8 by addition of 0.10 N HCl and the final volume made upto 100 ml with distilled water.

The assay system consisted of 1.960 ml buffer substrate solution in a test tube and 0.050 ml of the crude extract. The incubation was carried out at 37°C for 30 minutes. The reaction was terminated by

the addition of 10 ml of 0.05 N NaOH. The absorbance was read at 405 nm. A standard graph prepared with p-nitrophenol was used to convert the absorbance into amount of p-nitrophenol liberated and the enzyme activity is expressed as nano mole p-nitrophenol liberated/mg protein/minute under the specified conditions.

2.7 Protein determination

The protein concentration of the crude enzyme extract was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

CHAPTER 3

SURVEY AND DISTRIBUTION PATERN OF DIGESTIVE ENZYMES

3. SURVEY AND DISTRIBUTION PATTERN OF DIGESTIVE ENZYMES

3.1 Introduction

The mullets are widely cultured in India and several Mediterranean countries, south-east Asia, Japan and Hawai (Thomson, 1966). The tecchnology for its intensivse culture has to be developed to meet the increasing demand for animal protein in all parts of the world where they are distributed. Studies on feeding habits, nutritional requirements and activity of digestive enzymes are of paramount importance in the development of such a technology. Although literature is available on the food and feeding habits of mullets (Sarojini, 1951; Pillay, 1953; Thomson, 1963, 1966; Odum, 1968a,b, 1970; Hickling, 1970; Zismann et al., 1975; Das, 1977; DeSilva and Wijeyaratne, 1977; Brusle, 1981) there exists only limited knowledge on the process of digestion and digestive enzymes (Albertini-Berhaut and Alliot, 1978; Perera and DeSilva, 1978; Albertini-Berhaut, 1986). The digestive enzymes and their distribution pattern of a number of cultured fishes have been the subject of previous researchers (Fish, 1960; Lane, 1973; Dhaliwal, 1975; Ghosh, 1976; Chiu and Benitez, 1981; Benitez and Tiro, 1982; Clark et al., 1984, 1985a; Glass et al., 1987; Uys and Hecht, 1987). Since the mullets gained significance as a candidate species for intensive aquaculture operations, the knowledge on their digestive potential is imperative in feed formulations.

Digestive enzymes which are secreted into the lumen of the alimentary canal originate from the gastric mucosa, the pyloric

caeca, the pancreas, and the intestinal mucosa, which secrete a number of enzymes that hydrolyse proteins, peptides, carbohydrates and fats. In the present study the total complement of digestive enzymes available to the fish for the purpose of hydrolysis of the various components of the ingested diet are dealt with. Previous studies have demonstrated that different sections of the gut do not necessarily possess the same capacity for the digestion and absorption of a given nutrient (Fisher and Parsons, 1950; Manis and Schacter, 1962; Harrison and Harrison, 1974; Mathews, 1974). Hence, in the present study it was planned to investigate the digestive potential of the various sections of the gut.

3.2 Material and Methods

3.2.1 Prepation of enzyme extract

Material and methods followed for preparation of enzyme extracts were described earlier (Section 2.5). A brief summary of the procedure adopted is as follows:

The digestive tract of fishes were dissected out and divided into the following distinct regions: oesophagus, cardiac stomach, pyloric stomach, pyloric caeca, liver, gall bladder, spleen, anterior intestine and posterior intestine. The identical regions dissected out from 10 fishes of uniform size (15.3 ± 0.84 cm total length) were pooled, homogenised in chilled double distilled water and centrifuged at 25,000 g for 20 minutes. The supernatent was used for further study. Triplicates were run for all the assays.

3.2.2 Enzyme assay

All the enzymes were assayed as described in the earlier section(2.6).

3.2.3 Relative length of the gut (RLG)

The relative length of the gut (RLG) is expressed as the ratio of the length of the digestive tract to the total length of the fish. The length of the uncoiled intestine was measured and compared with total length of the fish. The RLG was calculated using the following formula:

$$RLG = \frac{\text{Length of the digestive tract (cm)}}{\text{Total length of the fish (cm)}}$$

3.3 Results

3.3.1 Relative length of the gut (RLG)

The RLG recorded for the adult mullets in the present study was 2.4 (± 0.32). The results of the present study is compared with other reports made for other fishes (Table-1)(Plate-2).

3.3.2 Survey of Carbohydrases

The distribution pattern of the carbohydrases in the digestive tract recorded in the present investigation is presented in Tables 2 and 3. The substrates listed in Table-2 have either alpha-glucosidic or alpha-galactosidic linkages. The subtrates with alpha-glucosidic linkages included maltose, dextrin, sucrose, trehalose and the synthetic substrate p-nitrophenyl-alpha-D-glucoside.

Table - 1. Feeding habits and relative length of the guts of fishes.

Species	Feeding habit & Nature of diet	RLG (Relative Length of Gut)	Author(s)
Anguilla japonica	Carnivorous	0.46 (± 0.050)	Hsu and Wu, 1979
Channa maculatus	"	0.58 (± 0.09)	Hsu and Wu, 1979
Clarias juscus	"	0.68 (± 0.11)	Hsu and Wu, 1979
Pyrrhulina filamentosa	Insectivorous	1.0	Jacobshagen, 1911, 1913, 1915
Elopichthys bambusa	Carnivorous	0.63	Verighina, 1963
Gobio gobio	Invertebrates	0.8 - 0.81	Vukovic, 1966
Leptocypris modestus	Invertibrates, plants	0.85 - 1.0	Matthes, 1963
Cyprinus carpio	Omnivorous	2.04 (± 0.35)	Hsu and Wu, 1979
Tilapia mossambica	Omnivorous	6.29 (± 0.19)	Hsu and Wu, 1979
Catla catla	Periphyton, plants, insect larvae	4.68	Kapoor, 1958
Garra dembensis	Algae, invertebrates	4.50	Matthes, 1963

Table - 1. Feeding habits and relative length of the guts of fishes (contd..)

Species	Feeding habit & Nature of diet	RLG (Relative Length of Gut)	Author(s)
Ctenopharyngodon idella	Herbivorous	2.15 (± 0.41)	Hsu and Wu, 1979
Ctenopharyngodon idella	Plants	2.5	Hickling, 1966
Amblypharyngodon mola	Plants	2.8	Khanna, 1961
Labeo calbasu	Plants, weeds, algae diatoms	3.75 - 10.33	Sehgal, 1966
Carassius auratus	Microphageous	5.15 (± 0.95)	Hsu and Wu, 1979
Hypothalmichthys molitrix	Microphageous	5.28 (± 0.30)	Hsu and Wu, 1979
Cirrhina mrighala	Algae, detritus	8.0	Jacobshagen, 1911, 1913, 1915
Labeo horia	Algae, detritus	15.5	Matthes, 1963
Liza parsia	Herbivorous, algae, diatoms, organic matter and detritus	2.41 (± 0.28)	Present investigation

Plate - 2. The uncoiled intestine of L. parsia

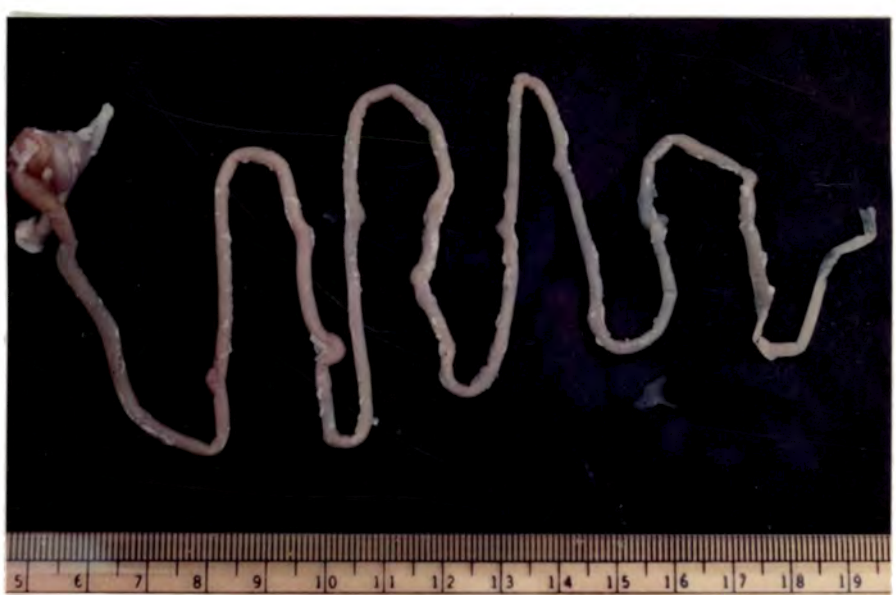

plate - 2

In general, crude enzyme extracts of intestine recorded maximal hydrolysis of maltose, dextrin, sucrose and trehalose, whereas extracts of pyloric caeca could record maximal hydrolysis of p-nitrophenyl-alpha-D-glucoside and significant levels of hydrolysis of maltose, dextrin, sucrose and trehalose. Extracts of gall bladder could hydrolyse, dextrin and sucrose, at significant level. Besides these regions, cardiac stomach also recorded significant level of hydrolysis of dextrin. All other regions hydrolysed the substrates at insignificant levels. The enzyme activity measured for the various substrates varied from 45.64 (± 12.97) to 184.52 (± 34.84) units for maltose, 89.53 (± 11.63) to 368.18 (± 47.86) units for dextrin, 3.83 (± 0.31) to 521.43 (± 83.42) units for sucrose, 15.20 (± 1.37) to 135.52 (± 27.78) units for trehalose and 2.58 (± 0.23) to 119.37 (± 16.71) units for p-nitrophenyl alpha-D-glucoside.

Of the two substrates with alpha-galactosidic linkages, melibiose and raffinose, only raffinose was hydrolysed by the extracts of pyloric caeaca and posterior intestine. However there was only a minimal hydrolysis. The hydrolytic activity towards raffinose varied from 3.25 (± 0.29) to 9.34 (± 1.02) units.

Results presented in Table-3 indicate the enzymatic hydrolysis of substrates with beta-glucosidic and beta-galactosidic linkages and complex polymeric substrates with alpha-glucosidic linkages and complex polymeric substrates with beta-glucosidic linkages.

In general the crude enzyme extracts obtained from pyloric caeca, anterior and posterir intestine recorded comparatively a maximal

Table-2. Hydrolysis of substrates with alpha-glucosidic and alpha-galactosidic bonds by crude extracts from the digestive tract of *L. parsia*

Substrate	Region of the digestive tract	Hydrolytic activity[a]
Maltose	pyloric caeca	97.62 (± 15.18)
	gall bladder	69.21 (± 18.23)
	spleen	45.64 (± 12.97)
	anterior intestine	154.87 (± 32.38)
	posterior intestine	184.52 (± 34.84)
Dextrin	oesophagus	92.33 (± 12.21)
	cardiac stomach	248.67 (± 38.93)
	pyloric stomach	89.53 (± 11.63)
	pyloric caeca	248.59 (± 39.28)
	gall bladder	346.19 (± 58.85)
	anterior intestine	311.43 (± 56.05)
	posterior intestine	368.18 (± 47.86)
Sucrose	pyloric caeca	288.27 (± 54.77)
	gall bladder	155.71 (± 14.01)
	spleen	3.83 (± 0.31)
	anterior intestine	443.02 (± 62.02)
	posterior intestine	521.43 (± 83.42)
Trehalose	cardiac stomach	31.00 (± 2.48)
	pyloric stomach	29.81 (± 3.28)
	pyloric caeca	130.38 (± 15.70)
	spleen	15.20 (± 1.37)
	anterior intestine	135.52 (± 27.78)
	posterior intestine	70.61 (± 12.00)

Table-2. Hydrolysis of substrates with alpha-glucosidic and alpha-galactosidic bonds by crude extracts from the digestive tract of L. parsia (contd.....)

Substrate	Region of the digestive tract	Hydrolytic activity[a]
P-nitrophenyl alpha-D glucoside	oesophagus	4.80 (± 0.49)
	cardiac stomach	7.08 (± 0.85)
	pyloric stomach	2.58 (± 0.23)
	pyloric caeca	119.37 (± 16.71)
	liver	6.75 (± 0.61)
	anterior intestine	30.56 (± 3.97)
	posterior intestine	30.23 (± 4.33)
Raffinose	pyloric caeca	3.25 (± 0.29)
	posterior intestine	9.34 (± 1.02)

[a] specific activity

Table-3. Hydrolysis of substrates with beta-glucosidic and beta-galactosidic bonds and starch and glycogen by crude extrats from the digestive tract of L. parsia.

Substrate	Region of the digestive tract	Hydrolytic activity[a]
Cellobiose	oesophagus	36.92 (± 4.79)
	cardiac stomach	15.53 (± 2.17)
	pyloric stomach	29.87 (± 4.18)
	pyloric caeca	97.62 (± 14.64)
	anterior intestine	129.34 (± 20.69)
	posteiror intestine	138.41 (± 19.37)
Salicin	oesophagus	4.68 (± 0.37)
	cardiac stomach	15.54 (± 1.39)
	pyloric stomach	9.39 (± 0.84)
	pyloric caeca	57.23 (± 6.86)
	spleen	7.16 (± 0.85)
	anterior intestine	34.43 (± 4.48)
	posterior intestine	27.64 (± 3.87)
P-nitrophenyl	oesophagus	3.91 (± 0.46)
beta-D glucoside	cardiac stomach	5.62 (± 0.51)
	pyloric stomach	2.00 (± 0.16)
	pyloric ceaca	90.32 (± 13.55)
	anterior intestine	43.72 (± 5.68)
	posterior intestine	12.68 (± 1.52)

Table-3. Hydrolysis of substrates with beta-glucosidic and beta-galactosidic bonds and starch and glycogen by crude extrats from the digestive tract of L. parsia. (contd.....)

Substrate	Region of the digestive tract	Hydrolytic activity[a]
P-nitrophenyl	oesophagus	5.21 (± 0.42)
beta-D galactoside	cardiac stomach	9.28 (± 0.84)
	pyloric stomach	2.94 (± 0.32)
	pyloric caeca	144.68 (± 18.80)
	liver	12.74 (± 1.15)
	spleen	4.18 (± 0.33)
	anterior intestine	137.98 (± 16.55)
	posterior intestine	64.53 (± 7.74)
Soluble starch	oesophagus	112.03 (± 15.68)
	cardiac stomach	124.29 (± 18.64)
	pyloric stomach	0
	pyloric caeca	227.74 (± 31.88)
	Gall bladder	36.62 (± 3.29)
	spleen	95.92 (± 11.51)
	anterior intestine	330.37 (± 39.64)
	posterior intestine	437.52 (± 61.25)
Glycogen	oesophagus	147.32 (± 20.62)
	cardiac stomach	155.36 (± 23.30)
	pyloric stomach	74.63 (± 6.72)
	pyloric caeca	232.48 (± 30.22)
	Gall bladder	48.52 (± 4.36)
	spleen	76.12 (± 6.08)
	anterior intestine	337.73 (± 54.03)
	posterior intestine	348.37 (± 62.70)

[a] specific activity

hydrolysis of cellobiose, salicin, p-nitrophenyl beta-D-glucoside and p-nitrophenyl beta-D-galactoside. The enzyme activity varied from 36.62 (± 3.29) to 437.52 (± 61.25) units. Similarly, excluding liver, all other regions demonstrated hydrolysis of glycogen. However, maximal hydrolysis was recorded by extracts of posterior intestine, anterior intestine and pyloric caeca. The hydrolytic activity varied from 48.52 (± 4.36) to 348.37 (± 62.70) units.

Complex polymeric substrates with beta-glucosidic linkages viz. microcrystalline cellulose and sodium carboxymethyl cellulose were not hydrolysed by the crude extracts.

3.3.3 Pepsin

The pepsin like activity tested with acid denatured haemoglobin as substrate, at pH 2.0, was detected in the extracts of cardiac stomach, pyloric stomach, pyloric caeca and oescophagus (Table-4). Except in cardiac stomach, which recorded an enzyme activity of 2.34 (± 0.28) units , all other regions exhibited only a trace activity.

3.3.4 Protease

The distribution pattern of the protease activity along the digestive tract is presented in Table-5. High protease activity was observed in the pyloric caeca folled by anterior intestine and posterior intestine. Comparatively significant levels of activity was also detected in the crude extracts obtained from the oesophagus. No activity could be detected in the extracts of the cardiac and pyloric stomach regions. The enzyme activity varied

Table-4. Hydrolysis of acid dentured haemoglobin by crude extracts from different regions of the digestive tract of L. parsia.

Region of the digestive tract	Hydrolytic activity (u mole tyrosine/mg/hr)
Cardiac stomach	2.34 (± 0.28)
Pyloric stomach	0.98 (± 0.08)
Others	0.21 (± 0.01)

The term 'others' include oesophagus, pyloric caeca, anterior and posterior intestines liver and gall bladder.

Table-5. Protease activity of crude extracts from various regions of the digestive tract of L. parsia.

Region of the digestive tract	Hydrolytic activity (u mole tyrosine/mg/hr)
oesophagus	1.01 (± 0.09)
cardiac stomach	0
pyloric stomach	0
pyloric caeca	12.89 (± 1.19)
anterior intestine	9.84 (± 1.08)
posterior intestine	8.95 (± 0.98)
liver	0.42 (± 0.03)

from 0.42 (± 0.03) to 12.89 (± 1.19) units.

3.3.5 Trypsin

The tryptic activity was estimated by using the synthetic substrate BAPNA and the distribution pattern of this enzyme is presented in Table-6. Highest activity was observed in the extracts of the pyloric caeca followed by anterior intestine and posterior intestine. Rest of the rgions did not show any activity. The enzyme activity ranged from 308.93 (± 37.07) to 716.11 (± 85.92) units.

3.3.6 Chymotrypsin

The chymotryptic activity was assayed with the synthetic substrate BTNA. Results presented in Table-7 indicate the presence of this enzyme only in three regions. Maximal hydrolysis of the substrate was observed in pyloric caeca, posterior intestine and anterior intestine. The enzyme activity for the regions ranged between 98.68 (± 11.84) and 138.22 (± 19.35) units.

3.3.7 Leucine aminopeptidase

Results presented in Table-8 indicate the distribution pattern of leucine aminopeptidase assayed by employing the synthetic substrate leucine p-nitroanilide. Most of leucine aminopeptidase activity was confined to the anterior and posterior intestines followed by the pyloric caeca. Oesophagus, cardiac stomach, pyloric stomach and liver evinced weak activity. The enzyme activity measured for the extracts varied widely from 12.81 (± 1.79) to 417.76 (± 66.84) units.

Table-6. Tryptic activity of crude extracts from selected regions of the digestive tract of L. parsia.

Region of the digestive tract	Specific activity
pylorioc caeca	716.11 (± 85.92)
anterior intestine	467.76 (± 65.48)
posterior intestine	308.93 (± 37.07)

Table-7. Chymotryptic activity of crude extracts from selected regions of the digestive tract of L. parsia.

Region of the digestive tract	Specific activity
pylorioc caeca	138.22 (± 19.35)
anterior intestine	98.68 (± 11.84)
posterior intestine	127.94 (± 19.19)

Table-8. Leucine aminopeptidase activity of crude extracts from various regions of the digestive tract of L. parsia.

Region of the digestive tract	Specific activity
oesophagus	22.34 (± 2.01)
cardiac stomach	36.29 (± 3.99)
pyloric stomach	12.81 (± 1.79)
pylorioc caeca	212.73 (± 31.91)
anterior intestine	380.42 (± 49.45)
posterior intestine	417.76 (± 66.84)
liver	40.67 (± 4.88)

3.3.8 Carboxypeptidase A and Carboxypeptidase B

Carboxypeptidase A and B activities were not recorded in many regions of the digestive tract except in pyloric caeca, anterior intestine and posterior intestine which recorded trace acctivity. Hence the three regions were pooled and tested for carboxypeptidase A and B activities. The enzyme activity observed for carboxypeptidase A and B were 0.025 (± 0.003) and 0.01 (± 0.001) units respectively (Table-9).

3.3.9 Acid phosphatase

The distribution profile of acid phosphatase activity along the digestive tract of mullet is presented in Table-10. The acid phosphatase activity was detected in all the regions tested. The highest activity was detected in the extracts obtained from cardiac stomach, pyloric stomach and pyloric caeca. Considerable amount of activity was also detected in the extracts of anterior intestine, posterior intestine, oesophagus and liver. In genral, the enzyme acativity varied from 2.82 (± 0.25) to 12.42 (± 1.74) units.

3.3.10 Alkaline phosphatase

The alkaline phosphatase activity was detected throughout the digestive tract of the mullet (Table-11). The highest activity was recorded in the extracts of anterior intestine, posterior intestine and the pyloric caeca. The oesophagus, cardiac stomach, pyloric stomach and liver also exhibited appreciable amount of alkaline phosphatase acativity. The activity for various regions varied from 33.54 (± 4.02) to 134.89 (± 18.88) units.

Table-9. Carboxypeptidase A and carboxypeptidase B activities in the digestive tract of L. parsia.

Region of the digestive tract[a]	Activity	
	Carboxypeptidase A	Carboxypeptidase B
pyloric caeca, anterior and posterior intestine	0.025 (± 0.003)	0.01 (± 0.001)

a since low level of activity all the three regions were pooled; No activity could be detected in other regions.

Table-10. Acid phosphatase activity of crude extracts from various regions of the digestive tract of L. parsia.

Region of the digestive tract	Specific activity
oesophagus	3.18 (± 0.38)
cardiac stomach	12.42 (± 1.74)
pyloric stomach	8.74 (± 1.04)
pylorioc caeca	10.38 (± 0.73)
anterior intestine	4.49 (± 0.49)
posterior intestine	2.82 (± 0.25)
liver	5.26 (± 0.63)

Table-11. Alkaline phosphatase activity of crude extracts from various regions of the digestive tract of L. parsia.

Region of the digestive tract	Specific activity
oesophagus	67.11 (± 8.05)
cardiac stomach	38.92 (± 5.45)
pyloric stomach	33,54 (± 4.02)
pylorioc caeca	134.89 (± 18.88)
anterior intestine	110.46 (± 13.25)
posterior intestine	128.29 (± 16.88)
liver	40.67 (± 6.10)

Table-12. Esterase activity of crude extracts from various regions of the digestive tract of L. parsia.

Region of the digestive tract	Specific activity
oesophagus	3.93 (± 0.35)
cardiac stomach	4.24 (± 0.50)
pyloric stomach	2.48 (± 0.34)
pylorioc caeca	15.76 (± 2.36)
anterior intestine	12.38 (± 1.98)
posterior intestine	13.46 (± 2.28)
liver	21.83 (± 3.27)

3.3.11 Esterase

Esterase activity was distributed almost throughout the digestive tract (Table-12). The highest activity was observed in the extracts obtained from pyloric caeca, anterior intestine, posterior intestine and liver. Compratively the extracts of oesophagus, cardiac stomach and pyloric stomach recorded lesser activity. The activity for various regions ranged between 2.48 (± 0.34) and 21.84 (± 3.27) units.

3.4 Discussion

In the present investigation the relative length of the gut (RLG) and the major digestive enzymes carbohydrases such as amylase, maltase, sucrase, dextrinase, raffinase and glucosidase; pepsin; alkaline proteases-trypsin, chymotrypsin, leucine aminopeptidase and carboxypeptidases A and B; alkaline and acid phosphatases and esterase present in the alimentary canal was studied using various substrates.

3.4.1 Relative length of the gut

From the results, it was noted that the RLG of the mullet ranged between 1.83 and 3.08 with a mean of 2.4 (± 0.32). When this RLG of *L. parsia* was compared with other reports for other fishes (Table-1) it is concluded that *L. parsia* is a herbivore.

Al-Hussaini (1947), Barrington (1957), Harder (1975) and Kapoor *et al*. (1975) pointed out that the relative length of the gut was clearly connected with the different feeding habits of fishes,

particularly related to the quantity of indigestible material ingested. According to them, the longest intestine is present in microphageous (algae-eating) and some herbivorous fishes and the shortest one in carnivores while the varied intestinal length in omnivorous fishes depends upon the proportion of the indigestible to digestible materials in the diet.

Present observations are in confirmity with the earlier investigations on food and feeding habits of mullets by virtue of RLG indicating herbivorous feeding. The bulk of the food of L. parsia consisted of decayed organic matter, green and blue green algae and some zooplanktons such as copepods and rotifers (Mookerjee et al., 1946; Sarojini, 1954). Pillay (1953) reported that the intestine in the mullet Mugil tade was several times longer than its body length. The mullet L. parsia by virtue of possessing an elongated intestine, which will lengthen the time to which food is exposed to digestive enzymes, increase the areas of absorption and effect complete and efficient hydrolysis and assimilation of ingested diet.

3.4.2 Carbohydrases

Carbohydrases assayed in the crude enzyme extract of the digestive tract included maltase, dextrinase, sucrase, trehalase, alpha-glucosidase, raffinase, melibiase, cellobiase, salicinase, beta-glucosidase, beta-galactosidase, amylase and cellulase. Results indicate that the crude extract could catalyse hydrolysis of the substrates tested. Considerable amount of this hydrolytic activity was confined to the pyloric caeca and both the anterior and

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Carbohydrases assayed in the crude enzyme extract of the digestive tract included maltase, dextrinase, sucrase, trehalase, alpha-glucosidase, raffinase, melibiase, cellobiase, salicinase, beta-glucosidase, beta-galactosidase, amylase and cellulase. Results indicate that the crude extract could catalyse hydrolysis of the substrates tested. Considerable amount of this hydrolytic activity was confined to the pyloric caeca and both the anterior and posterior intestines.

The rapid hydrolysis of the substrates with alpha-glucosidic linkages such as maltose, dextrin, sucrose, and trehalose suggests

that in addition to the alpha-glucosidase, the specific enzyme maltase, dextrinase, sucrase and trehalase are also present in the intestine and pyloric caeca of L. parsia. The maltase activity was maximum in the posterior intestine, followed by the anterior intestine, pyloric caeca, gall bladder and spleen and was absent in the rest of the regions. Vonk (1941) reported maximal maltase activity in the pancrease of carp and lota. However, Kawai and Ikeda (1971) reported maximal maltase activity in the posterior intestine of carp and in the pyloric caeca of ayu (Plecoglossus altivelis) and red se bream (Pugrus major). Milkfish also exhibited higher maltase activity in the intestine when compared to the pyloric caeca (Chiu and Benitez, 1981). Clark et al. (1984) demonstrated substantial activity of maltase in the whole gut extracts of dover sole, Solea solea. It is interesting to note that the pyloric caeca and intestinal extracts from Atlantic halibut, Hippoglossus hippoglossus, exhibited almost equal amount of maltase activity (Glass et al., 1987). Since starch is the main storage product of plants, and maltose being the breakdown producat of starch by hydrolytic action of amylase, the presence of maltase in substantial amounts would insure the complete utilisation of starch in L. parsia which is a a herbivore (Sarojini, 1954).

The rapid hydrolysis of dextrin by the extracts of cardiac stomach, pyloric caeca, gall bladder, anterior and posterior intestines suggest the presence of dextrinase (or dextrin-alpha 1-6)

glucosidase). Dextrinase activity has been reported in milk fish also (Chiu and Benitez, 1981). The exact site of origin of this activity is not clear. The activity associated with gall bladder may be due to contamination from the pancreatic tissue which is diffused in L. parsia as in most teleosts (Kapoor et al., 1975). The dextrinase activity found in stomach and oesophagus of L. parsia could be the result of regurgitated duodenal contents.

Sucrase activity was recorded in maximal levels in posterior intestine, anterior intestine, pyloric caeca and in gall bladder. Similar observation was made in the hepatophancreas and tissue extracts of digestive tract in gold gish Carassius auratus (Sarbahi, 1951), stomach extracts of Colisa, Gudusia and Barbus (Agrawal and Verma, 1966), Cyprinus carpio (Kawai and Ikeda, 1971) and in Chanos chanos (Chiu and Benitez, 1981). Weak sucrase activity was detected in Solea solea (Clark et al., 1984) and in Hippoglossus hippoglossus (Glass et al., 1987). Fange and Grove (1979) suggested that surcase enzyme is produced by the intestinal mucosa of fishes. Interestingly, the high activity of sucrase observed in the intestine of L. parsia in the present study adds evidence to the fact that intestine produces sucrase. The sucrase activity detected in pyloric caeca was about half of that detecated in the posterior intestine. The activity observed in the pyloric caeca might be due to the sucrase secreted by the adjoining diffused pancreatic cells or it might be due to the contamination

from the intestinal contents. Irrespective of the source of this enzyme, its presence in substantial quantities would ensure complete breakdown of the carbohydrate component of the ingested diet. Moreover, the presence of this enzyme can also be attributed to the herbivorous nature of the L. parsia a this enzyme was not detected in the carnivorous fishes investigated (Kawai and Ikeda, 1971).

The trehalase activity detected in the pyloric caeca, anterior intestine and posterior intestine of L. parsia was high. A similar pattern of distribution of this enzyme was also reported in milk fish (Chiu and Benitez, 1981).

In the present study specific activity of alpha-glucosidase acting on p-nitrophenyl-alpha-D glucoside was very high in the pyloric caeca followed by intestine. All other regions recorded poor activity of this enzyme. Higher activities of this enzyme for the same substrate was reported in intestine of milkfish (Chiu and Benitez, 1981). In Atlantic halibut (Hippoglossus hippoglossus) both pyloric caeeca and intestine recorded equal amounts of alpha-glucosidase activity (Glass et al., 1987).

When tested for melibiase and raffinase activity on substrates with alpha-galactosidic linkages, it was observbed that intestine and pyloric caeca extracts of L. parsia contained raffinase only in tracer amounts. Raffinase activity was not detected in any of the

fish groups studied (Kawai and Ikeda, 1971; Chiu and Benitez, 1981; Clark et al., 1984; Glass et al., 1987). Although melibiase activity was not detected in many fishes and in L. parsia during the present study slight activity has been reported in the intestine extracts of Salmo gairdneri (Kitamikado and Tachino, 1960a) and in carp (Kawai and Ikeda, 1971). The trace hydrolysis of raffinose and lack of hydrolysis of melibiose in L. parsia can be attributed to the lack of an alpha-galactosidase as in other groups of fishes discussed above. However, the specific enzymes raffinase, melibiase, and alpha-galactosidase were all reported in different groups of invertebrates such as Marinogammarus sp. (Van Weel, 1970); Carcinus maenas and Carngon crangon (Kristensen, 1972); Pecten maximus (Stark and Walker, 1983) and Macrobrachium idella (Palanisamy and Pillai, 1989). The absence of these enzymes in fishes may be due to evolutionary change leading to poor utilization of carbohydrates by fish, whereas the invertebrate groups have the potential to utilise a variety of carbohydrate foods (Stark and Walker, 1983).

Hydrolytic acticity of the extract on substrates with beta-glucosidic linkages was not significant for any region of the digestive tract of L. parsia when compared to those with alpha-glucosidic linkagees. Similar pattern of activity was reported earlier in milk fish (Chiu and Benitez, 1981) dover sole (Clark et al., 1984) and Atlantic halibut (Glass et al., 1987). The

hydrolysis of cellobiose, salicin and p-nitrophenyl-beta-D-glucoside evidences the presence of a beta-glucosidase of broad substrate specificity in L. parsia. In the present study, extracts of intestine and pyloric caeca could hydrolyse rapidly the cellobiose than other regions of the digestive tract. This observation indicate the presence of cellobiase in L. parsia. Cellobiase has also been reported earlier in carp (Kawai and Ikeda, 1971), dover sole (Clark et al., 1984) and in Atlantic halibut (Glass et al., 1987). In the present study salicin hydrolysis could be recorded by the pyloric caeca and intestinal extraccts of L. parsia. Similar reports of salicin hydrolysis were made in the intestinal extracts of Calotomus sp. and Salarius sp. (Ishida, 1936) and in Chanos chanos (Chiu and Benitez, 1981).

Hydrolysis of p-nitrophenyl beta-D-glucoside was observed in significant levels in pyloric caeca followed by anterior intestine in L. parsia in the present investigation. Chanos chanos (Chiu and Benitez, 1981), Solea solea (Clark et al., 1984) and Hippoglossus hippoglossus (Glass et al., 1987) have also showed marked hydrolysis of p-nitrophenyl beta-D-glucoside.

As reported earlier the three fishes, milk fish (Chiu and Benitez, 1981), dover sole (Clark et al., 1984) and Atlantic halibut (Glass et al., 1987) could not hydrolyse one or the other of the three substrates cellobiose, salicin and p-nitrophenyl beta-D-glucoside.

However the present study indicate the efficiency of L. parsia to hydrolyse all the three substrates. This observation suggest that L. parsia has the ability to hydrolyse wide range of carbohydrates.

The extracts of the digestive tract of L. parsia, in the present study, did not hydrolyse lactose. However, hydrolysis of lactose was reported in carp (Kawai and Ikeda, 1971). The hydrolysis of p-nitrophenyl-beta-D-galactoside, but not lactose, indicates the presence of a specific beta-galactosidase in L. parsia. Similar observation on the hydrolysis of p-nitrophenyl beta-D galactoside and absence of lactose hydrolysis was also reported in milk fish earlier (Chiu and Benitez, 1981). The presence of beta-glucosidase and beta-galactosidase, specific for the hydrolysis of nitrophenyl derivatives of glucose and galactose, has been demonstrated in the intestinal and liver extracts of some commonly cultured fishes in Japan (Nagayama and Saito, 1968). Present results with respect to these substrates are in agreement with the above mentioned report.

According to Sarojini (1954) the food of L. parsia generally include decayed organic matter, algae (chlorophyceae and myxophyceae) and diatoms from the benthic zones of their habitats. In the present study, cellulase which could hydrolyse the cell walls of algae and diatoms could not be detected in any region of the digestive tract of L. parsia. Though algae and diatoms form exclusive or partial diet of majority of fishes most of the earlier studies reported that fishes such as Tilapia mossambica (Fish,

1960) and Chanos chanos (Chiu and Benitez, 1981) lack indigenous cellulase which might lyse the cell walls of the algae and diatoms. Lobel (1981) in a review of fish herbivory stated: "The paradox in fish herbivory ..... is that fishes eat plant foods but are not known to produce cellulase or any other enzyme functioning to digest plant cell walls ..... Intestinal microoranisms serving the same function also have not yet been found".

Odum (1968a) suggested that bacteria and protozoans adsorbed on detritus particles of the diet of mullets may assist in the breakdown of plant materials. According to Hughes et al. (1971), the uniform size of the sand grains ingested by mullet may also increase the milling efficiency in the gizzard. Further changes in permeability due to the inducation of cracks in the cell envelop

during the grinding process are probably sufficient to lyse the cells. Role of gastric acid in the digestion of algal matter in Tilapia sp. was suggestted by Moriarty (1973) and Bowen (1976). In L. parsia, either or all of the mechanism mentioned above may be involved in the process of digestion. Since the food of L. parsia consists of considerbale amount of detritus, the microflora associated with it may be involved in the digestion. Mullets have an anterior thin walled stomach (Hickling, 1970). The gastric glands reported in this part of the stomach of M. capito and M. tade (Pillay, 1953) might secrete acid as in Tilapia. The

posterior part of the stomach is modified into gizzard in mullets which may play a grinding role. Thus even when the enzymes responsible for lysis of cell wall is not detected in L. parsia, it has some mechanism, as mentioned above, to utilize the ingested macroplant detritus, benthic and epiphytic microalgae and diatoms effectively.

Amylase was found to be a major digestive enzyme in L. parsia and its activity could be detected throughout the length of the alimentary tract. The amylase is likely of endoamylase or alpha type since it catalyses the hydrolysis of glycogen more easily than starch (Fischer and Stein, 1960). Maximal amylase activity was recorded in the posterior intestine followed by anterior intestine, pyloric caeca, cardiac stomach and oesophagus. High levels of amylase activity were detected in the pyloric caeca of rainbow trout (Kitamikado and Tachino, 1960a), in the distal part of the intestine than in proximal and central regions of intestine of carp; in the pyloric caeca alone of marine ayu and only in the hepatopancreas of redsea bream (Kawai and Ikeda, 1971), in the mid gut of flat fish, Limanda yokohamae (Yasunaga, 1972), in the intestine of Clarias batrachus (Mukhopadhyay, 1977) and in the pre gut regions followed by hindgut, midgut and stomach of dover sole (Clark et al., 1984).

Fange and Grove (1979) and Fraisse et al. (1981) have shown that starch digestion and glucose absorption occur mainlly in the

anterior part of the intestine of fishes which possess a stomach and that there is a decreasing gradient in amylase activity from the anterior region towards the posterior part of the intestine. This observation is known as the "descending proximo-distal gradient" (Kuzmina, 1985; Uys and Hecht, 1987).

The detection of amylase in pyloric caeca, anterior and posterior intestines of L. parsia is due to the fact that the pancreas is diffused and consists of ramified tubules or acini scattered in connective tissue of the intestinal surface, the mesenteries and between the intestinal caeca. In the mesenteries, the pancreas forms sheaths around blood vessels. Several small pancreatic ducts open into the intestine or intestinal caeca (Fange and Grove, 1979). The amylase activity in the intestine might be also due to that secreted from the intestinal mucosa (Fange and Grove, 1979). This might be the reason for the highest activity of amylasee in the intestine of L. parsia.

The high amylolytic activities observed in the digestive tract of L. parsia suggest that starch has an important role in the natural diet of the species. The equally high hydrolytic activity towards glycogen bears testimony for the ability of L. parsia to utilise the polysacchardies of plant and animal origin. The presence of maltase and dextrinase in L. parsia insure the complete and efficient hydrolytic breakdown of starch to glucose. Thus special

attention should be given to starch as a dietary component when formulating feeds for this species.

The observations made on the hydrolytic activity of extracts of the alimentary canal of L. parsia suggest that they can hydrolyse a broad range of low molecular weight substrates. Some of the hydrolytic activities recorded for few substrates appear to have no relevant role in digestion. For example, sucrase activity was observed in most parts of the digestive system. But it is unlikely that sucrose will ever be present in the natural diet of this mullet. It is likely that some of the enzyme activities observed are due to general glycosidases with a fairly broad specificity and an ability to hydrolyse substrates which are not usually met with in the natural state.

3.4.3 Proteases

Both biotic and abiotic factors influence the protein requirements of fish (Austreng and Refstie, 1979; Cowey and Luquet, 1983). Protein is useful to the animal only if it is digested and the degradation products (peptides and amino acids) are absorbed. This statement presupposes that intact protein absorption is nutritionally unimportant to the fish (Ash, 1985). Thus, the high dietary protein levels required to achieve optimal growth in most fish species (Cowey, 1979) might reflect either a low percentage efficiency of hydrolysis coupled with inefficient absorption of

degradation products or, either one of these individual components acting alone. Information regarding these physiological processes and factors which might affect their overall efficiency are therefore of considerable interest to all concerned with the development and management of aquaculture. In the present study, a survey of proteases in the digestive tract of L. parsia indicated the presence of the endopeptidases and exopeptidases which are notmally associated with protein digestion in mammals (Madge, 1975) and in many species of fish (Kapoor et al., 1975; Fange and Gove, 1979).

.4.3.1 Acid Protease

Pepsin undoubtedly is the major acid protease of all vertebrates, including fish. Peptic activity has been demonstrated in the stomachs of many teleosts of culture and or commercial importance (Barrington, 1957; Kapoor et al., 1975; Fange and Grove 1979).

Hsu and Wu (1979 observed pepsin in the stomachs of cultivable fishes such as Anguilla japonica, Channa maculatus, Clarias fuscus, Cyprinus carpio, Tilapia mossambica, Ctenopharyngodon idella, Carassius auratus and Hypothalmichthys molitrix and related it with the feeding habits of these fishes. They concluded that carnivorous fishes have high level of pepsin in their stomach, while omnivorous fishes have low level of pepsin. Jonas et al.(1983) compared the proteolytic digestive enzymes active in acid pH range of carnivore

Silurus glanis, herbivore Hypothalmichthys molitrix and omnivore Cyprinus carpio fishes. All these authors, who have compared peptic activity among fishes of different feeding habits, detected high pepsin activity in carnivores and with trace or no activity in herbivorous fishes. However, the crude extracts from the cardiac and pyloric stomach region of L. parsia, which is a herbivore, was found to hydrolyse the acid denatured haemoglobin at pH 1.80 to 2.00. Pepsin has been detected in other species of mullets also. Albertini-Berhaut and Alliot (1978) detected proteolytic activity in the stomach extracts of Mugil auratus and M. capito active at pH 2.0, which was considered to be pepsin.

In L. parsia, the detection of pepsin suggest that the digestion is initiated in the stomach itself. The combined action of acid in the stomach, pepsin and the grinding action of gizzard should be make the ingested diet in a completely accessible form to the subsequent pancreatic and intestinal ensymes which play a major role in hydrolysing the components of diet.

3.4.3.2 Alkaline protease

Proteolytic activity in the alkaline pH range was detected with casein as substrate from oesophagus, pyloric caeca, anterior and posterior intestines and liver. However, the proteases were most active only in the pyloric caeca, anterior and posterior intestines of L. parsia. Similar distribution pattern of protease activity was

also reported in other teleost fishes such as cod, haddock and mackeres (Johnston, 1941); Cyprinus sp., Rutilus sp. and Gobio sp. (Al-Hussaini 1949); Sebastes sp. (Stern and Lockhard, 1953); Perch and Tilapia (Fish, 1960); rainbow trout (Kitamikado and Tachino, 1960b); Yellow tail, eel, rainbow trout, carp and ayu (Morishita et al., 1964); ayu (Tanaka et al., 1972); Carp (Onishi et al., 1973a,b); cod (Overnell, 1973); Yellow tail (Kamoi et al., 1975); Mugil auratus and Mugil capito (Albertini-Berhaut and Alliot, 1978); milkfish (Benites and Tiro, 1982) and Atlantic halibut (Glass et al., 1987). In fishes the pyloric caeca and pancreas, either of diffuse or compact type, generally have a high concentration of digestive enzymes (Togaswa et al., 1959; prahl and Neurath, 1966; Ooshiro, 1968, 1971a, b; Lacko and Neurath, 1970; Imura, 1971; Jany, 1976; Simpson and Haard 1984 a,b,c and Glass et al., 1987). However, in the present study pancreas of L. parsia was not able to be dissected out and hence the ensymes of pancreas has not been studied. The pyloric caeca is usually rich in digestive ensymes. It is suggested that these enzymes may be synthesized as zymogens from it. (Vonk, 1927; Croston, 1960; Overnell, 1973). The highest amount of proteolytic activity detected in the pyloric caeca in the present study on L. parsia may be either due to a similar mechanism of synthesis in zymogen form from the pancreas-pyloric caeca complex or due to contamination of pyloric caeca complex or due to contamination of pyloric caeca with diffused pancreatic tissue. Since the protease were detected

in active form in L. parsia, their synthesis in pyloric caeca as zymogens is unlikely. In L. parsia, the pancreatic tissue was found to be adhered to the anterior intestinal wall and between pyloric caeca (Ghosh et al., 1987). This may be the reason for the detection of high amount of enzymes in the pyloric caeca in L. parsia. Hence the proteases of the pyloric caeca can be considered to be panreatic in origin (Croston, 1965; Bergot, 1979) and there is no doubt that these appendages, together with the associated pancreatic mesentry, provide a rich source of such enzymes (Bishop and Odense 1966); Overnell, 1973). The pyloric caeca in L. parsia has been considered as an extension of the intenstine which leads to an increase in the area of enzyme activity and absorption, besides prolonging the gut passage time of the ingested diet. This facilitates the exposure of the diet for a longer duration to the digestive enzymes to effect complete hydrolysis.

Microflora present in fish alimentary canal are additional source of digestive enzymes. Hamid et al., (1979) reported bacterial enzymes in the gut of grey mullet Mugil cephalus. Similar condition may exist in L. parsia also. However, since it was not investigated, further comments could not be made on this aspect.

The detection of protease activity and also carbohydrase activities in the oesophagus of L. parsia in the present study suggests a more

active role of this organ in the digestive process in the mullet. Kawai and Ikeda (1971) detected fairly high levels of protease activity and amylase and maltase activities in the oesophagus of carp and suggested that the stomachless fish such as carp, oesophagus may have important role in digestion. Protease activity was also detected in the oesophagus of Anguilla japonica and Ctenopharyngodon idella (Hsu and Wu, 1979). The oesophagus of milkfish exhibited proteolytic activity and hence an active and hence an active role for this organ in the digestive process in this fish was suggested (Benitez and Tiro, 1982).

In Plecoglossus altivelis the oesophagus has secretary glands composed of cuboidal cells with basal nuclei which probably produce enzymes (Iwai, 1962). Lines and Geyer (1968) electron microscopically examined the oesophageal mucosa in Esox lucinus and found cells resembling exocrine pancreatic cells and suggested that probably their secretion contains digestive enzymes. At this juncture, it is interesting to note that in mullets, Mugil capito (Ghazzawi, 1935) and Rhinomugil corsula (Thomson, 1866) the oesophagus was reported to possess rich gastric glands. The oesophagus in L. parsia resemble that of other mullets and play an active role in the digestive process.

The crude extract from the mullet liver had very little protease activity. No amylase activity was also detected in the liver of

L. parsia (Table-3). Whenever the liver in fishes was contaminated with the diffused pancreatic tissue, it was reported to exhibit digestive enzyme activity (Sarbahai, 1951; Bondi and Spandorf, 1954; Agrawal and Tyagi, 1963; Gohar and Latiff, 1963; Morishita et al., 1964; Lane; 1973; Jany, 1976. The liver in mullets is reported to be relatively free from attachment of the diffused pancreatic tissues (Seshadri, 1960; Ghosh et al., 1987).

Hence, no reason could be attributed for the trace of proteolytic activity detected in the liver since no detailed study was conducted in this regard.

In contrast, in Colisa fasciata, proteases and carbohydrases were reported to be secreted mainly by the liver (Agrawal and Singh, 1963).

The crude extracts from the anterior and posterior intestines of the mullet demonstrated considerable amount of proteolytic activity towards casein. However there was no difference among them. A similar condition was reported in other groups of fishes also. In gold fish protease activity was present in anterior and posterior intestines equally (Sarbahai, 1951). A comparative analysis of proteolytic activity in some cultured fishes (Morishita et al., 1964) revealed that proteolytic activity was invariably present throughout the intestine in yellowtail, eel, rainbow trout, carp and ayu. The white amur Ctenopharyngodon idella exhibited high

proteolytic activity in the mid intestine (Hickling, 1966). In perch, the crude extract from posterior part of the intestine showed greater proteolytic activity than pyloric caeca and anterior intestine in contrast to Tilapia sp. which recorded equal activity of protease in all these said regions (Fish 1960). Al-Hussaini (1949) reported proteases in the intestine of stomachless fishes Cyprinus sp., Rutilus sp. However, Jany (1976) could not record protease activity in the intestines of the stomachless fish Carassius auratus gibelio. The hind portion of the gut was found to hold high protease activity than the foregut and midgut in Salmo salar and Salmo gairdneri (Torrissen, 1984) and in Solea solea (Clark et al., 1985). In contrast, Uys and Hecht (1987) found that the pre-gut extracts of sharptooth catfish Clarias gariepinus displayed considerable amount of proteolytic activity when compared to hind gut. The activity in the hind gut was only 18% of that in the foregut. They described this phenomenon as descending proximo-distal gradient.

Although the literature abounds with reports that specific proteolytic activity in the digestive tract of the fish is proportional to the protein content of the diet, and thus it is higher in carnivorous than in herbivorous species (Fish, 1960; Nagase, 1964, 1964; Kawai and Ikeda, 1972; Mukhopadhyay, 1977; Hofer, 1979; Hofer and Schiemer, 1981; Hofer, 1982) it was shown that, if, besides specific activity, the volume of the gut fluid

and the number of gut fillings per day and RLG are also taken into account, food turns out to be exposed to higher proteolytic activity in the digestive tracts of herbivorous than in those of carnivorous fish (Hofer and Schiemer, 1981; Hofer, 1982). Thus the high protease activity recorded in L. parsia may be attributed to the efficient digestion of the complex diet of this fish.

3.4.3.3 Trypsin and chymotrypsin

Specific enzyme assay within an organ system relies on the characteristic ability of an enzyme to catalyse a particular kind of reaction (Dixon and Webb, 1964). Hence the proteolytic activity was further measured with specific synthesis substrates.

Trypsin and chymotrypsin are the dominant alkaline proteases in the post gastric alimentary canal of fish and other vertebrates (Barnard 1973, Fange and Grove, 1979). Trypsin is formed by the removal of a hexapeptide from the trypsinogen molecule as a result of the hydrolysis of a lysine-isoleucine bond. Trypsin is an endopeptidase with optimal activity at pH 7.00. It cleares peptide linkages whose carbonyl groups come from arginine or lysine (Lehninger, 1971). Chymotrypsin is formed by the action of trypsin or chymotrypsinogen. It is also an endopeptidase, that attacks peptide bonds with carbonyl from aromatic side chains (tyrosine, tryptophan and phenylalanine).

Both trypsin and chymotrypsin are reported in all the species of fish studied so far (Kapoor et al., 1975). Reports in the literature do not indicate the exact source of these enzymes, whether pancrease alone or parts of intestine are also involved. Owing to the diffused nature of the pancreas in many teleosts (Kapoor et al., 1975; Fange and Grove, 1979). This is the case with L. parsia also. The pancreatic tissue is partly situated along the portal veins, partly suspended in the mesenteries, often hidden in fat tissue and situated in between pyloric caeca. This inaccessible anatomical situation hampers the determination of the source of these enzymes. Because of this the tryptic and chymotryptic activity has been reported to be present in different regions of the gut in different groups of teleosts.

Highest activity of trypsin in L. parsia was detected in pyloric caeca while anterior and posterior intestine showed significant tryptic activity. Chymotrypsin recorded highest activity in the pyloric caeca and posterior intestion while the anterior intestine showed less activity (Table - 7). A similar distribution pattern for both the enzymes was observed in milkfish (Benitez and Tiro, 1982) and Atlantic halibut (Glass et al., 1987). Whereas mullets Mugil auratus and Mugil capito recorded highest activity of these enzymes in their intestines followed by pyloric caeca (Albertini-Berhaut and Alliot, 1978). As in L. parsia, the pyloric caeca invariably exhibited consistent activities of these enzymes among

other groups of fishes also (Sundaram and Sarma, 1960a,b; Croston, 1960, 1965; Creach, 1963; Overnell, 1973; Alliot et al., 1974; Kalac, 1975; Simpson and Haard, 1984a,b,c and Glass et al., 1987).

Jany (1976) investigated endopeptidases of the stomachless teleost Carassius auratus gibelio and concluded that both trypsin and chymotrypsin were synthesized as zymogens in the hepatopancreas and then secreted into the intestine. Whenever pancreas was able to be dissected out, it was shown to be the site of secretion of these enzymes (Lane, 1973; Cohen et al., 1981a; Khablyuk and Proskuryakov, 1983a; Yoshinaka et al., 1981a, 1984e). Hence it is concluded that the enzymes present in the gut of L. parsia might be secreted by the diffused pancreatic tissue and discharged into the lumen of pyloric caeca or intestine.

The activity of trypsin was comparatively higher than that of chymotrypsin in L. parsia. The preponderance of trypsin relative to chymotrypsin was also observed in other groups of fishes like Clarias batrachus (Mukhopadhyay et al, 1978); carp (Ragyanszki, 1980); milkfish (Benitez and Tiro, 1982) and white sturgeon (Buddington and Doroshov 1986a,b). In contrast, higher chymotrypsin values reltive to trypsin was also observed in Myxine glutinosa (Nilsson and Fange, 1970) and cod, Gadus morhua 9(Overnell, 1973).

Literature available on the feeding habits and activities of trypsin and chymotrypsin in fishes do not suggest any relationship

between the two factors because contrasting observations were made by different authors for different fishes (Hsu and Wu, 1979; Jonas et al., 1983). It is therefore difficult to correlate trypsin chymotrypsin activities to the feeding habits of L. parsia.

The existence of interspecies variations, in trypsin chymotrypsin ratio has been reported for various vertebrates (Zendzian and Barnard, 1967). In Clarias gariepinus both these enzymes were highly active (Uys and Hecht, 1987). Though the level of trypsin was higher than that of chymotrypsin in L. parsia comparison with other studies could not be made as the methodologyand the substrate used by different authors are varied. Further, these enzymes are reported to be influenced by diet composition (Kawai and Ikeda, 1972, 1973 a,b; Reimer, 1982), feeding intensity (Kapoor et al., 1975; Onishi et al., 1973a,b, 1976), feeding frequency (Stroganov and Buzinova, 1969), age (Buddington, 1985; Buddington and Doroshov, 1986a,b; Hofer, 1982) sex and spawning (Onishi and Murayama, 1970; Onishi et al., 1974; Kapoor et al., 1975). The trypsin chymotrypsin ratio in L. parsia was maintained in the same level in different size groups, different hours after feeding and hence appears to be constant for this species.

3.4.3.4 Elastase

Elastase is an endopeptidase, formed when the zymogen proelastase is activated by trypsin. Elastase activity was not detected in the

digestive tract extracts of L. parsia in the present study except for the trace of activity, in the pooled pyloric caeca and intestine extract, which was not quantifiable. This observation lends support to the view that elastase, in general is either absent in the fish alimentary canal (Nilsson and Fange, 1970; Jany, 1976) or present at low concentrations (Yoskinaka et al., 1978; Buddington and Doroshov, 1986b).

However, the fishes with discrete pancreas examined so far provided evidence for the presence of elastase (Lansing et al., 1953; Zendzian and Barnard, 1967; DeHaen and Gertler, 1974; Yoshinaka et al., 1982, 1985b). In L. parsia, the activity was not in dectable quantity either because of the scattered nature of pancreatic tissue or the enzyme might have occurred as zymogen form (Yoshinaka et al., 1985b). However, Albertini-Berhaut and Alliot (1978) could detect this enzyme in active form in the pyloric caeca and intestinal extracts of the mullets Mugil auratus and Mugil capito in French waters.

4.3.5 Exopeptidases

The exopeptidases assayed in the present study include carboxypeptidase A, carboxypeptidase B and leucine aminopeptidase. The contribution of exopeptidases and other peptidases to fish digestion are not clear owing to the poor attention paid on these enzymes. Despite this position, limited information is available

concerning the final stages of protein digestion and absorption in some economically important fishes. While endoproteases cause the initial hydrolysis of proteins to peptides sequential cleavage of peptides is effected by exopeptidases such as carboxypeptidase A and B, and leucine aminopeptidase. This is equally important in that it ensures the availability of free amino acids which can be readily absorbed from the gut lumen. An attempt was made in this study to survey the potential of various sections of the gut to hydrolyse peptides, and thus to ascertain whether terminal hydrolysis of dietary protein is confined to particular regions of the alimentary canal of L. parsia.

Both the carboxypeptidase A and B were detected only in low quantities in the pooled tissue extracts of pyloric caeca and intestine. Other regions did not exhibit any activity. This suggest that these enzymes are associated with post gastric alimentary canal in L. parsia as observed by Goel and Sastry (1975), Ash (1980) and Buddington and Doroshov (1986b) in other fishes. Carboxypeptidases have been found in several other species of fishes also (Nilsson and Fange, 1969; Lacko and Neurath, 1970; Reeck et al., 1970; Overnell, 1973; Uchida et al., 1973; Cohen et al., 1981a; Yoshinaka et al., 1984c,d 1985d; Clark et al., 1985a; Buddington and Doroshov, 1986b and Glass et al., 1987). Among mugilids, Mugil auratus and Mugil capito exhibited carboxypeptides A and B activities (Albertini-Berhaut and Alliot, 1978).

Results of research on other vertebrates suggest a co-evolution of carboxypeptidase A and B with chymotrypsin and trypsin respectively. Each carboxypeptidase hydrolyses peptide residues exposed by their associated endopeptidase (Barnard, 1973). Accordingly, higher carboxypeptidase B and tryptic activity relative to carboxypeptidase A and chymotryptic activity exist in white sturgeon (Buddington and Doroshov, 1986b), and such a relationship does not seem to exist in L. parsia.

Leucine aminopeptidase was observed to be secreted by intestinal tissue in fishes (Fange and Grove, 1979) and is generally associated with the brush border (Hirji and Courtney, 1982) and also described for other vertebrates (Barnard, 1973). Leucine aminopeptidase can act only upon the degradation products formed upon the action of endoproteases. In the present study, the enzyme activity could be detected almost throughout the alimentary canal with maximal activity at posterior intestine, anterior intestine and pyloric caeca. A similar distribution pattern was also observed in Ameiurus nebulosus and Cyprinus carpio (Fraisse et al., 1981) and Solea solea (Clark et al., 1987). Leucine aminopeptidase activity was also reported in the digestive tracts of various fishes (Nilsson and Fange, 1969, 1970; Lane, 1973; Alliot et al., 1977; Albertini-Berhaut and Alliot, 1978; Khablyuk and Proskuryakov, 1983a,b; Clark et al., 1985a; and Glass et al., 1987).

In the studies by overnell (1973), it was shown that in addition to the pyloric caeca, leucine aminopeptidase was also present in the spleen and kidney of the cod. It was therefore conncluded that in cod this enzyme might have some function other than digestion. The high activity of leucine aminopeptidase in the intestine and pyloric caeca of L. parsia endorses a digestive role for this enzyme in this mullet. The results of the present study also indicate a progressive increase in leucineaminopeptidase activity from anterior intestine to posterior intestine. Such a pattern was demonstrated in other vertebrates and also in fishes (Ash, 1980). Further, considerable amount of peptide hydrolysis appears to reside in the pyloric caeca also as this enzyme was present in significant quantities. Since these organs also possessed tryptic and chymotryptic activities they would appear to play a significant role in protein digestion and absorption in mullets.

3.4.3.6 Acid and Alkaline Phosphatase

Contribution of acid and alkaline phosphatases to digestion of food in fishes is not clearly understood. Acid phosphatase was detected in the striated border microvilli of fish intestine and caeca (Jansson) and Olsson, 1960; Jirge, 1970; Western, 1971). In L. parsia, this ensyme could be recorded throughout the alimentary canal. However, the highest activity was present in the cardiac stomach ~~stomach~~ followed by pyloric stomach and pyloric caeca. The

distribution of acid phosphatase at varied intensities has been recorded in the subepithelial connective tissue, muscularis and serossa of the stomach of Cottus gobio and Enophrys bubalis (Western, 1971) and in the subepithelial connective tissue of the entire intestine of Carassius auratus (Gauthier and Landis, 1972).

Khawaja and jafri (1968) stated that the concentrations of acid and alkaline phosphatases vary considerably in different regions of the alimentary canal of Ophiocephalus punctacus. Thus the activities of these enzymes were comparatively low in the oesophagus and rectum than in other regions. While a high acid phosphatase activity accompanied with a low activity of alkaline phosphatase was recorded in the stomach, a high alkaline phosphatase activity accompanied with low acid phosphatase activity has been observed in the intestine. The highest acid phosphatase activity occurred in the stomach and the highest alkaline phosphatase activity in the caeca. The present observations made on L. parsia are in accordance with the above observation.

Alkaline phosphatase activity was also detected throughout the alimentary canal of L. parsia but in significant level in the pyloric caeca, intestine and liver.

Alkaline phosphatase activity in the intestine and pyloric caeca of fishes has been detected by a number of authors (Al-Hussaini, 1948; 1949; Weinreb and Bilstad, 1955; Reznik, 1958; Verighina, 1961,

1963; Bullock, 1963, 1967; Luppa, 1966; Srivastava, 1966; Khalilov et al., 1963; Sivadas and Govindan, 1970; Western, 1971; Mester et al., 1972; Chao, 1973; Tanaka, 1973; Hirji, 1983; and Buddington and Doroshov, 1986b). In addition, considerable amount of this enzyme was also detected in oesophagus, stomach and liver of L. parsia. Such a distribution pattern of this enzyme was also reported by Prakash (1961) and Goel and Sastry (1973).

It is generally regarded that the alkaline phosphatase are involved in nutrient absorptive processes in fishes (Noaillac-Depeyre and Gas, 1973; Stroband et al., 1979; Buddington and Diamond, 1985 and Ferraris et al., 1987). Nutrient uptake rates in the stomach, intestine and spiral valve of white sturgeon correspond with distribution of alkaline phosphatase (Buddington and Diamond, 1985). Khalilov (1969) reported that the most intensive fat absorption takes place in the foregut and midgut of the tench Brachymystax lenok which is accompanied by an increase of the alkaline phosphatase activity. Thus it is concluded that the high amount of alkaline phosphatase activity encountered in the pyloric caeca and intestines of L. parsia are due to their requirement in the digestive process of the fish.

3.4.3.7 Esterase

Dietary lipids constitute an important energy source for fish and have a sparing effect on dietary protein in addition to being a

source of essential fatty acids. Information on the digestion and absorption of lipids in fish is still inadequate. The main hydrolytic enzyme active in the digestion of lipids in fish may not be a pancreatic lipase as proposed earlier (Barrington, 1957; Brockerhoff, 1966; Kapoor et al., 1975), but rather a non-specific lipase (Patton et al., 1975). Lipases and esterases are enzymes of low specificity, with only a general requirement for an ester linkage. Lipases are esterases which split ester bonds (Fange and Grove, 1979). In the present study no lipase activity could be detected in any region of the digestive tract of L. parsia. According to Chesley (1934), lipase is more abundant in fishes with a compact pancreas than in those with a diffuse pancreas. This might be the reason for the low levels of lipase activity which was not quantifiable by the method employed in the present study. Brockerhoff (1966) and Overnell (1973) failed to detect any lipase activity in extracts of the pyloric caeca and adjacent tissues of cod. Similarly, lipase activity could not be detected in Tilapia nilotica (Moriarty, 1973), Physailla pellucida, Eutropius niloticus and Schilbe mystus (Olatunde and Ogunbiyi, 1977). Nevertheless, a strong esterase activity was detected throughout the length of the alimentary canal in L. parsia. A similar kind of distribution of esterase was reported for Seriola quinqueradiata and Salmo gairdneri besides absence of lipase but strong esterase activity along the digestive tract of Mugil cephalus (Morishita et al.,

1967b). Similarly, Moriarty (1973) demonstrated the presence of strong esterase activity in the intestine of Tilapia nilotica and the absence of lipase. In L. parsia esterase activity was recorded in the intestinal extracts similar to that of other teleosts (Rahimullah, 1945; Barrington, 1957; Morishita et al., 1967a; Windell, 1967 and Kapoor et al., 1975). Esterase activity has also been demonstrated histochemically in the digestive tracts of few teleosts (Kitamikado and Tachino, 1960c; Luppa, 1966; Western, 1971; Mester et al., 1972; Patankar, 1973; Hirji and Courtney, 1983 and Ferraris et al., 1987).

Since fatty acids are essential dietary components for fish some form of lipase or esterase is essential to digest the fat component of the diet (Kanazawa, 1985). Further it has been suggested that fat droplets can pass through the mucosa of the intestine, and hence lipid digestion could be intracellular, not requiring a soluble lipase (Barrington, 1957). This seems to be the case in L. Parsia as intracellular lipid particles were detected along the intestines of other mullets Mugil cephalus and Liza ramada (Albertini-Berhaut, 1988).

Analysis of digestive enzymes enables easy detection of digestive capabilities of the fish . Mullets are unique by virtue of feeding low in the food chain, mainly on decayed organic matter, algae and diatoms, and thus avoid considerable loss of energy through

different trophic levels. The pohenomenon of relatively large fishes feeding directly from the first trophic level has been referred to by Hiatt (1944) as "telescoping of the food chain". The present study evidences the adaptation the fish has undergone in extracting the available nutrients from the diet effectively. The extensive length of the intestine facilities complete digestion and absorption of the nutrients by prolonging the gut passage time and thereby exposing the ingested diet to the enzymatic hydrolysis for a longer duration. Further, the mullets which feed on algae and detritus, have an anterior thin walled stomach. Hence it may be suggested that the production of acid in this part may aid in lysis and digestion of the bluegreen algae and the grinding action of gizzard may improve the efficiency of acid lysis and enzymatic hydrolysis of the algal cells. The full complement of various enzymes detected endorse the view that the grey mullets are efficient convertors of energy in the food chain.

CHAPTER 4

CHARACTERISATION OF DIGESTIVE ENZYMES

4. CHARACTERISATION OF DIGESTIVE ENZYMES

4.1 Introduction

An essay for a specific enzyme within an organ system relies on the characteristic ability of an enzyme to catalyse a particular kind of reaction (Dixon and Webb, 1964). Work done in the past detecting proteolytic enzymes from tissue extracts involved the use of a complete protein molecule as a substrate. Casein was commonly used as substrate for tryptic and chymotryptic assays, while haemoglobin was used for analysis of pepsin. Casein is rapidly hydrolysed by trypsin but it is also hydrolysed to a lesser extent by other proteolytic enzymes. This discrepancy in analytical procedures probably resulted in many of the erroneous conclusions that exist in the literature. However, even by employing same substrate the activities of different enzymes can be detected by studying the optimum pH required by each activity. Thus, each enzyme has a specific requirement for pH and so many other such parameters. To carry out any experiment involving assaying of enzymatic activity, it is a prerequisite to establish the optimum assay conditions required by each enzyme so that consistent results could be obtained from the experimental studies. In this chapter, an attempt is made to characterise the important digestive enzymes and the results obtained are discussed. The enzymes exhibiting high levels of activity were only chosen for this study. Further, the region of the digestive tract showing maximum activity with respect to particular enzyme was only selected as the source of enzymes.

4.2 Material and Methods

4.2.1 Amylase

Investigations on the carbohydrases of fish was largely confined to the identification of amylolytic activity. In the present study, the intestine of the fish exhibited maximum amylase activity. Hence the intestinal amylase was characterised with respect to the following physico-chemical parameters.

4.2.1.1 pH

The activity profile of intestinal amylase at various pH was studied using the crude extracts obtained from anterior intestines of *L. parsia*. Mixtures of 0.20 ml of crude extract, 0.20 ml. of 1.0% starch solution and 1.0 ml of the respective pH buffer were incubated at 37°C for 1 hr. At the end of 1 hr incubation period, the amount of glucose formed was estimated following the methods suggested by Nelson (1944) and Somogyi (1952). The buffer systems consisted of 0.1 M acetate buffers for pH 4.0 to 5.6; 0.01 M Tris-citrate buffers for pH 6.0 to 6.8; 0.1M Tris-HCl buffers for pH 7.0 to 8.0, and 0.2 M Tris-HCl buffers for pH 8.2 to 9.0. Amylase activity is expressed as microgram glucose liberated/mg protein/hr.

4.2.1.2 Temperature

The activity profile of intestinal amylase at various incubation temperatures was studied at optimum pH6.6. Mixtures of 0.20 ml of crude extract of intestine, 0.20 ml of 1.0% starch solution and 1.0 ml of 0.1 M Tris-citrate buffer at pH 6.6 were incubated for 1 hr at different temperatures in a water bath. At the end of the 1 hr

incubation period, the amount of glucose liberated was estimated as mentioned in general material and methods (Section 2.6)

4.2.1.3 Chloride ion concentration

The effect of chloride concentration on amylase activity was determined after desalting the crude extract of anterior intestine by dialysis in the cold against several changes of distilled water for 24 hrs. Mixtures of 0.25 ml crude extract, 0.50 ml of 1.0% starch solution in 0.1 M Tris-citrate buffer pH 6.6 and 0.75 ml of NaCl solution of appropriate concentration were incubated at 37°C for 1 hr. The final chloride concentration in the reaction mixture was within the range of 0-90 ppt. Amylase activity is expressed as microgram glucose liberated/mg/protein hr.

4.2.2. Proteases

Characterization of proteases with reference to physico-chemical factors was performed with appropriate substrates for acid protease and alkaline protease. The activities of trypsin and chymotrypsin were assayed with synthetic substrates.

4.2.2.1 Acid protease

Pepsin is considered to be the major acid protease in the gut of fishes. Pepsin like activity was determined as mentioned in the general material and methods (2.6.2) by employing acid denatured haemoglobin as substrate. The digestion mixture consisted of acid denatured 2% haemoglobin solution (0.5 ml), cardiac stomach extract (0.25 ml) and appropriate pH buffer (1.25 ml). After 30 minutes

incubation at 37°C, 10.0 ml of 5% TCA was added and then centrifuged at 2000 g for 10 minutes. The amount of tyrosine formed in the supernatant was estimated and expressed as micro mole tyrosine liberated/mg protein/hr. The buffers used were glycine - HCl (pH 1.0 - 1.7) and citrate-phosphate (pH 1.7 - 7.8).

1.2.2.2 Alkaline protease

Alkaline protease activity at various pH and temperature was tested with the crude extracts of anterior intestine.

1.2.2.2.1 pH

The buffer systems used in the assay consisted of: 0.1 M citrate buffers for pH 4.0 to 5.5; 0.01 M Tris-0.2 M citric acid for pH 5.9 to 6.8; 0.01 M Tris-HCl for pH 7.0 to 8.0 and 0.2 M Tris-HCl for pH 8.5 to 11.0.

Enzyme assays were performed as mentioned in the section general material and methods (2.6.3). The assay systems were incubated for 30 minutes at 37°C. Protease activity is expressed as micro mole tyrosine liberated/mg protein/hr.

Trypsin and chymotrypsin

The optimum pH required for tryptic and chymotryptic activity was detected using the synthetic substrates BAPNA and BTNA respectively. The procedures given in the material and methods (Section 2.6.4 and 2.6.5) were followed with little modifications. The BAPNA substrate solution was made up to the required volume with distilled water instead of buffer solution. The assay mixture

for trypsin consisted of 1.4 ml of the substrate solution, 1.5 ml of the respective pH buffer and 0.1 ml of the crude enzyme extract. All other procedures were the same, except for the change in pH buffers. The buffers used were 0.1 M citrate buffer for pH 4.0 to 5.5; 0.01 M Tris-0.2 M citric acid for pH 6.0 to 6.8; 0.01 M Tris-HCl for pH 7.0 to 8.0 and 0.2 M Tris-HCl for pH 8.5 to 11.0.

4.2.2.2.2 Temperature

Influence of temperature on the activity of intestinal alkaline protease was determined at an optimum pH of 8.0. Enzyme assays were performed at different temperatures as described under the section 2.6.3 using crude extracts of the anterior intestine. The buffer system consisted of 0.2 M Tris-HCl adjusted to the optimum pH of the activity. Protease activity is expressed as micro mole tyrosine liberated/mg protein/hr.

4.2.2.2.3 Inhibition studies

Inhibition of tryptic activity by SBTI

The assay for tryptic activity was performed as mentioned in the general material and methods (Section 2.6.4). The crude extract was preincubated with the inhibitor solution of appropriate concentration for 5 minutes at room temperature at pH 8.0 prior to its addition to the assay system. All assays were run for 10 minutes at room temperature. The tryptic activity in response to inhibition is expressed as relative activity by assigning a value of 100 for uninhibited activity.

Inhibition of intestinal chymotrypsin by TPCK

The assays for chymotryptic activity was performed as described in the general material and methods (section 2.6.5). The crude extract was preincubated with the inhibitor solution of different concentrations for 5 minutes at room temperature prior to its addition to the assay system. All assays were run for 10 minutes at room temperature. The chymotryptic activity in response to inhibition is expressed as relative activity by assigning a value of 100 for uninhibited activity.

Effect of inhibitors on the activities of total protease, trypsin and chymotrypsin

As described above, the intestinal crude extract was preincubated with the following inhibitor solutions PMSF, TLCK, TPCK, EDTA and SBTI for 5 minutes at room temperature at 8.0 and then their residual activity was determined as described elsewhere (2.6.3, 4,5). The concentration of the inhibitors employed are given in Table-13. All the enzyme activities in response to inhibitors are expressed as percent inhibition by comparing with uninhibited assay system.

Effect of bivalent-ions on the activities of total protease, trypsin and chymotrypsin.

The crude enzyme extract was dialysed against several changes of distilled water in cold and then preincubated with the metal ions (Table-14) for 30 minutes at room temperature at pH 8.0 and then the residual activity was determined as described earlier

(2.6.3,4,5). The activity of uninhbited assay system was assigned a value of 100 and the activity in response with bivalent ions are expressed as relative activity.

4.2.2.3 Leucine aminopeptidase

4.2.2.3.1 pH

The leucine aminopeptidase activity profile in relation to pH was studied with the crude extracts obtained from the anterior intestine. Enzyme assays were performed as mentioned in section 2.6.9 with suitable pH buffers. The buffers used were 0.1 M HCl (pH 1.0), glycine - HCl (pH 1.0 - 1.7), citrate-phosphate (pH 1.7 - 7.8), glycine - NaoH (pH 7.8 - 10.5). The assay systems were incubated at 37°C for 20 minutes.

4.2.2.3.2 Temperature

The influence of temperature on the activity of leucine aminopeptidase of the crude extracts of anterior intestine was studied by conducting enzyme assays at pH 8.3 at different incubation temperatures as mentioned earlier (2.6.9).

4.3 Results and discussion

The different group of digestive enzymes such as amylase, pepsin, total alkaline protease, trypsin, chymotrypsin and leucine aminopeptidase were characterised with respect to pH, temperature, chloride ion concentration, effect of inhibitors and metal ions. The results of these studies are preseented and discussed in the ensuing section.

4.3.1 Amylase

The effect of pH on the activity of intestinal amylase is shown in Fig.2. A well defined optimum activity was recored at pH 6.6. The enzyme remained active over a wide range of pH (5.0-9.0). A similar optimum pH was observed for amylases extracted from other fishes (Takahashi, 1960; Morishita et al., 1964; Ikeda and Kawai, 1966; Yasunaga, 1972; Brigaudeau, 1981; Chiu and Benitez, 1981; Clark et al., 1984 and Glass et al., 1987). Optimal pH for the amylases of the digestive tracts of fish appears to be species specific. Thus the optimum pH of amylase was 8.5 for salmon, Oncorhynchus keta (Ushiyama et al., 1965); 7.5 for flat fish (Yasunaga, 1972); 7.0 for Periophthalmus koelreuteri (Dhange and Mohamed, 1977) and 7.8 for catfish Clarias gariepinus (Uys and Hecht, 1987). According to Bernfeld (1955) amylases have a wide pH range over which they are active.

Amylases from Tilapia mossambica remained active over a wide range of pH 5.0 to 8.0 (Fish, 1960). Moriarty (1973) also reported that the optimum for amylase activity of Tilapia nilotica occurs between pH 7.0 and pH 8.0 and the enzyme was found to be active over a wide range of pH from 6.0 to 9.0. The results of the present study with reference to influence of pH on amylase activity is in agreement with earlier reports in this regard.

In sharp contrast to other vertebrates, fishes consume a great variety of food (Kapoor et al., 1975). Mullets are also known to feed on a variety of items such as Zooplankters and detritus along with fine sand particles, diatoms and blue green algae (Sarojini,

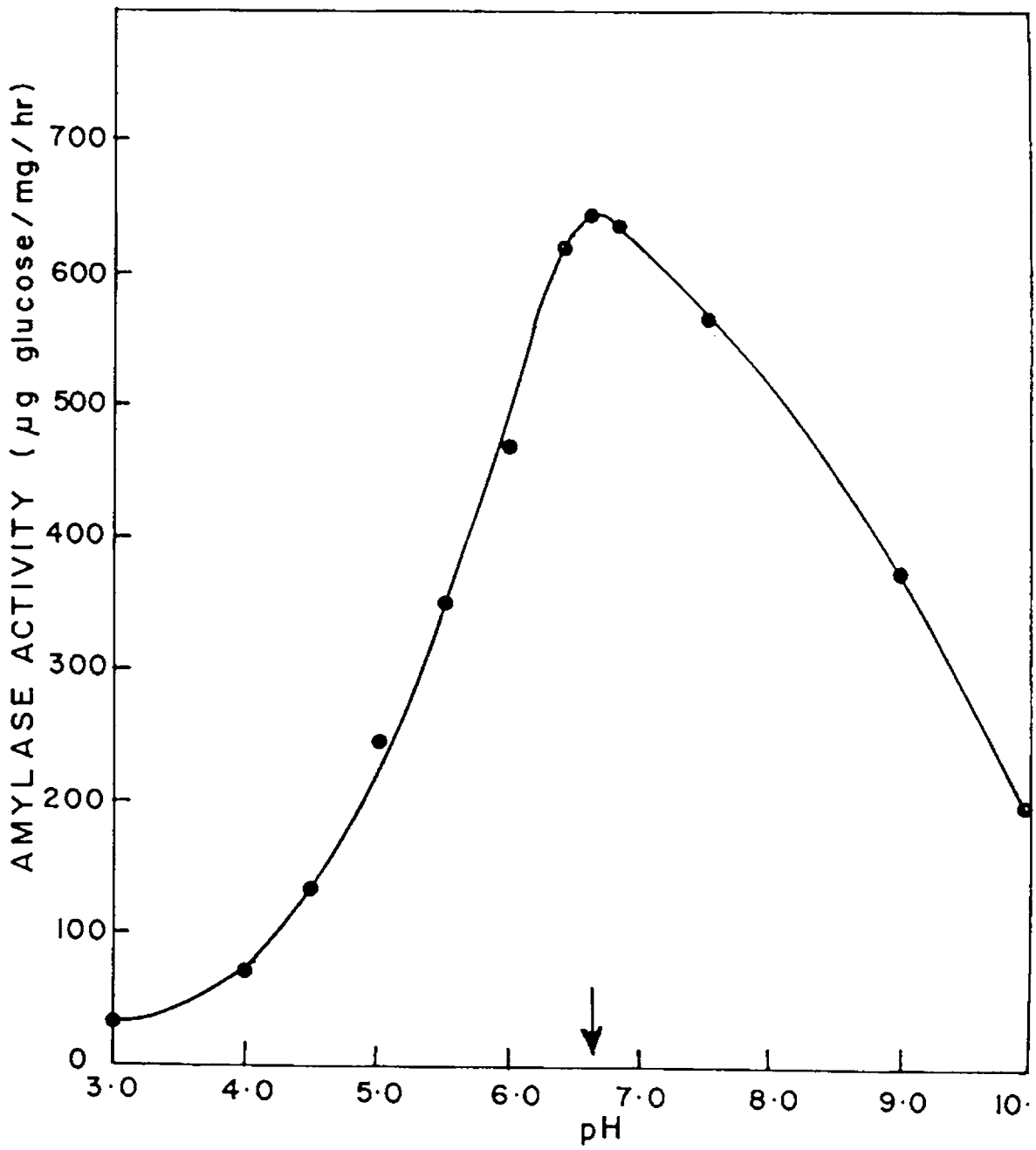

Fig.2 Effect of pH on intestinal amylase activity of L. parsia.

1954). This omnivorous habit, coupled with dilution of the gut fluid, might cause changes in physico-chemical properties of the entire gut contents thereby changing the optimal conditions required for the digestive enzyme activities. To overcome such shortcomings the amylase activity remain active over a wide pH range there by assuring that the process of starch digestion could proceed at any event which is evident during the present investigation. Further, it is suggested that in practical diet formulations, the pH of the diet should be adjusted in such a way to coincide with the optimal pH requirements of the digestive enzymes to ensure better utilisation of the diet.

The temperature activity profile of intestinal amylase is given in Fig. 3. The amylase had a temperature optimum of about 52°C. Although a high amylase activity could be observed at 65°C, further increase in the incubation temperature led to rapid decline in the enzyme activity. Similarly temperature less than 15°C also resulted in a decline in the enzyme activity. Results of similar nature with temperature optimum of 50°C or above have been reported earlier for amylase activity in other fishes (Kitamikado and Tachino, 1960a; Takahashi, 1960; Morishita et al., 1964; Chiu and Benitez, 1981). However, the amylase activity in Clarias gariepinus had an optimum at 35°C and significant loss of activity at 45°C (Uys and Hecht. 1987).

The temperature optimum (55°C) observed for the intestinal amylase in the present study is well above the usual range of ambient temperature observed in fish ponds or in natural environments. This

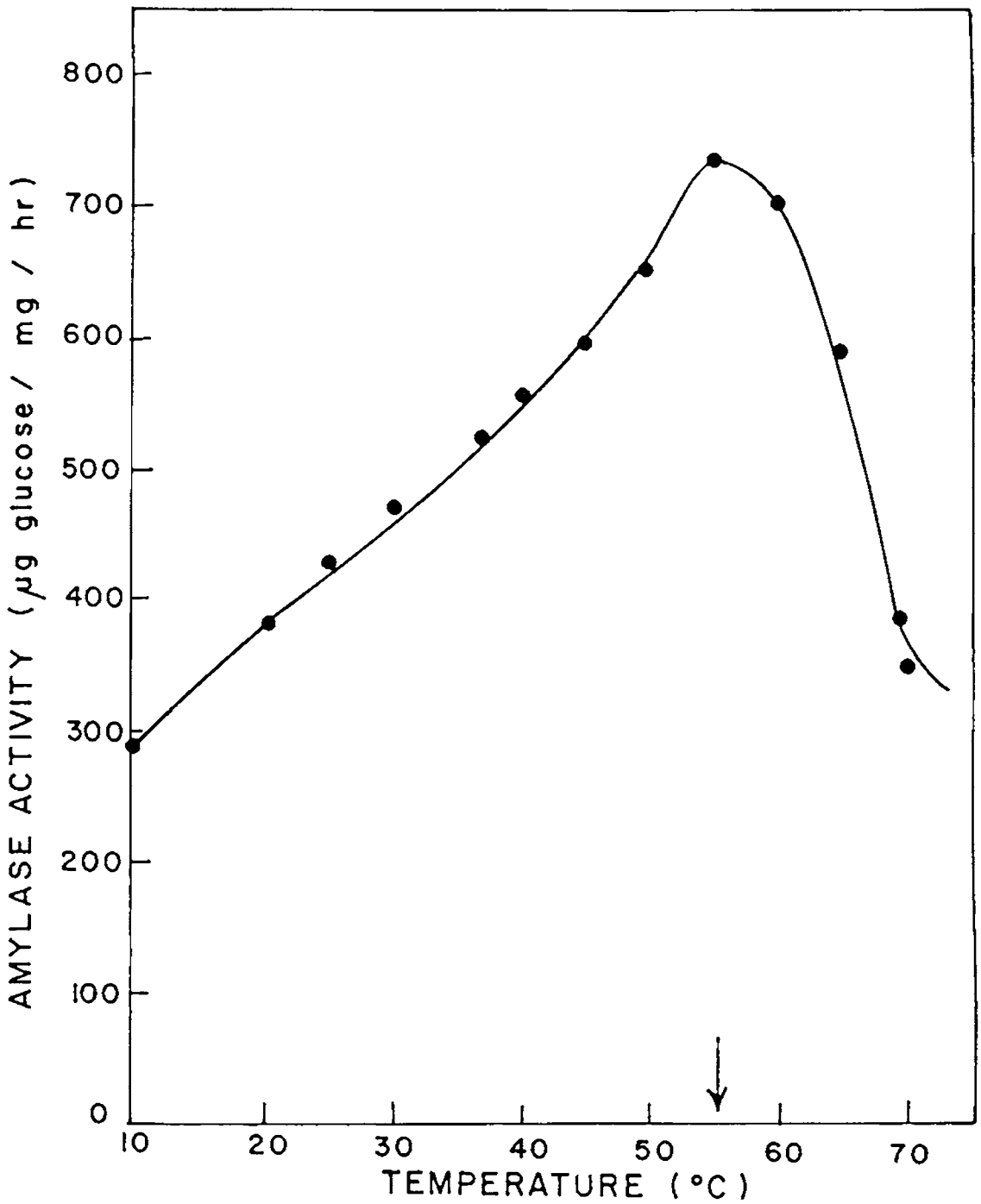

Fig.3 Effect of temperature on intestinal amylase activity of L. parsia.

suggest a possible digestion of starch even at high temperature in the body of fish which is the reflection of environmental temperature. Furhter, it clearly explains how the mullets which are eurythermal in nature (Bardach et al., 1972) are adapted to survive and grow in extremes of temperature, a character much desirable for a candidate species for aquaculture operations.

The effect of chloride concentration on amylase activity was determined employing a range that is frequently encountered in fish ponds. Prior to use in this experiment, the crude extracts were desalted by exhaustive dialysis in the cold against several changes of distilled water for 24 hours. A consistent trend in the activity profile of intestinal amylase at various chloride concentrations was observed as shown in Fig. 4. Slight activation of the amylase was observed initially as the chloride concentration increased from 0 to 10 ppt. The amylase activity remained fairly constant for the further increase in the chloride concentration from 10 to 50 ppt. However, a slight decrease in activity was observed, when the chloride concentration was increased above 50 ppt. A similar trend in the activity of amylase with regard to temperature and chloride ion concentrations were observed for milk fish (Chiu and Benitez, 1981). This explains the fact that both milkfish and grey mullets share similar habitats for their feeding and growth.

Although high amylase activity is observed even at high salinity and high temperature, these conditions may not be ideal for the growth of natural food organisms in the ponds. It may be suggested that digestion of starch could however proceed even under such

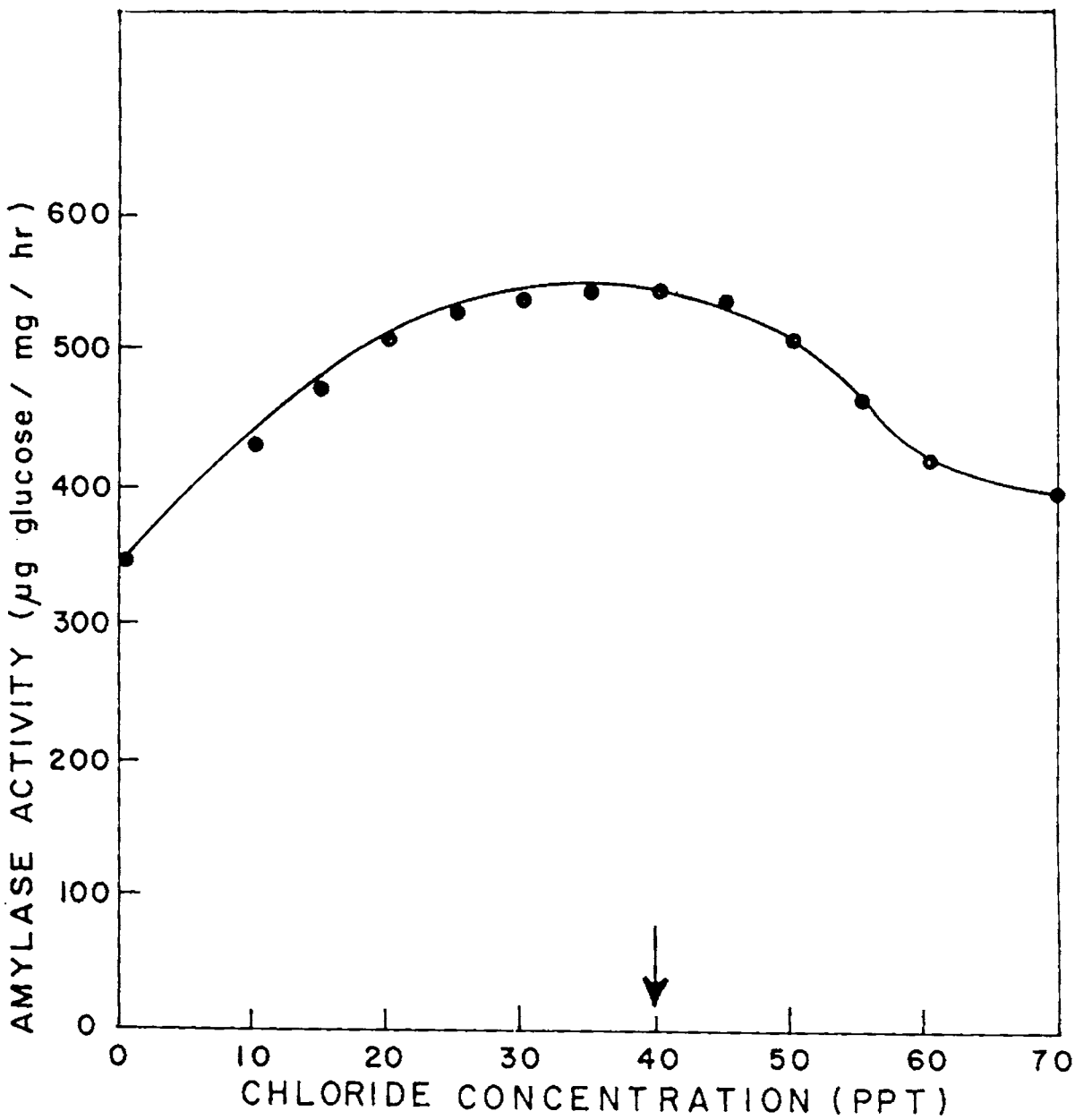

Fig.4 Effect of chloride concentration on intestinal amylase activity of L. parsia

adverse environmental conditions. Further, the ability of the enzyme to remain active over a wide range of chloride concentration explains the euryhaline nature of the fish which is a desirable character of candidate species for aquaculture (Bardach et al., 1972).

1.3.2 Pepsin

Pepsin is secreted as a zymogen (pepsinopgen), which is activated by the acid in the stomach region to an active form of the enzyme. The hydrolysis of haemoglobin tested at acidic pH range showed that there was an active pepsin like activity in the cardiac stomach region of the mullet. Pepsin like enzymes are the major acid proteases in many species of fish (Fange and Grove, 1979). Fig. 5 shows the pH activity profile of pepsin like activity. The optimum activity of pepsin seems to be at pH 2.0. There was also a small peak in the pH range between 4.0 and 5.0. Several pepsins and pepsinogens have been examined from the stomach regions of fish including dogfish (Merret et al., 1969), bonito (Kubota and Ohnuma, 1970) and rainbow trout (Twining et al., 1983). In these studies the optimal activity was generally in the region of pH 2.0 for the pepsin activity. Albertini- Berhaut and Alliot (1978) found that the stomach extracts of the grey mullets Mugil auratus and Mugil capito exhibited maximum activity at about pH 2.0 with haemoglobin as substrate. The pH optimum for pepsin in the present finding is similar to the observations mentioned above. However, in certain fishes the pH optima of peptic activity was reported as 3.0, which include rainbow trout (Twining et al., 1983) Atlantic cod (Brewer

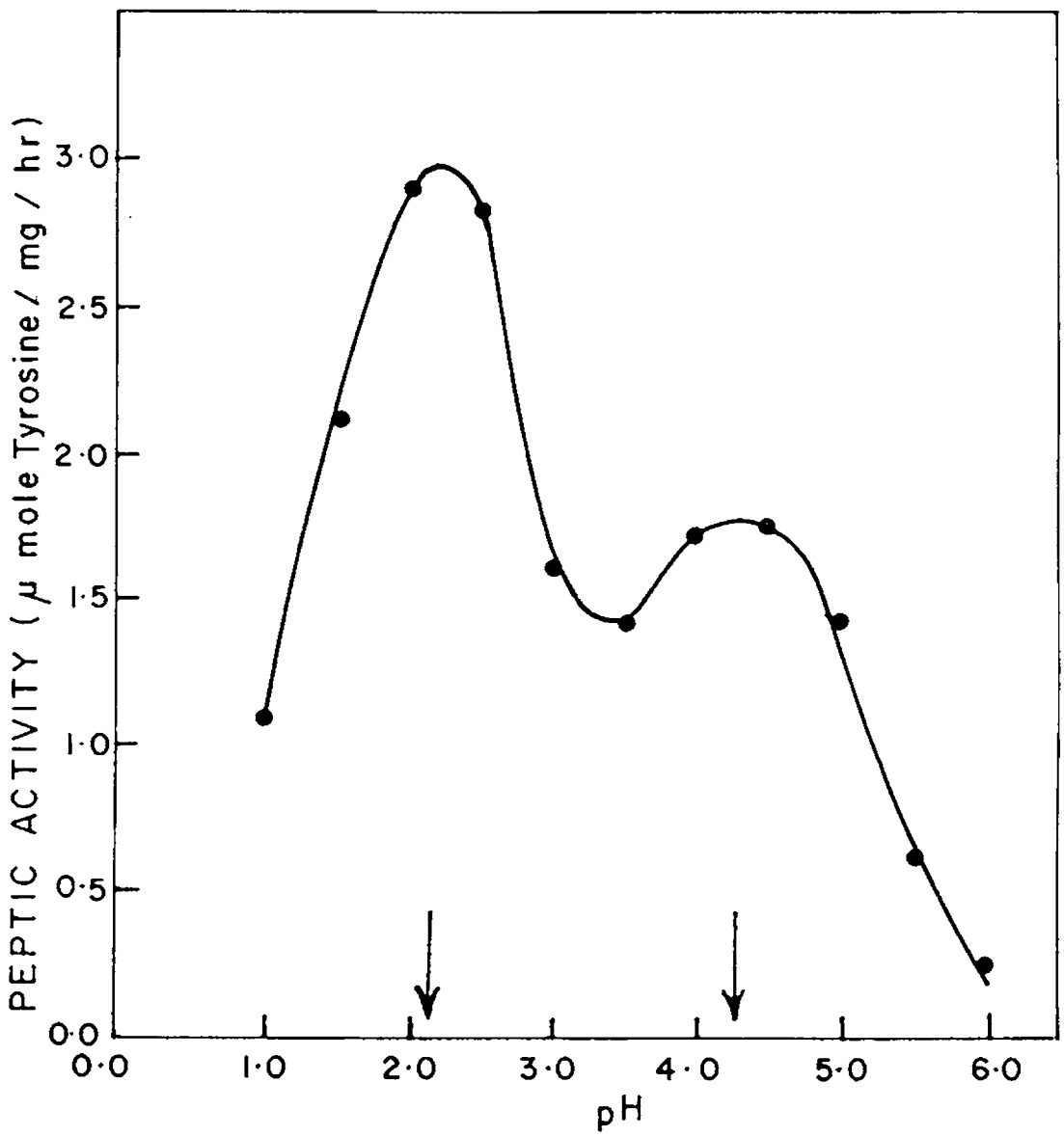

Fig.5 Effect of pH on peptic activity of L. parsia.

et al., 1984), polar cod (Arunchalam and Haard, 1985), sturgeon (Buddington and Doroshov, 1986b) and catfish (Uys and Hecht, 1987).

The fact that optimal proteolytic activity in the acidic pH range has been reported at different values of pH (2.0, 3.0, 5.0) indicates that the gastric fluid of fishes contains several types of pepsin (Creach, 1963). The small peak observed at the pH range 4.0 to 5.0 in the present study might be due to a different kind of protease other than pepsin which might be present in the extract. Norris and Elam (1940) (for salmons) and Norris and Mathies (1953) (for tuna) found double peaked curve of activity for pepsin, a sharp peak occuring at pH 1.3 and a broader optimum between pH 2.5 and 3.5 using haemoglobin as substrate. This kind of double peaked curves are also known for mammalian pepsins, which have been investigated more extensively than those of fish (Taylor, 1959). According to Taylor (1962), the two optima are not based on the activity of two proteolytic enzymes; instead, the pepsin molecule has two enzymatic active sites attacking the substrates maximally at different values of pH.

The digestive power of the gastric juice depends on the amount of pepsin and on the pH. At a given enzyme concentration, proteolytic activity of the juice will be maximal at pH values lower than 5 as this enzyme was found to be active over a wide pH range. The occurrence of a second peak of peptic activity in some fish, and also in grey mullet in the present study, at pH values upto 4 or 5, suggests that the pH of the chyme is not extremely critical, proteolytic digestion proceeding at a level of considerable

intensity through a rather wide pH range, possibly from 1 to 5. Further, the pH fluctuations caused by dilution by food will not affect the action of pepsin in L. parsia as this enzyme was found to be active over a wide pH range.

4.3.3 Alkaline Protease

The activity profile of proteases in the crude extracts obtained from the anterior intestines was determined in relation to various pH and temperature and the results are presented in Fig. 6 and 7.

Data presented in Fig. 6 indicate a maximum activity between pH 7.5 and 8.5. In addition a smaller but broader peak between pH 9.4 and 10.0 also could be noted. The presence of alkaline protease with similar pH activity profile has been observed in other species of fishes also. The optimum pH for proteolytic activity in the pyloric caeca and intestines of the rainbow trout was found to be 9.5 (Kitamikado and Tachino, 1960b). The protease from mackerel was most active at pH 9.0 (Ooshiro, 1971 a,b,c). An optimum pH of 10.0 was reported for all the three alkaline proteases purified from the pyloric caeca of sardines (Murakami and Noda, 1981). In the milk fish the optimum pH for proteolytic activity was found to be between 7.2 and 9.3 (Benitez and Tiro, 1982). The whole gut homogenates of dover sole indicated three main pH regions of activity. At pH 1-2 a low level of pepsin like activity could be detected, but the majority of protease action was at pH 7 to 8 and 9.5 to 10.5 (Clark et al., 1985a).

The pH profile for protease activity in silver carp (Ragyanszki

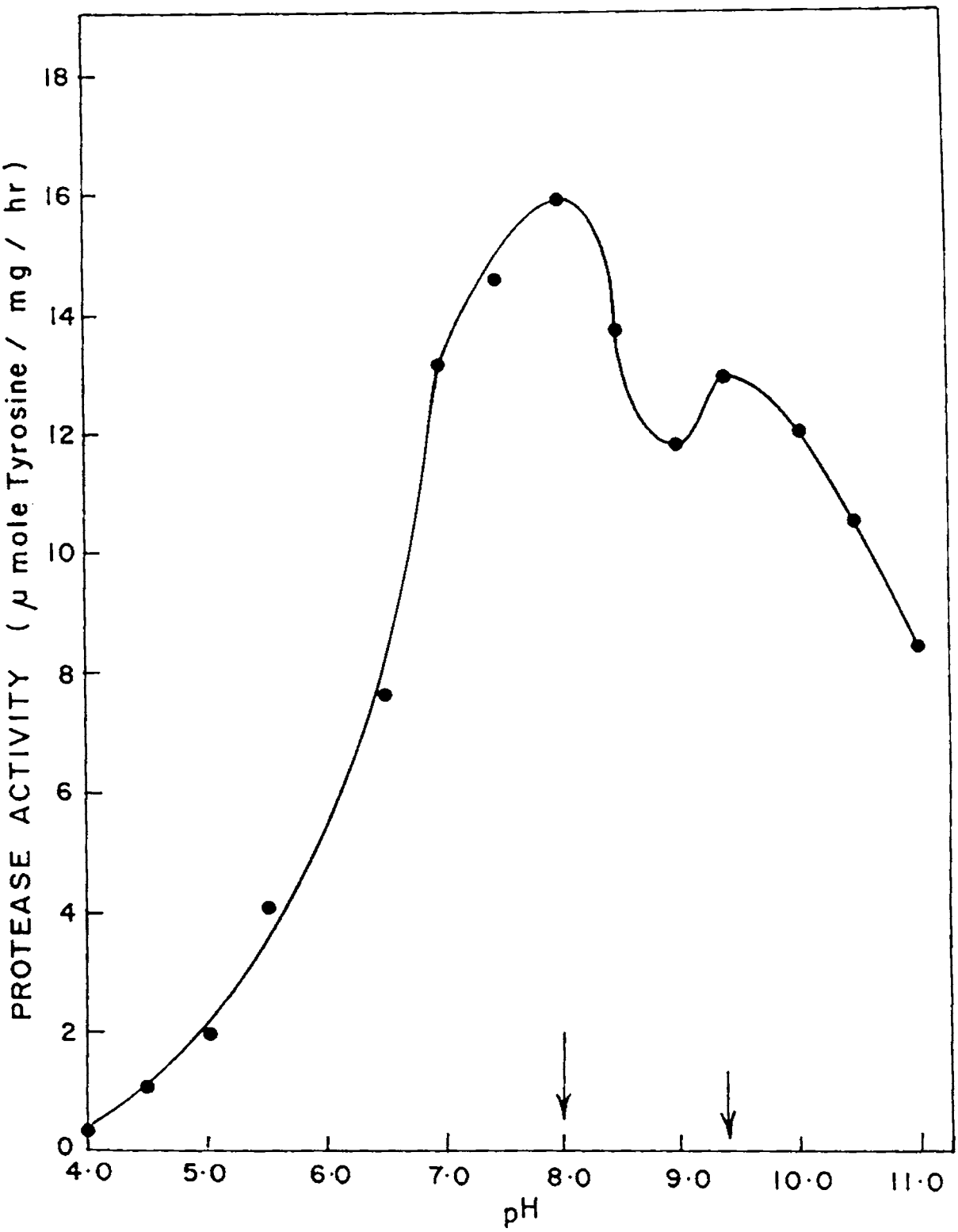

Fig.6 Effect of pH on intestinal protease activity of L.parsia

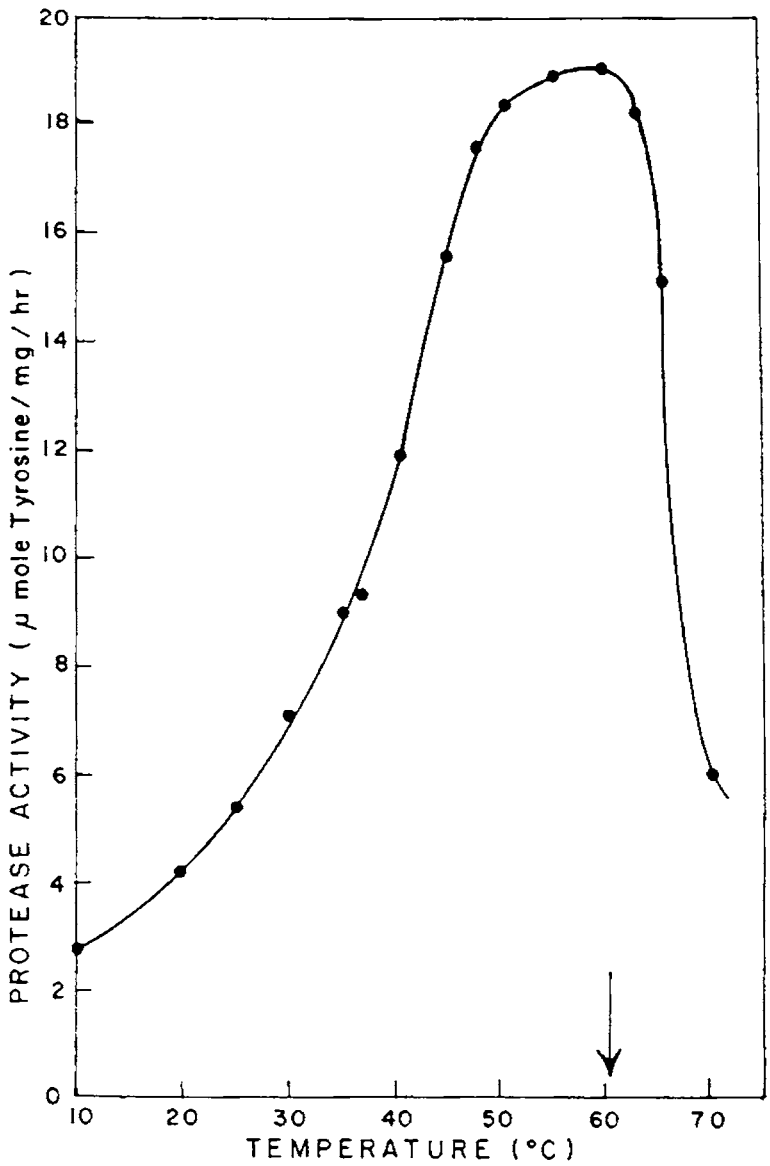

Fig.7 Effect of temperature on intestinal protease activity of L.parsia.

et al., 1977) had a peak at about pH 10.0 and a shoulder at pH 7.5. An even clearer indication of double peak profiles was recorded by the proteases of the milkfish with optima at pH 7.0 - 7.6 and 9.5 to 10.0 respectively (Benitez and Tiro, 1982). Similarly, Clark et al., (1985a) working on dover sole proteases observed two peaks one at pH 7-8 and the other at pH 9.5-10.5. Similar activity profile was also observed in whole gut homogenates of Atlantic halibut (Glass et al., 1987). Similarly double peak was observed for the activity of protease in L. parsia in the present study. Since the pH activity profile (Fig. 6) shows more than one peak in the neutral alkaline region, it is presumed that there exist more than one enzyme active in the neutral alkaline region.

To understand the different peaks observed in the pH activity profile of protease tested with casein as substrate, further experiments were conducted with synthetic substrates. The pH profiles for trypsin and chymotrypsin using BAPNA and BTNA respectively as substrates are shown in Figs. 8 and 9. The results indicate optimal pH values of 8.0 and 7.5 for trypsin and chymotrypsin respectively. From catenary of the curves it appears that these activities contribute mainly to the neutral (pH 7.0 - 7.8) peak on the casein profile.

Similar results for the pH optimum of trypsin have been reported for other groups of mullets which include Mugil auratus and Mugil capito (pH 8.1 to 8.5) (Albertini-Berhaut and Alliot, 1978). Trypsin from other groups of fish also exhibited similar pH activity profiles, chum salmon (Uchida, 1973), herring and capelin

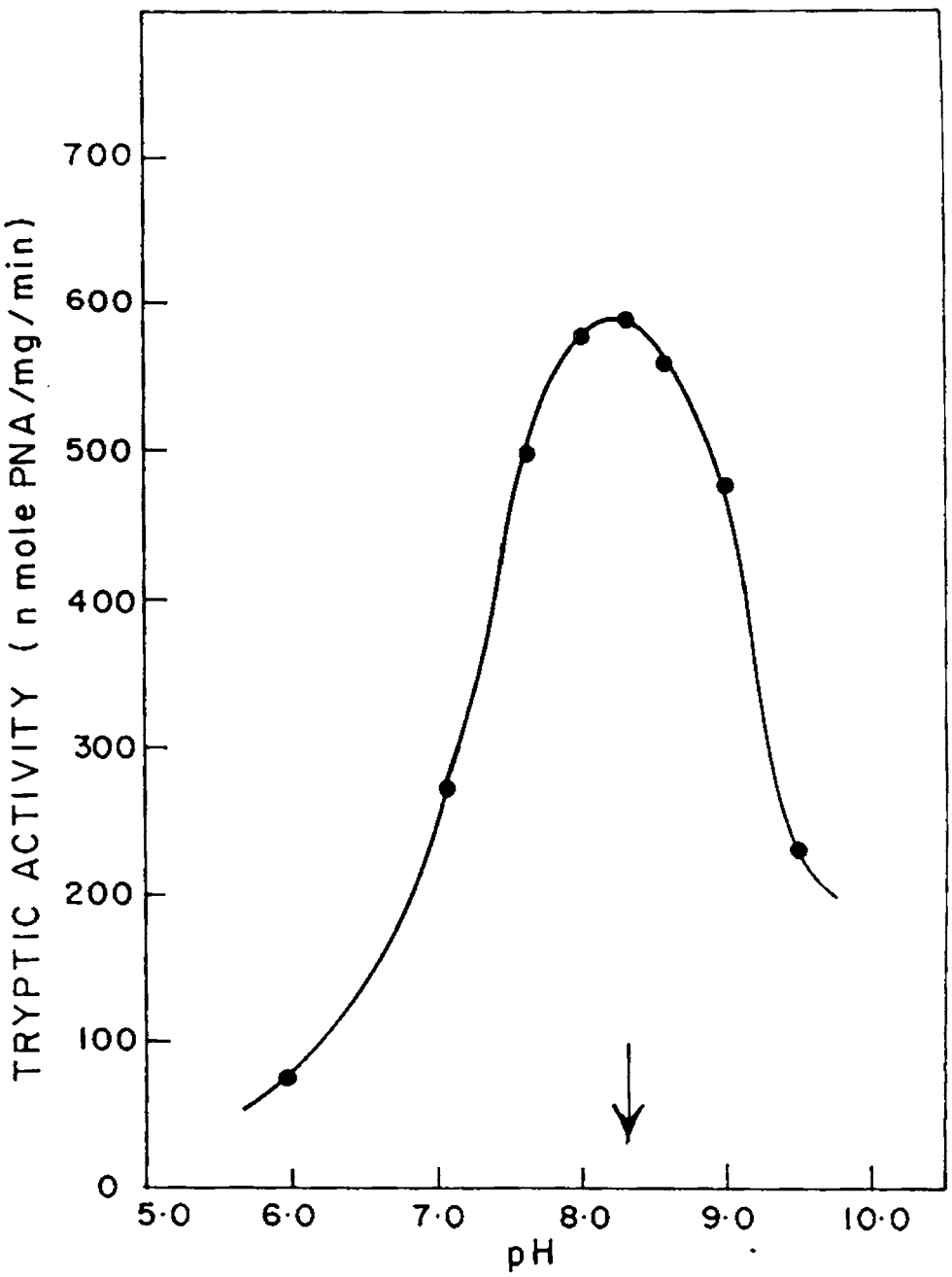

Fig.8 Effect of pH on intestinal tryptic activity of L. parsia

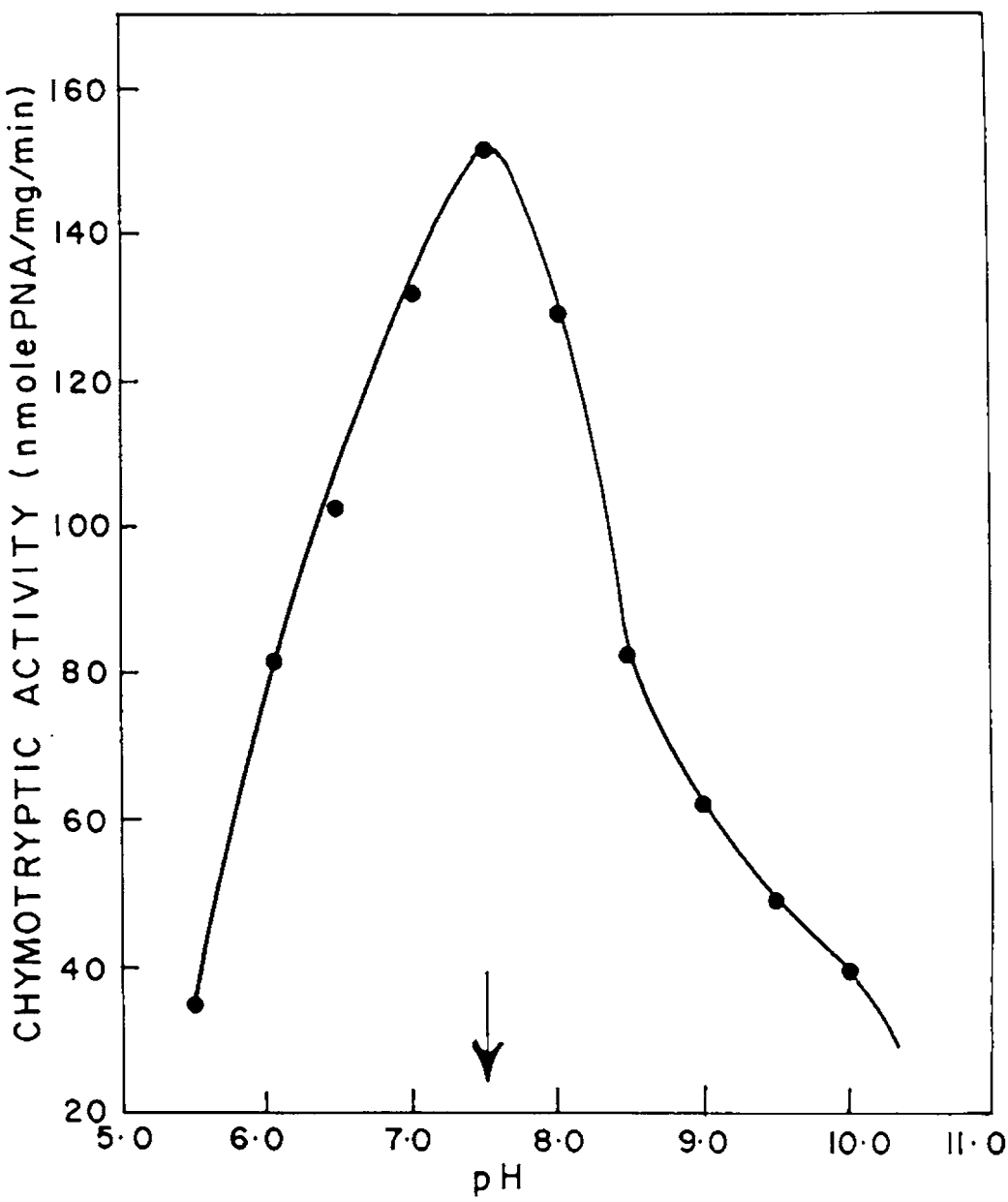

Fig.9 Effect of pH on intestinal chymotryptic activity of L. parsia

(Kalac, 1976, 1978a,b), carp (Cohen et al., 1981a,b), greenland cod (Simpson and Haard, 1984a,b,c), catfish (Yoshinaka et al., 1984b) and eel (Yoshinakka et al., 1985e).

To understand the major peak at pH 9.3 to 10.0, elastase activity was assayed. The optimal activity assayed at pH 9.7 using elastin-orcein as substrate indicate that elastase possibly accounted for atleast some of the alkaline protease peak produced when casein was used as substrate.

Reports available on the elastase activity in fish include African lungfish (deHaen and Gertler, 1974) cat fish (Yoshinaka et al., 1983a, 1984a), dover sole (Clark et al., 1985b) and in several species of fish (Yoshinaka et al., 1985b). Among Mugilidae, Albertini- Berhaut and Alliot (1978) found elastase activity in the alkaline range in the extracts of digestive tract of Mugil auratus and Mugil capito.

The presence of proteases which can remain active over a wide range of pH in L. parsia suggest that the process of protein digestion will not be affeected by minor fluctuation in the pH of the chyme. The fluctuation in the pH of the chyme may be caused by dilution of the same by the ingested diet or due to varied nature of the ingested diet at different times. Further, in the manufacturing of diets for culturing mullets, the pH of the diet may be maintained over a broad range since the enzymes involved in the digestive process can be active over a wide pH range. Adjustment of the pH of the food to optimal pH of digestive enzyme activity was emphasized by Ceccaldi (1982). It appears in L. parsia that the pH of the diet

over a range will not be a limiting factor in the process of digestion.

The temperature - activity profile of the protease in the crude extracts from the anterior intestines is shown in Fig. **7** . A broad peak of activity could be recorded for temperature between 47°C and 60°C. The temperature activity profile of protease in mullet is similar to that observed in other teleosts. A temperature optimum of 45°C was observed for three alkaline proteases of sardine (Murakami and Noda, 1981). In milk fish, the protease had a broad activity peak between temperatures 45°C and 60°C (Benitez and Tiro, 1982). The proteolytic enzymes studied by Jonas et al. (1983) in carnivorous (Silurus glanis), herbivorous (Hypophthalmichthys molitrix) and omnivorous (Cyprinus carpio) fishes exhibited optimum temperatures in the range of 53°C, 40°C and 55°C respectively.

The proteases from the intestines of Salmo salar and Salmo gairdneri had temperature optimum between 45°C and 52.5°C (Torrissen, 1984). Clark et al. (1985a) determined optimum temperature for both acidic and alkaline proteases from the whole gut extracts of dover sole. They observed that while the optimum temperature was about 40°C for acid protease, it was at 50°C for alkaline protease. Recently, Uys and Hecht (1987) investigated the temperature dependence profiles of protease in Clarias gariepinus and found that the optimum temperature was around 40°C. This profile has, however, a wider base than profiles drawn for Salmo gairdneri (Kitamikado and Tachino, 1960b), Clupea harengus (Kalac, 1978a,b) and Cyprinus carpio and Hypophthalmichthys molitrix (Jonas

et al., 1983). The temperature optimum of the grey mullet L. parsia recorded in the present study is in conformity with above mentioned findings.

The protease of L. parsia was active over a wide range of temperature between 15°C and 60°C with maximal activity between 47°C and 60°C. This indicates that eventhough these extremes of temperature may not be encountered in nature, the fishes posses the mechanism to maintain their activity in these temperatures. Since the body temperature of fish will be very much influenced by the environmental temperature (poikilothermic), the fluctuations in the environmental temperature and thus in the fish's body temperature will not affect the digestive process as the enzymes are active over a wide range of temperature. It is interesting to note in this respect that mullets are eurythermal (Bardach et al., 1972) a character much desired for a candidate species in aquaculture.

Effect of inhibitor substances on the activity of alkaline protease, trypsin and chymatrypsin was conducted using PMSF, TLCK, TPCK, EDTA and SBTI.

Data presented in Table-13 indicate that PMSF could inhibit the activity of total protease (80% inhibition), trypsin (74% inhibition) and chymotrypsin (77% inhibition) significantly at a minimal concentration of 10^{-4} M.

TLCK inhibited total protease (65%) and trypsin (97%) activities significantly at 10^{-4} M concentration whereas the chymotryptic activity was not inhibited by this inhibitor. In contrast to TLCK,

Table-13: Effect of some inhibitors on total proteolytic, tryptic an chymotryptic activities of *L. parsia*.

Inhibitor	Concentration	Percent inhibition		
		Total proteolytic activity	Trypsin	Chymotrypsin
Without		0	0	0
PMSF	10^{-4} M	80	74	77
	10^{-3} M	85	100	99
TLCK	10^{-4} M	65	97	0
	10^{-3} M	60	100	0
TPCK	10^{-4} M	40	0	79
	10^{-3} M	34	0	97
EDTA	10^{-3} M	0	1	0
	10^{-2} M	12	0	0
SBTI	25 g	84	92	83
	50	98	100	98

TPCK inhibited the activities of total protease moderately (40%) and chymotrypsin significantly (79%) at 10^{-4} M concentration when compared to the trypsin which was not inhibited by this inhibitor. On the other hand, EDTA could not inhibit activities of these enzymes at the maximal concentration tested (10^{-2} M). In the case of SBTI, enzyme inhibition could be observed at a significant level for total protease, trypsin and chymotrypsin at concentration of 25 microgram.

Similar reports on PMSF inhibition on endoproteases of teleost fishes were made earlier on Carassius auratus (Jany, 1976); Mallotus villosus (Hjelmeland and Raa, 1982); Gadus ogac (Simpson and Haard, 1984b); Parasilurus asotus and Anguilla japonica (Yoshinaka et al., 1984b, 1985e). PMSF has been described by various workers as serine protease inhibitor which react with essential serine residue at the active centre of these proteinases (Gold and Fahrney, 1964). In the present study both trypsin and chymotrypsin has been categorised as serine proteases since they are inhibited by PMSF. Serine proteases are remarkable in that they are widely distributed among animal kindgom ranging from the lower invertebrates to the higher mammals (Neurath et al., 1970; Neurath, 1984). Hartley et al. (1965)remarked that the sequence around this serine residue is remarkably constant. It would seem that these proteins would have been arosen by multiplication of some common ancestral gene, followed by independent mutations to allow divergence of sequence, and ultimately of enzyme specificity.

SBTI and TLCK inhibited the tryptic activity significantly (Fig. 10

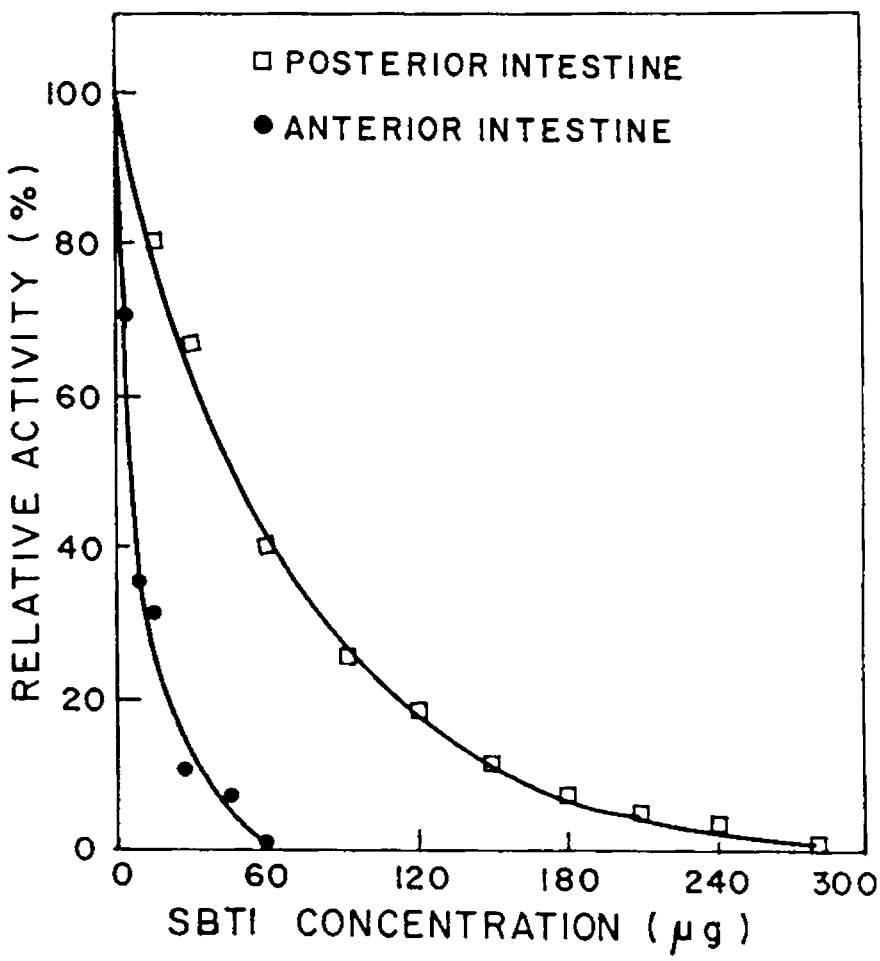

Fig.10 Effect of SBTI on intestinal tryptic activity of L. parsia

and Table - 13). While the tryptic activity of the extracts of anterior intestine could be completely inhibited at a concentration of 60 microgram of SBTI, the extracts of posterior intestine required 280 microgram of SBTI to get completely inhibited. Inhibition of trypsin by SBTI was shown in many species of teleosts as follows:

Rastrelliger Kanagurta and Etroplus suratensis (Sundaram and Sarma, 1960a, b);Oncorhynchus tshawytscha (Croston, 1960, 1965); Gadus morhua (Overnell, 1973); Carassius auratus gibelio (Jany, 1976); Cyprinus carpio (Cohen et al., 1981b); Chanos chanos (Benitez and Tiro, 1982); Gadus ogac (Simpson and Haard, 1984b) Parasilurus astous and Anguilla japonica (Yoshinaka et al., 1984b, 1985e).

Blow et al., (1974) have shown that SBTI's are proteins which bind strongly to trypsin, blocking its active site in the process. A similar mechanism might be involved in the process of inhibition of trypsin in L. parsia by SBTI.

The inhibition of trypsin by TLCK as observed in the present investigation was also reported for many species of teleost fishes investigated such as Scomber scombrus, Clupea harengus and Mallotus villosus (Kalac, 1975, 1978a,b); Sardinops melanosticta (Murakami and Noda, 1981); Cyprinus carpio (Cohen et al.,1981b); Parasilurus asotus and Anguilla japonica (Yoshinaka et al., 1984b, 1985e).

TLCK is a very specific inhibitor for bovine trypsin, reacting with histidine in the active centre (Shaw et al., 1965). The inhibition

of trypsin in L. parsia by TLCK shows that histidine residues are involved in the active centre of its trypsin also.

Based upon the pH, temperature activity profile and the inhibition studies, the trypsin in L. parsia can be categorized as a serine proteinase (as inhibited by PMSF) (Gold and Fahrney, 1964) with histidine as the active centre, (as inhibited by TLCK) (Shaw et al., 1965) as observed in other groups of fish.

The chymotryptic activity of the extracts of the anterior intestine would be completely inhibited at a concentration of 150 micro mole of TPCK while the extracts of the posterior intestine required double the amount of inhibitor, as required for anterior intestine extracts, for complete inhibititon. (Fig.11). Inhibition of chymotryptic activity by TPCK was also reported in other groups of fish such as Scomber Scombrus, Clupea harengus and Mallotus villosus (Kalac, 1975 , 1978a,b); Carassius auratus gibelio (Jany. 1976) Sardinops melanosticta (Murakami and Noda, 1981) and Chanos chanos (Benitez and Tiro, 1982).

TPCK specifically and completely inhibits mammalian chymotrypsin by alkylation of a histidine residue in the active site of the enzyme (Schoellman and Shaw, 1963; Neurath, 1984). In the present study, inhibition of chymotrypsin by TPCK indicates that its active site may be structurally similar to the mammalian enzyme and the inhibition by PMSF also indicates the role of histidine and serine residues in the proteolytic process.

Effect of various metal ions on the activities of total protease,

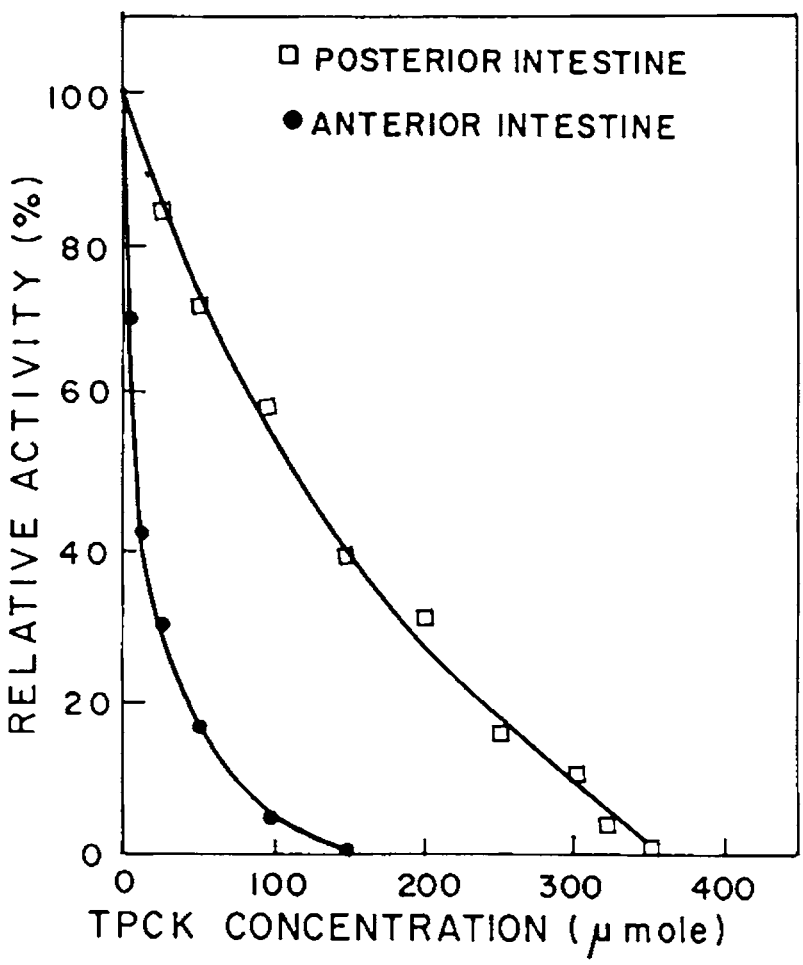

Fig.11 Effect of TPCK on intestinal chymotryptic activity of L. parsia.

trypsin and chymotrypsin in the extracts of anterior intestine was studied and the results are presented in Table-14. The dialysed intestinal extract and the metal ions were preincubated together, and then the residual activity was determined. All the metal ions showed inhibition of the activity at varied levels for different metal ions except Ca^{2+} which induced chymotrypsin activity marginally at 5mM concentration. Of all the metal ions tested, Cu^{2+} could record almost complete inhibition of all the enzymes tested when compared to Hg^{2+} and Ni^{2+} which recorded significant levels of inhibition at 1mM concentration. Among Co^{2+}, Mn^{2+}, Mg^{2+}, and Ca^{2+} which were tested at 5mM concentration, Co^{2+} alone could effect significant inhibition while others showed poor levels of inhibition. Similar results were obtained for the total protease, trypsin and chymotrypsin from Carassius auratus gibelio with respect to these metal ions (Jany, 1976). Most of the trypsins require calcium ions for their stability. In the present study, the absence of enhancement of enhancement of enzyme activity by calcium ion suggest that they might have remained bound to trypsin and hardly removed by dialysis. Yoshinaka et al. (1984b) experimentally demonstrated that calcium ions are required for the stability of trypsin in catfish and that calcium bound to it was not removed by dialysis. Similar observations have been reported for trypsins from chum salmon (Uchida, 1973).

In contrast, the stability and activity of another group of trypsin was found to remain unaffected in the presence of calcium ions. This group includes trypsins from the African lungfish, Protopterus aethiopicus (Reeck and Neurath, 1972), the capelin, Mallotus

Table 14: Effect of some bivalent - ions on total proteolytic, tryptic and chymotryptic activities of L. parsia.

Ion Concentration		Relative activity (%)		
		Total proteolytic activity	Trypsin	Chymotrypsin
Without	-	100	100	100
Hg^{2+}	1	19	5	10
Cu^{2+}	1	8	0	7
Ni^{2+}	1	40	36	24
Zn^{2+}	1	56	53	41
Co^{2+}	5	24	5	16
Mn^{2+}	5	86	88	98
Mg^{2+}	5	82	90	96
Ca^{2+}	5	87	91	108

villosus (Hjelmeland and Raa, 1982). Out of the two fractions of trypsin purified from pancreas of Anguilla japonica, one fraction required Ca^{2+} for stability whereas the other fraction did not require Ca^{2+} for its stability (Yoshinaka et al., 1985e). The activation of trypsinogen and chymotrypsinogen into their respective active forms were accelerated by $MgCl_2$ which completely inhibited the process in Parasilurus asotus (Yoshinaka et al., 1981a) and Anguilla japonica (Yoshinaka et al., 1984e). The results of the present investigation also show the strong inhibition of both tryptic and chymotryptic activities by heavy metals.

From the results obtained in this study it may be said that these proteases of L. parsia require the metal ions tested neither for enhancement of activity nor for maintaining stability. Instead, it appears that presence of these metals in the food may reduce these enzyme activities.

Leucine aminopeptidase

Results presented in Fig. 12 indicate that leucine aminopeptidase of the extracts of anterior intestine prefers pH 8.4 for its maximal activity under the assay conditions. Clark et al. (1987) reported that the leucine aminopeptidase in the digestive tract of dover sole preferred pH 8.3 to record maximal activity on leucinamide. The results obtained by Clark et al. (1987) are very much similar to those observed during the present investigation. Eventhough the substrate used in the present study is different from that of Clark et al. (1987), the optimal pH obtained for maximal activities are very close. Further, Albertini-Berhaut and

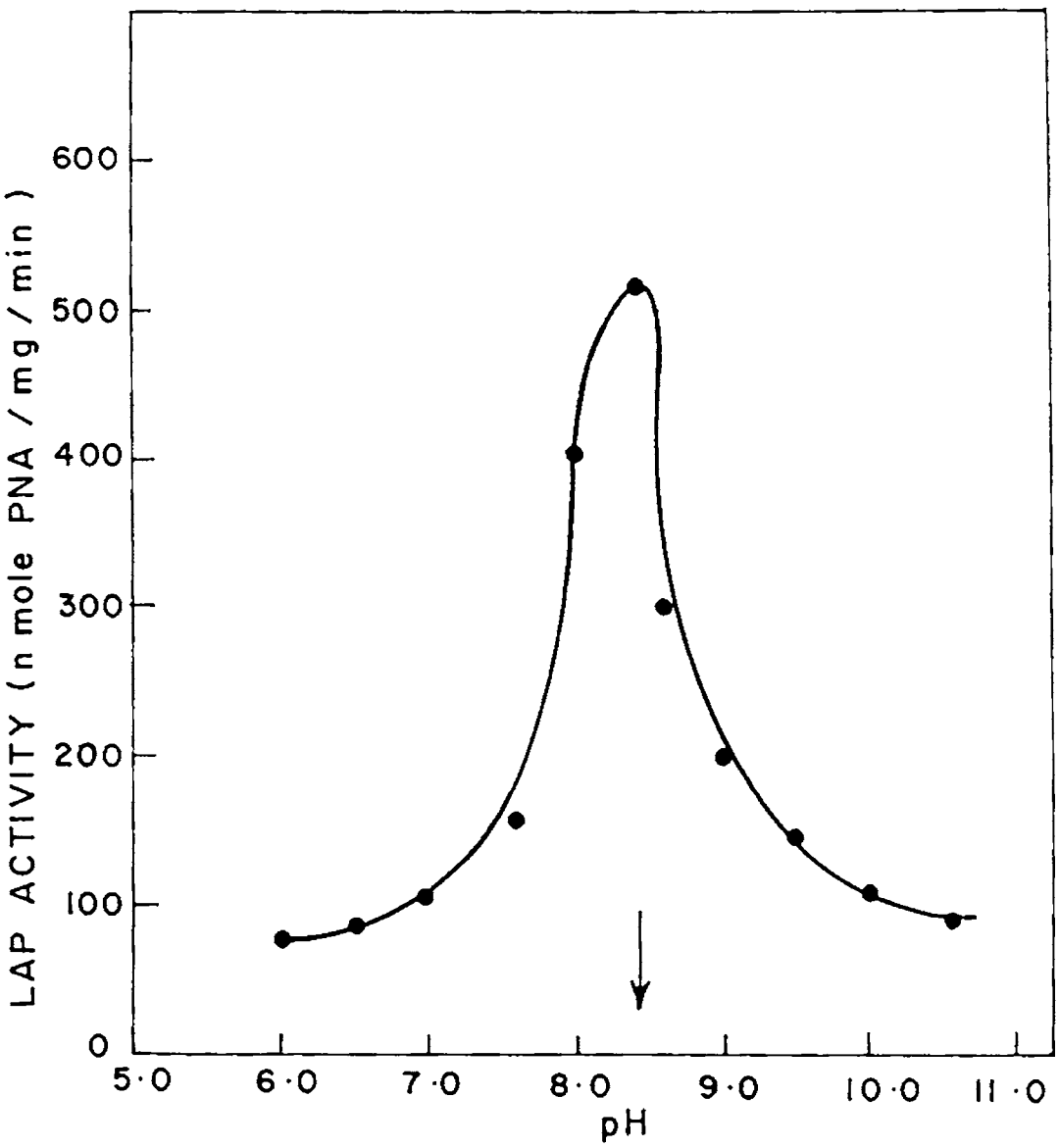

Fig.12 Effect of pH on intestinal leucine aminopeptidase activity of L. parsia.

Alliot (1978) found a pH optimum of 7.5 for leucine aminopeptidase from mullets Mugil auratus and Mugil capito using L-leucyl-2-naphthylamide as a substrate. The leucine aminopeptidase from carp had a pH optimum of 7.4 (Khablyuk and Proskuryakov, 1983a,b).

In the present study, under the conditions tested the enzyme leucine aminopeptidase showed maximum activity at 52°C (Fig. 13). A similar temperature optimum for leucine aminopeptidase was also reported earlier for dover sole by Clark et al. (1987).

It is interesting to note that the pH optimum and temperature optimum of leucine aminopeptidase coincide well with those recorded for total protease with casein as substrate in the present study. This suggests that leucine aminopeptidase is a component of total alkaline protease systems in the digestive system.

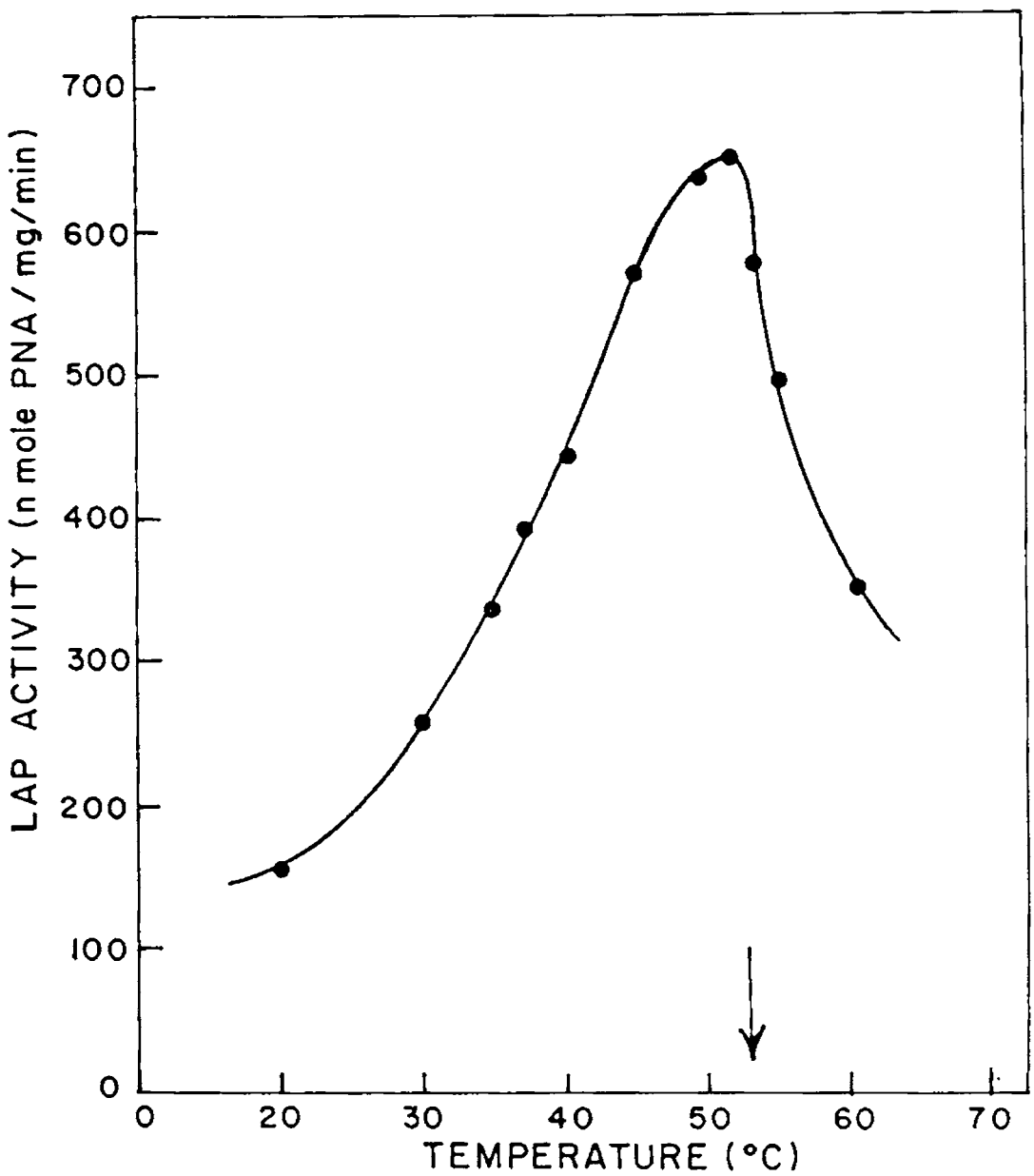

Fig.13 Effect of temperature on intestinal leucine aminopeptidase activity of L. parsia.

CHAPTER 5

DIGESTIVE ENZYME ACTIVITY IN RELATION TO SIZE OF THE FISH

5. DIGESTIVE ENZYME ACTIVITY IN RELATION TO SIZE OF THE FISH

1 Introduction

Acquisition of sufficient nutrients to satisfy the metabolic requirements is dependent on the ability of an organisam to select and subsequently digest suitable feeds. Therefore, the development of digestive functions can influence survival of fish in its early life history stages. At hatch, alimentary canals of teleost fishes are undifferentiated and generally remain stomachless throughout the larval phase. The digestive system which is one of the most important parts of a young and rapidly growing fish remains as simple as any other organ of the larvae (Storband and Dabrowski, 1979). The short and less differentiated intestine of the larvae is adapted to accept an animal diet, mainly zooplankton which is of high nutritive value and easily digestible. With the differentiation of the digestive tract the fish change gradually to their species specific feeding habits. Many changes results in the fish anatomy and physiological process along with gradual changes in the feeding habits imparting ability to survive and grow with changing feeding habits in the fish.

The gut of larval and early post-larval mullet is a simple loop. Jacot (1920), Bazzani (1932) and Hotta (1955) have described the transformation of this loop into a coiled one, typical of the adults. Bazzani accounts this change to the adoption of an iliophagus diet succeeding the planktivorous diet of the young. The number of loops in the intestine gradually increases with age upto sexual maturation (Suyehiro, 1942). Correspondingly, there occurs a

change of diet in young Mugilidae starting from about 2.5 cm in standard length (Albertini-Berhaut, 1973, 1985, 1986). Such changes in morphology of digestive system, feeding habits, and corresponding shift in nutritional requirements have been reported earlier in other fishes (Caloianu-Iordachel, 1966; Baranova and Mirshinichenko, 1969; Tanaka, 1971; Garling and Wilson, 1976; Detlef et al., 1981 and Buddington, 1983).

Digestive processes have been extensively studied in the terrestrial vertebrates, particularly the homoiotherms. The data from these studies have been applied to agricultrual research and have led to improvements of feed and husbandry technology. Nevertheless, the limited research has revealed that the digestive enzyme complement of fish are qualitatively similar to those of other vertebrates (Barnard, 1973) and they are influenced by age (Hofer, 1982; Buddington, 1985; Buddington and Doroshov, 1986a) and other factors (Hofer, 1979a,b) besides being influenced by dietary changes (Olatunde and Ogunbiyi, 1977; Reimer, 1982; Patra and Ray, 1987; Phadate and Srikan, 1987). In several species the larval period is characterised by a more or less drastic increase of enzyme activities (Tanaka et al., 1972; Kawai and Ikeda, 1973a, b; Dabrowski, 1979; Lauff and Hofer, 1984) and sometimes by changes of enzyme patterns (Mahr et al., 1983; and Lauff and Hofer, 1984). Nevertheless, little is known about the development of digestive enzyme activities from larvae to adult fish (Torrissen and Torrissen, 1984). Information on the changes in the digestive secretions and enzyme activities are mandatory to draw an insight on the larval digestive mechanisms which consequently assist in the

studies of feeding habits and nutritional requirements. This knowledge can also be applied towards improvement of larval rearing and growout culture of fish by providing appropriate feed in different growth stages.

This study was aimed at monitoring and recording changes in digestive functions of L. parsia during different growth stages.

5.2 Material and Methods

5.2.1 Animals

The fishes used in this study were grouped into five different size groups according to total length viz. (1) 1 -2 cm (2) 3 - 4 cm, (3) 6 - 8 cm (4) 11 - 12 cm and (5) above 15 cm; the fifth size group collected from the chinese dipnet catches along the Vypeen Island near the Cochin Bar mouth area. The rest of the four size groups were collected from the shallow canals and creeks in the Fisheries Experimentation Station of Kerala Agriculture University at Puduveypu in Vypeen island by cast nets and small hand nets. Immediately after catch, the fishes were transferred into polythene bags and transported to the laboratory under iced condition and kept in deep freezer for subsequent studies.

5.2.2 Preparation of crude enzyme extract

The fishes were weighed, total length measured and then dissected out to separate the digestive tract. For all the fish groups the whole gut was used to prepare the crude enzyme extract. For the group I, (1-2 cm total length) about 500 numbers of fry were dissected out and the whole gut was separated and pooled to prepare

the enzyme extract. For the group II (3-4 cm total length) about 200 fingerlings were used. For the III (7-8 cm total length), IV (11-12 cm total length) and V (total length above 15 cm) groups 30 fish each were used. In the latter 3 groups the whole gut from individual fish were used separately. The whole gut, either pooled from respective size groups or separately used, were washed gently with ice cold double distilled water, blot dried and weighed. The guts were homogenized using double distilled water with an Potter - Elvehjem homogeniser to give a 1:10 (W/v) homogenate. The homogenates included the lumen contents also. The homogenates were then centrifuged at 19000 g, at 4°C, and the clear supernatants, designated as crude enzyme extract, were used for enzyme assays with appropriate dilutions as required by specific enzyme assays. The protein content of the crude enzyme extract was determined by the Lowry's method with bovine serum albumin as the standard (Lowry et al., 1951).

5.2.3 Enzyme assays

The enzymes assayed include amylase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, pepsin, total protease, trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B, leucine aminopeptidase, esterase, acid phosphatase and alkaline phosphatase. All these enzymes were assayed as per the detailed methods given elsewhere (Section 2.6)

5.2.4 Statistical analysis

Polynomial regressions computed for a 'best fit' were used to

describe the relationship between the digestive enzyme activities and size of the fish.

5.3 Results

The different enzymes such as amylase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, pepsin, total protease, trypsin, chymotrypsin, carboxypeptidases A and B, leucine aminopeptidase, esterase, acid and alkaline phosphatases were analysed in different size groups of *L. parsia* ranging from 1-2 cm to 19 cm in total length. All the enzymes were assayed at an optimum pH.

The amylase activity showed a direct relationship with the size of the fish by registering increased activity along with increase in the size of the fish (Fig. 14). Polynomial fit computed for fourth degree had an r^2 of 0.947. The amylase activity which was 95 units in I size group was observed to increase to 437 units in V size group.

The activity of alpha-glucosidase registered a rise along with increase in size of the fish (Fourth degree polynomial fit; r^2 = 0.829). The activity increased from 26 units (I group) to 126 units (V group) (Fig. 15). Beta-glucosidase activity increased from 18 units (I group) to 90 units (V group) (Fig. 16, third degree polynomial fit; r^2 = 0.79). The activity of beta-galactosidase also showed a uniform rise with increase in size (Fig. 17, third degree polynomial fit, r^2 = 0.66). The activity ranged from 55 units (I group) to 162 units (V group).

In general, peptic activity was observed to increase along with

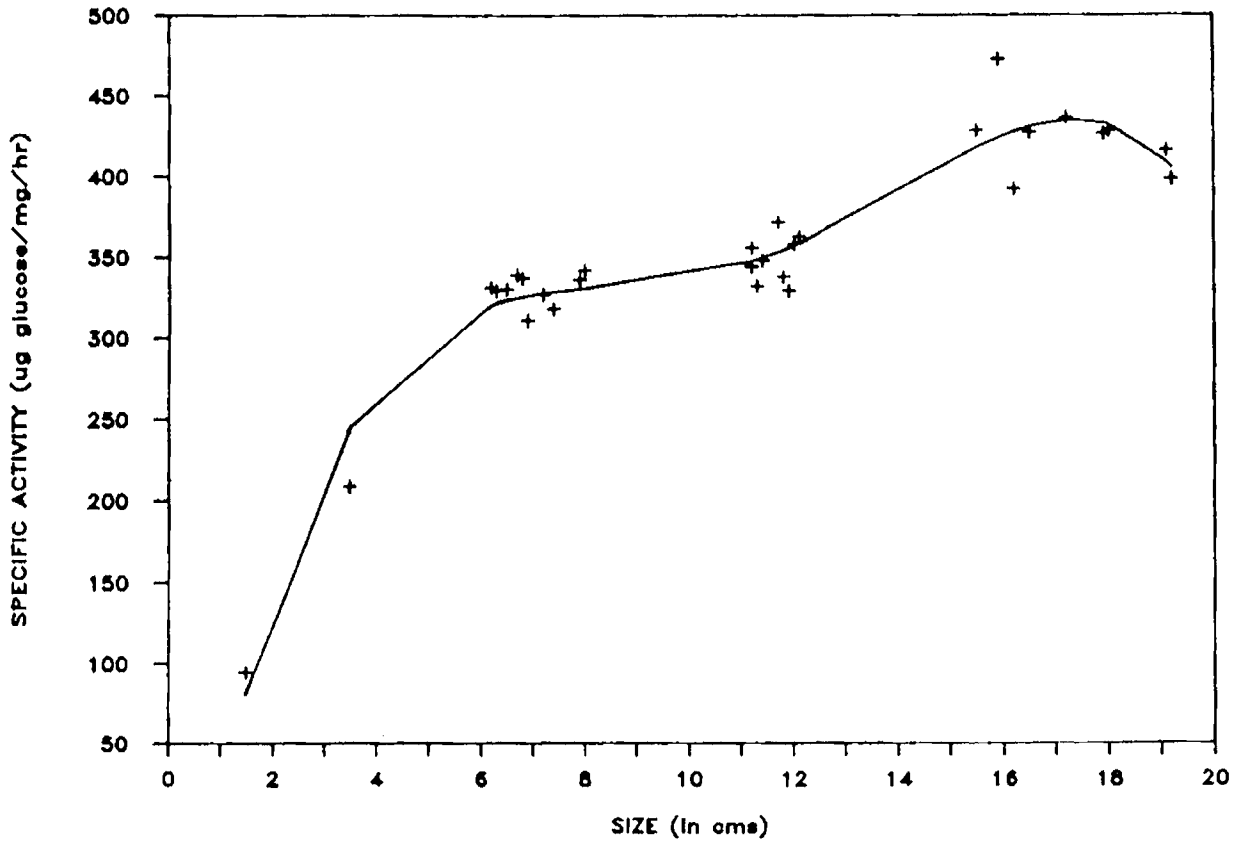

Fig.14 Polynomial regression curve showing the relationship between amylase activity and size of the fish.

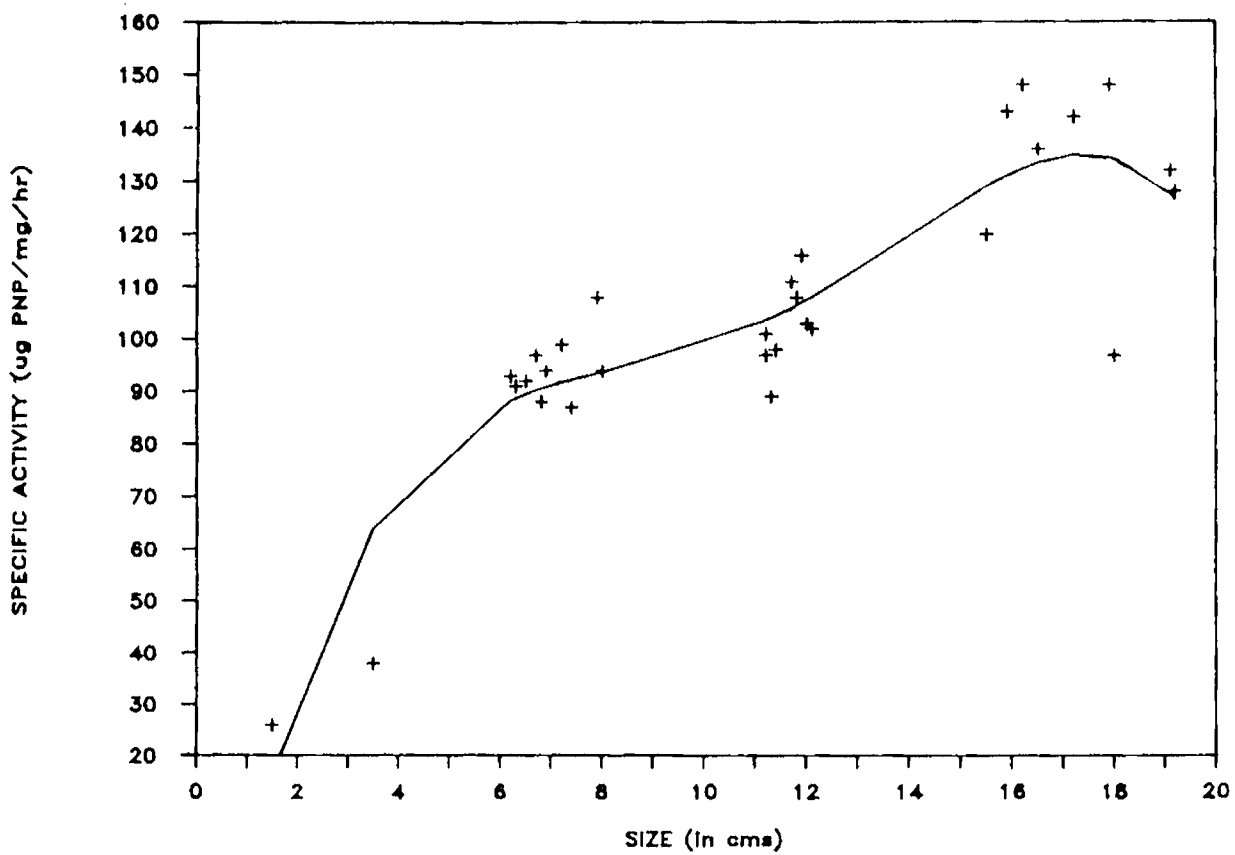

Fig.15 Polynomial regression curve showing the relationship between alpha-glucosidase activity and size of the fish.

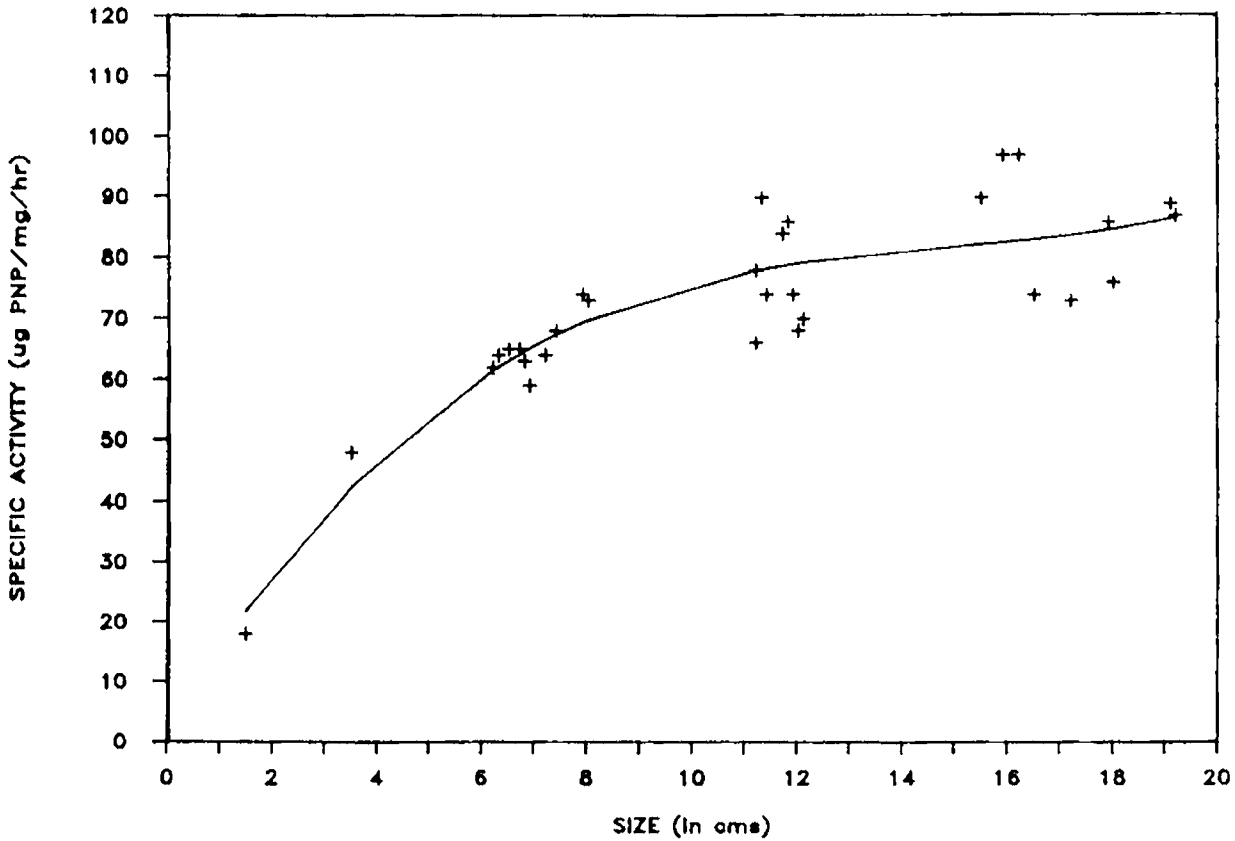

Fig.16 Polynomial regression curve showing the relationship between beta-glucosidase activity and size of the fish.

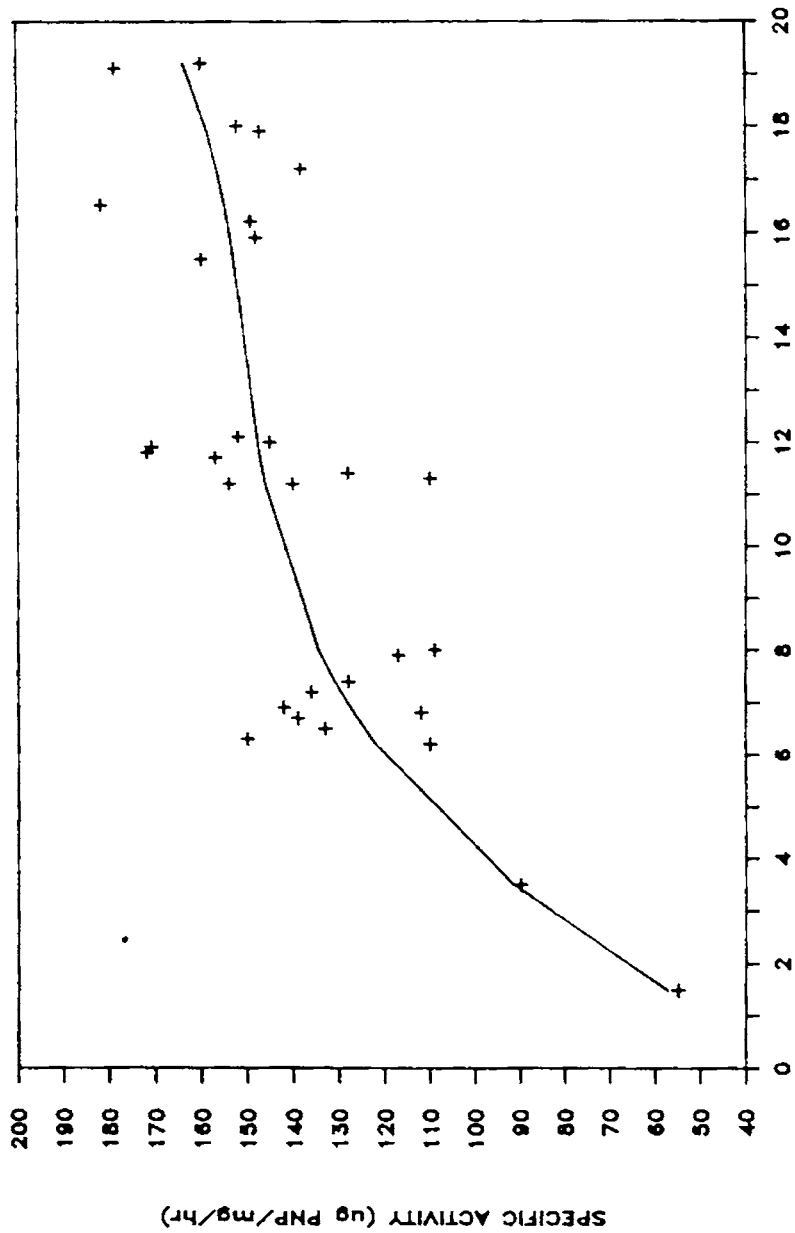

Fig.17 Polynomial regression curve showing the relationship between beta-galactosidase activity and size of the fish.

increase in fish size (Fig. 18) (r^2 = 0.834, fifth degree polynomial). There was no difference between I and II groups whose activities were 1.40 and 1.41 units respectively. However, in the III group the activity dropped to a minimum of 0.891 units. In the fourth and fifth groups, the activity raised to 2.0 units and 1.83 units respectively indicating an overall increase in activity when compared to groups I and II.

The total alkaline protease activity showed a different trend from that of pepsin. The proteolytic activity showed a decreasing trend along with increase in the size of fish (Fig. 19, r^2 = 0.753). It was observed that while I and II group of fishes recorded increased enzyme activities of 4 units and 12 units respectively, the III, IV and V groups gradually declined their enzyme activity with increase in size of fish.

The activity of trypsin measured with specific synthetic substrate followed a similar profile as that of total alkaline protease. Except for the initial increase from 80 units of I group to 410 units of II group the activities reccorded for the larger size groups showed a decreasing trend along with increase in size (143 units in IV and V groups) (Fig. 20, r^2 = 0.932).

However, the activity of chymotrypsin exhibited a different trend from that of the total protease and trypsin by recording increased activity along with increase in fish size. The activity increased from 32 units (I group) to 123 units (IV group) (Fig. 21, r^2 = 0.616).

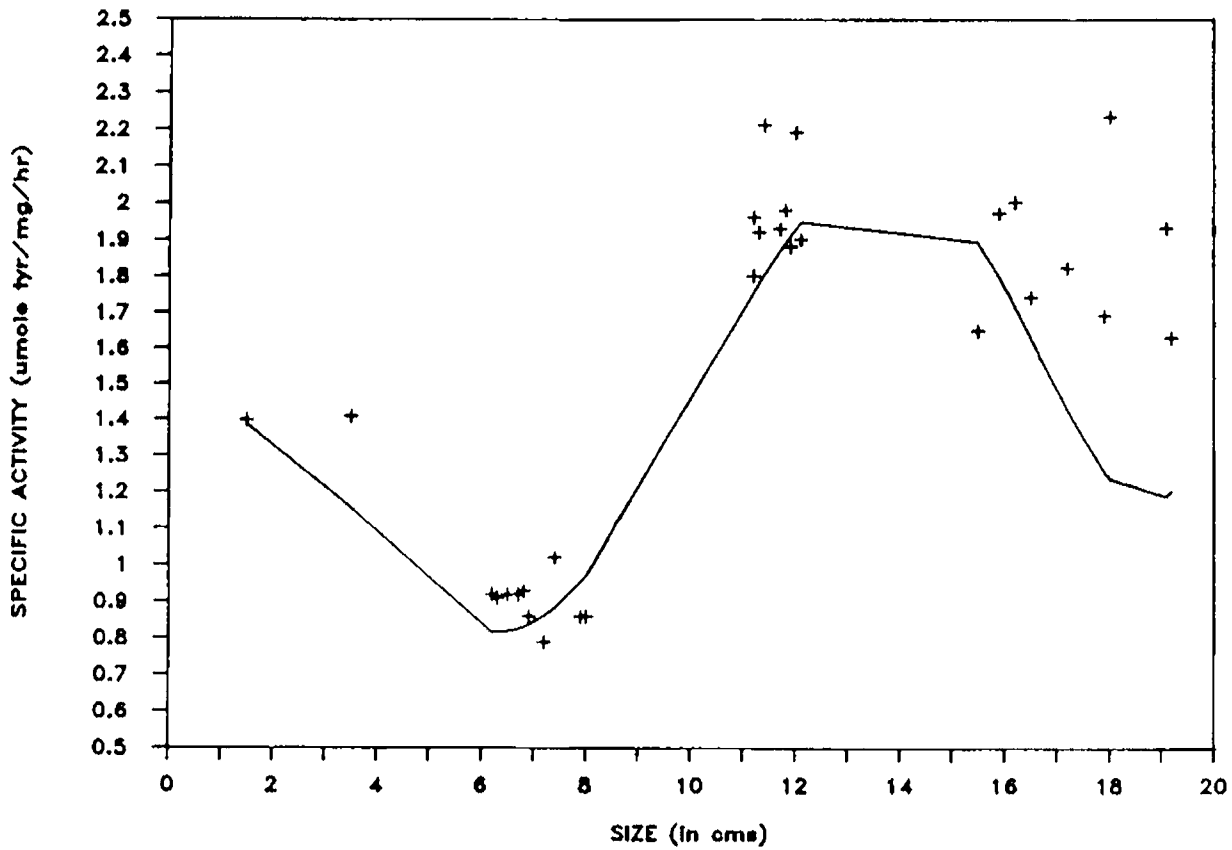

Fig.18 Polynomial regression curve showing the relationship between peptic activity and size of the fish.

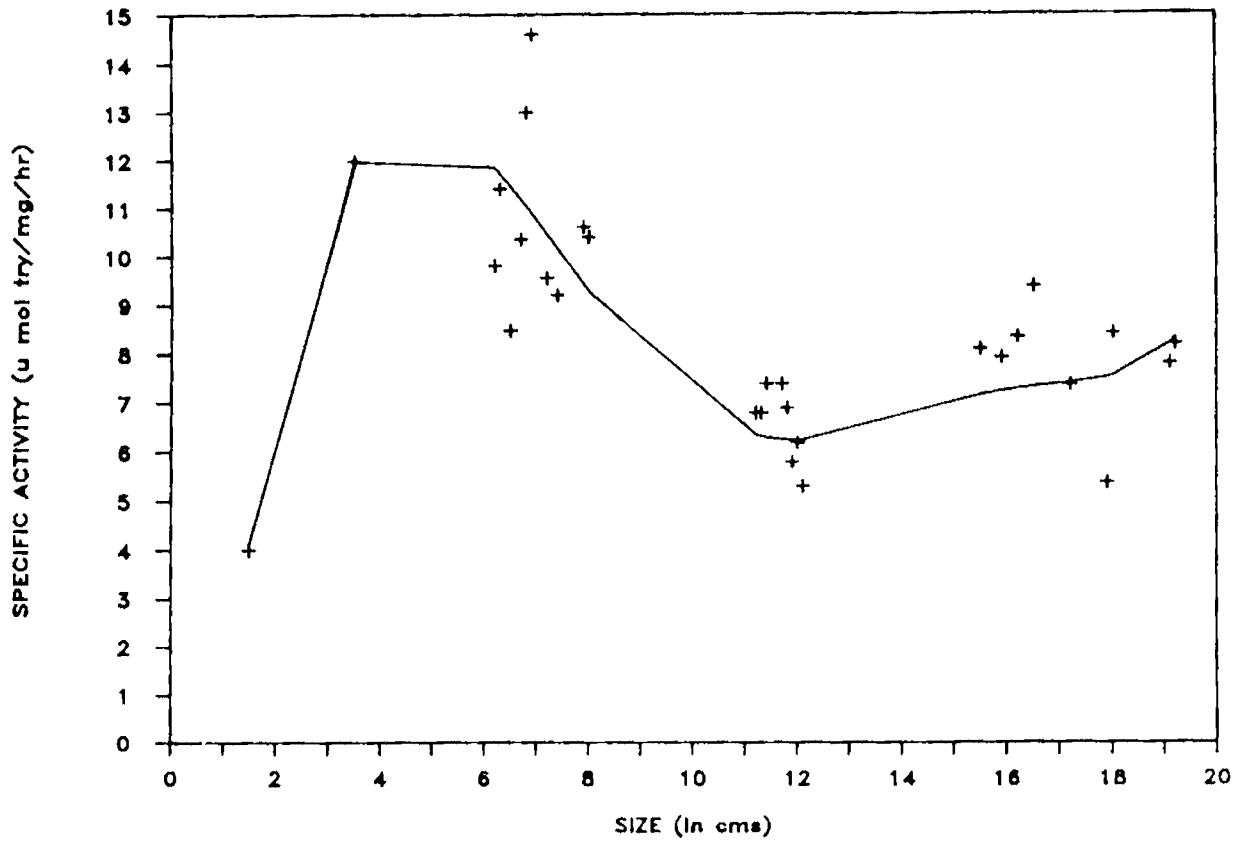

Fig.19 Polynomial regression curve showing the relationship between protease activity and size of the fish.

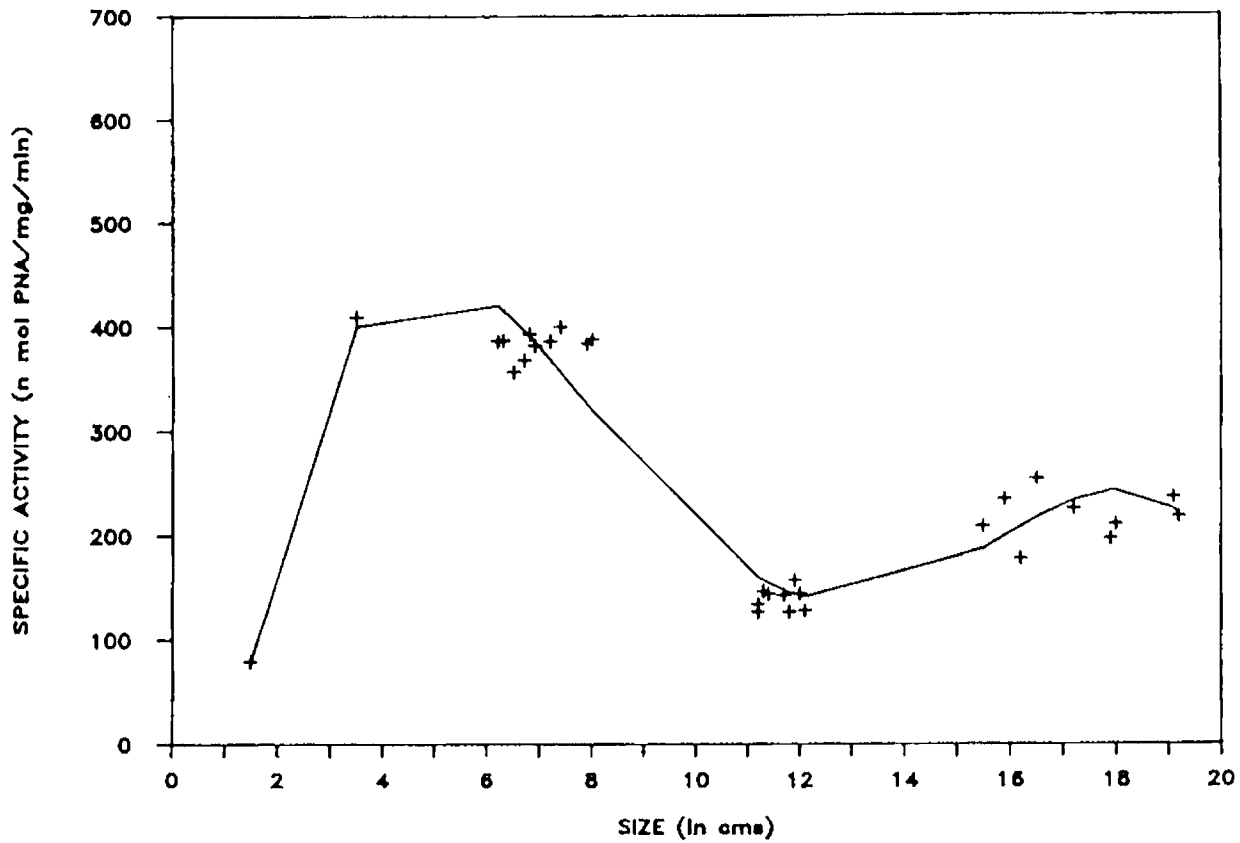

Fig.20 Polynomial regression curve showing the relationship between tryptic activity and size of the fish.

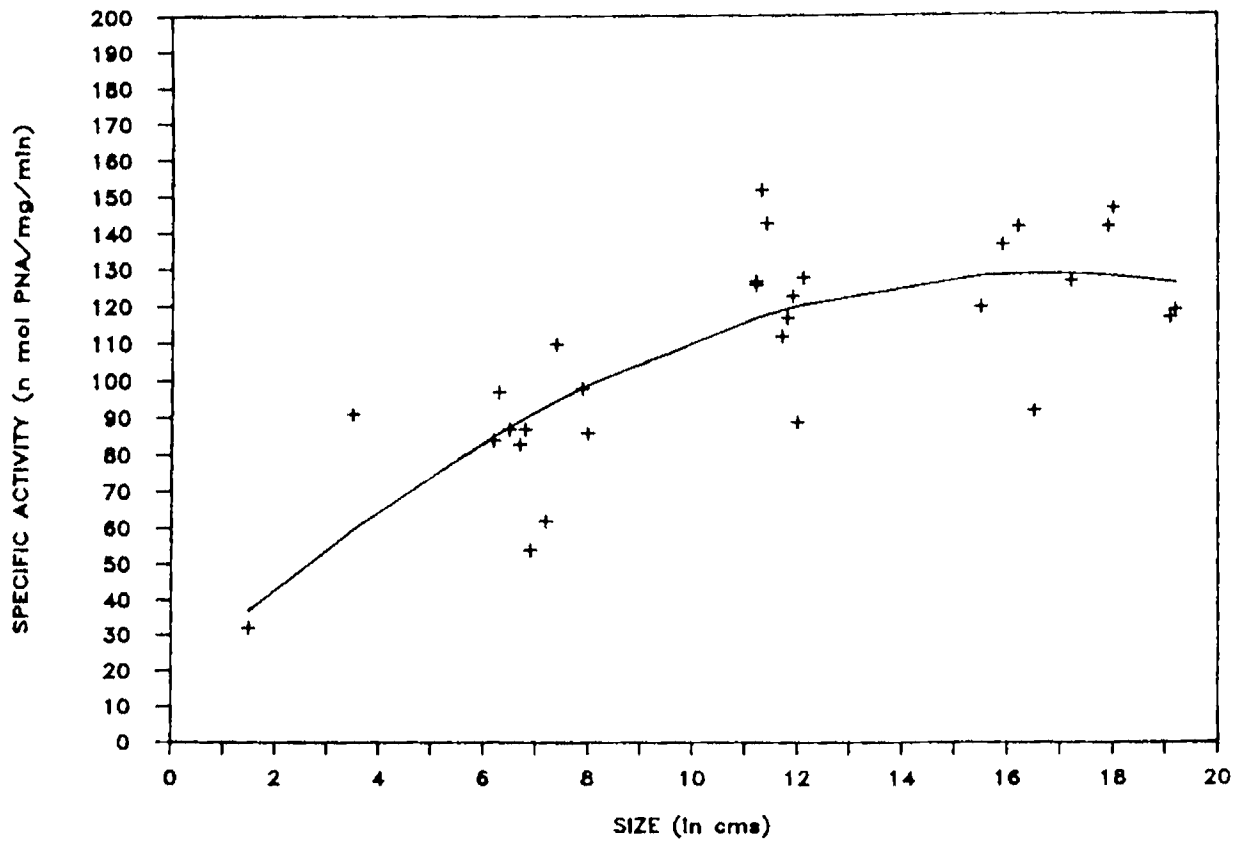

Fig.21 Polynomial regression curve showing the relationship between chymotryptic activity and size of the fish.

The activities of carboxypeptidase A and carboxypeptidase B decreased as the size increased and showed a poor significant correlation with size. The carboxypeptidase A activity showed an initial increase from 0.005 units (I group) to 0.23 units (II group) and gradually decreased subsequently and remained stabilized at 0.15 units in adults (Fig.22, fourth degree polynominal r^2 = 0.73). Similarly, the actvity of carboxypeptidase B also showed an initial increase form 0.001 in I group to 0.007 units in II group and afterwards gradually decreased to 0.004 units in adults and remained constant (Fig. 23, r^2 = 0.53).

Leucine aminopeptidase activity showed a consistent decreasing trend as the size increased (Fig. 24, r^2 = 0.956). The activity showed a sharp decline from 380 units in I group to 282 units in II group. In larger size groups there was a gradual decline in the activity (144 units in the V group).

The activity of non-specific esterase showed consistent increasing trend along with increase in size of the fish (Fig.25, r^2 = 0.683).

The activities of acid phosphatase and alkaline phosphatase showed different trends. Acid phosphatase activity rose steeply from 2.28 units in I group to 12.37 units in the III group, and further increase in size of the fish led to gradual decline in the activity (8.5 units in adults) (Fig. 26, r^2 = 0.622). The alkaline phosphatase activity increased along with increase in size of the fish (Fig. 27, r^2 = 0.967). The activity initially showed sharp rise from 56.0 units in Group I to 100.3 units in Group II and later plateaued with some increase in adult reaching 110.0 units.

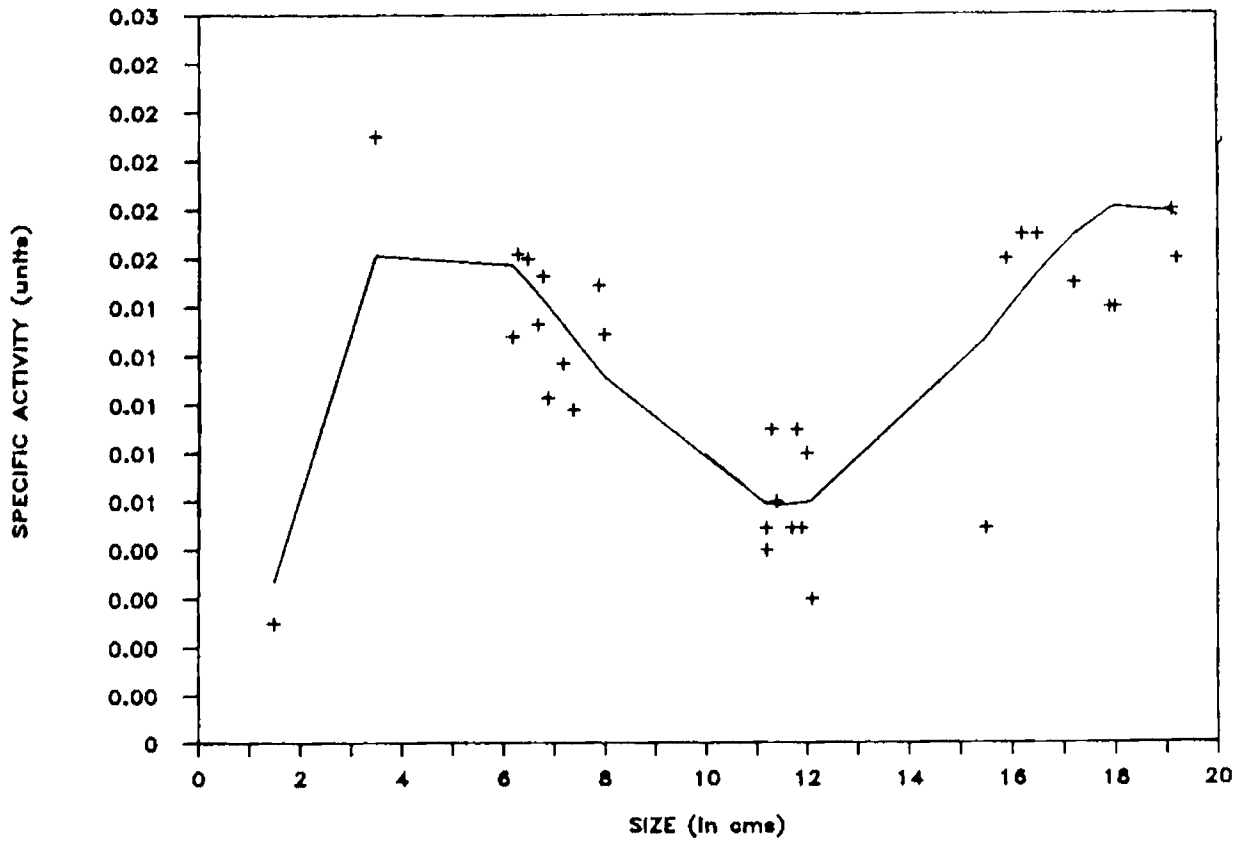

Fig.22 Polynomial regression curve showing the relationship between carboxypeptidase A activity and size of the fish.

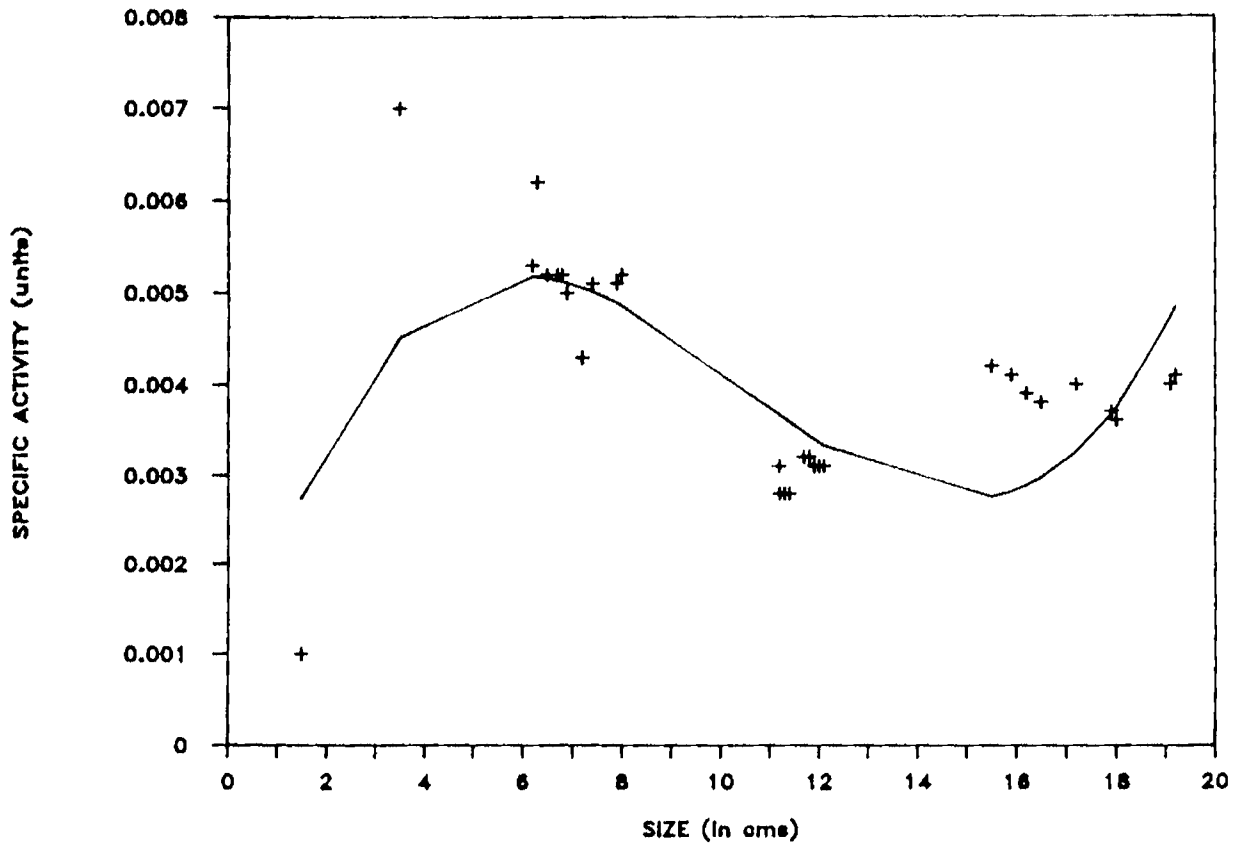

Fig.23 Polynomial regression curve showing the relationship between carboxypeptidase B activity and size of the fish.

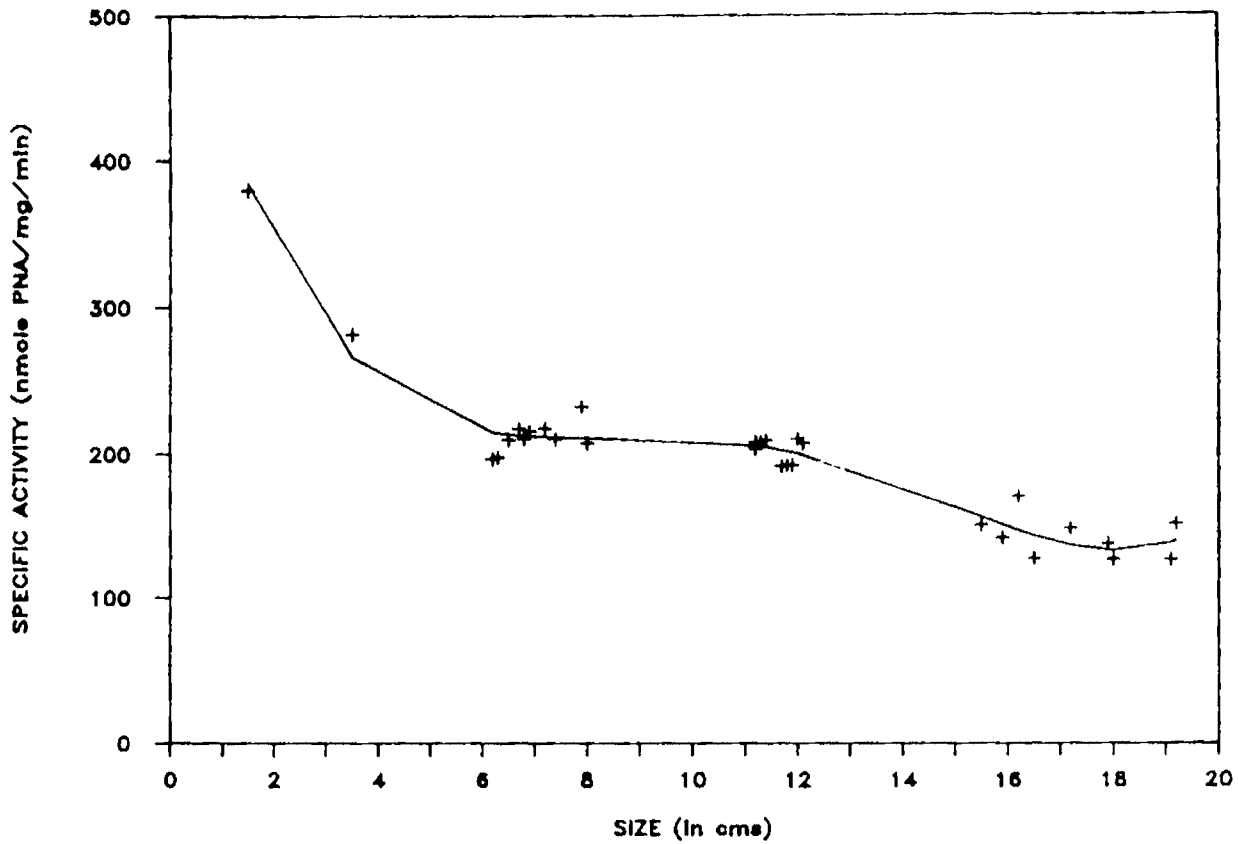

Fig.24 Polynomial regression curve showing the relationship between leucine aminopeptidase activity and size of the fish.

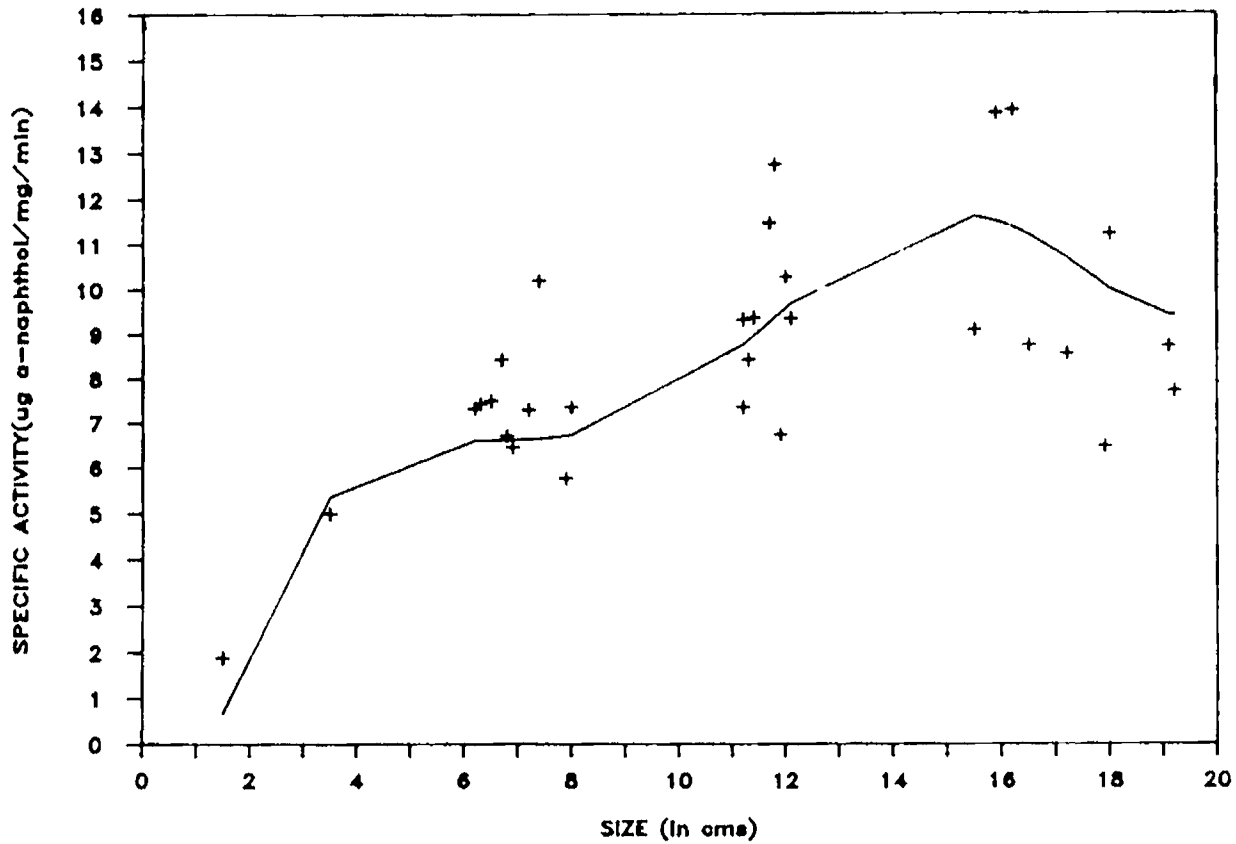

Fig.25 Polynomial regression curve showing the relationship between esterase activity and size of the fish.

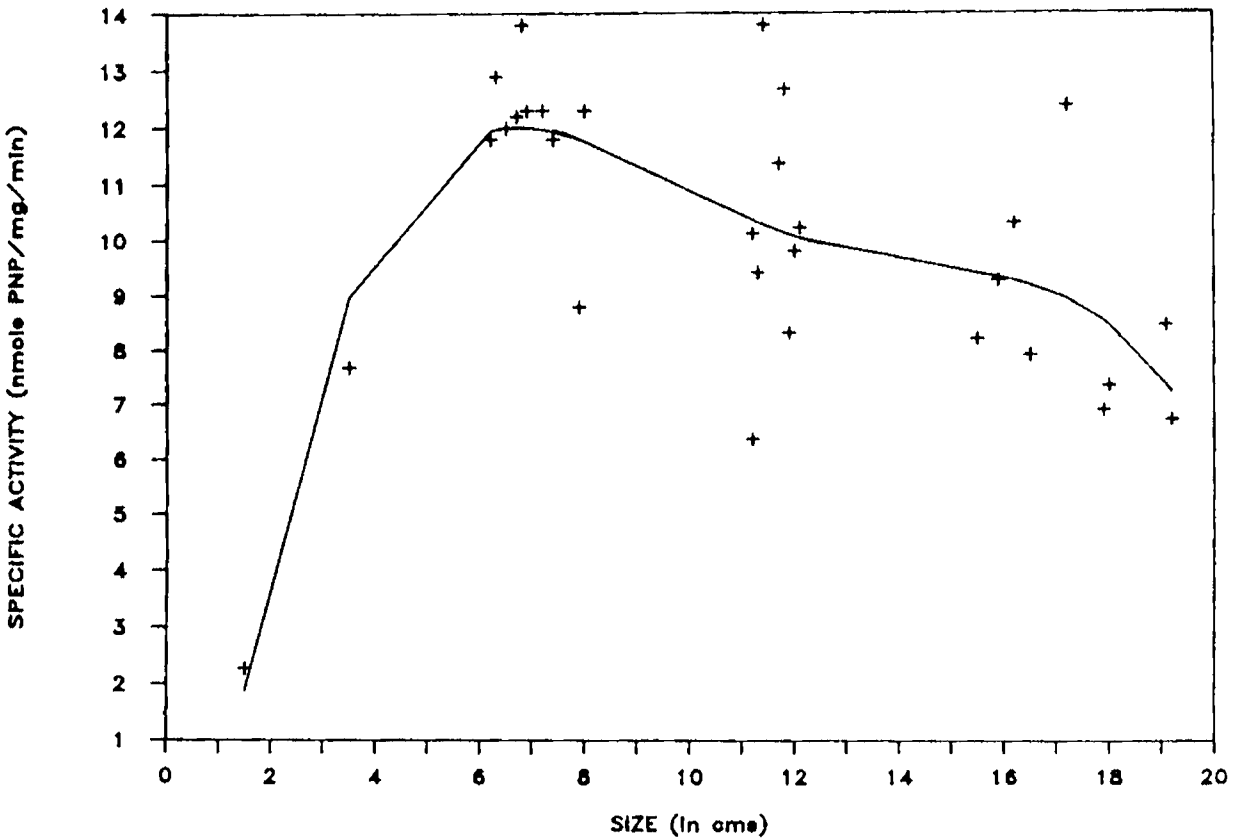

Fig.26 Polynomial regression curve showing the relationship between and phosphatase activity and size of the fish.

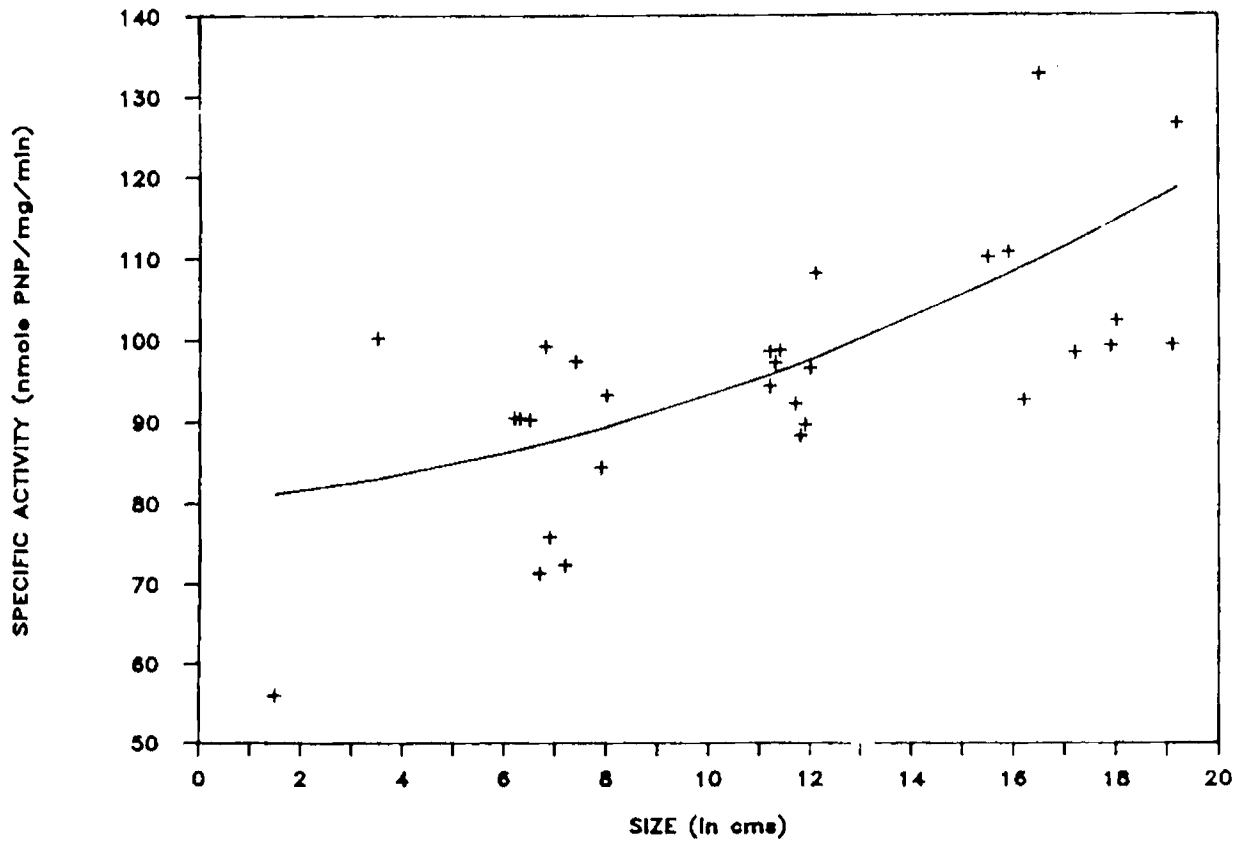

Fig.27 Polynomial regression curve showing the relationship between alkaline phosphatase activity and size of the fish.

5.4 Discussion

In the present study, different digestive enzymes, involved in the hydrolysis of carbohydrates, proteins, lipids and phosphates such as amylase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, pepsin, total protease, trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B, leucine aminopeptidase, esterase, acid phosphatase and alkaline phosphatase were assayed in different size groups of L. parsia. The main criteria for choosing different sizes of fishes was their availability. Since the larvae less than 1 cm in length were not available from wild, no attempt was to study this aspect on such group. Results of the present study indicate an increase in enzyme activity along with increase in fish size for amylase, alpha-glucoside, beta-glucosidase and beta-galactosidase. This is in agreement with the general pattern reported in most other studies. In Mugil capito, the activity of carbohydrase increased with size as reported by Richard et al (1982) who studied the changes in activities of 16 carbohydrases in 1.8, 6.3 and 15.0 cm size groups of M. capito and all the activities which were present showed positive correlation with increase in size. Similarly in other mullets such as L. aurata, L. saliens and L. ramada the existence of a positive correlation between amylase activity and size of the fish were reported (Albertini - Berhaut, 1986). The maltase and amylase activities in the digestive organs of rainbow trout, Salmo gairdneri increased with increase in size (Kawai and Ikeda, 1973a). Interestingly, even in carnivorous fishes the carbohydrases increased with increase in size. Similarly, in the carp Cyprinus carpio and black sea bream

Acanthopagrus schlegelii also the maltase and amylase activities increased as the fish grew larger in size (Kawai and Ikeda, 1973b).

Kuzmina (1980) observed an inverse relationship between amylase activity and size of the bream, Abramis brama. In the lake sturgeon Acipenser flurescens, (Buddington, 1985) and white sturgeon Acipenser transmontanus (Buddington and Doroshov, 1986a) also, the amylase activity, which was highest during larval feeding phase, declined in concentration as the larvae metamorphosed and grew further.

However, the direct positive relationship between increased enzyme activity and increase in size in L. parsia and related groups assumes a vital role for these enzymes in the digestive processes of these fishes which take more carbohydrates in their diet as they increase in size.

The activity of pepsin showed irregular patterns in the increasing trend along with increase in size. Similar pattern was also reported for rainbow trout, Salmo gairdneri and bream, Acanthopagrus schlegelii (Kawai and Ikeda, 1973a, b), white sturgeon Acipenser transmontanus (Buddington and Doroshov, 1986a). In dover sole Solea solea (Clark et al., 1986) the peptic activity developed only in one year old juveniles and later increased with increase in size. Contrastingly in the carp Cyprinus carpio, which is a stomachless fish, pepsin did not show any increase along with increase in size, but decreased and disappeared in the adult (Kawai and Ikeda, 1973b).

The presence of high activity of pepsin in 1-2 cm and 3-4 cm size of L. parsia and sudden drop in activity in 6-8 cm size and then the gradual increase in activity may be attributed to the feeding habit of L. parsia. The high activity in the earlier period might be accomplished by the dietary exogenous enzymes. Further, the intake of zooplankers get reduced with growth of fish which is accompanied by a drop in peptic activity in 6-8 size group. By then, the stomach might have developed gastric cells which secrete pepsin and HCl, and facilitate digestion. The present observation is in conformity with the reports on the late development of gastric cells and the peptic activity observed in lake sturgeon (Buddington, 1985), white sturgeon (Buddington and Doroshov 1986a) and dover sole (Clark et al., 1986) and the role of exogenous enzymes in digestion in fish larvae (Jancarik, 1964; Dabrowski and Glogowski, 1977; Lauff and Hofer, 1984).

The total alkaline protease activity assayed with casein as substrate showed a significant negative correlation with size of the fish. Except for the initial increase in proteolytic activity in the smaller size groups the activity evinced decrease with size. A similar pattern of declining protease activity was also reported in other species of Mugilidae. In Mugil capito all the 34 different proteolytic activities, studied in three different size groups viz. 1.8, 6.3 and 16 cm, all the activities which were present showed a decreasing trend along with increase in size (Richard et al., 1982). More recently, Albertini-Berhaut (1986) showed that in Liza aurata, L. ramada and L.saliens the proteolytic activity was negatively correlated with size of the fish.

Other proteases assayed include trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B and leucine aminopeptidase. All these proteases were assayed on specific substrates since casein is a non-specific substrate for these enzymes. All the enzymes tested showed a similar trend like that of total proteolytic activity except chymotrypsin which increased in its activity along with fish size.

Except for these observations and the present one on L. parsia, majority of other studies indicated that the proteolytic activity increased along with increase in size of the fish. In the rainbow trout, Salmo gairdneri (Kawai and Ikeda, 1973a) protease activity increased gradually corresponding to the growth. A similar observation was made for carp Cyprinus carpio and bream Acanthopagrus schlegelii (Kawai and Ikeda, 1973b). Fishes such as Coregonus sp., Salmo gairdnei and Rutilus rutilus also showed an increase in the activity of trypsin, chymotrypsin and aminopeptidase with increase in size (Lauff and Hofer, (1984). In the lake sturgeon, Acipenser fulvescens (Buddington, 1985) and the white Sturgeon, A. transmontanus (Buddington and Dorsoshov. 1986a) trypsin and chymotrypsin continued to increase with increase in size of fish. In the dover sole, Solea solea the total proteolytic activity, trypsin, chymotrypsin, elastase, leucineaminopeptidase, carboxypeptidase A and B were reported to increase gradually and continuously as the fish increased in size (Clark et al., 1986).

The esterase activity of L. parsia exhibited an increasing trend with increase in size upto 11-12 cm and remained the same in

adults. While the esterase activity in the carp Cyprinus carpio increased with increase in size as observed in the present study, the activity declined in yellow tail, eel and rainbow trout (Morishita et al., 1964). Further, in lake sturgeon and white sturgeon also a decline in the activity of lipase was observed as the fish increased in size (Buddington, 1985; Buddington and Doroshov, 1986a).

The activity of acid phosphatase and alkaline phosphatase showed decreasing and increasing trends respectively along with increase in size of fish. However, the changes were not significant. Since there is no report on the activities of these enzymes with respect to size of fishes no comparison could be made.

The changes in the digestive enzyme profile can be attributed to the changes in feeding habits of L. parsia from larvae to adult. It has been reported that juveniles of most fish species are carnivores, feeding mainly on zooplankters. As they grow they assuem their species specific feeding habits. Such a situation has also been reported in mullets (Suzuki, 1965; Odum, 1970; Zismann et al., 1975; De Silva and Wijeyaratne, 1977; Ferrari and Chieregato, 1981). A change of the diet occurs in young Mugilids starting from 25mm in standard length; a mixed diet, more or less vegetal origin and depending on the species, succeeds the exclusively animal diet (Albertini-Berhaut, 1985, 1986). In the present study, the fishes of 1-2 cm size recorded higher protease and lesser carbohydrase activities. This might be due to the fact that a diet of zooplankters need more of proteases than carbohydrases to digest them. As the fish grows further the faunal components decrease in

the diet leading to a decrease in protease activity. Such a relationship between faunal components in diet and protease activity was described by Hofer (1979b) for Rutilus rutilus and Scardinius erythrophthalmus where both species had a higher proteolytic activity when feeding on animals when compared to detritus feeds. As the diet of plant origin succeeds that of animal origin, the carbohydrases assume a major role in digestion in adult L. parsia. The change in diet during the development of mullet is also associated with changes in gut morphology and anatomy, besides the changes observed in enzymes (Albertini-Berhaut, 1987, 1988). The gut of larval and early post larval mullet is a simple loop (Thomson, 1966). This evidences carnivorous feeding habit at this stage. As there occurs a shift in diet from predominantly carnivorous to herbivorous with diatoms and detritus forming the major components of diet, correspondingly the intestine increases in length and become coiled. Jacot (1920), Bazzani (1932) and Hotta (1955) have described the transformation of the simple loop of the intestine of larvae into convolutions typical of the adult. However, in mullets, the change in feeding habits observed in young stages has no effect on the histological, ultrastructural and cytophysiological aspects of the intestine other than an increase in its relative length significantly (Albertini-Berhaut, 1988). Further, the changes in digestive enzyme activities at different growth stages might reflect changes in nutritional requirement of the fish, as such a shift at different stages of growth has already been reported (Garling and Wilson, 1976).

The activity of chymotrypsin showed positive correlation with size in L. parsia in contrast to other proteases. As the size increased, the diatoms and micro algae also increased in the diet component of Mugilids (Albertini - Berhaut, 1986). It has been reported that trypsin is the major protease in carnivores compared to chymotrypsin in microphagus (algae-eating)fish with long guts (Hsu and Wu, 1979). In carnivorous fishes such as lake sturgeon and white sturgeon while lipase, amylase and chymotrypsin levels decreased after metamorphosis pepsin and trypsin continued to record an increase in the levels (Buddington, 1985; Buddington and Doroshov, 1986a). In L. parsia, although the level of trypsin at a time is always greater than chymotrypsin, the tryptic activity declined significantly as the size of the fish increased while the chymotryptic activity exhibited positive correlation with size. Trypsin is specific for the hydrolysis of the peptide linkages in which the carbomyl function is contributed by lysine and arginine (Rick, 1965). On the contrary, chymotrypsin is not only specific for the hydrolysis of the peptide linkage in which the carbomyl function is contributed by aromtic amino acid residues, such as tyrosine, tryptophan and phenylalanine, but also hydrolyse the amides and esters of these aromtic amino acids (Rick, 1965). In otherwords, chymotrypsin has broad spectrum of protein digestion than trypsin. The result is that the fish need to induce more chymotrypsin secretion for complete digestion of protein and peptides. This may explain for the continuous and steady increase of chymotrypsin in L. parsia which largely feeds on algae, diatoms and detrital matters as they grow to adults.

Though the total protease and trypsin activities decreased as the size increased, the diet is exposed to these enzymes for a longer duration in larger size groups as they have lengthy intestine which will prolong passage time in the gut. In larval forms, by virtue of their carnivorous nature, the intestine is a simple loop (Thomson, 1966). Because of this the passage time in gut may be shorter in larval forms (R. Hofer, personal communication). This is compensated by high amount of proteolytic activity as in L. parsia frys. As the size of the fish increased, the degree of undigestible materials in the diet also increased which resulted in lengthening of the intestine (Kapoor et al., 1975). The increased RLG will enhance the time for the gut passage of food (R. Hofer, personal communication) and also the duration of the exposure time of diet to the enzymes. Thus, in the mullet L. parsia though there is a decline in proteolytic activity as the size increased it may be suggested that the efficiency of protein digestion is not altered.

CHAPTER 6

DIGESTIVE ENZYME ACTIVITY IN RELATION TO FEEDING HABIT

6. DIGESTIVE ENZYME ACTIVITY IN RELATION TO FEEDING HABIT

6.1 Introduction

The digestive physiology of teleost fishes has been reviewed by Bernard (1952), Barrington (1957) and Kapoor et al. (1975). Physiological process of digestion are dependent on a number of environmental and endogenous factors and also on the quality and quantity of food ingested. Although few reports on food and feeding of certain mugilids are available (Sarojini, 1954; Luther, 1962; Suzuki, 1965; Odum, 1968a,b; 1970; Desilva and Wijeyaratne, 1976, 1977; Ferrari and Chieregato, 1981; Brusle, 1981) there is no information available on any aspects of digestion in grey mullets (Perera and Desilva, 1978). Albertini-Berhaut (1985 observed in Mugilidae that there is a clear preference in the juvenile stages for a diet of animal origin, while studying the feeding habits in relation to the size. A change of the diet occurs in young Mugilidae starting from 25 mm in standard length; a mixed diet more or less of vegetal origin and depending on the species, succeeds the exclusive animal diet (Albertini-Berhaut, 1985).

The effect of body size and salinity on the rate of digestion in young grey mullet was studied using the "sacrifice' method (Perera and De Silva, 1978). The rate of digestion was found to be dependent on salinity, being slower at lower salinities, and on body size, increasing with increasing body weight (Perera and De Silva, 1978).

Results of investigations in the past although limited reveal that

the digestive enzymes of fish are qualitatively similar to those of other vertebrates (Barnard, 1973) and they are influenced by dietary changes (Nagase, 1964; Kawai and Ikeda, 1972, 1973a; Olatunde and Ogunbiyi, 1977; Mukhopadhyay et al., 1978; Hofer, 1979a, b; Hsu and Wu, 1979; Niederholzer and Hofer, 1979; Reimer, 1982 and Danulat, 1986); age (Morishita et al., 1964; Hofer, 1982; Richard et al., 1982; Buddington, 1985; Budddington and Doroshov, 1986a and Clark et al., 1986), and other factors such as season and temperature (Lane, 1973; Hofer 1979a, b and Niederholzer and Hofer 1979), rearing temperature and sexual maturation (Torrissen and Torrissen 1984, 1985). Runguangsak and Utne (1981) studied the effect of different acidified wet feeds on protease activities in the digestive tract of the rainbow trout, Salmo gairdneri.

Similarly the digestive processes in fishes are reported to be influenced by other factors also. It was considered that feeding periodicity to be one among them. In milkfish, the digestive process has been correlated with feeding periodicity (Chiu and Benitez, 1980). Odum (1970) suggested that, under normal conditions, mullet appears to feed almost continuously throughout the day, though at different intensities. There are several reports regarding the feeding periodicity of mullets in natural conditions (Balber, 1976; De Silva and Wijeyaratne, 1977; Marais, 1980; and Collins, 1981). However, there is no information available on the relationship between feeding periodicity and digestive process in mullets. A knowledge of the digestive process in relation to feeding periodicity of the fish will help in the manipulation of

the feeding schedule in such away to coincide with the active phase of physiological processes of digestion. This study is aimed at filling the gap in our knowledge on digestive process in mullets in relation to feeding periodicity.

6.2 Material and methods

6.2.1 Fish samples

Liza parsia were sampled from culture pond at Narakkal Prawn Culture Laboratory of the Central Marine Fisheries Research Institute. Random sampling of fish was done at six hour interval over a 24 hrs period starting from 0600 hrs. The fishes were of almost uniform length and weight (16-19 cm; 135-143g.) and each sampling included 20 fishes. Immediately after capture, the fishes were covered with ice in plastic bags, and transported to the laboratory in an ice box and stored in a deep freezer for subsequent analysis.

6.2.2 Preparation of crude enzyme extracts

The fish samples were dissected, after recording total length and weight, to separate out the alimentary canal from each fish separately. The intestine was uncoiled and divided into two equal portions, designated as anterior and posterior intestines (care was taken to avoid mixing of the contents in the lumen of the intestine). The adjoining mesentric tissue and other lipid deposits were removed. Each portion of the intesine was cut open and the contents were collected in a preweighed aluminium foil and then

weighed. The intestinal tissue was then washed gently with chilled double distilled water, blot dried, weighed and then included with the respective contents. Crude enzyme extracts were prepared from the anterior and posterior intestines along with their contents as given in the general material and methods section (2.5).

6.2.3 Intestinal Feeding Index (IFI)

The intestinal feeding index, defined in this experiment as the weight of food in the anterior or posterior intestines expressed in terms of percentage of body weight , was calculated as per the formula given below according to Chiu and Benitez (1981).

$$IFI = \frac{\text{Weight of food in the anterior or posterior intestine (g)}}{\text{Weight of the fish (g)}} \times 100$$

The digestive enzyme activities were estimated in the crude extracts of anterior and posterior intestines and compared with the intestinal feeding index at different sampling timings.

6.2.4 Determination of enzyme activities

All the enzyme activity determinations were performed at the optimum pH of each enzyme, as per the detailed methods given in general material and methods section (2.6). The protein content of the crude extract was determined by the Lowry's method with bovine serum albumin as the standard (Lowry et al., 1951).

6.2.5 Statistical analyses

The relationship between intestinal feeding index and digestive enzyme activity was computed by linear and polynomial regression analyses.

6.3 Results

Intestinal feeding index was calculated for anterior and posterior intestines of all the fish samples collected at different hours of the day. The digestive enzymes *viz*. amylase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, total protease, trypsin, chymotrypsin and leucine aminopeptidase were assayed and their activities compared with intestinal feeding index.

6.3.1 Intestinal Feeding Index (IFI)

The intestinal feeding index gives the relative weight of food in the intestine in relation to the body weight of the fish. The intestinal feeding index calculated for fishes sampled at different hours of the day shows the feeding periodicity of *L. parsia* (Table-16). Results indicate that the fishes caught during midnight (2400 hrs) and early morning (0600 hrs) had their intestines almost empty. Those sampled during mid day (1200 hrs) and evening (1800 hrs)had their intestines full with maximum being in the samples collected at 1200 hrs. The value of intestinal feeding index ranged from 0.689 (± 0.07) to 4.21 (± 0.25) at different hours.

Table - 16. Intestinal Feeding Index (IFI) of *L. parsia* collected at different hours of a day.

Sampling time (Hrs)	Average wt of fish samples (gm)	Intestinal Feeding Index	
		Anterior intestine	Posterior intestine
0600	138.4 (± 18.6)	0.689 (± 0.07)	0.89 (± 0.09)
1200	141.0 (± 16.4)	4.210 (± 0.25)	3.91 (± 0.15)
1800	135.8 (± 19.3)	3.250 (± 0.36)	4.16 (± 0.31)
2400	143.2 (± 16.7)	1.540 (± 0.24)	1.99 (± 0.17)

6.3.2 Enzyme activity in relation to intestinal feeding index

The intestinal feeding index, calculated for fishes sampled at different hours of the day showed that there is a peak feeding activity at a particular time of the day. The digestive enzymes amylase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, protease, trypsin, chymotrypsin and leucine aminopeptidase were assayed from the anterior and posterior intestines and compared with the feeding index.

6.3.2.1 Anterior intestine

The results presented in Fig. 28 suggest that amylase activity was positively correlated (r^2 = 0.919) with intestinal feeding index. This suggest that the enzyme activity had only one peak at 1200 hrs over the 24 hrsof the day. The activity in general ranged between 285.4 (± 19.76) and 735.2 (± 41.29) units.

The alpha-glucosidase of anterior intestine exhibited maximum activity in the samples of 1200 hrs indicating a high positive correlation (r^2 = 0.843) with intestinal feeding index (Fig. 29). In general, the activity ranged between 24.0 (± 2.45) and 61.3 (± 8.65) units.

The beta-glucosidase activity of anterior intestine also followed the pattern of other carbohydrases reported above with a peak activity coinciding with high intestinal feeding index in the samples of 1200 hrs. The correlation analysis showed high correlation (r^2 = 0.871) with intestinal feeding index (Fig. 30).

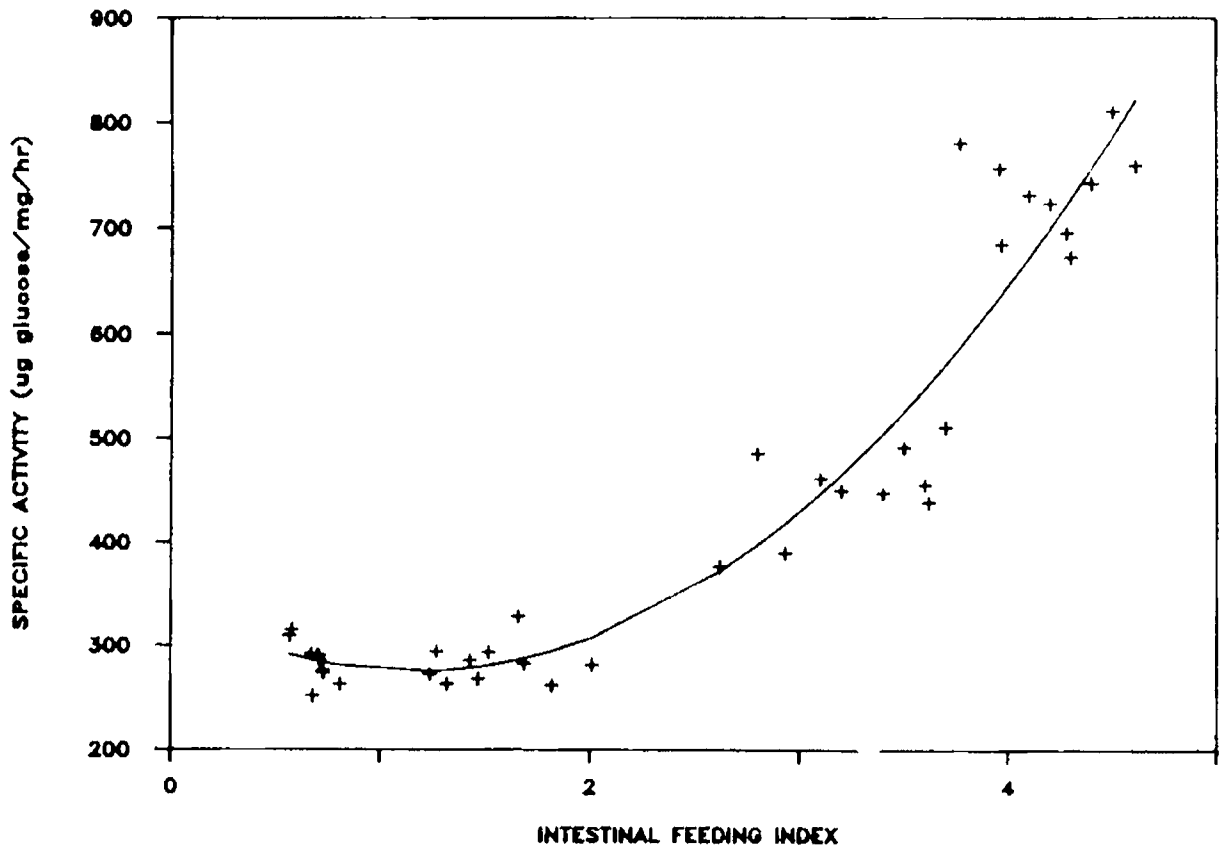

Fig.28 Relationship between intestinal feeding index and amylase activity of anterior intestine in L. parsia.

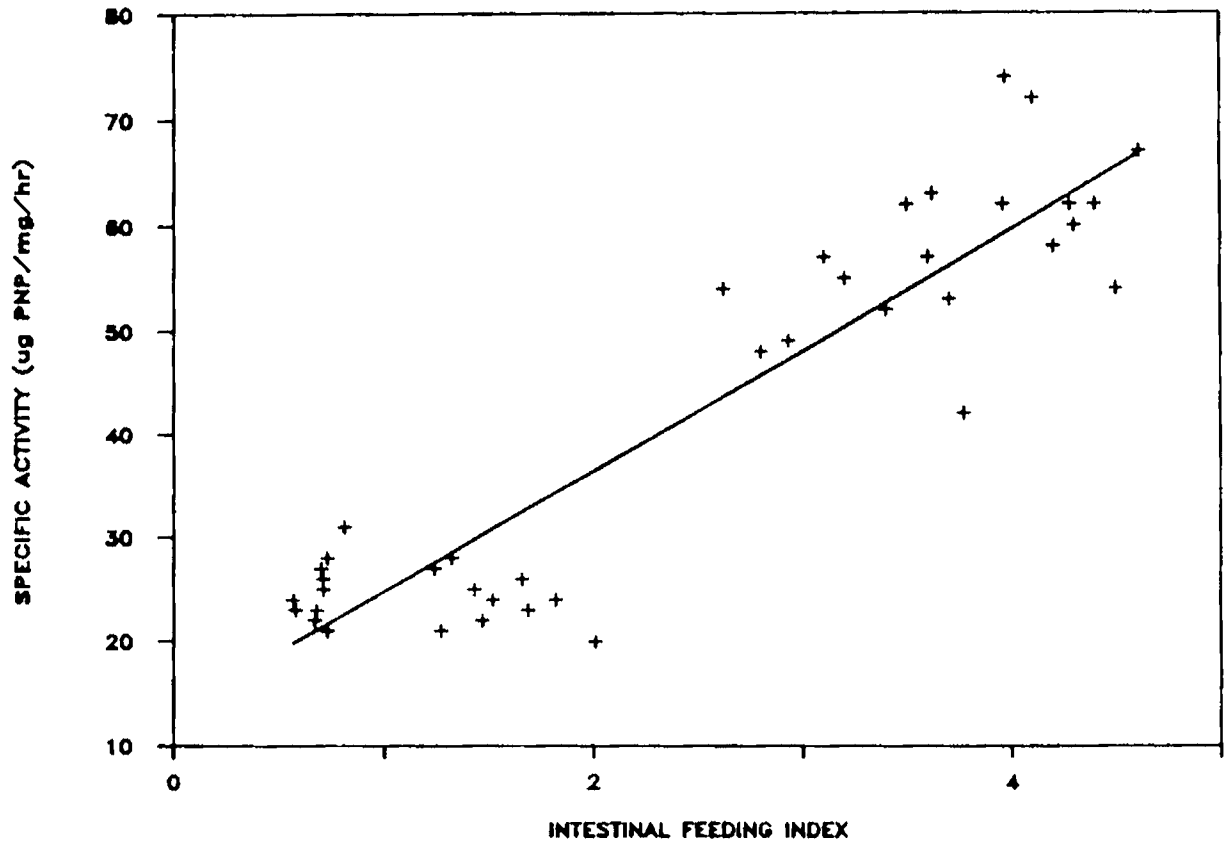

Fig.29 Relationship between intestinal feeding index and alpha-glucosidase activity of anterior intestine in L. parsia

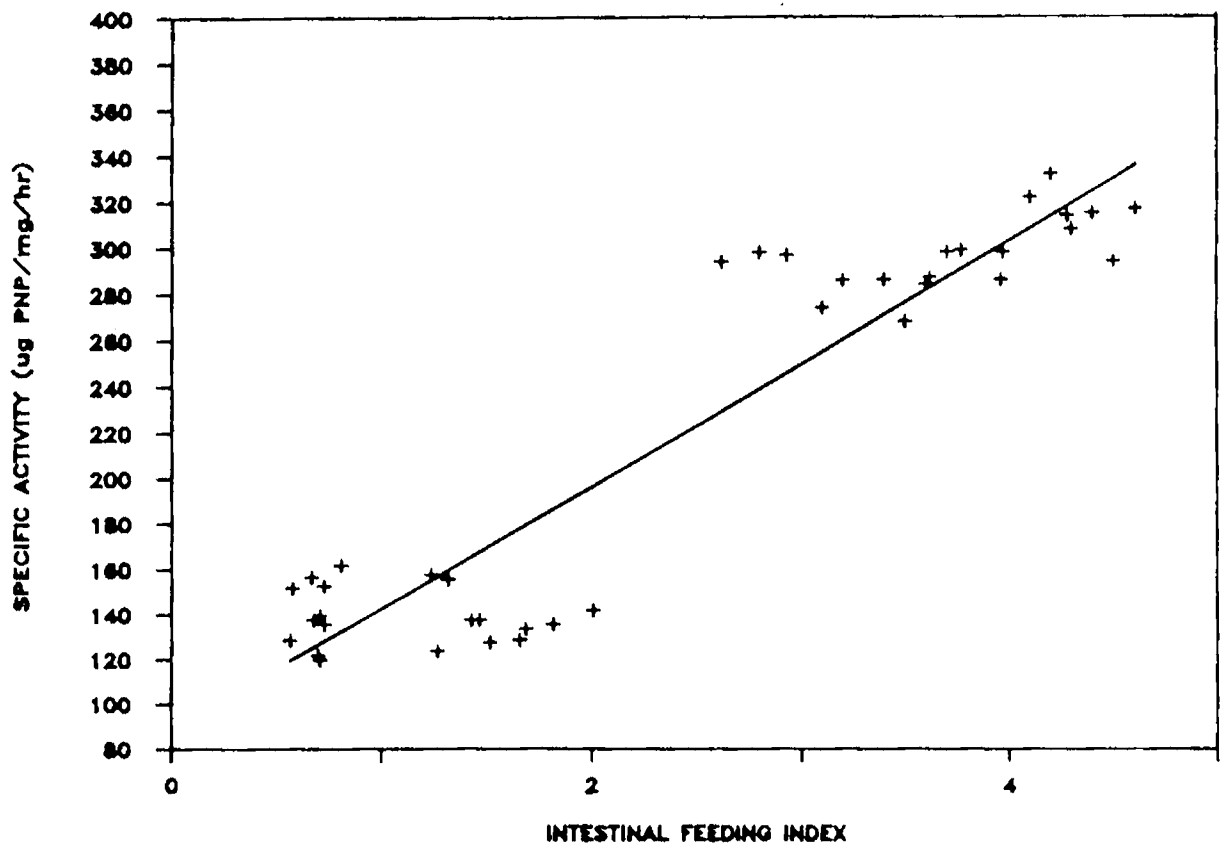

Fig.30 Relationship between intestinal feeding index and beta-glucosidase activity of anterior intestine in L. parsia.

The activity was observed to range between 32.9 (±4.99) and 84.5 (± 3.88) units.

The beta-galactosidase activity of the anterior intestine exhibited a peak in the samples of 1200 hrs which coincided with the peak of intestinal feeding index. The activity showed high positive correlation (r^2 = 0.872) with intestinal feeding index (Fig. 31). In general, the activity varied from 138.3 (± 10.66) to 308.5 (± 14.13) units.

The total proteolytic activity of the anterior intestine showed a positive relationship with intestinal feeding index (r^2 = 0.822) (Fig. 32). Similar to carbohydrases the protease activity also exhibited one peak in the samples of 1200 hrs. The activity varied from 5.24 (± 1.05) to 14.02 (± 1.99) units.

The tryptic activity assayed with specific substrates exhibited a peak in the samples collected at 1200 hrs. Correlation analysis between trypic activity and feeding index showed a high correlation (r^2 = 0.833), (Fig. 33). However, the activity ranged between 143.7 (± 14.6) and 415.8 (± 12.26) units.

The chymotryptic activity of the anterior intestine assayed with specific substrate also exhibited a high positive correlation (r^2 =0.750) with intestinal feeding index indicating that this enzyme follows a similar pattern of other proteases (Fig. 34). The activity ranged between 60.8 (± 10.67) and 129.5 (± 8.44) units.

The leucine aminopetidase, an exopeptidase assayed with specific

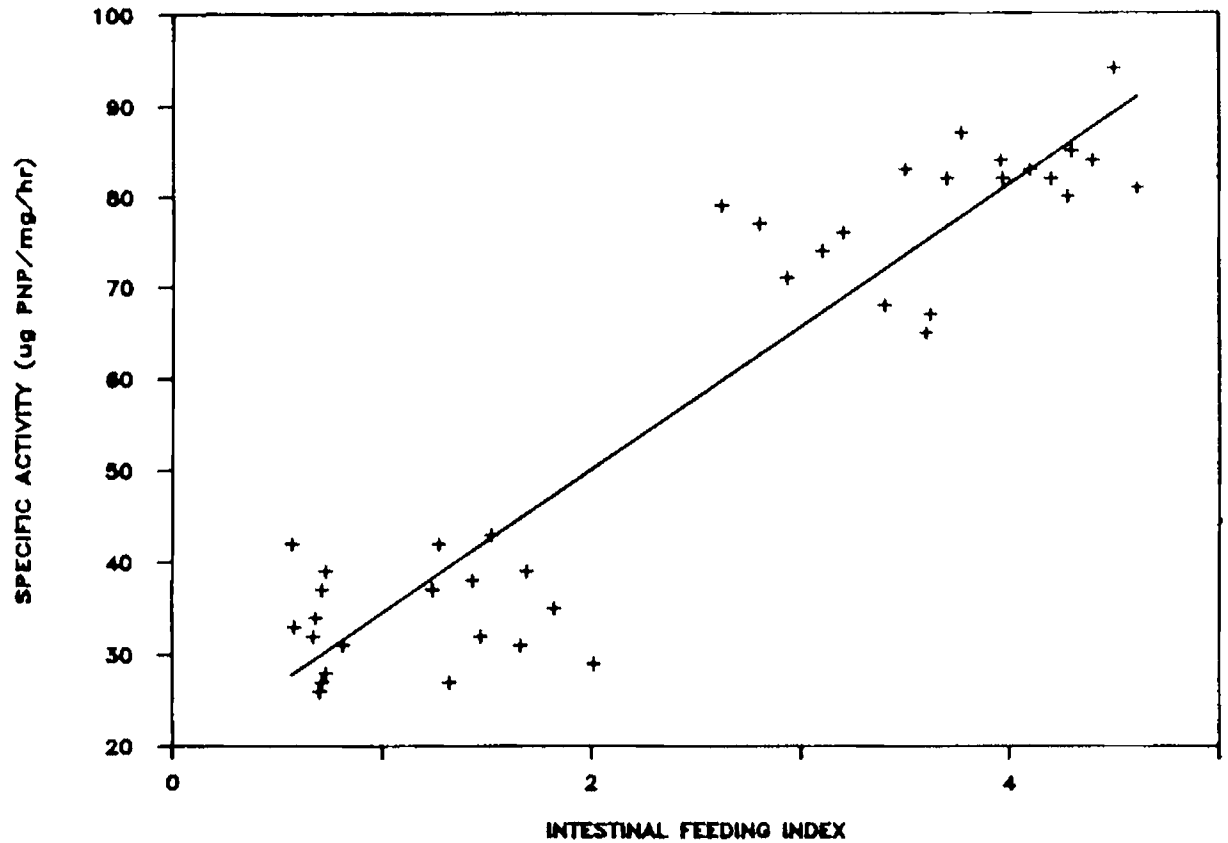

Fig.31 Relationship between intestinal feeding index and beta-galactosidase activity of anterior intestine in L. parsia.

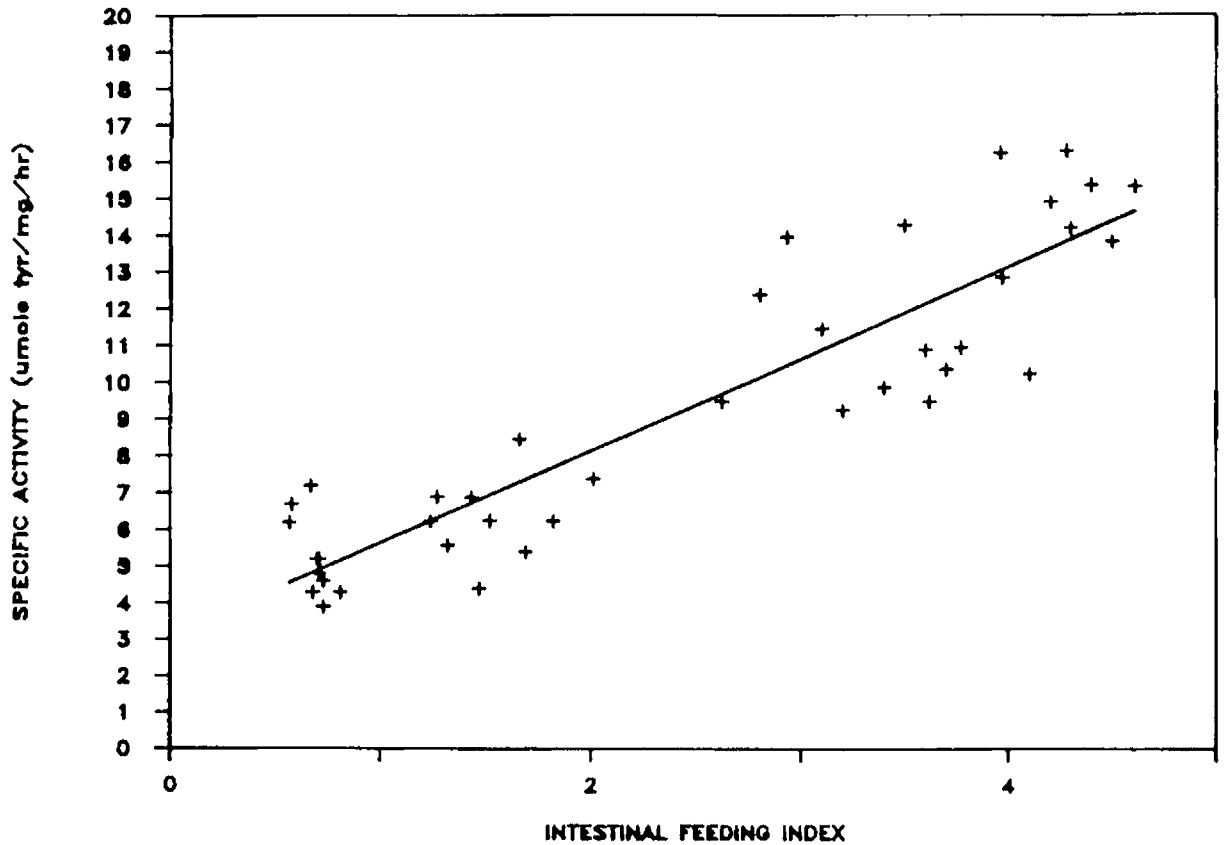

Fig.32 Relationship between intestinal feeding index and protease activity of anterior intestine in L. parsia.

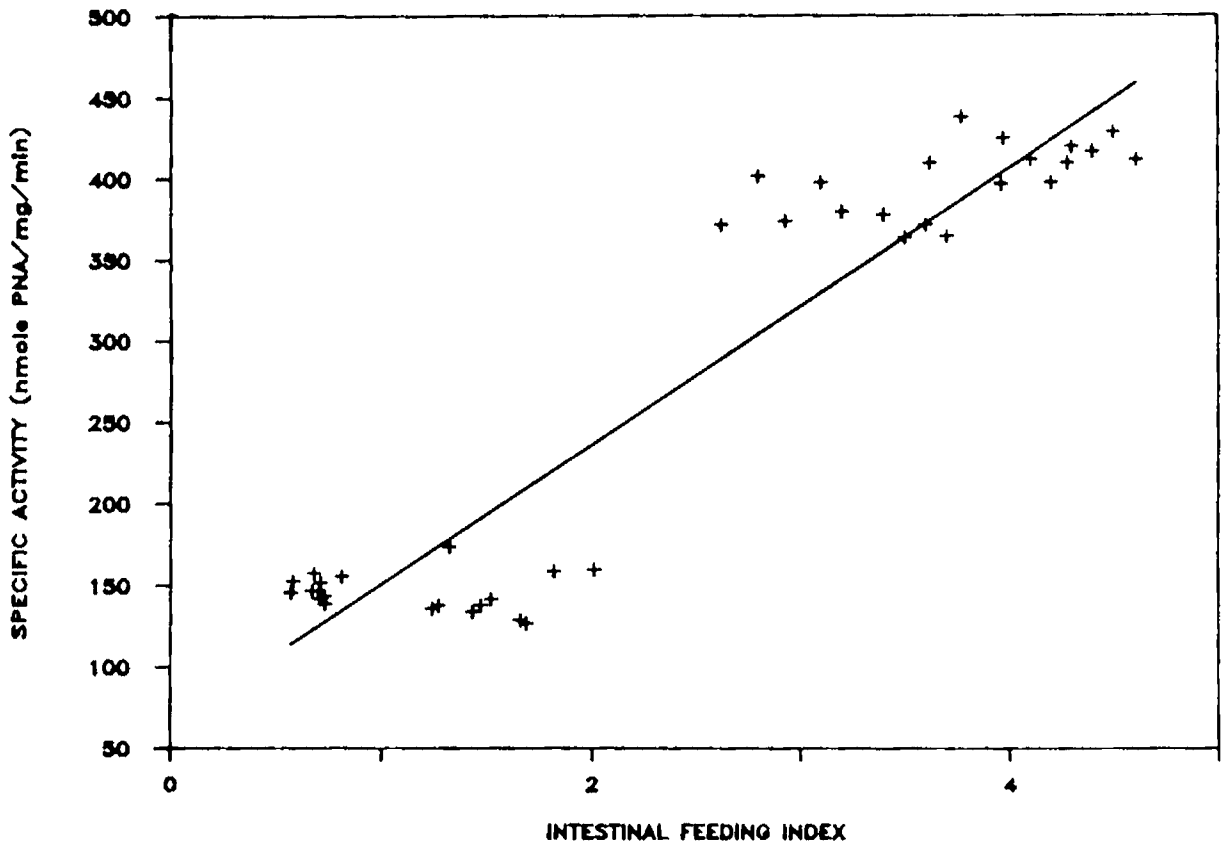

Fig.33 Relationship between intestinal feeding index and tryptic of activity of anterior intestine in L. parsia.

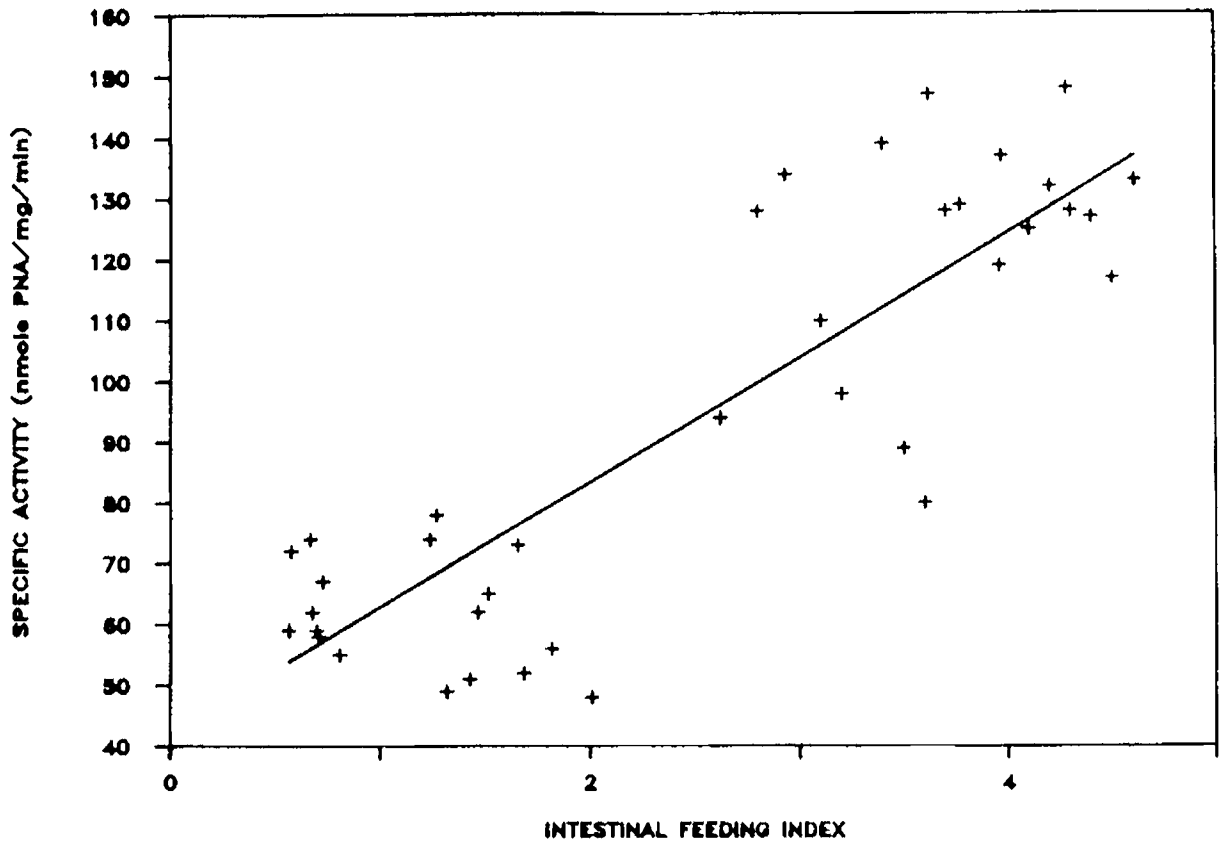

Fig.34 Relationship between intestinal feeding index and chymotryptic activity of anterior intestine in L. parsia.

substrate in the anterior intestines of fish samples exhibited high positive correlation (r^2 = 0.869) increasing with increase in feeding index (Fig. 35). The activity ranged between 159.1 (± 10.74) and 384.7 (± 17.4) units.

6.3.2.2 Posterior intestine

All the enzymes that have been assayed in the anterior intestine were also assayed in the posterior intestine. Comparatively the enzyme activity in the posterior intestine was lesser than the former.

The amylase activity in the posterior intestine also exhibited a peak over the 24 hrs in samples collected at 1200 hrs. The activity showed a high correlation (r^2 = 0.724) with feeding index (Fig. 36). In general, the activity ranged between 279.6 (±25.19) and 567.2 (± 36.64) units.

The alpha-glucosidase activity, like the amylase activity showed a peak over the 24 hours in samples collected at 1200 hrs. The activity showed a high correlation (r^2 = 0.723) with feeding index (Fig. 37). The activity was observed to range between 23.2 (± 4.07) and 61.2 (± 4.83) units. The activity observed in the samples of 1800 hrs was almost the same as that of 1200 hrs, which indicate that there was no change in the activity in the later hours.

Similarly the beta-glucosidase also demonstrated a positive correlation (r^2 = 0.788) with feeding index (Fig. 38). The activity observed for 1200 hrs samples remained in the same level for 1800 hr samples also. In general, the activity ranged from 10.8 (± 2.48)

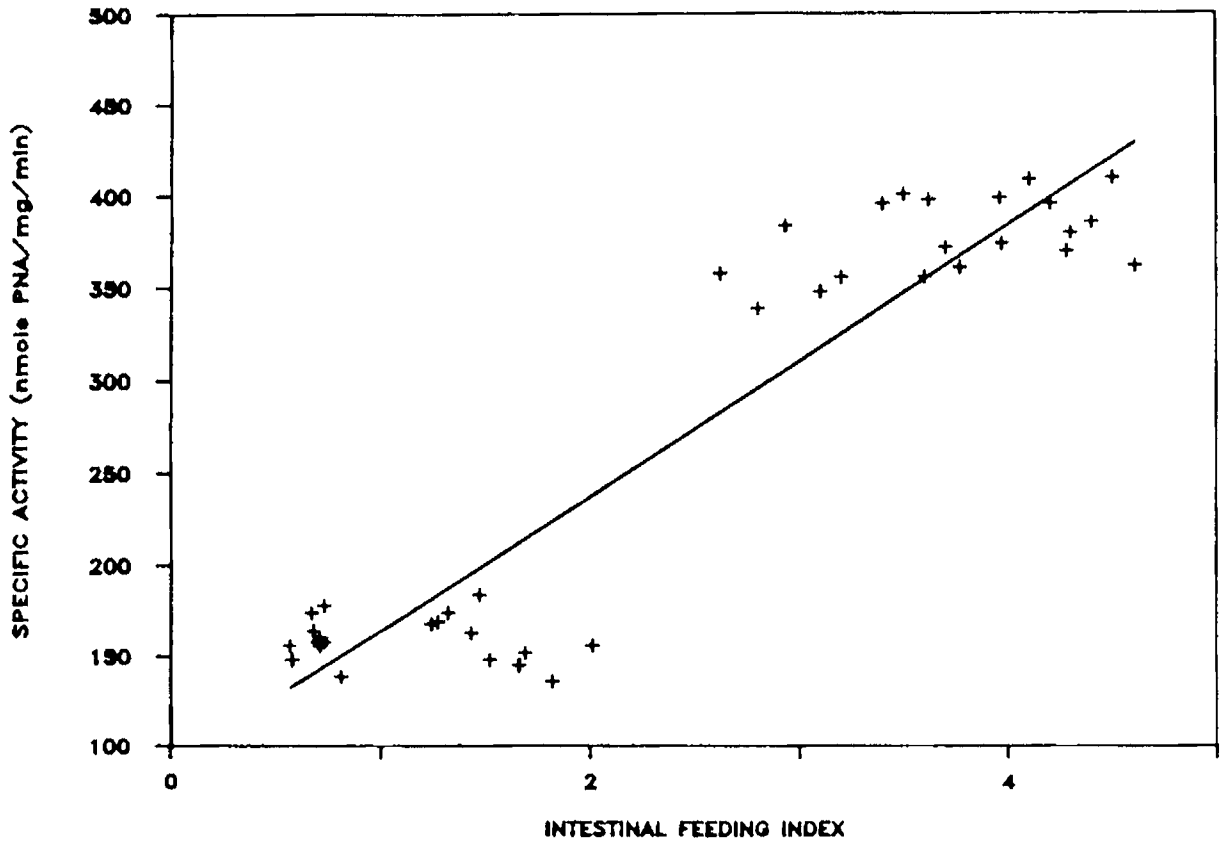

Fig.35 Relationship between intestinal feeding index and leucine aminopeptidase activity of anterior intestine in L. parsia.

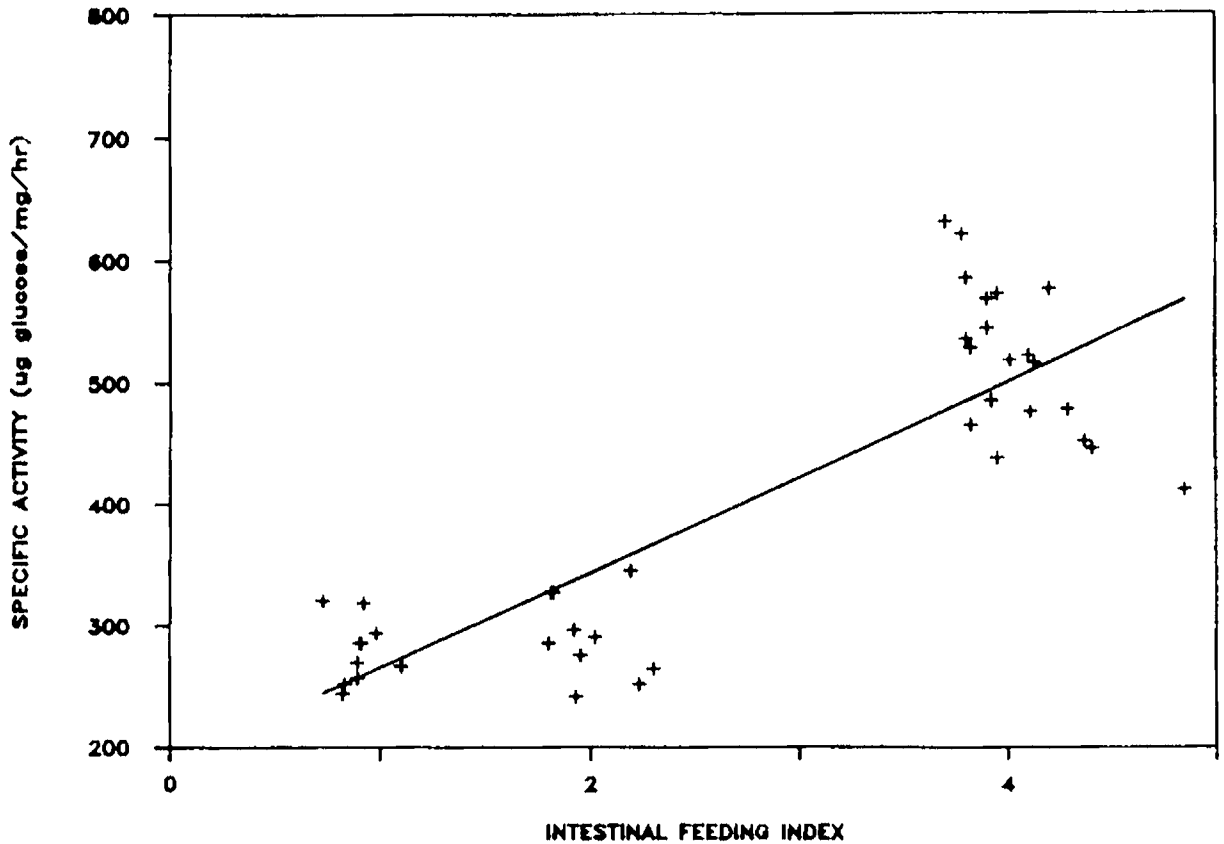

Fig.36 Relationship between intestinal feeding index and amylase activity of posterior intestine in L. parsia.

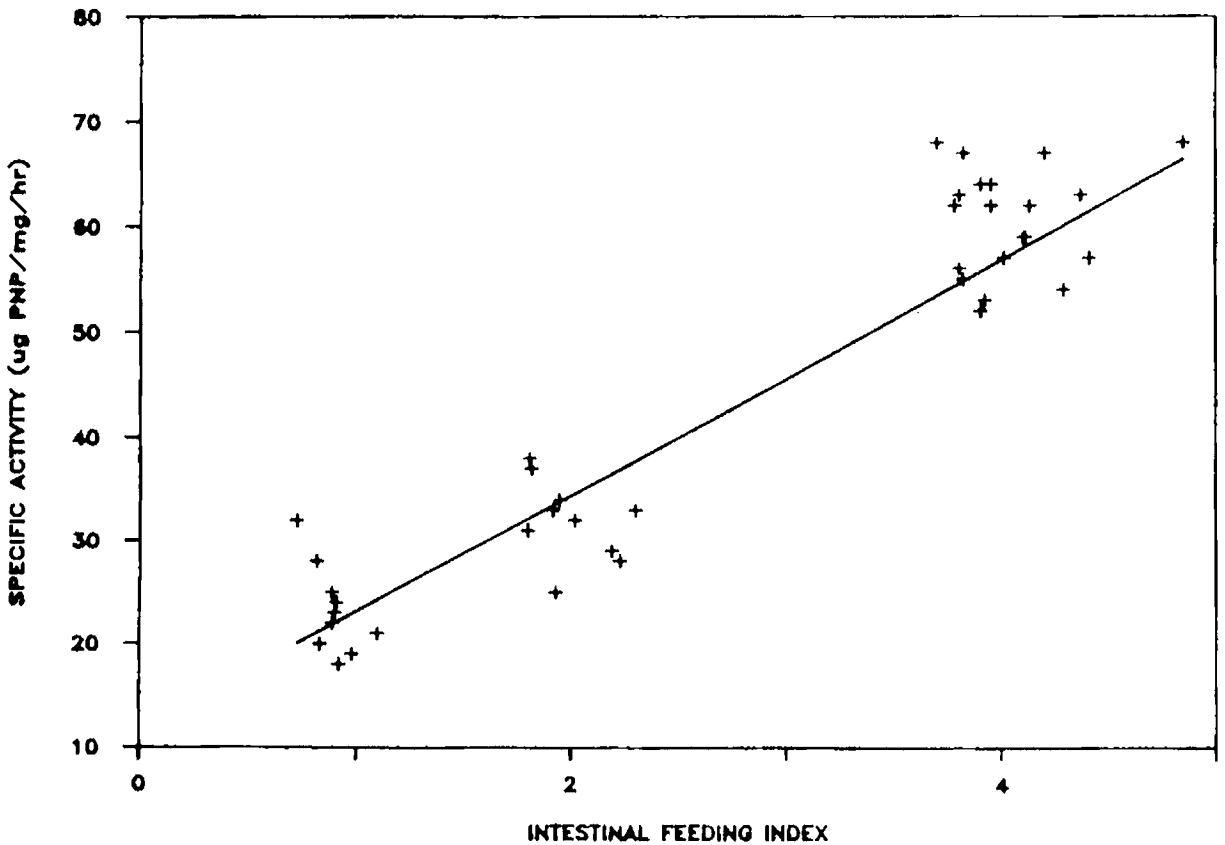

Fig.37 Relationship between intestinal feeding index and alpha-glucosidase activity of posterior intestine in L. parsia.

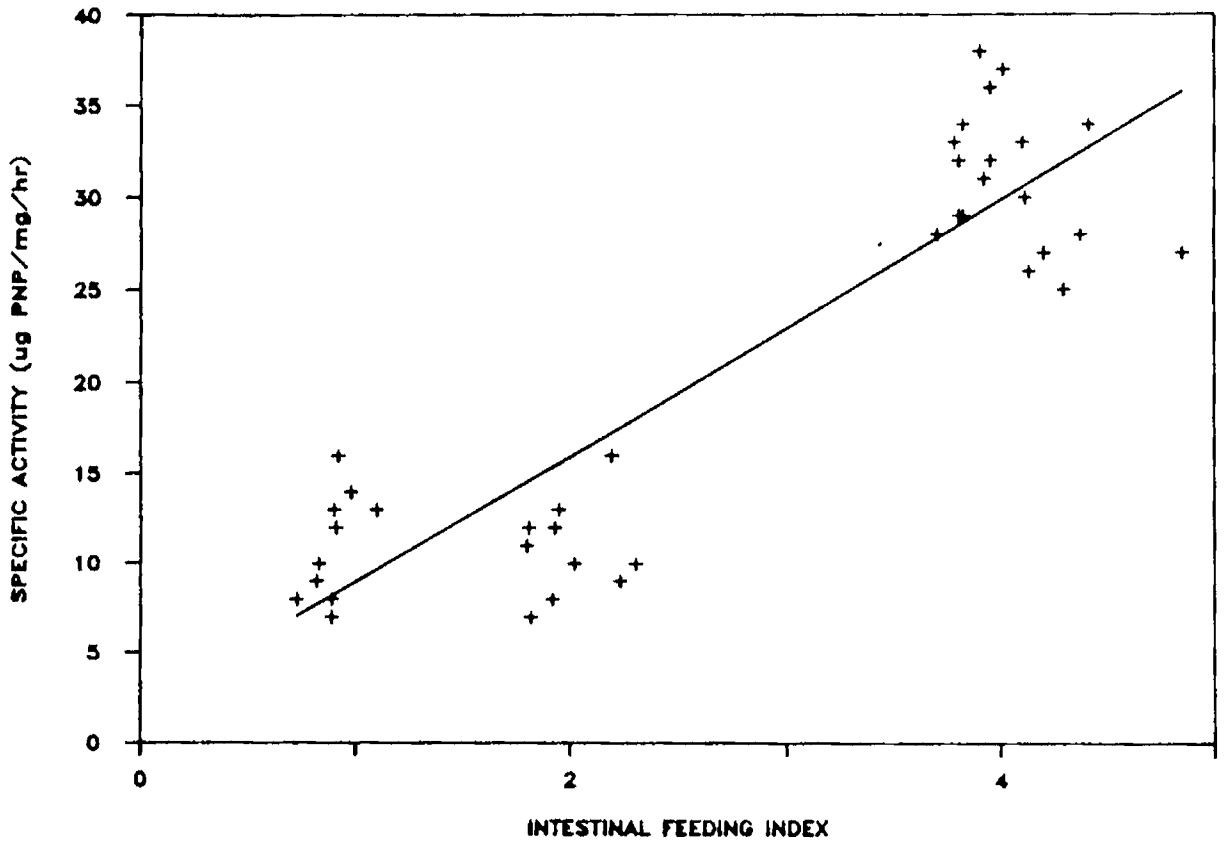

Fig.38 Relationship between intestinal feeding index and beta-glucosidase activity of posterior intestine in L. parsia.

to 33.1 (± 3.9) units.

In line with other carbohydrases, the beta-galactosidase also exhibited high positive correlation (r^2 = 0.787) with feeding index (Fig. 39). Unlike alpha-glucosidase and beta-glucosidase activities, the beta-galactosidase activity dropped sharply from the peak level in the samples made at 1200 hrs to a significantly low level in the samples of 1800 hrs. The activity ranged between 65.0 (± 5.0) and 181.5 (± 5.29) units.

The total protelytic showed a prominent peak in the samples made at 1200 hrs indicating a positive correlation (r^2 = 0.770) with feeding index (Fig. 40). The activity ranged between 4.35 (± 0.82) and 11.1 (± 1.51) units.

The tryptic activity exhibited a high positive correlation (r^2 = 0.869) with feeding index with a peak activity in samples collected at 1200 hrs (Fig. 41). It was observed that the activity ranged between 136.4 (± 6.89) and 377.1 (± 11.99) units.

Similar to trypsin, the chymotryptic activity also displayed a positive correlation (r^2 = 0.779) with feeding index with a peak activity in samples collected at 1200 hrs (Fig. 42). In general, the activity varied from 71.8 (± 6.29) to 126.7 (± 8.39) units during the different hours of sampling.

Leucine aminopeptidase, similar to other proteases, also exhibited high positive correlation (r^2 = 0.891) with feeding index (Fig. 43). The activity showed one peak in the samples of 1200 hrs and

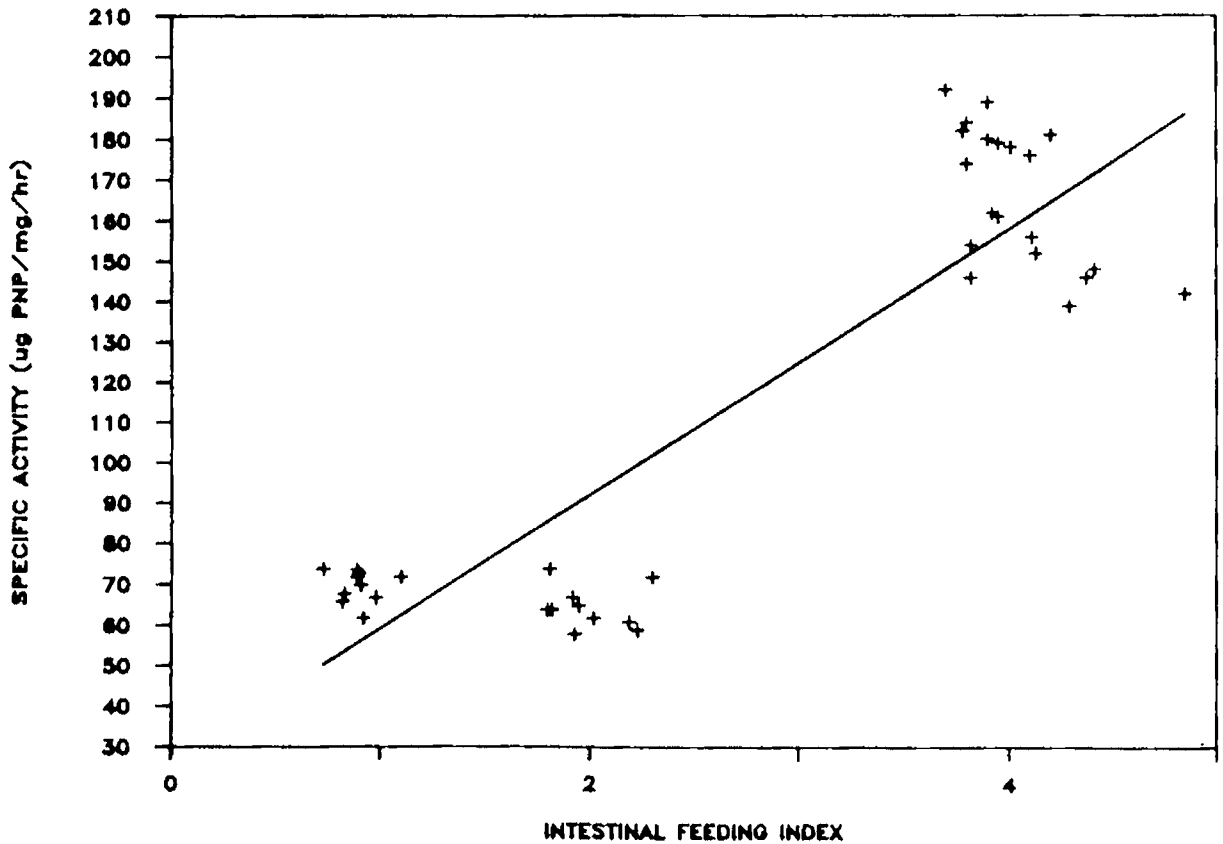

Fig.39 Relationship between intestinal feeding index and beta-galactosidase activity of posterior intestine in L. parsia.

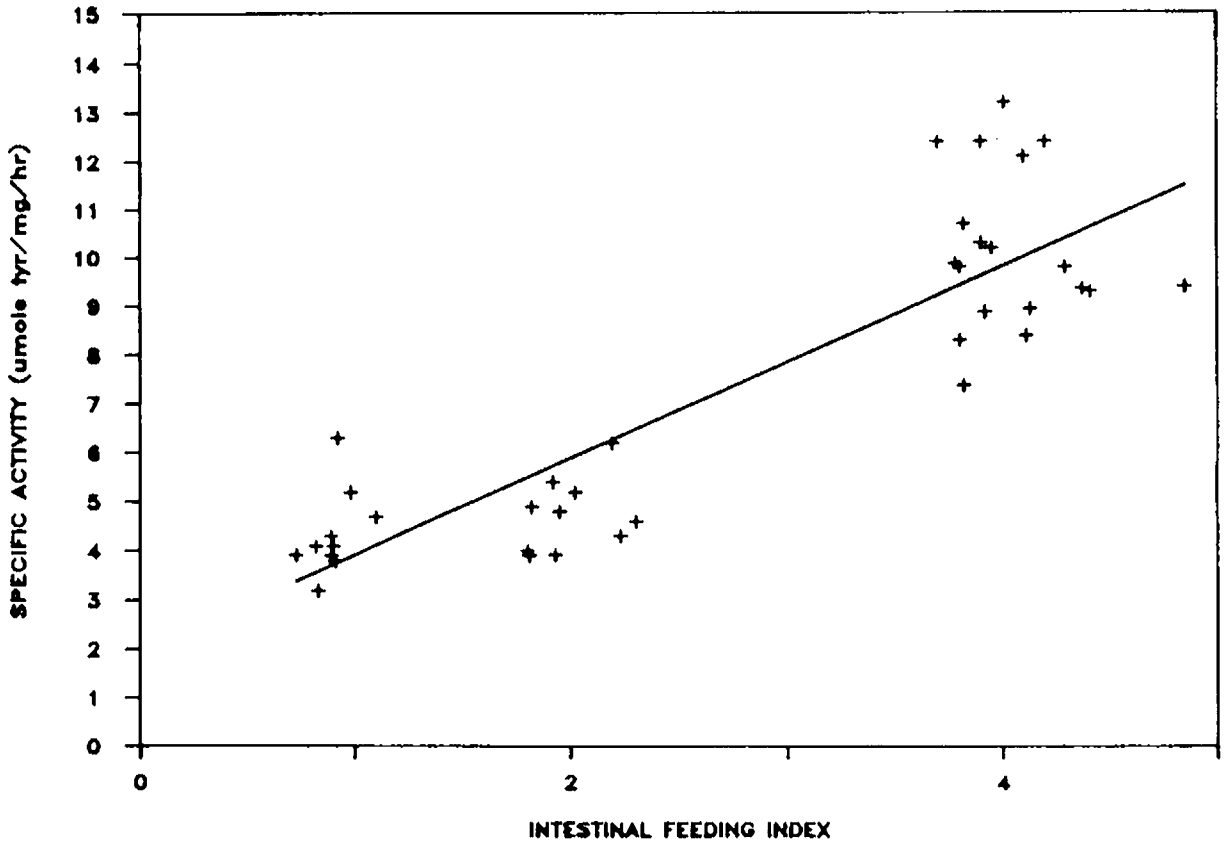

Fig.40 Relationship between intestinal feeding index and protease activity of posterior intestine in L. parsia.

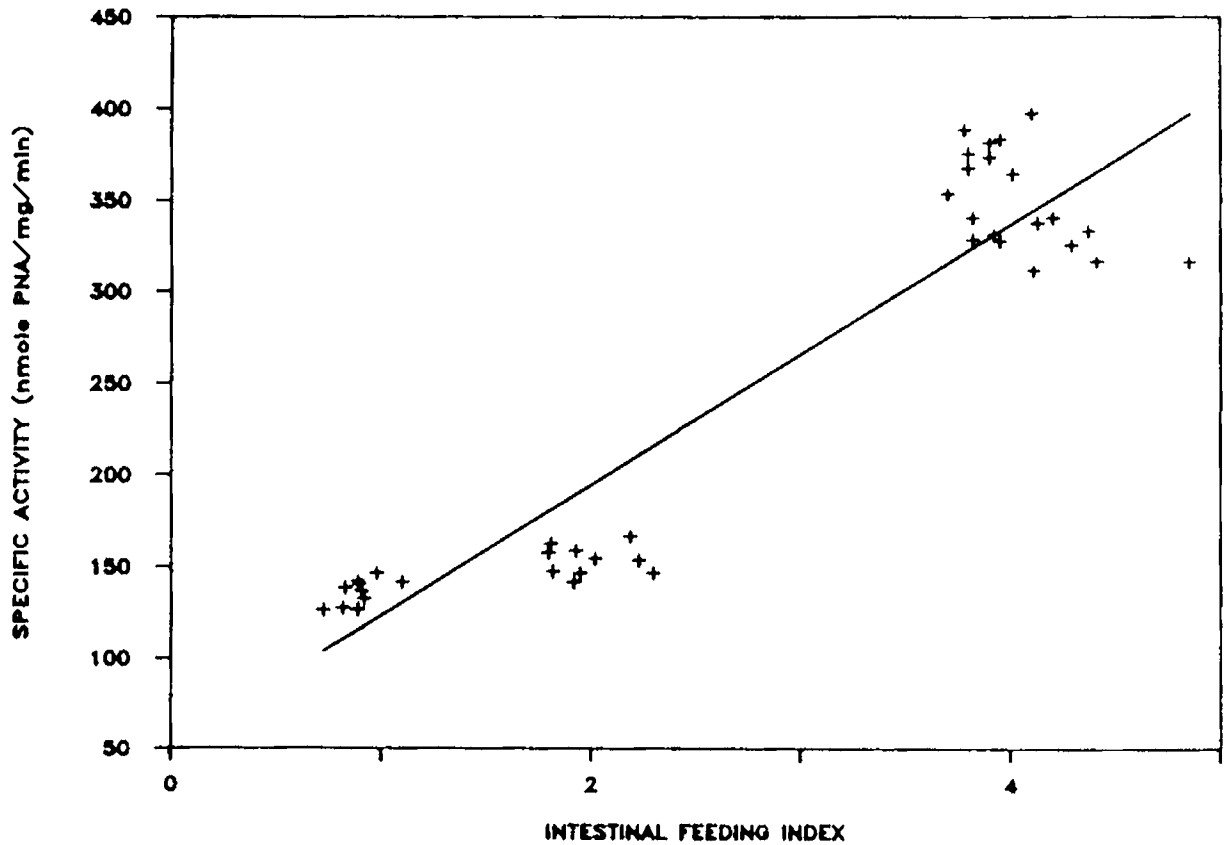

Fig.41 Relationship between intestinal feeding index and tryptic activity of posterior intestine in L. parsia.

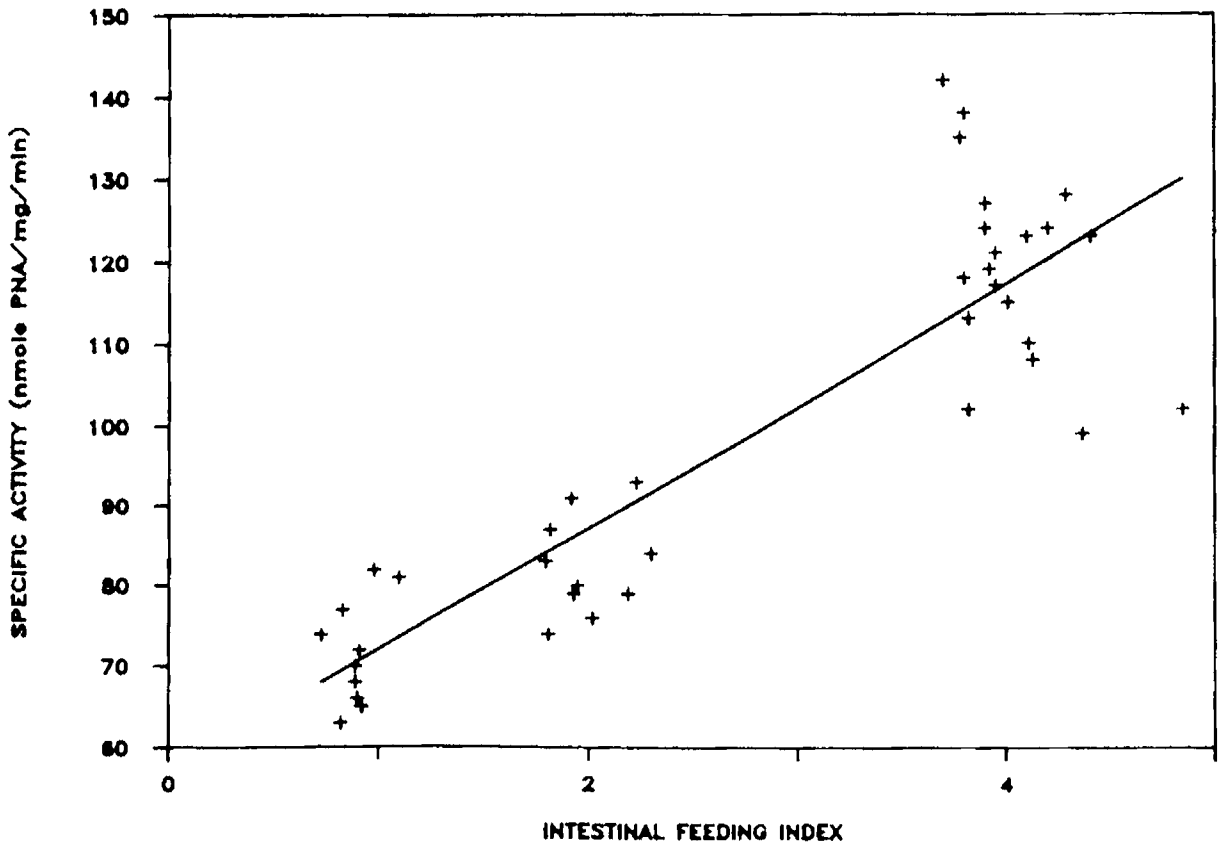

Fig.42 Relationship between intestinal feeding index and chymotryptic activity of posterior intestine in L. parsia.

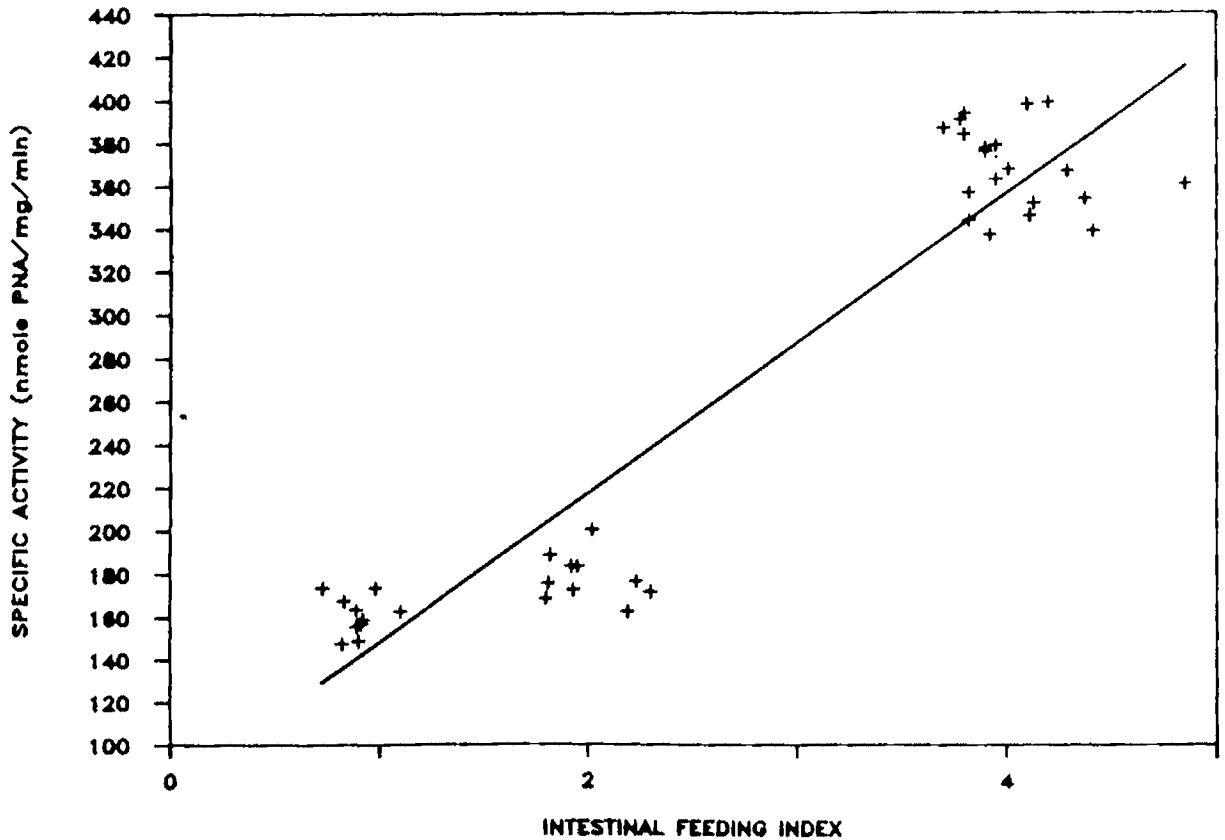

Fig.43 Relationship between intestinal feeding index and leucine aminopeptidase activity of posterior intestine in L. parsia.

varied from 161.2 (± 8.7) to 385.4 (± 9.69) units during the different hours of sampling.

6.4 Discussion

The relationship between feeding periodicity and secretion of digestive enzymes is an important aspect in aquaculture operations. Since fishes show variations in their feeding periodicity, and their digestive ability is maximal at specific times, it is mandatory to adjust the given feed and feeding hours to suit the digestive potential of the fish. The fish L. parsia were caught from the grow out ponds at six hour intervals of the 24 hrs. period and the quantity of diet in the anterior and posterior intestines were estimted and expressed as percentage of body weight. The digestive enzymes such as amylase, alpha- glucosidase, beta-glucosidase, beta galactosidase, protease, trypsin, chymotrypsin and leucine aminopeptidase were estimated and compared with the amount of diet in the intestine.

The intestinal feeding index, estimated in both anterior and posterior intestines as shown in Table-16, varied at different hours of the day. The results suggest that L. parsia feeds during day time only and there is no significant food intake during night. There has been a great deal of information regarding the feeding periodicity in mullets. Kuthalingam (1966) found no variation in feeding intensity for M. cephalus in relation to time of day. Thomson (1966) and Odum (1970) concluded that under normal conditions mullet feed almost continuously, but the intensity of

feeding may vary, and in areas under tidal influence there is a definite reltionship between feeding intesntiy and the state of the tide, with intensity being highest at high tide. Blaber (1976) found that Liza dumerili and M.cephalus feed at maximum intensity during the day. Moriarty (1976) has shown that mullets feed only during the day and not at night. Mullets caught at night were not observed to be feeding, and all had empty guts whereas most mullets caught during the day had full guts. Different species of Adriatic mullets fed intensively by daylight, whereas at night they ceased feeding (Homen, 1976). De Silva and Wijeyaratne (1977) carried out a detailed investigation of feeding periodicity of M. cephalus and concluded that, in contrast to the observations of Odum (1970) the mullet feed during the day, starting around dawn. Their peak of feeding activity was around midday, when the light intensity was at its highest. Similarly, both L. dumerili and L. richardsoni fed predominantly during the day and had a lower food intake during night (Marias, 1980). Collins (1981) concluded that peak feeding intensity at two different localities studied occured at midday and was not influenced by tidal stage. The present investigation on L.parsia adds support to the view that the mullets feeding periodicity vary diurnally with a peak at midday. In this respect it is interesting to note that Yashov and Ben-Shachar (1970), based on a feeding experiment on M. capito and M. cephalus recorded that these fishes rely primarily on vision in capturing food organisms such as copepods, caladocerans etc. This might be accounted for the maximal feeding intensity in the daytime in the L. parsia.

With respect to the level of digestive enzyme activities in both the regions of the alimentary canal, all the enzymes tested exhibited high correlations with the intestinal feeding index. This indicates that the secretion of digestive enzymes are in tune with the feeding intensity of the mullet. In other words, the enzyme activities per gram of intestinal contents are relatively constant at any time after feeding, and suggest therefore that the amount of enzymes active in the intestine is in proportion to the amount of food in the intestine. The physiological phenomenon behind this regulative process is not clearly understood in any group of fishes and is also beyond the scope of the present investigation.

The secretion of amylase in tune with feeding activity was also reported in milk fish (Chiu and Benitez, 1981). Milkfish is also a day time feeder and the amylase activity, both in the anterior and posterior intestines showed one peak at around 12.30 hrs over a 24 hrs. period. In a related study, a high correlation was found between the feeding index in the milk fish and the concentration of dissolved oxygen in the pond; both parameters were also observed to rise at about midday (Lin, 1968). Vallet et al. (1970) suggested that mugilids spend more energy in search of food and this behaviour is reflected in a higher basic metabolic rate than that of other teleost fishes (Davis, 1968; Davis and Warren, 1968). The higher metabolic rate, resulted due to search for food during midday demands higher oxygen. This additional oxygen requirement is satisfied from the pond which is known in general to have higher oxygen level in the midday.

The present investigation also supports an earlier study on eel where there was significant correlation between percent intestinal content and total activities of amylase and protease (Takii et al, 1985).

The secretion of digestive enzymes in phase with the feeding periodicity will maximise the digestive potential of the fish. The reduction of enzyme activity at midnight (2400 hrs) and early morning (0600 hrs) may be due to the absorption of enzymes by the intestine after the effective phase of digestion. Reabsorption of enzymes as a measure to conserve protein in herbivorous fishes was suggested in many species of fishes with herbivorous feeding habits (Hofer and Schiemer, 1981; Hofer, 1982; and Hofer and Nasir Uddin, 1985 unpublished). Further, Hofer and Schiemer (1981) reported that the efficiency of reabsorption of enzymes is attained at a gut length of 2.5 to 3 fold body length. It is interesting to note that the L. parsia also has a gut length around this value (2.4 ± 0.32). Evidence for reabsorption of enzymes was provided by Stroband and Vd Veen (1981) in grass carp. They showed that the absorption of protein takes place in the anterior 40-50% of the gut. The second intestinal segment has the ability to absorb macromolecules of protein by pinocytosis. The process of pinocytosis was also demonstrated in some species of mullets; Mugil cephalus and L.ramada exhibited pinocytosis in their posterior intestines (Albertini-berhaut, 1988).

Similar to the secretion of digestive enzymes in L. parsia in relation to feeding periodicity, digestive enzymes in fishes are

reported to adapt to the changes in the various parameters. Attempts were made to relate the action or properties of the digestive enzymes to the physiology of the fish and its responses to the environment. Predominant among these are the investigations on the effect of dietary components on the digestive enzymes (Kawai and Ikeda, 1973a,b; Mukhopadhyay et al., 1978; Hofer and Schiemer, 1981; Hofer, 1982; Reimer, 1982, and Danulat, 1986), and the effect of temperature (Smit, 1967; Owen and Wiggs, 1971 and Hofer, 1982). Further, the activities of digestive enzymes were also correlated with feeding intensity, sex and spawning (Kapoor et al., 1975); age and feeding frequency (Stroganov and Buzinova, 1969; Onishi et al., 1976; Buddington, 1985; Buddington and Doroshov, 1986a), and season (Chepik, 1964) and salinity (Macleod, 1977). In the present study, it is shown that the feeding periodicity does influence the level of digestive enzyme activities.

Based on the observations made on L. parsia with reference to feeding habits and enzyme activity, especially maximal intestinal feeding index and maximal enzyme activity attained at noon hours, it is suggested that in aquaculture practice feeding schedule for this species should be appropriately adjusted to the day time to have best results.

CHAPTER 7

CHANGES IN DIGESTIVE ENZYME ACTIVITIES AFTER FEEDING

7. CHANGES IN DIGESTIVE ENZYME ACTIVITIES AFTER FEEDING

7.1 Introduction

Nutritional studies on fish have mainly concentrated on qualitative nutrient requirements. Attempts at reducing the cost of feed in commercial aquaculture have usually focussed on the substitution of expensive ingredients with less expensive ones (Atalk et al., 1979; Jackson et al., 1982; Viola and Arieli, 1982 and Viola et al., 1984).

Nutritional studies conducted with fishes were classically confined to empirically designed dietary trials. However, investigations on the bioenergetics and digestive physiology of the organisms have received less emphasis. Since ration and feeding frequency can strongly influence the growth and conversion efficiency of cultured fish (Lovell, 1980; Dupree 1984; Singh and Srivastava, 1984, 1985) it is stated that more attention should be paid to elucidate the physiological basis for the relationship between feeding frequency and feed utilization (Foltz, 1984).

Objective of studying this physiological premise is to determine the rhythmicity of digestive and other metabolic processes in relation to feeding frequency. De Silva and Perera (1983, 1984) observed that the digestibility of dry matter and protein in two cichlid species varied from day to day, depicting a rhythmic pattern. DeSilva (1985) subsequently found that feeding of Oreochromis niloticus alternately on high and low protein content feeds resulted in better protein utilization, and consequently

reduced feeding costs. De Silva's (1985) observations illustrate how data on digestive physiology can be applied to provide economical benefits for the commercial aquaculturist.

Works on the response of digestive enzyme activity to feed ingestion has also elucidated the relationship between temporal digestive processes and feeding frequency in fishes. Onishi et al. (1973 a,b, 1976) investigated the sequence of digestive enzyme levels in Cyprinus carpio after feeding. Takii et al. (1985) observed changes in digestive enzyme activities in the eel (Anguilla japonica) after feeding. The results of the above observations suggest that irregular meals will result in poor utilisation due to the slow secretary response of digestive enzymes in these fishes. Studies on these lines were also carried out in Clarias gariepinus (Uys et al., 1987).

It was against these background, the present investigation, to assess the secretary response time in the mullet L. parsia, has been carried out to establish a better understanding of its temporal digestive abilities. This knowledge could, in turn, help establish correct feeding frequencies in commercial aquaculture.

7.2. Material and Methods

7.2.1 Experimental set up

Juveniles of L. parsia (4-6 cm TL; mean weight 6 gm) were maintained in plastic circular troughs of 50 l capacity, 3/4th filled with filtered seawater of appropriate salinity (17-20 ppt), at room temperature 28°C (± 2°C). The troughs were provided with

continuous aeration. Six groups (15 nos each) of fishes were maintained in duplicates. Each group of the first set was designated as E0, E1, E2, E3, E4 and E5 for the purpose of convenience. The groups of second set were designated as C0, C1, C2, C3, C4 and C5. The fishes were fed on the following test diet for 60 days before the commencement of the experiment.

7.2.2 Diet

A moist diet was prepared by mixing the dry ingredients (Table-17) with appropriate amount of water. Gelatin taken in a container, was added with cold water, mixed well and then dissolved by stirring at 80°C in a water bath. The container was then removed from heating and starch followed by fishmeal and casein were added while the contents were stirred properly. As the temperature decreased, mineral mixture, oil and vitamin mixtures were added and mixed well until the temperature dropped to 40°C. The warm diet was then transferred into a suitable container and stored in a deep freezer.

The troughs were cleared of faecal matter daily and the water was changed in alternate days. Care was taken not to disturb the fish much while cleaning or changing water. The fishes were fasted one day prior to the experiment after which 2 samples of fishes (each sample contained 10 numbers) from E0 and C0 were collected for enzymes analysis. Later the C series set was kept unfed and the E series was fed to satiation. Further samples were drawn from each trough at specific time intervals of 1, 3, 5, 12 and 24 hrs after feeding.

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Table 17. Dietary ingredients used in the feed to determine the sequential changes of digestive enzyme activities after feeding in L. parsia.

Ingredients	gm
Gelatin	10
Casein	30
Fishmeal	30
Vitamin Mixture[1]	1
Mineral Mixture[2]	3
Potato Starch	20
Codliver Oil	3
Sunflower Oil	3

1. Choline chloride, Inositol, L. Ascorbic acid, Nicotinic acid, calcium - pantothenate, riboflavin, Thiaminehydrochloride, Poyridoxinehydrochloride, Menadione (K), Folic acid (Vitamin Cyanocobalamine, B_{12}, Biotin, (L. tocopherol acetate (E)

2. Calcium biphosphate, calcium lactate, Ferric citrate, Magnesium sulfate, Dibasic potassium phsophate, sodium biphosphate, sodium chloride, Aluminium chloride, Zinc sulphate, Cuprous chloride, Manganese sulphate, Potassium iodide, Cobaltous chloride.

Preparation of crude enzyme extract

The sampled fishes were sacrificed and the stomach contents and intestinal contents were individually withdrawn from each fish. At '0' hr, there was no content in the stomach and intestine and only the mucus present was used for the preparation of crude extracts. Care was taken to avoid contamination between samples. Gastric and intestinal tissues were then gently washed with a stream of ice cold double distilled water and then blot dried. All the samples, the contents and tissues, were immediately kept in ice or in freezer till further steps were taken. All the samples were weighed individually and homogenised in an Potter-Elvehjem type homogeniser with chilled double distilled water to get a 1:5 (w/v) homogenate. The homogenates were then centrifuged in a refrigerated centrifuge at 19000 g for 15 minutes at 4°C. The clear supernatant obtained was stored in vials kept in a deep freezer and used as crude enzyme extract for the ensyme assays.

Determination of enzyme activities

All the enzymes were assayed at their optimum pH as per the detailed methods given earlier (Section 2.6). The protein content of the crude extract was determined by the Lowry's method with bovine serum albumin as the standard (Lowry et al., 1951).

7.2.5 Statistical analysis

The change in the enzyme activity, at different hours, after feeding was tested with Analysis of variance (Snedecor and Cochran, 1967).

7.3 Results

The sequential changes in the activities of amylase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, pepsin, alkaline protease, trypsin and chymotrypsin in the stomach tissue and contents, intestinal tissue and contents of juveniles of *L. parsia* at various timings (0, 1, 3, 5, 12 and 24 hrs) after feeding with a moist diet were investigated and the results are presented in Figs.44 to 51.

The changes in amylase activity in the intestinal tissue and intestinal contents are presented in Fig.44. The changes in amylase activity in the intestinal tissue at different hours were highly significant ($P < 0.01$). The amylase activity in the intestinal tissues recorded a rapid decline from the initial activity (388.1) to a minimum of 279 units by 5 hrs after feeding. However, the activity regained momentum after 5 hrs and raised significantly, and uniformly to a maximum of 333.0 units by 24 hrs. In general, the amylase activity ranged from 279.1 (± 11.73) to 388.1 (± 23.16) units.

The amylase activity in the intestinal content (Fig.44) displayed a different pattern when compared to that in intestinal tissue. The

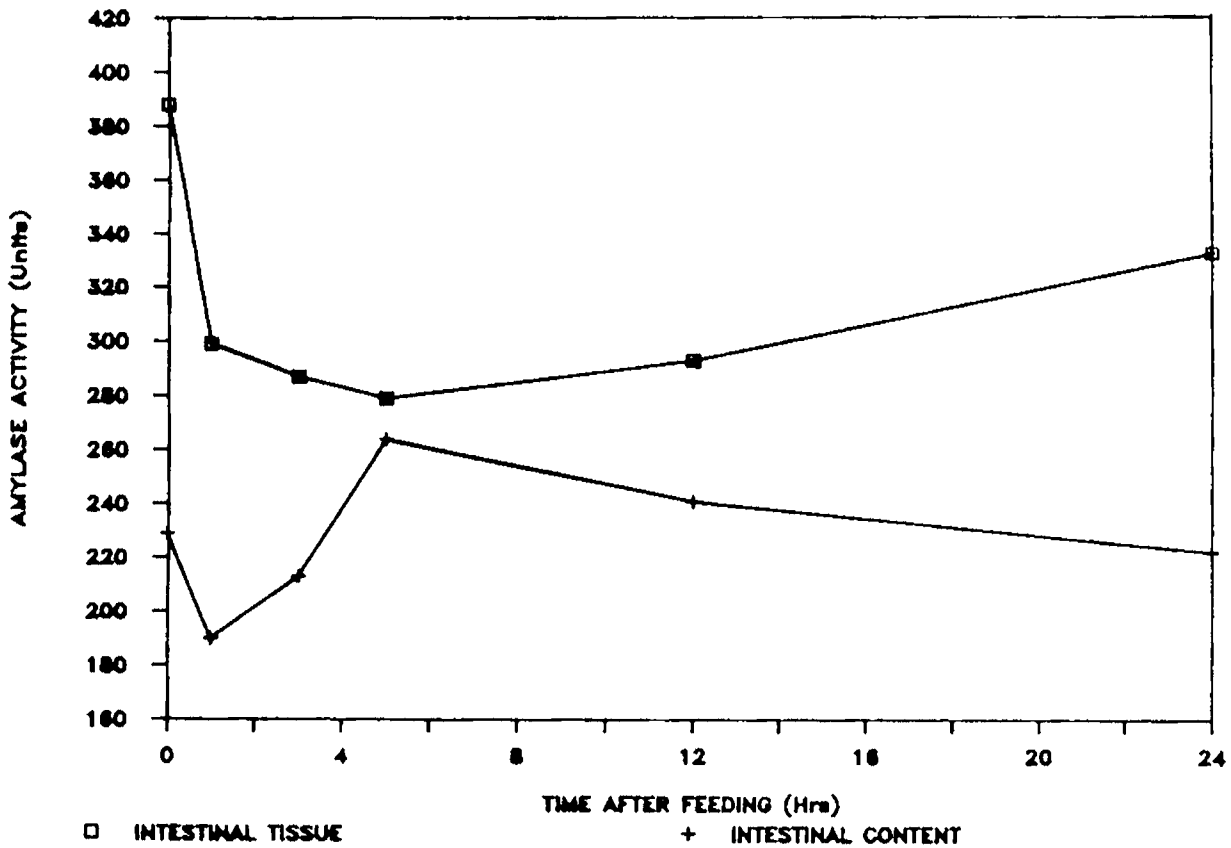

Fig.44 Changes in amylase activity of intestinal tissue and intestinal contents of L. parsia with time after feeding.

changes at different hours were highly significant ($P<0.01$). The activity in the contents were at a lesser level when compared to that in intestinal tissue. In contrast to that of intestinal tissue, in the intestinal contents a peak in the amylase activity was observed around 5 hrs after feeding (264.0 units). During the initial and the later hours after 5 hrs of feeding, the activities of the enzyme recorded a decline ($P < 0.01$). Interestingly, the activity value obtained at 24 hrs was almost the same with that of the prefeeding level ($P < 0.01$). The activity ranged between 190.4 (± 12.13) and 264.0 (± 15.0) units.

The sequential changes in alpha-glucosidase in the intestinal tissue and contents of L. parsia are presented in Fig.45. The changes in the activity at different hours were highly significant in the intestinal tissue ($P < 0.01$). The enzyme in the intestinal tissue followed a similar pattern of fluctuation around 5 hours after feeding as that of amylase in the intestinal tissues. It declined from 40 units (prefeeding) to a level of 29 units (5 hrs) and recorded uniform raise in the later hours to a level of 39 units at 24 hrs. It may be noted that the alpha-glucosidase activity at 24 hrs was almost the same as that of prefeeding. It was observed that the activity varied between 29.1 (± 5.36) and 40.5 (± 6.96) units.

The intestinal contents showed significant changes for alpha-glucosidase activity at different hours after feeding ($P < 0.01$). The activity in the intestinal contents also demonstrated a similar pattern of activity as that of amylase, by recording a peak (36.0

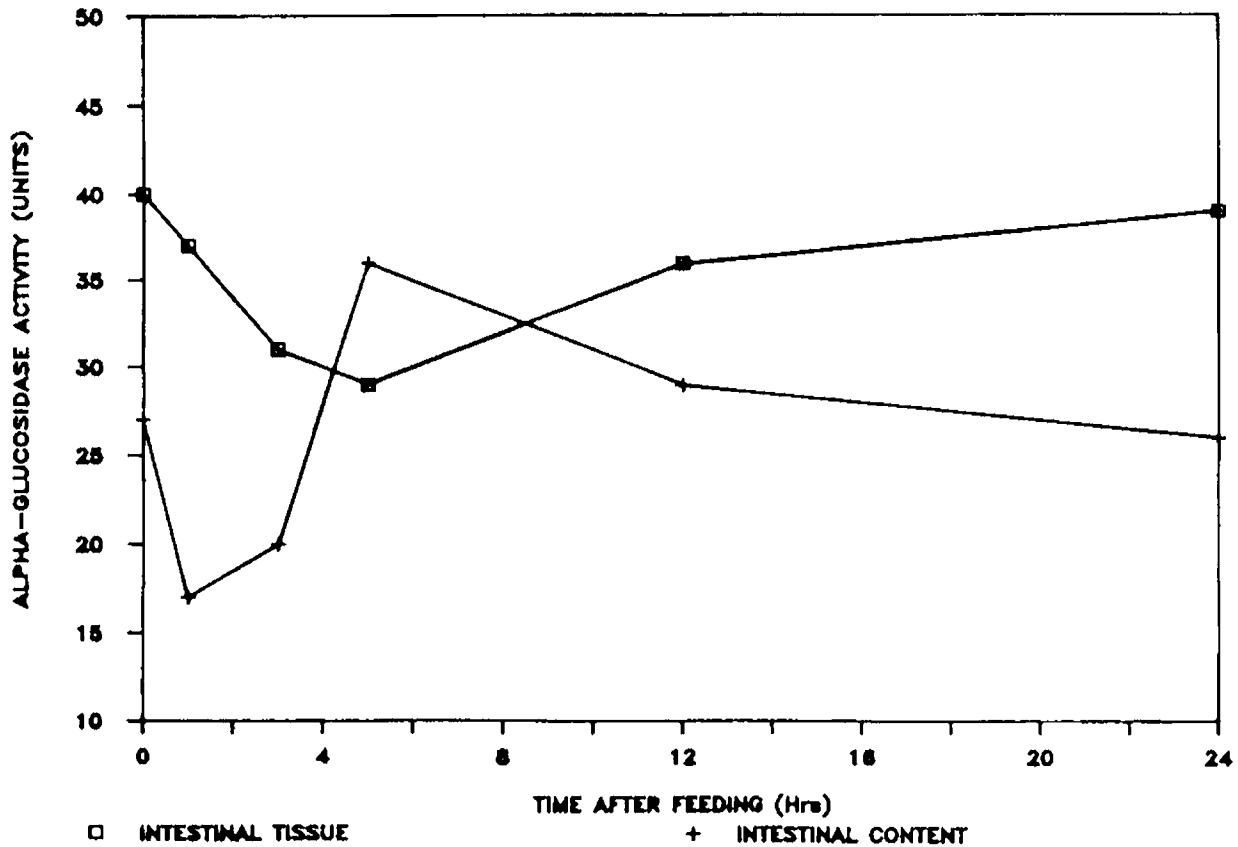

Fig.45 Changes in alpha-glucosidase activity of intestinal tissue and intestinal contents of L. parsia with time after feeding.

units) around 5 hrs after feeding, which later declined (26.0 units) significantly to the level of prefeeding (27.0 units) (Fig.45). In general, the activity varied from 17.7 (± 3.6) to 36.9 (± 3.14) units which is comparatively lower than that of intestinal tissue.

The profile of beta-glucosidase in the intestinal tissue at different hours of feeding are presented in Fig.46. The overall change in activity at different hours were highly significant ($P < 0.01$). Beta-glucosidase also demonstrated a similar pattern of activity as that of amylase and alpha-glucosidase in the intestinal tissue by recording a decline around 5 hrs (29.0 units) after feeding from the prefeeding (39.0 units) level and later raised to the level of prefeeding at 24 hrs (39.0 units). In general, the activity of the enzyme varied from 29.6 (± 3.89) to 39.0 (± 5.5) units.

The beta-glucosidase activity in the intestinal contents remained comparatively at a lower level in comparison with that of the intestinal tissue. The activity displayed significant changes ($P < 0.01$), at different hours, after feeding, very similar to that of amylase and alpha-glucosidase in the contents. Immediately after feeding the activity started increasing gradually reaching a maximum at 5 hrs (Fig.46). Later, the activity declined significantly to the prefeeding level at 12 hrs and remained almost same at 24 hrs. The activity ranged between 18.7 (± 4.74) and 28.6 (± 4.97) units.

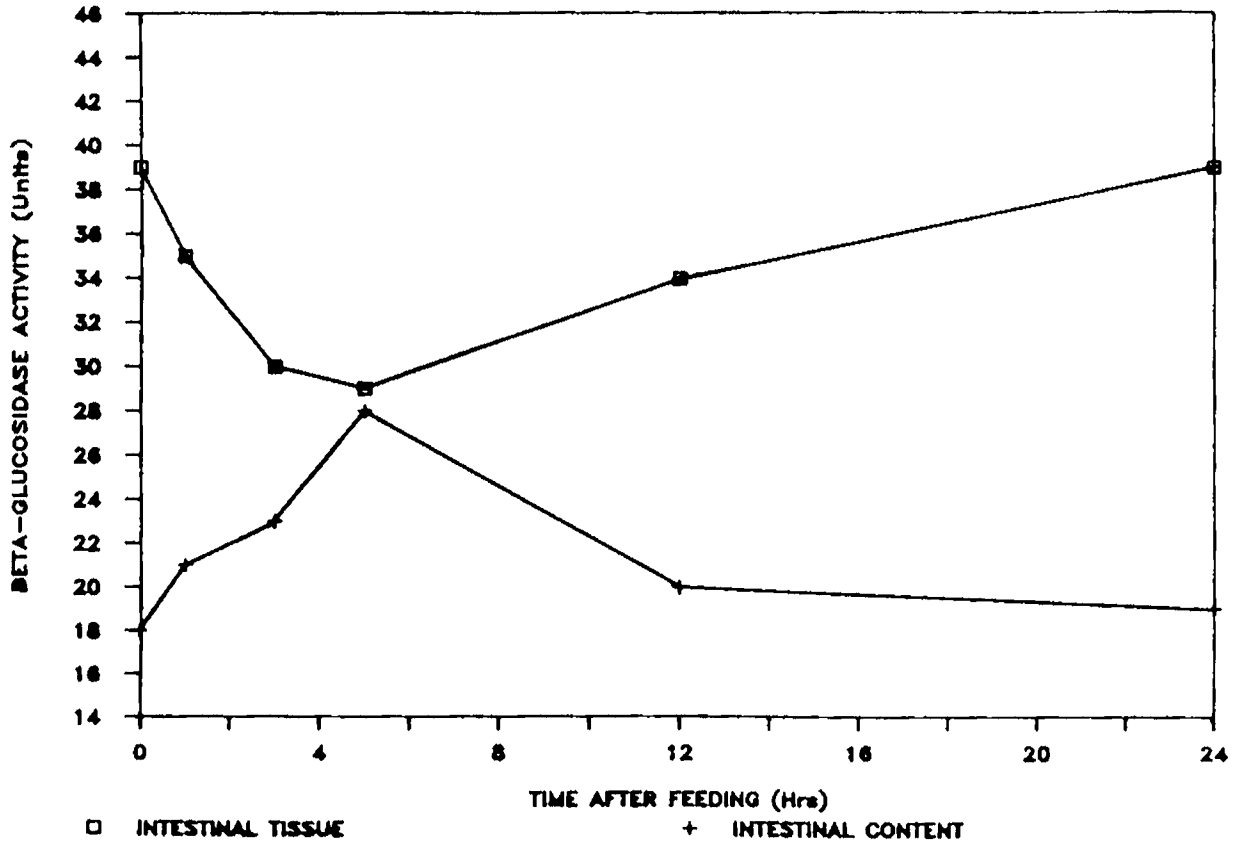

Fig.46 Changes in beta-glucosidase activity of intestinal tissue and intestinal contents of L. parsia with time after feeding.

The beta-galactosidase activity in the intestinal tissue varied significantly after different hours of feeding ($P < 0.01$) (Fig.47). Unlike other carbohydrases studied, this enzyme showed a sudden significant increase from 139 units of prefeeding level to 157 units at 1 hr of feeding. However, later it recorded a rapid decline to a minimum of 117 units around 5 hrs and thereafter an increase to a level of 136 units at 24 hrs as that of other carbohydrases tested. The activity varied from 117.7 (± 7.35) to 157.6 (± 11.79) units.

The beta-galactosidase activity in the intestinal content varied significantly ($P < 0.01$) at different hours after feeding very similar to that of other carbohydrases (Fig.47). Although there was an initial decline in the activity at 1 hr after feeding, there was a gradual raise in the activity reaching a peak at 5 hrs (139 units). However, in the later hours this value declined to a level of 116 units similar to that of the prefeeding level at 24 hrs. In general it was observed that the level of activity ranged between 104.4 (± 10.76) and 139.8 (± 9.04) units.

The peptic activity was assayed in the stomach tissue and contents and the changes in the activity after feeding are given in Fig.48. The activity in the stomach tissue demonstrated an irregular pattern of raise and fall. However, a significant high activity was observed at 5 hrs., which rose from a level of 0.742 units at 3 hrs. to 2.042 units. Comparatively, the level of decline observed in the later hours after 5 hrs upto 24 hrs was less than that observed in the early hours immediately after feeding. In general,

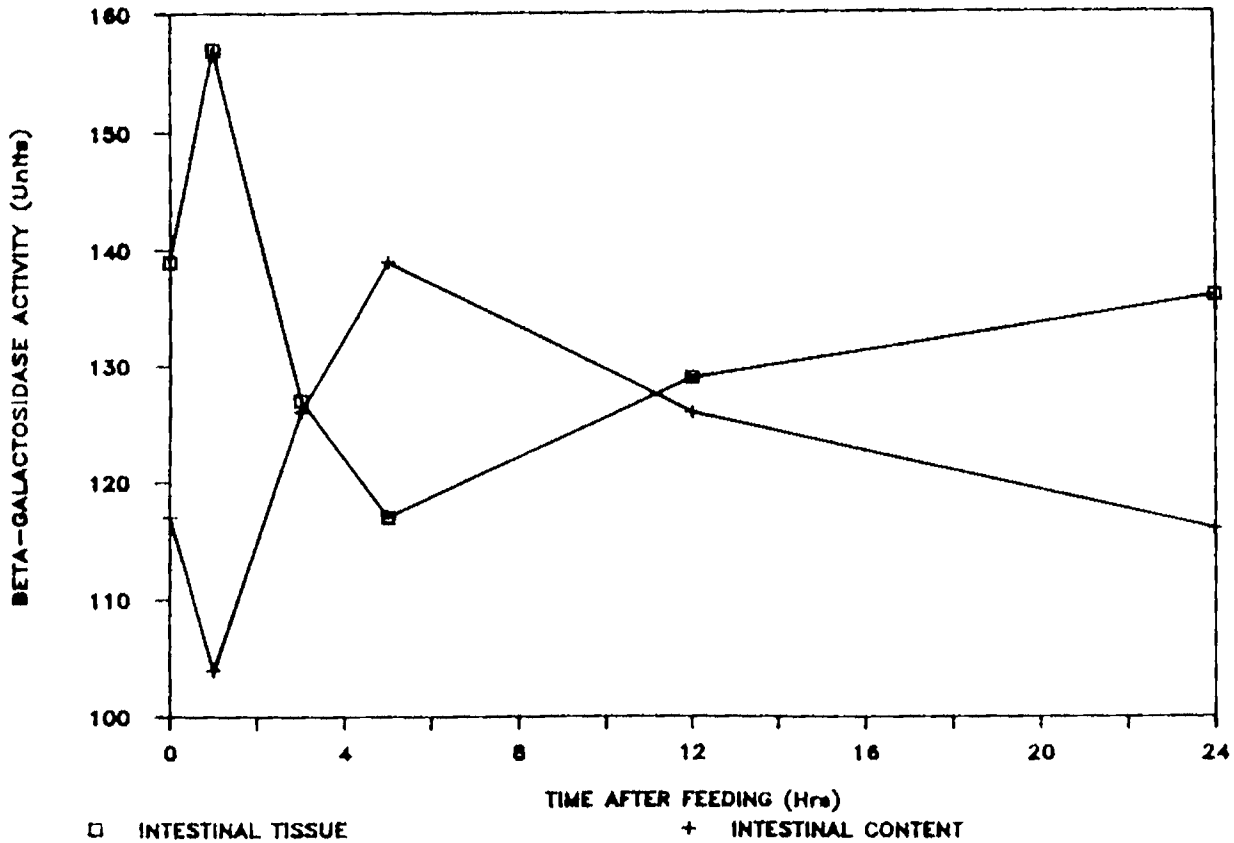

Fig.47 Changes in beta-galactosidase activity of intestinal tissue and intestinal contents of L. parsia with time after feeding.

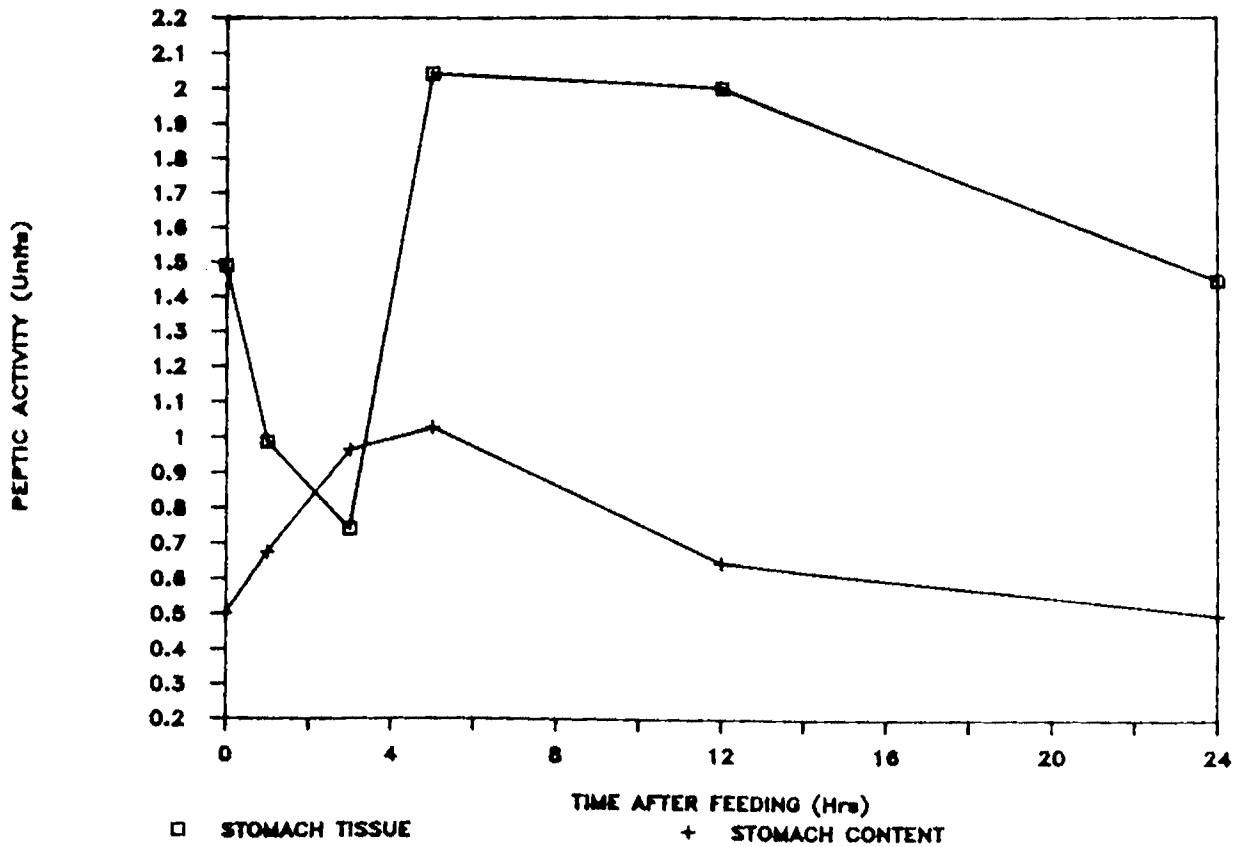

Fig.48 Changes in peptic activity of stomach tissue and stomach contents of L. parsia with time after feeding.

the activity varied between 0.742 (± 0.13) and 2.042 (± 0.345) units.

The total proteolytic activity estimated with casein as substrate in the intestinal tissue and contents varied significantly at different hours after feeding ($P < 0.01$). After feeding the activity in the intestinal tissue started declining gradually reaching the minimum level at 5 hrs. Later the activity gradually raised to prefeeding level at 24 hrs (Fig.49). The activity ranged between 15.5 (± 3.20) and 38.5 (± 5.5) units).

The protease activity in the intestinal contents showed significant changes over the 24 hrs period ($P < 0.01$) (Fig.49). On the contrary to the intestinal tissue, the activity in the contents significantly increased to a maximum at 5 hrs and then declined gradually during the later hours reaching the prefeeding level at 24 hrs. In general, the activity ranged from 10.01 (± 1.96) to 40.89 (± 5.54) units.

The tryptic activity in the intestinal tissue varied significantly ($P < 0.01$) over the 24 hrs period after feeding (Fig.50). The activity in the intestinal tissue exhibited a significant decline at 5 hrs, very similar to that of protease. The activity gradually declined from the prefeeding level (403 units) to a minimum of 239 units at 5 hrs and later increased gradually to a maximum of 417 units at 24 hrs. The activity in general ranged between 239 (± 18) and 417 (± 16) units.

The tryptic activity in the intestinal content displayed

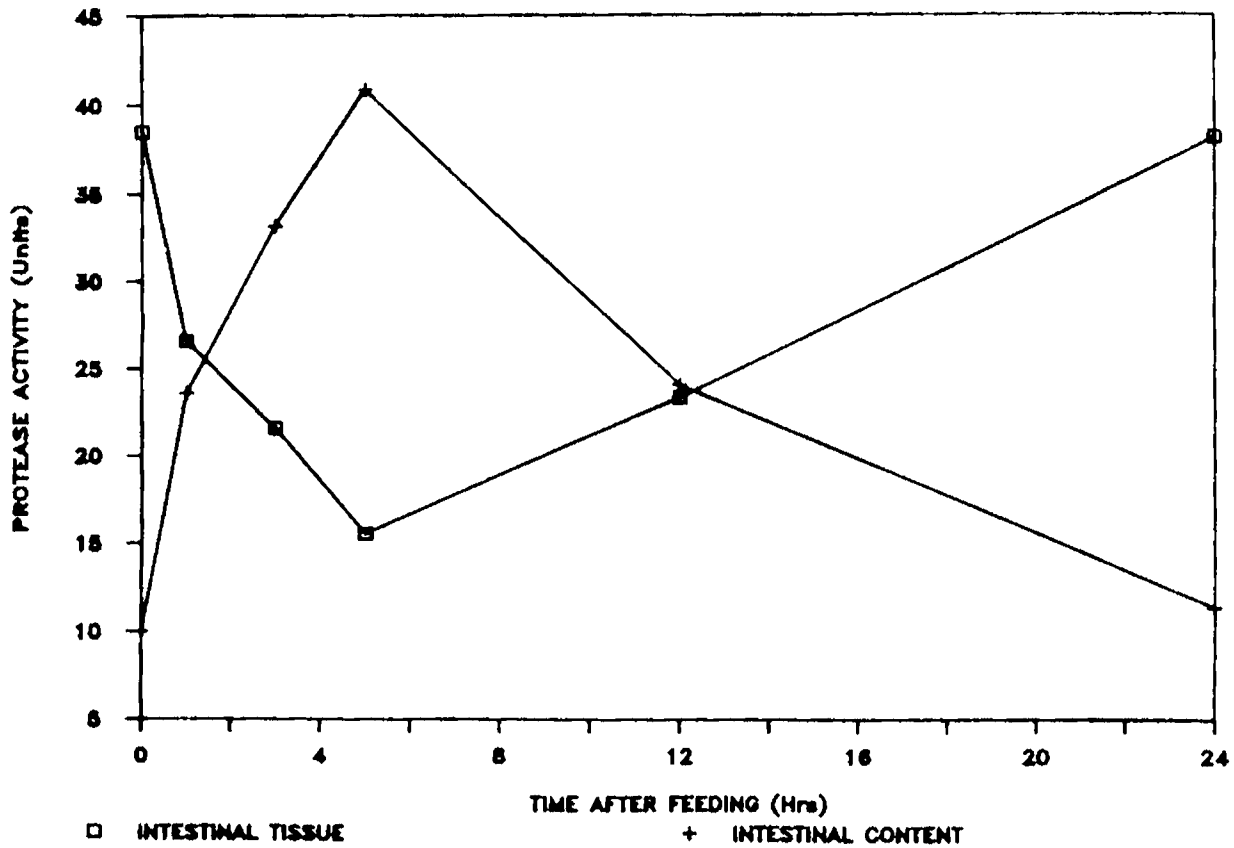

Fig.49 Changes in protease activity of intestinal tissue and intestinal contents of L. parsia with time after feeding.

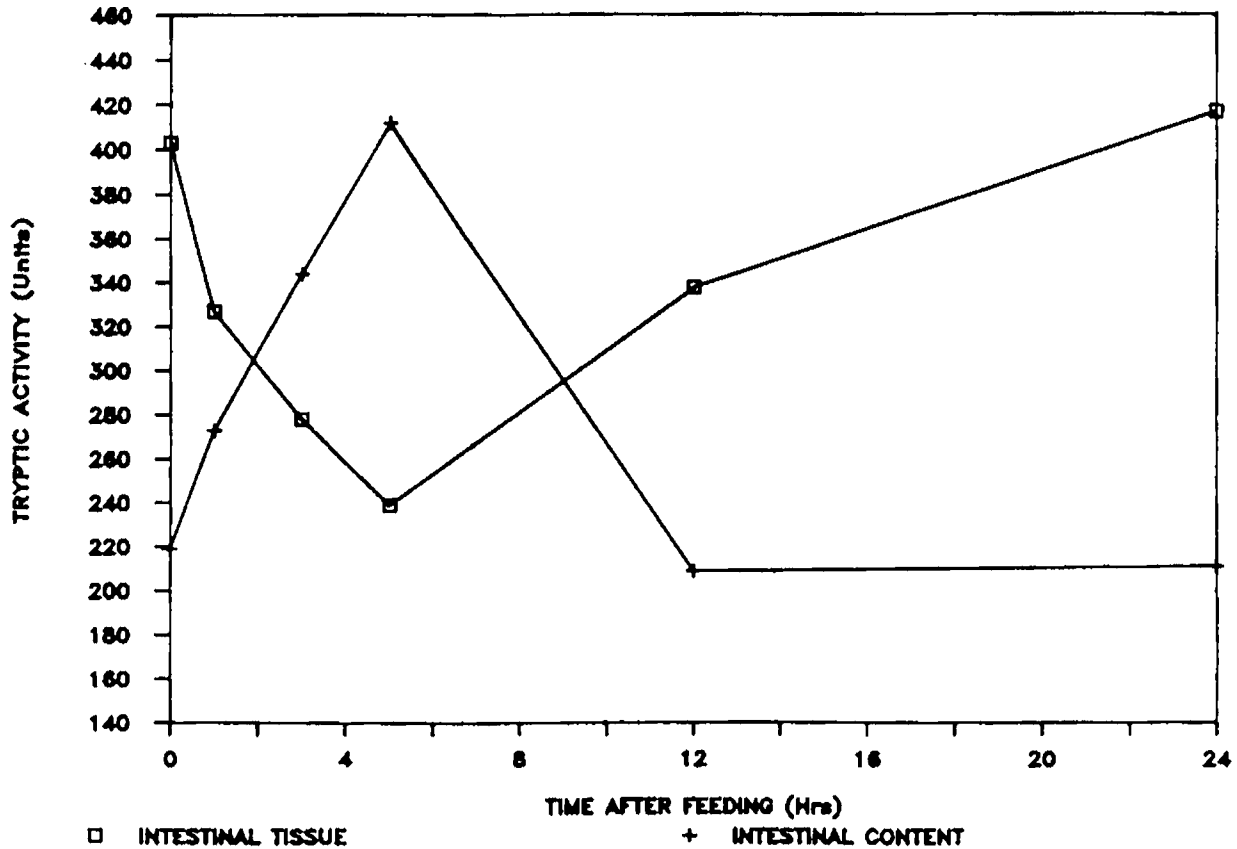

Fig.50 Changes in tryptic activity of intestinal tissue and intestinal contents of L. parsia with time after feeding.

significant changes ($P < 0.01$) over the 24 hr period (Fig.50). Unlike the intestinal tissue, the activity in the intestinal content started increasing gradually reaching a maximum level at 5 hrs and thereafter the activity declined to a minimum of 209 units at 12 hrs. Later, although a marginal increase in the activity (211 units) was observed at 24 hrs, it was only negligible. In general, the activity ranged between 211 (± 12) and 412 (± 12) units.

The activity of chymotrypsin in the intestinal tissue (Fig.51) showed significant changes ($P < 0.01$) over the 24 hr period very similar to that of trypsin. The activity started declining significantly after feeding, and reached the lowest level at 5 hrs (67.0 units). Later, the activity rose significantly and regularly to a level of 122 units at 24 hrs which was close to that of the prefeeding level. The activity varied between 67.4 (± 7.27) and 129.0 (± 10.0) units.

The chymotryptic activity in the intestinal contents was comparatively low and showed significant changes ($P<0.01$) during the 24 hr period, very similar to that of trypsin in the intestinal contents (Fig.51). The enzyme activity after feeding increased steadily and reached a maximum at 5 hrs (119.0 units). Subsequently, the activity dropped gradually and significantly to a level of 64 units at 24 hrs. The level observed at 24 hrs was significantly higher than that of prefeeding. In general, activity ranged between 53.9 (± 9.73) and 119.3 (± 12.4) units at different hours.

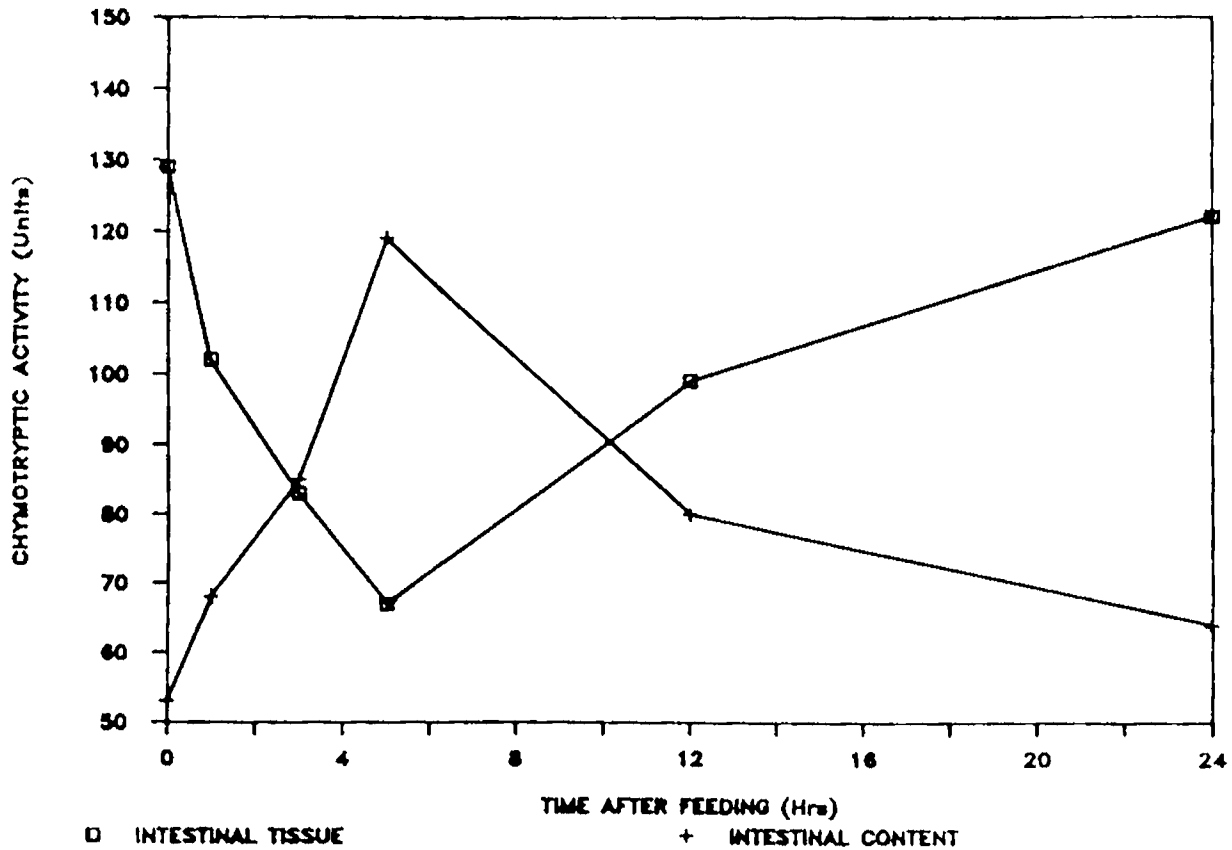

Fig.51 Changes in chymotryptic activity of intestinal tissue and intestinal contents of L. parsia with time after feeding.

7.4 Discussion

The sequential changes in the digestive enzyme activity in the digestive tract of juveniles of *L. parsia* after feeding was investigated by monitoring the pepsin in the stomach tissue and contents and the amylase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, total protease, trypsin and chymotrypsin in the intestinal tissue and its contents. It was observed that, in general the activities of all the enzymes tested were higher in the tissue of the organs than that of their contents.

All the carbohydrases viz. amylase, alpha-glucosidase, beta-glucosidase, and beta-galactosidase recorded a decline at 5 hrs after feeding in the intestinal tissue while recording a peak in the intestinal contents. Further, in both the samples the final activity observed at 24 hrs was very similar to that of pre-feeding level. The high prefeeding activity of carbohydrases in the intestinal tissue indicates that high levels are maintained despite the absence of food in the intestine. Immediately after feeding the activity in the intestinal tissue started declining. This change may be due to the secretion of this enzyme into the lumen of the intestine. A peak in the enzyme activity in the contents, at the time of low activity in the tissue, adds evidence to the fact that the enzyme secreted into the lumen is in response to the ingested diet. Hofer and Schiemer (1981) suggested that the free enzymes in the lumen are reabsorbed by the intestinal wall. In this context, the regaining of the level of enzyme activity to a prefeeding level at 24 hrs, in both tissue and contents, suggest that after the

completion of the digestion of concerned diet the free enzymes in the lumen would have got reabsorbed into the tissue (Bergot, 1976; Noaillac Depeyre and Gas, 1979; Storband and Kroon, 1981; Stroband and vd Veen, 1981; Hofer and Schiemer, 1981; Hofer, 1982; Albertini -Berhaut, 1988). In contrast to the present observation, in the eel Anguilla japonica the amylase activity of the intestinal tissue was reported to maintain an approximately constant level during the 12 hr period after feeding (Takii et al., 1985).

The activity of all the carbohydrases tested decreased sharply at 1 hr after feeding in the intestinal content of L. parsia. However, the activity gradually increased to the maximum level at 5 hrs after feeding and then declined again to reach the prefeeding level at 24 hrs after feeding. The temporary decrease in amylase activity at 1 hr after feeding may be due to dilution by the ingested food and an inadequate rate of secretion. The highest activity attained at 5 hrs after feeding indicates that the enzyme is secreted continuously after feeding to counteract the dilution caused by the food. It seems, therefore, that amylase activities in the intestine of L. parsia are maintained at more or less constant levels and that the temporary decrease after feeding is caused only by the dilution effect. The highest activity detected at 5 hrs after feeding suggests that the digestive process is at its maximum around this time. In the carp, the amylase activity in the intestinal content increased gradually after feeding to reach a maximum at 5 hrs (Onishi et al., 1973 a,b). They also studied the response of digestive enzyme levels to twice-a-day feeding and

found that even in such cases the amylase activity in the intestinal content of carp attained its maximum at 5-7.5 hrs after first feeding (Onishi et al., 1976). They also observed the amylase activity to decrease temporarily just after feeding. They suggested that this might be due to the loss of enzyme excreted with the undigested residue of the previous day. The same suggestion may also hold good for the present study, as, such a decrease was observed immediately after feeding. In the eel Anguilla japonica the amylase activity of the intestinal content showed slight increase from 3 to 5 hrs after feeding but maintained a significantly low level compared with that in the tissue (Takii et al., 1985). In the catfish Clarias gariepinus the amylase activity of the intestinal contents, after feeding also displayed similar sequential changes to that of the present investigation (Uys et al., 1987). Thus the sequential changes in amylase in the intestinal content of L. parsia after different hours of feeding exhibits striking similarities with the other groups of fishes investigated in this respect.

The changes in the activity of other carbohydrases such as alpha-glucosidase, beta-glucosidase and beta-galactosidase were not reported in other groups of fishes. Hence no comparison could be made with other reports. Although these enzymes followed the same pattern of activity exhibited by amylase, the changes in the activity were very much gradual being not significantly different at different hours in most cases. Except for minor details all the carbohydrases followed a similar pattern of activity which is more

or less comparable with such studies in other groups of fishes (Onishi *et al.*, 1973 *a, b*, 1976; Takii *et al.*, 1985; Uys *et al.*, 1987).

The activity of pepsin in the stomach tissue after recording a gradual decrease during initial 3 hrs, rose to the maximum at 5 hrs and remained almost at a similar level at 12 hrs. Later, it reached the prefeeding level at 24 hrs. This observation is in agreement with the observations made in eel (Takii *et al.*, 1985) where the peptic activity in the stomach tissue decreased immediately after feeding and reached a minimum level at 3 hrs which later rose gradually at 12 hrs after feeding. The initial decrease in the activity in the stomach tissue might be due to the enzymes secreted into the stomach lumen in response to the ingested diet. It is known that in mammals gastric secretion is evoked by distension of the stomach wall which activates the neuro-hormonal mechanism. Secretary response to stomach distension is also known in *Rana* (Smit, 1968). A similar kind of mechanism might be involved in the present case also. Krayukhin (1959) found that in the bullhead (*Ictalurus nebulosus*) the act of swallowing leads to abundant secretion of gastric juice. Further, the effectiveness of the distension stimulus in producing gastric secretion has also been demonstrated in the Cottidae (*Cottus gobio* and *Enophrys bubalis*) and in the brown trout (*Salmo trutta*) by Western and Jennings (1970). The peak peptic activity attained at 5 hrs after feeding in *L. parsia* indicates that more enzymes are secreted with the elapse of a period of about 5 hrs after feeding. Based on the experiments

on bluegill, Ashir (1967) concluded that gastric secretion, produced by distension, reached a peak after a lag period of 2-6 hrs. Thus it is probable that a similar kind of secretary mechanism also operates in L. parsia. Further, the maintenance of the higher activity even at 12 hrs after feeding indicates the capacity to digest relatively large quantity of diet by prolonging the digestive process to effect complete and efficient digestion.

The peptic activity in the stomach contents increased gradually and reached a peak at 5 hrs after feeding. However the level of activity was significantly low when compared to the activity in the stomach tissue. In eel, Takii et al. (1985) reported a slight increase in peptic activity in the gastric contents soon after feeding which later maintained a significantly low level. In the sharptooth catfish the activity in the stomach contents increased gradually and reached a maximum at 2.5 to 4 hrs after feeding (Uys et al., 1987). Later, it declined to the prefeeding level at 10 hrs. It is evident from the present results that peptic activity responds quickly to food intake. That might be the reason for the initial increase in peptic activity in the stomach contents, observed as early as at 1 hr after feeding, despite the dilution of the enzyme by food as it is believed. The decline in the activity after 5 hrs indicates that little or no more enzyme is secreted into the stomach and they are partially inactivated. Probably this phenomenon might be due to the denaturation of enzyme or their binding irreversibly to the substrates. However, it may be suggested that the enzymes along with the diet are pushed into the

intestine where the pepsin may get denatured due to suboptimal pH in the intestine.

Similar to that of carbohydrases, protease activity recorded a decline at 5 hrs after feeding in the tissue when compared to a peak observed in the contents. Trypsin and chymotrypsin also exhibited a similar phenomenon following protease in the intestinal tissue and contents. EXcept for a few instances, at all other occasions all the three enzyme activities demonstrated a uniform pattern in their activities. An almost similar type of changes in the enzyme activity was reported for eel (Takii et al., 1985). The decline in activity after feeding shows that the enzyme is secreted into the lumen from the intestinal tissue or the adjoining pancreatic tissue which is diffused almost throughout the length of the intestine in L. parsia (Ghosh et al., 1987).

In contrast to intestinal tissue, the protease activity in the intestinal content sharply increased soon after feeding. The activity almost doubled at 1 hr and became four times higher at 5 hrs after feeding. Even at 12 hrs after feeding the activity was maintained at twice that recorded before feeding. As in the present study, the protease activity in the intestinal contents peaked at 5 hrs after feeding in carp (Onishi et al., 1973 a, b, 1976) and in eel (Takii et al., 1985). In the sharptooth catfish the protease activity peaked at 4.5 hrs after feeding and subsequently decreased quite rapidly to reach the prefeeding level at 10 hrs after feeding (Uys et al., 1987). In contrast to catfish, where the prefeeding level was attained as early as 10 hrs after feeding the protease in

L. parsia maintained high levels of activity (twice that of prefeeding) even at 12 hrs after feeding thereby evidencing the ability to maintain an active phase of digestion over a longer duration.

The increase in activity after feeding in the contents, despite the dilution caused by the arrival of food might be due to an increased secretion from the tissue into the lumen which consequently resulted in a declined activity in the tissue. Further, the secretary response of protease seems to be faster in L. parsia which can be inferred from doubling of activity at 1 hr after feeding.

Sequential changes in the ratio of amylase activity to protease activity in the intestinal tissue and contents after feeding as presented in Table-18, suggest that proteases were secreted rapidly in larger quantity when compared to amylases after ingestion of diet. The ratio of amylase to protease for the tissue increased gradually and continuously reaching a maximum value at 5 hrs. In contrast, the ratio for the contents decreased rapidly within 3 hrs and then increased gradually reaching only 84% of the prefeeding level but twice the value of 1 hr. In the carp, a stomachless fish, Onishi et al. (1973 a) observed the ratio of these enzyme activities in intestinal contents decreased within 3 hrs after feeding and increased thereafter. In eel, Takii et al (1985) reported that the ratio of these enzymes in intestinal tissue increased rapidly and reached a maximum value at 5 hrs after feeding while the ratio for the intestinal contents decreased at 3

Table -18. Ratios of amylase activity to protease activity in intestinal tissue and contents at intervals after feeding in *L. parsia*

Time after feeding (hrs)	Activity Ratio[a]	
	Intestinal tissue	Intestinal content
0	10.07	22.87
1	11.24	8.04
3	13.28	6.42
5	17.91	6.46
12	12.52	9.99
24	8.71	19.43

a The activity ratio was calculated from the following formula:

$$\text{Activity Ratio} = \frac{\text{Total activity of amylase}}{\text{Total activity of protease}}$$

hrs and increased subsequently. It is interesting that L. parsia showed similar changes in the ratio in intestinal contents to carp and eel and in the intestinal tissue to eel inspite of their varied feeding habits and gut morphology.

Onishi et al. (1976) suggested that there is a daily rhythm in digestive enzyme activity in carp. Since no change in enzyme activity was observed in un-fed group of L. parsia ('C' series), it appears that digestive enzyme activity cycles are induced by feed intake. The presence of food in the intestine is known to stimulate secretion of enzymes in mammals (Corring, 1980; Girard-Globa et al., 1980) and amphibians (Scapin, 1982). The digestive enzyme secretary response in L. parsia is similar to that reported in carp (Onishi et al., 1976) eel (Takii et al., 1985) and sharptooth catfish (Uys et al., 1987). These findings imply that L. parsia is physiologically equipped to utilise frequent and irregular meals effectively. Since L. parsia has no intrinsic digestive enzyme cycle and have a rapid digestive enzyme secretary response, optimum ration and feeding frequencies can be established purely on the basis of maximum growth and most favourable food conversion ratios.

CHAPTER 8

EFFECT OF DIET ON DIGESTIVE ENZYME ACTIVITIES

8. EFFECT OF DIET ON DIGESTIVE ENZYME ACTIVITIES

8.1 Introduction

Fishes can be distinguished as herbivores, omnivores and carnivores based on their nature of diet (Al-Hussaini, 1947; Barrington, 1957; Kapoor *et al.*, 1975). The digestive enzymes can be induced by appropriate diet. Thus it is assumed that the carbohydrases would be more copiously secreted in herbivores, while proteases would be quantitatively more in carnivores. There is indeed some evidence that such a relationship does exist, although it is by no means clearly established. Vonk (1927) was the first to record that an adaptation of digestive enzymes to feeding habits does exist in fishes. Nagase (1964) showed that trypsin activity increased with a diet rich in protein while lipase and pepsin did not adapt to food. Other studies on these lines were made by Kawai and Ikeda (1972, 1973 a,b) in carp and rainbow trout; Mukhopadhyay (1977) and Mukhopadhyay *et al.* (1978) in *Clarias batrachus*; Hofer (1979 a,b) and Niederholzer and Hofer (1979) in roach; Reimer (1982) in *Brycon melanopterus* and Danulat (1986) in *Gadus morhua*.

8.2 Material and Methods

8.2.1 Experimental animals

Juveniles of *L. parsia* (10.3 ± 1.8 g) were collected from the Fisheries Experimentation Centre, Kerala Agricultural University, Puduvypu in the Vypeen Island of Cochin. The fishes were transported in oxygen packed transportation bags to the laboratory.

In the lab they were gradually acclimatised to the experimental conditions and fed with a standard diet, the composition of which is given in Table-19.

8.2.2 Experimental diets

Three moist diets were formulated by changing the relative content of protein, carbohydrate and fat and designated as protein rich (P), carbohydrate rich (C) and fat rich (F) diets. The P, C, and F diets were formulated as shown in Table-19. The moist diets were prepared by mixing the dry ingredients as per the proportions given in Table-19 with appropriate amount of water. Gelatin taken in a container was added with cold water, mixed well and then heated (with continuous stirring) in a water bath at 80°C to dissolve gelatin. The container was then removed from the water bath, starch was added with stirring followed by fish meal and casein, and mixed properly. As the temperature decreased, mineral mixture, oil and vitamin mixtures were added and mixed throughly until the temperature dropped to 40°C. The warm diets were transferred into suitable labelled (P, C, F) containers and stored in a deep freezer.

8.2.3 Experimental set up

The acclimatised juveniles were transferred to plastic troughs containing appropriately diluted filtered sea water (20 ppt). Three sets of fishes, each in triplicate, were randomly arranged and the containers were labelled as P1, P2 and P3 (Set-I), C1, C2 and C3 (Set-II), F1, F2 and F3 (Set-III). Each group contained about 20

Table - 19 Dietary ingredients used in the preparation of different diets to study the effect of composition of diet on digestive enzymes in L. parsia.

Ingredients	Diets		
	P	C	F
Gelatin	20	20	20
Fish Meal	88	9	30
Casein	68	7	25
Starch	16	156	74
Codliver Oil	4	4	26
Sunflower Oil	4	4	26
Vitamin mixture[a]	2	2	2
Mineral mixture[b]	6	6	6

a, b the composition of vitamin mixture and mineral mixture are as given in Table-17.

fishes and were fed with the respective diets, P, C and F *ad libitum* twice a day. The containers were cleaned every day by siphoning out the left over feed and faecal matter. Water was changed on alternate days. Throughout the experimental period the salinity of the water was kept constant (20 ppt). The fishes were fed for 60 days and the feeding was discontinued on the penultimate day of termination of the experiment. After concluding the feeding trials, the fishes were killed by cervical dislocation and the intestines were dissected out.

8.2.4 Preparation of enzyme extract

The intestine along with pyloric caeca from each fish was dissected out separately. From each group 10 fishes were dissected out. Care was taken to avoid contamination among the samples. The separated intestines, along with their contents were homogenised individually in ice cold double distilled water to give a 1:10 (w/v) homogenate. The homogenates were centrifuged in a refrigerated centrifuge at 19000 g at 4°C for 15 minutes. The clear supernatant was used as crude enzyme extract.

8.2.5 Assay of Enzyme activities

All the enzymes were assayed at their optimum pH at 37°C as per the detailed procedures given in general Material and Methods (Section 2.6). The protein content of the crude extract was determined by the Lowry's method with bovine serum albumin as the standard (Lowry *et al.*, 1951).

8.2.6 Statistical Analysis

Changes in the enzyme activities with respect to different diets were tested using Analysis of variance (Snedecor and Cochran, 1967).

8.3 Results

Effect of composition of diet on the activities of the digestive enzymes of the intestinal tract was studied by giving diets with different composition of protein, carbohydrate and fat. Results presented in Table-20 suggest that all the enzymes tested in the present study such as amylase, maltase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, protease, trypsin, chymotrypsin and esterase evinced increased activity with the respective diet significantly, i.e. exhibited compensatory changes in the relative contribution of different digestive enzymes in response to changes in diet composition.

All the carbohydrases including amylase, maltase, alpha-glucosidase, beta-glucosidase and beta-galactosidase recorded highly significant changes ($P < 0.01$) in their activities with respect to varied diet composition. Thus all the above mentioned enzymes could demonstrate comparatively a higher activity in fishes fed with carbohydrate rich diet (C) followed by those fed with fat rich diet (F) and protein rich diet (P) (Table-20).

Total protease, trypsin and chymotrypsin, similar to carbohydrases, recorded highly significant changes ($P<0.01$) in their activities

Table - 20 Effect of diet composition on the activities of various digestive enzymes in L. parsia.

Enzymes	Enzyme activity[a] Diet Treatments		
	P	C	F
1. Amylase	209.03 (± 20.72)	640.26 (± 66.13)	318.86 (± 39.63)
2. Maltase	65.46 (± 12.89)	202.8 (± 21.50)	103.0 (± 14.89)
3. Alpha-glucosidase	43.5 (± 8.09)	156.33 (± 27.90)	94.86 (± 12.57)
4. Beta-glucosidase	18.8 (± 4.87)	95.6 (± 14.18)	38.4 (± 8.74)
5. Beta-galactosidase	50.33 (± 11.76)	196.53 (± 20.70)	102.1 (± 23.04)
6. Protease	18.74 (± 3.79)	10.92 (± 1.95)	12.56 (± 23.12)
7. Trypsin	377.73 (± 39.82)	188.43 (± 26.8)	280.06 (± 29.10)
8. Chymotrypsin	111.63 (± 18.05)	70.03 (± 9.12)	90.1 (± 11.34)
9. Esterase	12.74 (± 2.60)[b]	13.27 (± 3.03)[b]	27.49 (± 5.01)

a all the means differ significantly unless otherwise stated at $P < 0.01$

b not different at $P > 0.01$

galactosidase, protease, trypsin, chymotrypsin and esterase were assayed from each individual of the group and compared with the group which received other diets. The results clearly showed that these digestive enzymes adapted to the relative composition of the diet. Vonk (1927) was the first to record that an adaptation of digestive enzymes to feeding habits exist in fishes. Yonge (1937) has stated that there exists a definite correlation between the food of any animal and the nature and relative strength of its digestive enzymes.

It could be seen from the Table-20 that the diet rich in protein (P) has induced more proteolytic enzymes viz. protease, trypsin and chymotrypsin. Similarly the fish group that received the diet rich in carbohydrate (C) exhibited higher levels of amylase, maltase, alpha-glucosidase, beta-glucosidase and beta-galactosidase when compared with the other two groups. The diet rich in fat (F) has also induced more esterase activity in groups that have received it. Further, carbohydrases and proteases also recorded high activities in the fishes which were fed with fat rich (F) diet, second only to their respective diets C and P. This might be due to the presence of carbohydrate and protein components in significant levels in the diet F (Table-19). Nagase (1964) confirmed this type of adaptation with amylase and proteases in a similar experiment on Tilapia mossambica. Volya (1966) investigated the proteolytic, lipolytic and amylolytic activities in the alimentary canal of Trachurus, Scomber, Mullus, Pleuronectes and Mugil. The highest proteolytic and lipolytic activities were found in the predatory

species, Trachurus and Scomber. The lowest protease activity coupled with the highest amylolytic activity was found in Mugil. Moitra and Das (1967) presented some evidence to the fact that in Tilapia mossambica the relative activity of the digestive enzymes be correlated with the actual diet composition of the fish. In carp intestine, the activities of amylase, maltase and protease increased in accordance with the content of starch and protein in the diet respectively showing an adaptation to the diet composition (Kawai and Ikeda, 1972). In contrast to the present results and those mentioned above, in the rainbow trout Salmo gairdneri, fed with different diets the activities of pepsin, trypsin, maltase and amylase were all high in the high protein diet and were generally proportional to the protein content (Kawai and Ikeda, 1973a). The preponderance of protease in rainbow trout may be attributed to its carnivorous feeding habits. In the air-breathing cat fish Clarias batrachus the activities of protease and amylase showed significant changes with change in the dietary constituents while cellulose and lipase showed no such changes (Mukhopadhyay, 1977). Later, in a similar experiment on Clarias batrachus itself Mukhopadhyay et al. (1978) demonstrated that the protease activity was maximum in fishes maintained with a diet of 50% protein when compared with the fishes that were maintained with 25% and 75% protein in the diet. Albertini-Berhaut (1978) showed that in young mullets (Mugil capito) there was an enzymatic adaptation to the nature of diet. Further, the correlation between the digestive enzyme activity and the diet was emphasized in roach and rudd with respect to amylase (Hofer, 1979 a), protease (Hofer, 1979 b), cellulase (Niederholzer

and Hofer, 1979) and in several species of fishes (Hofer and Schiemer, 1981).

In a similar experiment as that of the present one, Reimer (1982) demonstrated that the activity of amylase, trypsin and lipase can be increased respectively with a diet rich in carbohydrate, protein and fat in Amazon fish Matrincha (Brycon sp.). Pepsin, however, did not show any change in activity. This was the first experiment to show that the lipase activity was influenced by the composition of fat in the diet. The present investigation also support this observation. The previous studies indicate that there is no lipase adaptation. Chesley (1934) noted there was no correlation between the fat content in the food of a fish and lipase activity. Nagase (1964) found no increase of lipase activity in Tilapia mossambica fed with a fat rich diet. Agrawal et al. (1975) stated as "Lipase activity ..... did not seem to be correlated to the fat content of the diet".

Chitinase activity in cod, Gadus morhua, was found to be influenced by the chitin content of the food consumed while beta-glucosidase revealed no such dependence on food quality (Danulat, 1986). Interestingly in L. parsia the influence of diet composition on the beta-glucosidase is marked. Patra and Ray (1987) demonstrated that the protease activity in Anabas testudineus was influenced by the quality and source of protein in the diet. Similarly in Cyprinus carpio, Catla catla and Hypophlathalmichthys molitrix the protease activity was shown to be influenced by the composition of the diet (Phadate and Srikar, 1987). Stevens and McLeese (1988) concluded

that the composition of diet is a more potent stimulus than the amount of food available in effecting changes in the amount of trypsin produced, since they could not find any change in enzyme activity in rainbow trout fed on various levels of diet. The present results add support to the above conclusion.

There are also reports on digestive enzyme levels in various fishes that could not be correlated with their feeding habit and diet composition. Cheslay (1934) found differences in proteolytic action in the alimentary canals of a number of fish species, that could not be correlated with their diet. Fish (1960) reported that as far as proteases are concerned, differences between Tilapia sp. and Perca sp. are small and insignificant. Morishita et al. (1964) also found differences in proteolytic action in the alimentary canals of a number of fish species that could not be correlated with their diet. The anchovy (Patton and Benson, 1975) which was fed with a diet containing 70% wax ester on a dry weight demonstrated no superior ability to incorporate wax ester when compared to the coral reef fish whose diets probably do not contain more than 2-4% wax ester (Lee and Hirota, 1973). In rainbow trout, ration had no effect on the specific activity of trypsin and chymotrypsin eventhough those fed adlib consumed more than twice as much as those on the low ration diet (McLeese and Stevens, 1982).

The influence of enzyme inhibitors on the levels of digestive enzymes were reported in milk fish. Benitez and Tiro (1982) demonstrated that a group of milk fish reared in ponds dominated by the filamentous green algae Chaetomorpha brachygona, exhibited no

tryptic activity in the digestive tract. Later, they showed that the algae contained a powerful trypsin inhibitor. Yet another evidence for the influence of quality of diet on digestive enzymes and growth was provided by Wilson and Poe (1985) who observed reduced growth rate and protein efficiency ratio in the channel cat fish fingerlings fed with raw and inadequately heated soyabean meal. This might be due to decreased digestive enzyme activity, as this meal contained trypsin inhibitor. Further, a number of trypsin inhibitors have been isolated from various plants (Laskowski and Laskowski, 1954; Mikola and Suolinna, 1969; Kanamori et al., 1976; Swartz et al., 1977; Tashiro and Maki, 1979). Seeds of leguminous plants are particularly rich in trypsin inhibitors (Kunitz, 1947; Wagner and Riehn, 1967; Wilson and Laskowski, 1973; Odani and Ikenaka, 1977). In this context it is suggested that since the digestive enzymes in L. parsia are affected by diet composition and also by enzyme inhibitors, care should be exercised while compounding feed for its culture operations. Amylase inhibitors from wheat could not produce any significant difference in amylase activity in the intestinal fluids of carp probably due to an increased production of pancreatic amylase to compensate the effect of inhibitors (Sturmbauer and Hofer, 1985).

It is interesting to note that L. parsia being herbivorous and feeding mainly on detritus which contain only very little protein are able to satisfy their requirements for protein. As it is expected, since the natural diet contains little protein the enzymes of protein digestion should also be less. From an

evolutionary stand point of view, fish apparently have at least two digestive strategies for exploiting an increased or hard-to-digest food supply. They can modify the amount and character of their digestive enzymes and/or lengthen the time in which food is exposed to enzymes. L. parsia exhibits both these tendencies to exploit and utilize the available dietary components efficiently. Natural selection has produced two gross morphological characteristics of the fish alimentary canal which increases the time of food retention as well as increases the area of absorption. One of the characteristics is the elongated intestine which is about 2.5 times longer than the total length of the fish in L. parsia. The second characteristic is the development of blind finger like out-growths of the anterior end of the intestine called pyloric caecum numbering 5 in L. parsia. These interesting structures vary greatly in number, ranging from one to over a thousand in different groups of fishes (Suyehiro, 1941; Rahimullah, 1945). Their lack of taxonomic significance strongly supports their role in an nutritional strategy for increasing retention time of food in the intestine (Rahimullah, 1945).

Hofer and Schiemer (1981) and Hofer (1982) have shown that, if, besides specific activity, the volume of the gut fluid and the number of gut fillings per day are also taken into account, food turns out to be exposed to higher proteolytic activity in the digestive tracts of herbivorous than those of carnivorous fish.

Thus, it may be suggested that the mullet L. parsia possess the biochemical mechanism to adapt the secretion of the digestive enzymes to the composition of the diet and also possess the anatomical structure (pyloric caeca and lengthy intestine) to allow significant retention of diet for complete and effective digestion and assimilation.

SUMMARY

SUMMARY

1. The present investigation entitled "Studies on the digestive enzymes of the cultivable grey mullet Liza parsia (Hamilton Buchanan, 1822)" was carried out with a view to understand and suggest solutions to the following problems:

   a. What kind of qualitative and quantitative distribution profiles the digestive enzymes envince in L. parsia;

   b. How does the digestive enzymes respond to the changes in the physiochemical factors prevailing in the habitats;

   c. Are the digestive enzymes linked up with the size, food and feeding habits of the fish, and

   d. Is there any change in the relative contribution of various digestive enzymes in response to changes in the dietry composition.

2. The present study included the following programmes of work:

   a. Assessment of the distribution pattern of digestive enzymes along the various regions of the alimentary canal.

   b. Characterisation of digestive enzymes in relation to different physicochemical parameters.

   c. Studies on the relationship between digestive enzyme activity and size of the fish.

d. Observations on changes in the digestive enzymes in relation to feeding habit.

e. Investigation on the changes in digestive enzyme activity after different hours of feeding, and

f. Assessment of the effect of dietary composition on the digestive enzymes.

3. A thorough review of literature revealed that works on nutrition and digestion of mullets are rather scanty and limited to few reports from abroad and in India.

4. The Gold-spot mullet L. parsia were collected from Cochin Backwater nearby Vypeen Island using Chinese dipnets, and the fishes were transported to the laboratory and subjected to further studies. During feeding experiments, they were maintained at room temperature in FRP tanks filled with filtered sea water.

5. The relative length of gut for the adult mullet was 2.4 (± 0.32) times of the length of the fish.

6. a. The crude enzyme extracts of the digestive tract was prepared using the different regions, oesophagus, cardiac stomach, pyloric stomach, pyloric caeca, liver, gall bladder, spleen, anterior intestine and posterior intestine.

b. The crude extract from each region was assayed for the various carbohydrases, proteases, lipases and phosphotases.

c. Of all the regions, pyloric caeca followed by the intestine were the major areas of enzyme activity.

7. Carbohydrases assayed in the crude enzyme extract of the digestive tract included maltase, dextrinase, sucrase, trehalase, alpha-glucosidase, raffinase, melibiase, cellobiase, salicinase, beta-glucosidase, beta-galactosidase, amylase, and cellulase. However maltase dextrinase, sucrase, trehalase, alpha-glucosidase, raffinase, cellobiase, salicinase, beta-glucosidase, beta-galactosidase, and amylase were present in the digestive tract. Of all the carbohydrases, amylase was the major enzyme found in the various regions.

8. a. Proteases assayed in the present study included acid protease-pepsin, total alkaline protease, endoproteases-trypsin, chymotrypsin, and elastase, exoproteases-carboxypeptidase A, carboxypeptidase B and leucine aminopeptidase. All the enzymes were present in various regions of digestive tract. Pyloric caeca and intestine were the major areas of enzyme activity. Pepsin was the major enzyme in the cardiac stomach region. Elastase and carboxypeptidases A and B were found only in meagre quantities.

b. Acid phosphatase, alkaline phosphatase and esterase activities varied in their intensities in different regions.

9. a. The different group of digestive enzymes such as amylase, pepsin, total alkaline protease, trypsin, chymotrypsin and leucine aminopeptidase were characterised with respect to pH, temperature, chloride ion concentration, effect of inhibitors and metal ions.

b. Amylse activity was optimum at pH 6.6 and temperature 52°C. Chloride ion was found to have no significant effect.

c. Pepsin activity was optimum at pH 2.0.

d. Total alkaline protease activity was optimum between pH 7.5 and 8.5 and there was also a smaller peak between 9.4 and 10.0. The optimum temperature was between 47°C and 60°C. Trypsin and chymotrypsin had optimal pH at 8.0 and 7.5 respectively.

10. Effect of inhibitor substances on the activity of alkaline protease trypsin and chymotrypsin was conducted using PMSF, TLCK, TPCK, EDTA and SBTI. PMSF inhibited total protease, trypsin and chymotrypsin activities. TLCK inhibited total protease activity partly and trypsin completely but not chymotrypsin. TPCK inhibited total protease moderately and chymotrypsin significantly. Whereas trypsin was not inhibited. EDTA had no effect while SBTI inhibited all the three activities.

11. a. Effect of various metal ions on the activities of protease, trypsin and chymotrypsin was studied. Hg^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+} strongly inhibited the enzyme activities.

b. Leucine aminopeptidase had a pH optimum of 8.4 and temperature optimum of 52°C.

12. The different digestive enzymes such as amylase, alpha-glucosidase, beta - glucosidase, beta-galactsidase, pepsin, total alkaline protease, trypsin, chymotrypsin, carboxypeptidase A and B, leucine aminopeptidase, esterase, acid and alkaline phosphatases, were analysed in different size groups of L. parsia ranging from 1-2 cm to 19 cm in total length.

- Activities of amylase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, and pepsin, were found to increase with increasing size;

- Activities of total protease, trypsin, carboxypeptidase A and B, leucine aminopeptidase and acid phosphatase decreased as the size of the fish increased.

13. Studies on intestinal feeding index showed that the fish feeds only during the day with high intensity during the midday.

14. The digestive enzymes, amylase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, protease, trypsin, chymotrypsin and leucine aminopeptidase assayed from the anterior and posterior intestines exhibited a positive correlation with intestinal feeding index indicating that the active phase of digestion is in tune with feeding intensity.

15. The sequential changes in the digestive enzyme activity in the digestive tract of jeuveniles of L. parsia after feeding was investigated by monitoring the pepsin in the stomach tissue and contents and the amylase, alpha-glucosidase, beta-glucosidase, beta-galactosidase, total protease, trypsin and chymotrypsin in the intestinal tissue and its contents.

- All the enzyme activities tested were higher in the tissues of the organs than that of their respective lumen contents.

- All the enzymes recorded a decline at five hours after feeding in the intestinal tissue while recording a peak activity in the intestinal contents indicating that these enzymes are secreted into the lumen of the alimentary canal in response to the ingested diet.

- Active digestive process takes place around five hours after feeding.

- L. parsia is physiologically equipped to utilize infrequent and irregular meals effectively and has rapid digestive enzyme secretary response and has no intrinsic digestive enzyme cycle.

16. Effect of dietary composition on the relative contribution of various digestive enzymes was studied by feeding diets with different relative composition of protein, carbohydrate and fat in different groups of juveniles of L. parsia maintained in triplicates. The level of digestive enzymes were found to be adapted to the dietary composition.

The results of the present investigation embodied in this thesis, besides enhancing our knowledge on the digestive physiology of mullets, throws more light on the following aspects when this species is desired as a candidate species for aquaculture. L. parsia have full complement of various digestive enzymes, which can hydrolyse a variety of substances, and increased RLG which would increase the gut passage time and maintain an active phase of digestion over a longer duration. They posses the biochemical mechanism to adapt the secretion of the digestive enzymes to the composition of the diet. Since L. parsia has no intrinsic digestive enzyme cycle, other than that induced by the ingestion of diet, and have a rapid digestive enzyme secretary response, optimum ration and feeding frequencies can be established purely on the basis of maximum growth and most favourable food conversion ratios.

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