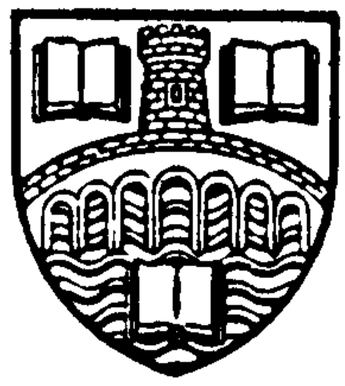
EFFECTS OF AMYLASE INHIBITOR ALBUMIN FROM WHEAT ON THE ALPHA-AMYLASE ACTIVITY IN CARP AND TILAPIA



THESIS
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BY
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M.N

ABSTRACT

The amylolytic activities of alpha-amylase extracted from Mirror carp (Cyprinus carpio) and Nile tilapia (Oreochromis niloticus) were significantly reduced by purified amylase inhibitor albumin of wheat when tested under in vitro conditions. The action of this inhibitor was rapid and maximum levels of inhibition were attained within 20 minutes. For both carp and tilapia, the enzyme residual activities after inhibition were found to be related inversely to inhibitor concentration and positively to the initial enzyme activity levels. The curvilinear relationships between these parameters were explained by deriving equations of the type:

$$A_2 = a + b A_1 - c I + d I^2$$

where a, b, c and d are constants,

A_1 = Initial amylase activity (mU/min),

A_2 = Residual amylase activity (mU/min),

I = Inhibitor concentration as ug protein.

Inhibitions were greatest for amylases from gut tissue and lowest for amylases from gut fluids. 1 ug of purified inhibitor was found to contain a potency to

reduce 298 Units of carp gut tissue alpha-amylase and 532 Units of tilapia intestinal tissue alpha-amylase, by 50%.

When amylase inhibitor extracted from wheat was incorporated in the feed of carp in its active form for three weeks, it caused a significant reduction in the specific growth rate to only 0.16%/day, while in carp fed autoclaved inhibitor, such reduction in growth was not seen and the SGR was maintained at over 1.00%/day. However, despite the presence of active inhibitor in the intestine, the fish were able to maintain alpha-amylase activities in the gut contents at a level similar to that in fish fed denatured inhibitor. This was achieved by hyperactivation of enzyme secretions in the tissues of hepatopancreas and intestine. Hepatopancreas from fish fed active inhibitor exhibited more than two-fold increase in amylase activity compared to those fed denatured inhibitor. By the third week of the experiment this difference in enzyme activity levels was not apparent but there were also no indications of adaptation or improvement in growth rate. Degenerations in hepatopancreas were also not apparent.

Feeding carp with diet containing wheat with its inherent content of inhibitor also caused pancreas hyperactivity and some reduction in growth rate for a short period in comparison to those fish fed autoclaved

wheat. In carp, the alpha-amylase activity did not vary depending on the raw or gelatinized nature of starch, both forms elicited equal increases in enzyme activity. However, autoclaving wheat, though effective in inactivating the inhibitor, was found to lower the biological value and digestibility of wheat proteins.

Contrary to the result of the carp trials, in Nile tilapia, the growth was not significantly reduced by feeding on diet containing active inhibitor and a SGR of 1.57%/day was recorded in comparison to 1.81%/day in tilapia fed denatured inhibitor. Samples of stomach and intestinal contents collected 4 hours after feeding did not reveal the presence of active inhibitor. Apparently the acidic protease, pepsin, in the stomach of tilapia caused the total destruction of the inhibitor in the diet before the contents were passed into the intestinal region. The presence of active amylase inhibitor in tilapia feed did not affect the digestibilities of starch and protein in the diet. Both the groups were able to digest carbohydrates and protein to levels of over 90%.

The implications of these results are discussed in relation to feed formulation and fish nutrition.

The work presented in this thesis is the result of my own investigations and has neither been accepted nor is being submitted for any other degree.

....Natarajan.M..... Candidate

....Barbara.Ross.... Supervisor

....L.G.Ross..... Supervisor

CHAPTER 1

GENERAL INTRODUCTION.

CHAPTER 1

GENERAL INTRODUCTION.

Ingestion of food by animals, including fish, is the immediate stimulus for a large number of enzyme systems in different organs and tissues. A change in the amount or composition of the diet leads to a change in the activity of the various systems involved in the nutrition of the animal (Fabry, 1969). Thus nutritional adaptation could be viewed as the adaptation of enzyme systems to a changing diet. Most animals have the general capability to adapt their enzyme systems and even their regulatory mechanisms in order to tolerate short term or even long term dietary changes (Fabry, 1969). The degree of this nutritional adaptability could however vary with different species of fishes (Walton, 1986).

The importance of carbohydrates as an energy source to spare proteins in compounded diets of all cultivated fishes is well recognised (Erman, 1969; Cowey et al., 1975; Adron et al., 1976). Most of the carbohydrate sources used in the preparation of animal diets, including those of fish, are of plant origin. Considerable effort has been directed towards the evaluation of the nutritional value of starch to fish including carp, trout, catfish and tilapia (Page and Andrews, 1973; Ogino et al., 1976; Pieper and Pfeffer, 1978; Anderson et al., 1984). Attempts have been made, with varying success, to reduce the proportion of costly protein in fish feed (usually 40% for most fish) and

incorporate higher percentages of easily available and cheaper carbohydrates (Singh and Nose, 1967; Takeuchi et al., 1979). Studies have indicated that common carp, Cyprinus carpio, are capable of utilising higher levels of carbohydrates in their diets compared to carnivorous fishes such as yellow tail (Seriola quinqueradiata) and sea bream (Chrysophrys major) (Shimeno et al., 1977; Furuichi and Yone, 1971a, b; 1980), without any appreciable loss in growth. Anderson et al. (1984) showed that the Nile tilapia, Oreochromis niloticus, can utilise up to 25%, and possibly even more, carbohydrate in their diet without loss of growth. This general ability of carp and tilapia to tolerate higher levels of carbohydrates in the diet may be attributable to an ability to adapt or modify the enzyme systems involved in digesting and assimilating the increased carbohydrate levels.

Discovered by Leuchs (1831), amylases were originally called 'diastase'. The major classes of amylases are endoamylases, such as the alpha-amylase, and exoamylases such as beta-amylase and gamma or glucoamylase (Thoma et al., 1970). Alpha-amylase (alpha-1,4 glucan 4 glucanohydrolase; EC 3.2.1.1) was defined by Kuhn (1925) as a carbohydrase which yields saccharide products with the alpha configuration. Since it is an endoamylase, it does not require free ends of amylose chains for activity. It randomly cleaves the alpha-1,4- glucoside bonds in the amylose polysaccharide chain. Beta-amylases on the other hand attack the terminal

alpha-1-4 linkages. The alpha-1,6- glucosidic linkages in the branching (amylopectin) portions of starch cannot be broken by either the alpha or beta forms of this enzyme. Consequently, the products of hydrolysis of starch in the gut lumen are maltose (glucose-glucose), maltotriose (glucose-glucose-glucose) and a mixture of dextrins containing on average 6 glucose units per molecule and the alpha-1-6- branches of amylopectin (Davenport, 1982). The hydrolysis of polysaccharide chains occurs via the principle of 'multiple attack': once the enzyme-substrate complex is formed, the enzyme can hydrolyse several bonds of the polymer successfully before the enzyme is again liberated in the free form (Robyt and French, 1967). Amylase activity in general is measured either by monitoring the disappearance of starch substrate (amyloclastic method) or the release of maltose (saccharogenic method) from the hydrolysis of starch.. One International Unit (U) of amylase is the enzyme activity that transforms 1 uMole of substrate in 1 minute, under optimal conditions.

Alpha-amylase is secreted by all animals. It is synthesised <u>de novo</u> in the salivary glands, the acinar cells in the pancreas and the intestinal mucosal cells. Amylolytic activity has been found in extracts of pancreas, liver, stomach, intestine and pyloric caeca of several species of fishes (Battle, 1935; Vonk, 1937; Al-Hussaini, 1949; Sarbahi, 1951; Nagayama and Saito, 1968; Kawai and Ikeda,. 1971). Jancarik (1964) claimed that various parts of

the digestive tract of common carp are involved in secretion of amylase and Cockson and Bourn (1973) state that the amylolytic activity of the posterior part of the intestine of Barbus paludinosus (a variety of carp) is equal to that of the anterior part. In tilapia, (O.mossambicus), amylase activity is dispersed along the entire alimentary canal (Fish, 1960).

Unlike higher animals most fish do not have a compact pancreas, rather it is usually dispersed in the liver and along the intestine (Barrington, 1957). Vonk (1937) found that in common carp the amount of amylase in pancreatic tissue was about 50 - 100 times as large as in the gut extracts. He considered that amylase detected in the gut extracts had originated from the pancreas. Bondi and Spandorf (1954) pointed out that most of the amylolytic activity in common carp was found in the pancreas. Yamane (1973 a, b) studied the localization of amylase activity in common carp, tilapia (O.mossambicus) and bluegill sunfish (Lepomis macrochirus) by a histochemical procedure. He found that, although weak amylolytic activity is detectable in various parts of the digestive organs, the activity appeared only on the luminal surface of the tract and not in the mucosa or submucosa. In addition, no activity was found in the cells of the liver but strong amylase activity was found in the pancreatic exocrine cells. Therefore it is evident that it is the pancreas and not the intestine that is the main amylase secretory organ in fish. In the intestine

of fish, as is the case in the small intestine of higher animals and mammals (Davenport, 1982), the digestive enzymes, including alpha-amylase, are an integral part of the brush border membrane and are not secreted. When epithelial cells desquamate, intracellular enzymes as well as the enzymes of the brush border are shed into the lumen.

Amylase is considered to be relatively easy to purify (Karn and Malacinski, 1978). Yet fish alpha-amylases have not been purified and therefore their specific kinetic properties are not known. However animal amylase in general is a single polypeptide chain with a native molecular weight of approximately 50,000 - 55,000 daltons (Karn and Malacinski, 1978). Based on the work of MacDonald et al. (1977), it has been suggested by Karn and Malacinski (1978) that amylase might be synthesised in the pancreas as a higher molecular weight precursor molecule which is subsequently modified to the size of the native enzyme. Amylase often occurs as multiple forms. Malacinski and Rutter (1969) noted multiple forms (isozymes) of amylase in lungfish pancreas.

All amylases, regardless of their biological origin, contain calcium ions associated within the enzyme molecule. Removal of calcium results in decreased activity which can be restored upon readdition (Vallee et al., 1959; Hsiu et al., 1964). Chlorides are also required for maximum enzyme activity. A concentration of approximately 10 mM chloride ions has been determined as optimum for the catalytic activity of human salivary amylase (Muus et

al., 1956) and porcine pancreatic amylase (Meyer et al., 1947). Calcium and chloride ions improve the stability of amylase at extremes of pH.

Animal alpha-amylases exhibit maximal activity around neutral pH (Karn and Malacinski, 1978). Their pH/activity profile is always bellshaped and at extremes of pH they display no activity. The optimum pH of alpha-amylase in most fish examined by Kuzmina and Nevalennyy (1983) was in the range of 7 to 8. According to Nagase (1964) tilapia amylase has a pH optimum of 6.71. Thus the amylase detected in the acidic stomach of tilapia cannot always be expected to be functional because of the very acidic conditions there with records of pH's as low as 1.0 (Moriarty, 1973; Bowen, 1976). Ushiyama et al. (1965) compared the amylase activities of salmon, carp, cod and flounder and found that salmon alpha-amylase had an optimum activity at a pH of 8.5 and an optimum temperature of 20°C. Agrawal et al. (1975) found that the optimum pH value for carbohydrases from the cat fishes, Wallogo attu and Clarias batrachus and the Indian major carp, Labeo rohita were in the range of 5.0 and 7.0.

Like pH, highest activity of amylase is usually seen at a certain optimum temperature. Above the optimum temperature a sharp drop in enzyme activity can be expected. Morishita et al. (1964) found that digestive enzymes from salmonids were more active at lower temperatures than those of yellowtail (S. quinqueradiata), eel (Anguilla

japanica) and ayu (Plecoglossus altivelis). Therefore this suggests that the optimum temperature for warm water fish may be higher than that of cold water fishes. Chiu and Benitez (1981) reported that the optimum temperature for the activity of intestinal amylase in milkfish (Chanos chanos) was about 50°C which is above the lethal limit for most fishes.

Amylase activity may change with fish age, physiological state and season. Kitamikado and Tachino (1960) found that amylolytic activity in young rainbow trout is high and increases as the fish grow, reaching a peak at a weight of around 100g. Above this weight the activity decreases and in large fish of about 1000g the amylolytic activity is considerably lower than in young fish. Decreasing amylolytic activity with age was also found by Morishita et al. (1964), Stroganov and Buzinova (1969) and Kuzmina (1980). In common carp, amylase activities have been found to develop as the larva develops, starting at a point 7 to 10 days after hatching (Kawai and Ikeda, 1973). Sinha (1978) noticed weak amylase activity in the fry stage of Cirrhinus mrigala which became pronounced in the fingerling and adult stages of this fish, and these changes closely correlated with the changing food habit from animal to plant food.

Ananichev (1959) found that amylase activity of burbot (Lota lota), pike-perch (Stizostedion vitreum) and bream (Abramis brama) was at a maximum during the period

of preparation for spawning. In rainbow trout the activity ratio of amylase and protease increases during maturation (Onishi et al., 1974) which appeared to indicate a higher utilisation of carbohydrates during this period.

Seasonal variation has also been observed in the activities of carbohydrases and, in general, maximum activities coincide with periods of intensive food intake (Ananichev, 1959). Chepik (1964) found that the activity of digestive enzymes of common carp was higher in spring than in any other seasons. Hofer (1979a) showed that under natural conditions amylolytic activities in the roach (Rutilus rutilus) were correlated with seasonal food intake and temperature cycles.

Amylases have been found in omnivorous fish such as cyprinids (Sarbahi, 1951; Bondi and Spandorf, 1954), in herbivores such as tilapias or grass carp (Al-Hussaini and Kholy, 1953; Fish, 1960; Nagase, 1964; Moriarty, 1973, Das and Tripathi, 1986) and also various species of carnivores such as salmonids, eels, yellowtail and black bass (Kitamikado and Tachino, 1960; McGeachin and Debnham, 1960; Ushiyama et al., 1965, Nagayama and Saito, 1968). Many authors have suggested that carnivorous fishes have limited secretions of amylase compared to omnivores or herbivores. As early as 1925, Kenyon found an abundance of starch-digesting enzyme in the intestinal mucosa of carp but almost none in pickerel (cited by Phillips, 1969). Kitamikado and Tachino (1960) found that the amylolytic

activity in rainbow trout was lower than in common carp but higher than that in eel (A. japonica). Ushiyama et al. (1965) found an amylase activity in the pyloric caeca of salmon that was 1/411 that of carp intestine, 1/29.5 of cod pyloric caeca and 1/95 of flounder intestine. Cockson and Bourn (1972) compared the amylase activities in a herbivorous Tilapia sp. with that of the omnivorous Clarias sp., and found the activity in tilapia was higher than that in the catfish. Kapoor et al. (1976) reported that in carnivorous fish, the ratio of amylolytic and proteolytic activity was about 0.125 while in herbivores and omnivores, ratios of 1.1 to 3.4 were obtained. Similarly Shimeno et al. (1979) compared the amylase activity in common carp with yellowtail and found the activities in the former species many times higher than in the latter.

Amylase production by the pancreas is strongly affected by the composition of the diet. Rats synthesise eight times as much amylase in the pancreas when fed starch-rich diets compared to those fed on casein-rich diets (Marchis-Mouren et al., 1963; Reboud et al., 1966). This phenomenon of 'substrate-induced enzyme synthesis' has also been demonstrated in fishes (Fish, 1960; Nagase, 1964; Agrawal et al., 1975; Hofer, 1979a, b; Hofer and Schiemer, 1981; Reimer, 1982).

Nagase (1964) found that amylase activity in O. mossambicus can be positively correlated with the carbohydrate content of the diet. Kawai and Ikeda

(1972) showed that feeding carp with diets containing starch induced secretion of maltase and amylase and that the adaptation of carbohydrase activities to the composition of the diet occurred in less than a week.

Onishi et al. (1973) measured the sequence of amylase activity in the intestinal contents and hepatopancreas of common carp after feeding. They found that the activity in the intestinal contents increases gradually after feeding to reach a maximum level five hours after the meal. Concurrent with this increase, amylase levels in the hepatopancreas decreased sharply after feeding, probably due to the release of all the stored enzyme into the intestinal lumen. The activity in the hepatopancreatic tissues showed minimum activity two hours after feeding and then recovered slowly to attain a maximum level after 24 hours.

Onishi et al. (1976) reported that the activities of digestive enzymes in carp reach their peak levels when the absorption of the digested food materials has already commenced. It appears that the products of enzyme hydrolysis themselves induce synthesis and secretion of enzymes from the pancreas in animals (Karn and Malacinski, 1978). Ben Abdeljlil et al. (1965) showed that excess glucose and excess amino acids had the same effect on amylase induction and repression as did starch-rich and casein-rich diets, respectively. Pancreozymin, the hormone that stimulates the secretion of pancreatic enzymes and

contraction of the gall bladder in animals, is itself induced by the presence of protein-digestion products and essential amino acids such as L-tryptophan in the intestinal lumen (Davenport, 1982).

Onishi et al. (1976) also showed that feeding carp twice a day induced higher levels of digestive enzyme secretion when compared to fish fed a single daily meal.

Recently attempts have been made to explain the poor utilisation of carbohydrates by fish such as trout. Spannhof and Plantikow (1983) reported that soluble starch was found to increase amylase activity in trout intestine whilst raw starch adsorbed the enzyme and reduced the activity leading to poorer utilisation. Others have found that gelatinised starch is more easily digestible than raw starch (Chiou and Ogino, 1975), probably because of the rapid hydrolysis of gelatinized starch by amylase compared to raw starch (Bergot and Breque, 1983). Chiou and Ogino (1975) reported that alpha-starch is better utilised by carp than beta-starch. Beta-starch or cellulose is composed of beta-1,4'- linkages between the glucose units and amylase is not capable of breaking them. Further the cellulolytic activity is low in fish (Niederhalzer and Hofer, 1979; Lesel et al., 1986). There are also reports correlating the complexity of the polysaccharide chains to their digestibility in fish (Singh and Nose, 1967, Pieper and Pfeffer, 1978).

In complete feeds for domestic animals, the efficiency

of various grains as basic ingredients has been studied extensively and their merits as sources of energy and amino acids have been documented (Gohl, 1975; NRC, 1981, 1983). Cereal grains such as wheat, although an important human food, are generally accepted as a dietary ingredients for fish in many countries. Wheat grains, wheat milling fractions, wheat shorts, wheat germ, wheat bran, wheat flour by-product and wheat gluten have all been suggested as suitable ingredients for fish feed preparations (NRC, 1981, 1983). Wheat and wheat by-products are mainly included in the fish feed as an energy source. Although wheat can contain up to 20% protein, it is deficient in some amino acids essential for animal nutrition, lysine being the most deficient amino acid (Kasarda et al., 1971).

In addition, the albumin portion of the wheat proteins has the property of inhibiting digestive enzymes. The anti-nutritional property of this albumin is a factor that could affect the digestion and utilisation of wheat by animals.

Amylase inhibitors in wheat were first discovered by Kneen and Sandstedt (1943, 1946). Since then this inhibitor has received considerable attention by biochemists as well as nutritionists. The biochemical aspects of this inhibitor, its structure, mechanisms of action, nutritional significance and possible role in vivo have been reviewed by Marshall (1975), Saunders (1975), and Buonocore et al. (1977).

Amylase inhibitor can be extracted from wheat using

water or dilute salt solutions. Since the amylase inhibitor is a protein albumin, it can be precipitated by ammonium sulphate or high concentrations of ethyl-alcohol (Kneen and Sandstedt, 1946; Militzer et al., 1946a, b). Although it is stable at 70°C or when boiled for short periods, autoclaving at 121°C for 30 minutes causes complete loss of the inhibitory activity (Kneen and Sandstedt, 1946). This thermostability is used during the extraction of the amylase inhibitor albumin from wheat to destroy the contaminating alpha and beta-amylase enzymes which are denatured at temperatures below 70°C. This inhibitor is nondialyzable, highly sensitive to a number of oxidising and reducing agents and is rendered inactive by pepsin (Kneen and Sandstedt, 1946; Militzer et al., 1946a, b; Shainkin and Birk, 1970; Petrucci et al., 1976; O'Connor and McGeeney, 1981).

Wheat amylase inhibitor is active against alpha-amylases from various origins, including bacterial, fungal, insect, avian, mammalian and aquatic species (Shainkin and Birk, 1970; Silano et al., 1973; 1975; Saunders and Lang, 1973; Bedetti et al., 1974; Petrucci et al., 1974) but does not inhibit the activities of any plant alpha and beta-amylases. Silano et al., (1975) tested the activity of this inhibitor on alpha-amylases from scorpion fish (Scorpaena ustulata Raf.) and golden grey mullet (Mugil auratus Risso) and reported that the wheat amylase inhibitor albumin does not

inhibit these fish amylases. However, alpha-amylases from other marine species such as molluscs and crustraceans were easily inhibited which led Silano et al. (1975) to classify marine species of animals as having susceptible enzymes to wheat amylase inhibitor albumin. Hofer and Sturmbauer (1985) suggested that the reduction in amylase activities observed in carp and trout gut fluid samples incubated with ground wheat was due to the inhibition by the wheat albumin. With the exception of the work of Silano et al. (1975) and Hofer and Sturmbauer, (1985), the susceptibility of fish amylases to wheat amylase inhibitor has not been investigated.

Research by several workers has revealed that this amylase inhibitor albumin in wheat is highly heterogeneous and can be separated into fractions with different electrophoretic mobilities and with differing inhibition specificities (Shainkin and Birk, 1970; Saunders and Lang, 1973; O'Donnell and McGeeney, 1976). The review of Buonocore et al. (1977) indicates that these inhibitors can be classified into at least three major groups on the basis of their molecular weights: 60,000, 24,000 and 12,500 daltons. The multiple forms of the same amylase inhibitor can probably be considered as isoinhibitors, analogous to isoenzymes.

Enzyme inhibition can be competitive, non-competitive or a mixed type known as uncompetitive inhibition. In competitive inhibition the substrate of the enzyme and the inhibitor compete with each other for the same reactive

points on the enzyme molecule. In non-competitive inhibition the substrate of the enzyme and the inhibitor occupy different sites on the enzyme but the conversion of the enzyme-substrate complex into the reaction products is affected. In uncompetitive inhibition a combination of both the above processes appears to occur (Bergmeyer, 1978).

Militzer et al. (1946b) thought that the reaction between the wheat inhibitor and amylase enzyme was non-competitive in nature. Marshall (1975) supported this finding stating that since this inhibitor affects different alpha-amylases at different rates the nature of inhibition is of the non-competitive type. Marshall (1975) further considered that, if the inhibitor acted by competing with starch for the active site of the enzyme, all amylases could be expected to be affected. Only certain alpha-amylases have sites which can combine with the inhibitor molecule while some others do not have such binding sites. All amylases however, have sites for binding with starch.

By contrast Saunders and Lang (1973) reported that the alpha-amylase inhibitors exhibited reversible linear uncompetitive inhibition kinetics against chick pancreatic alpha-amylase with K_i values of 5×10^{-8} M. Buonocore et al., (1976) also stated that the inhibitor binds with alpha-amylase in a 1:1 molar ratio and the inhibition is uncompetitive.

It was noted during the in vitro studies of O'Connor and McGeeney, (1981) that the amylolytic activity in

the amylase and inhibitor mixtures is never totally reduced. Hence they suggest that the enzyme-inhibitor complex might have some amylolytic activity. This indicates that the inhibitor molecule attaches to the enzyme at a site which is different from the enzyme-starch binding site.

The inhibition of amylase by the wheat inhibitor albumin is reversible under certain conditions. This was demonstrated by Kneen and Sandstedt (1946) by the addition of 70% alcohol which precipitated the amylase from the enzyme-inhibitor complex while the inhibitor remained in solution. The enzyme recovered by this method of dissociation was found to retain its amylolytic activity. Maltose and starch interfere with the inhibitory activity of this albumin (O'Connor and McGeeney, 1981).

The nutritional significance of this amylase inhibitor in wheat has also been studied in animals. Addition of protein extracts from wheat bran to the synthetic diet of yellow meal worm (Tenebrio molitor) adversely affected larval development and increased mortality. This inhibitor has also been shown to inhibit the growth of rats (Lang et al., 1974) and chicks (Marci et al., 1977), an effect that was generally accompanied by low starch digestibility. Puls and Keup (1973) reported that an alpha-amylase preparation from wheat retarded the digestion of raw starch in rats, dogs and man. The inhibitor was not effective in retarding the breakdown of cooked starch. Amylase inhibitor was even initially thought to be

responsible for coeliac disease in man (Strumeyer, 1972) where impaired ability to metabolise starch is observed but this theory was later disproved (Auricchio et al., 1974). Hofer and Sturmbauer (1985) suggested that amylase inhibitor in wheat could play a part in determining the carbohydrate utilisation of carp and trout.

Despite their susceptibility to heat, the wheat amylase inhibitors may persist through bread baking since large amounts have been detected in the centre of loaves (Bessho and Kurosawa, 1967) and in some wheat-based breakfast cereals (Marshall, 1975). In view of their possible action in vivo, Liener (1980) considered the presence of these inhibitors in wheat-based food products as undesirable and recommended them to be excluded from the diets of infants and patients. In animal feeds such care is not usually taken and often raw wheat is used to prepare artificial diets for fish.

All varieties of wheat contain amylase inhibitor (Kneen and Sandstedt, 1946; Bedetti et al., 1974; Vitozzi and Silano, 1976). The production of the inhibitor protein in wheat kernel reaches a maximum concentration when the kernels reach full maturity (Sandstedt and Beckord, 1946; Pace et al., 1977). Saunders (1975) assayed the inhibitor content of different wheat milling fractions and showed that the inhibitor concentrations were markedly lower in wheat bran than in whole ground wheat. It has therefore been suggested that the inhibitor albumin is closely

associated with the starch granules and is probably located in the endosperm.

It has been further stated that over two-thirds of the albumin fraction of wheat has the property of inhibiting the activities of amylase enzyme (Petrucci *et al.*, 1974; Marci *et al.*, 1977). Petrucci *et al.* (1974) also reported that the total albumin extracted by him from wheat constituted about 0.3% of the seed weight. Other workers have indicated that wheat albumin extractable with water amounts to 1% of the kernel weight and that the albumin inhibitor should thus correspond to about a third of the total albumins (Minetti *et al*, 1975, cited by Kasarda *et al.*, 1971). Despite the discrepancy in the values, these reports nevertheless indicate that a considerable proportion of the wheat protein has the property of reducing the activities of amylase.

Unfortunately the presence of amylase inhibitor in wheat has been largely ignored because raw wheat is rarely consumed by man and a large proportion of the inhibitor is destroyed or inactivated during the cooking or baking of wheat products. In fish feed manufacture however, the temperature rarely exceeds 100°C (except in extrusion) to minimise loss of protein quality and the destruction of added vitamins. Therefore the effect of wheat amylase inhibitor is probably more prevalent in animal nutrition than in human nutrition, although little attention has been paid to the possible implications. Heat treatment has been shown

to improve the utilisation of wheat by animals, including fish, and this beneficial effect has generally been attributed to the gelatinization of the starch in wheat (Tacon, 1981). But this improvement in digestibility could also be partially due to the effects of the destruction of the inhibitors it contains.

In view of the possible *in vivo* effect of the amylase inhibitor on fish amylases, the full nutritional potential of raw wheat may not be being realised. Furthermore it has not been conclusively demonstrated that the amylase inhibitor in wheat is effective against fish alpha-amylases. Thus the aim of this thesis was to investigate the effects of wheat amylase inhibitor on two omnivorous freshwater tropical fish species, the Mirror carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*).

CHAPTER 2

GENERAL MATERIALS AND METHODS.

CHAPTER 2

GENERAL MATERIALS AND METHODS.

2.1 Fish.

Mirror carp (Cyprinus carpio) fingerlings (Plate 1) weighing between 15 and 25g, and of mixed sex, were obtained from Humberside Fisheries, Cleaves farm, Skerne, Driffield, England. On arrival at the Institute of Aquaculture the carp were quarantined in static aquaria at 25°C for a month. After this period they were treated with a formalin bath (160 ppm for 40 minutes) as a prophylactic measure against parasites before transfer to the main tropical aquarium house, where they were maintained in a recirculatory stock system until required for experiments.

Fingerling Nile tilapia, Oreochromis niloticus (Plate 2) in the size range of 10 to 15g, of mixed sexes were obtained from pure stock bred at the Institute of Aquaculture.

Prior to the experiments all fish were fed twice daily to satiation on commercial trout diets (Ewos Baker). The compositions of these holding rations are given in Appendix I.

PLATE 1 Mirror carp

PLATE 2 Nile tilapia

PLATE 3 Stock system

PLATE 4 Experimental system

2.2 Stock system.

The stock system (Plate 3) was used for maintaining fish until required for experiments when they were transferred to the tanks in the experimental system.

This recirculatory, self-cleaning, system consisted of eight 400 litre capacity square fibreglass tanks (L.M.Glasfibre, Denmark) stocked with fish at a maximum density of 25 g/litre of water. The water temperature in the system was maintained constant between 27 and 28°C.

2.3 Experimental system.

The experimental system consisted of 24 circular white plastic tanks (50 cm diameter, height 32 cm) of 50 litre capacity arranged in two tiers (Plate 4, Fig.2.1). Each of these tanks was provided with a central stand pipe and a collar to maintain water level and to provide a self-cleaning action. Water, at a flow rate of 1.23 $\pm$ 0.16 litres per minute, entered each tank tangentially from an inlet pipe which resulted in continuous water circulation and thus aided tank cleaning. The effluent water passed through a series of six, 114 litre black polypropylene settling/biofilter cisterns ('Wizard' PC25, Tanks and Drums Ltd., Bradford) before reaching the pump tank. The biological filter tanks were packed with a

1	Fish holding tanks
2	Settling tanks
3	Pump tank
4	Gravel filter
5	Heater/Header tank
6	Thermostat

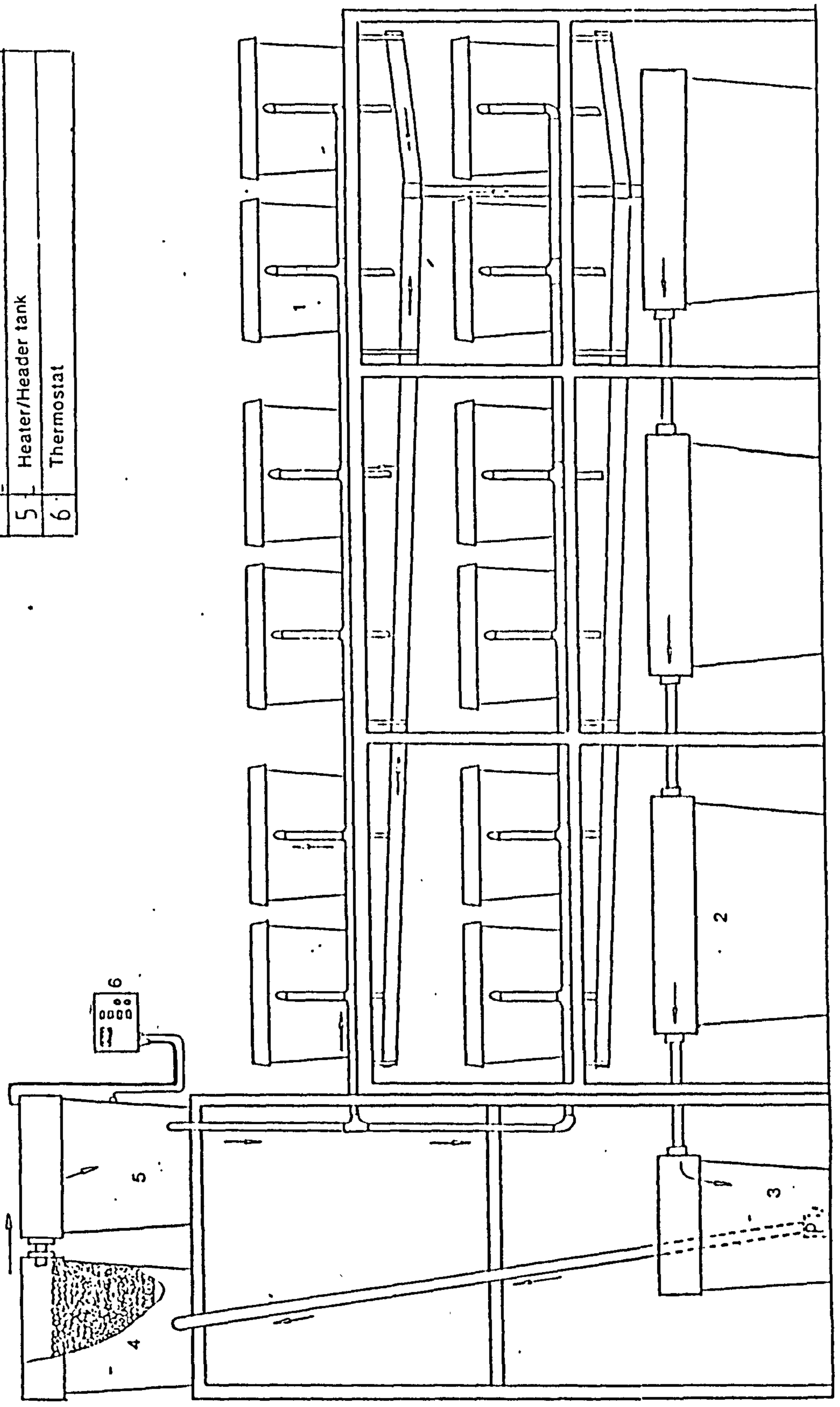

FIGURE 2.1 Diagramatic representation of the experimental recirculatory system used in this study.

total of four cubic metres of filter rings (Cascade Filterpak type CR50 media, Mass transfer International, Haversham Cumbria, England), which provided a substratum for the growth of microflora, essential for the functioning of the biological filter.

A submersible pump ('NOVA' 300, maximum pumping rate 150 litres per minute) lifted the water through a non-return valve pushing through an overhead gravel filter contained in a 227 litre (PC50) cistern. The gravel in this filter was mixed with shell pieces to help maintain the water at a neutral pH.

The water then overflowed into a heater tank (PC25) fitted with a three kilowatt immersion heater (Howden Engineering) controlled by a thermostat which maintained the temperature to within $\pm$ 1 degree Celsius of the set temperature, of 27°C. The heater was also connected to an alarm system which signalled any unusual fall in the water level in the heater tank. From the header tank, the heated clean water flowed by gravity back into each of the 24 experimental tanks. Excess water from the header tank overflowed back into the biological filter.

Compressed air was bubbled continuously through airstones in every fish tank. Fresh make-up water entered the system at a point close to the pump tank, at a rate of one litre per minute. A time switch regulated the photoperiod (12 hours dark/12 hours light) by switching on the fluorescent tube lights from 8.00 am to

8.00 pm, every day.

The entire system was cleaned and refilled with fresh tap water a week before the start of each experiment and in addition the fish tanks were brushed clean on every sampling day, during the experiment.

2.4 Water quality.

Certain water quality parameters in the experimental system were monitored periodically during all experimental periods. Dissolved oxygen content, pH and specific conductivity of water were measured using an oxygen meter (YSI model 57), pH meter (Philips model PW 9409) and conductivity meter (Fox pHOX 52), respectively. Automated procedures using an autoanalyser (Technicon II) were followed for determining the nitrate, nitrite and ammonia content of the water samples. The methods used were adapted from those of Stephens and Brandstaetler (1983).

The water quality in the experimental recirculatory system was within the limitations and requirements for both carp and tilapia (Muir, 1982).

The range of values recorded with their means are given in Table 2.1.

Table 2.1 Water quality in the recirculatory system during the experimental periods.

Parameter	Range	Mean ± SD
Temperature °Celsius	27 - 28	27.81 ± 0.37
pH	5.97 - 7.26	6.89 ± 0.42
Conductivity x10 uS	10 - 31	20.50 ± 14.85
Dissolved oxygen mg/l	5.58 - > 10	7.92 ± 2.91
Nitrate nitrogen mg/l	1.33 - 6.11	2.69 ± 1.81
Nitrite nitrogen mg/l	0.002 - 0.03	0.015 ± 0.014
Ammonia nitrogen mg/l	0.007 - 0.39	0.143 ± 0.153

2.5 Diet formulation and preparation.

All experimental diets were formulated, with the aid of a spreadsheet on a BBC microcomputer, to contain around 30% crude protein, 25% hydrolysable carbohydrates and 10% crude lipid, on a moisture free basis. The exact diet formulations and compositions of the prepared test diets for each of the experiments in this study are considered in the relevant chapters. However, the general method of preparation of the diets was common for all the trials and is therefore described here.

A comprehensive list of the ingredients with their sources, is presented in Table 2.2.

Fishmeal is one of the best protein sources for fish (Cowey and Sargent, 1979), supplying almost all the essential and non essential amino acids in proper proportions and hence it was used in all diets as the main or sometimes the only protein source. Pure cod liver oil and corn oil supplied additional fish and vegetable oils, respectively. Alpha-cellulose (99.5% fibre) was used in all the diets as the bulking agent. Since the dietary ingredients used in this work generally have poor binding qualities it was considered essential to incorporate 1% carboxymethyl cellulose as a binder. Diets were supplemented with 4% mineral premix (Table 2.3) and 2% vitamin premix (Table 2.4). When nutrient digestibilities were to be evaluated, 0.5% chromic oxide

Table 2.2 Sources of ingredients used in the experimental diets.

Herring meal (2 batches)	Ewos Aquaculture International
Wheat (white)	Ewos Aquaculture International
Wheat Starch unmodified, type I	Sigma Chemical Co.Ltd., Poole, England.
Dextrin (corn, type III, 80 % water soluble)	Sigma chemical Co. Ltd., Poole, England.
Alpha-Cellulose (GPR)	BDH Chemicals Ltd., Poole, England.
Carboxymethyl Cellulose (GPR)	BDH Chemicals Ltd., Poole, England.
Chromic oxide (GPR)	BDH Chemicals Ltd., Poole, England.
Cod liver oil	Boots Company plc, Nottingham, England.
Corn oil	Shaco Oil Products Ltd., Bolton, Lancashire.
Mineral mixture	After the formula of Tacon et al. (1982)
Vitamin mixture	After the formula of Tacon et al. (1982).

Table 2.3 Mineral supplement (after Tacon et al., 1982)

Minerals		g / kg
Calcium orthophosphate	($CaHPO_4 .2H_2O$)	727.7775
Magnesium sulphate	($MgSO_4 .7H_2O$)	127.5000
Sodium chloride	(NaCl)	60.0000
Potassium chloride	(KCl)	50.0000
Iron sulphate	($FeSO_4 .7H_2O$)	25.0000
Zinc sulphate	($ZnSO_4 .7H_2O$)	5.5000
Manganese sulphate	($MnSO_4 .4H_2O$)	2.5375
Copper sulphate	($CuSO_4 .5H_2O$)	0.7850
Cobalt sulphate	($CoSO_4 .7H_2O$)	0.4775
Calcium iodate	($CaIO_3 .6H_2O$)	0.2950
Chromic chloride	($CrCl_3 .6H_2O$)	0.1275

Table 2.4 Vitamin premix (after Tacon et al., 1982)

Vitamins	g/kg of premix
Thiamine (B_1)	2.5
Riboflavin (B_2)	2.5
Pyridoxine (B_6)	2.0
Pantothenic acid	5.0
Inositol	100.0
Biotin	0.3
Folic acid	0.75
Para amino benzoic acid	2.5
Choline	200.0
Niacin (nicotinic acid, B_3)	10.0
Cyanocobalamine (B_{12})	0.005
Retinol palmitate (A)	100000 IU
Alpha tocopherol acetate (E)	20.1
Ascorbic acid (C)	50.0
Menadione (K)	2.0
Cholecalciferol (D_3)	500000 IU
Bulking agent	up to 1 kg

Table 2.5 Proximate composition (moisture free %) of dietary ingredients.

Values in parenthesis show the SE.

Ingredients	Moisture	Crude protein	Crude lipid	Crude fibre	Hydrolysable carbohydrate	Total ash
Fishmeal I	9.18 (0.36)	75.31 (1.51)	9.16 (0.92)	0.80 (0.12)	0.06 (0.01)	14.61 (0.90)
Fishmeal II	6.93 (0.90)	77.93 (1.47)	9.81 (0.25)	0.62	0.06	12.52 (0.60)
Wheat	13.46 (0.03)	11.92 (0.08)	1.13 (0.03)	4.68 (0.15)	60.01 (5.69)	2.01 (0.16)
Wheat (autoclaved)	7.05 (0.64)	11.85 (0.12)	0.44 (0.01)	2.12 (1.16)	61.25 (1.20)	2.21 (0.14)
Wheat Starch	10.71 (0.27)	0.27 (0.06)	----	----	87.42 (1.99)	0.14 (0.02)
Wheat Starch (autoclaved)	6.83 (0.90)	0.29 (0.05)	----	----	89.07 (2.40)	0.08 (0.04)
Dextrin	8.03 (0.01)	----	----	----	81.83 (7.16)	0.03 (0.007)
Alpha-Cellulose	5.05 (0.82)	----	----	77.48 (0.68)	1.86 (0.43)	0.10
Carboxymethyl-Cellulose	10.76 (0.77)	----	----	----	----	----
Mineral mix	6.29 (0.59)	----	----	----	----	----
Vitamin mix	13.71 (1.46)	----	----	----	----	----

was incorporated in the diets.

After formulating diets on a moisture-free basis, the proportions were corrected to compensate for the moisture contained in each ingredient. The compositions of the main dietary ingredients used in this study are tabulated in Table 2.5.

All dry ingredients, which had previously been ground and sieved through a 1 mm mesh, were weighed out to the nearest 0.1 gram using a Sartorius 3716MP top pan balance. They were then transferred into the bowl of a Hobart 'A' 200 series food mixer with mincer (No.12) attachment (Hobart Manufacturing Co. Ltd., New Southgate, London) and were mixed thoroughly for about ten minutes to obtain a homogeneous mix. The oils were then added gradually with continuous mixing. Finally, 250 to 400 ml of water for each kilogram of dry mixture was added gradually to prepare a dough suitable for extruding. After the addition of the water, mixing was continued for a further 5 - 10 minutes until the dough attained the required consistency. The dough was then extruded through a 3 mm die, spread out on perforated trays and dried overnight in a forced air drier at 32 - 40°C, which was sufficient to reduce moisture content to less than 10%.

After drying, the pellet strings were broken by hand to lengths of 5 - 10 mm and stored at -20°C in airtight plastic containers until required.

2.6 Feeding regime.

The experimental groups of fish were fed daily at three percent body weight (feed dry weight/fish live weight) in two equal instalments at 9.00 - 9.30 am and 5.00 - 5.30 pm. On weekly sampling days when the fish were anaesthetised and handled, the second instalment of feed was omitted. The feeding rate was adjusted on a weekly basis to take account of increased fish weights.

2.7 Fish growth.

The experimental fish were anaesthetized using benzocaine (ethyl-p-aminobenzoate) at 50-100 mg per litre of water before handling for recording weights. Fish were first gently blotted with paper towels and weighed individually on a Mettler PC400 balance to the nearest 0.01g. Specific growth rate (SGR), which defines the growth rate of experimental fish in terms of change in weight as percentage per day, were calculated using the formula:

$$SGR\ (\%/day) = \frac{\log_e (W_T) - \log_e (W_t)}{T - t} \times 100$$

(Brown, 1957)

where,

W_T = Final weight at time T in days,

W_t = Initial weight at time t in days.

2.8 Analytical techniques.

2.8.1 Proximate analysis of diet ingredients and test diets.

Triplicate samples of diet ingredients and prepared test diets were ground finely in a Braun grinder (model No.4-041) and analysed for their proximate compositions using standard AOAC (1980) methods. Analysis of ingredients was always performed before diet formulation.

2.8.1.1 Moisture.

Moisture content was determined by drying triplicate preweighed samples overnight in a Gallenkamp hot air oven (Model OVE 300) at 105°C (AOAC, 1980).

2.8.1.2 Total ash.

Total ash content was determined by ashing a known weight of sample at 450°C in a muffle furnace (Gallenkamp) for 12 hours, (AOAC, 1980).

2.8.1.3 Crude fibre.

The residue after consecutive digestions with hot 0.255N sulphuric acid and 0.313N sodium hydroxide solutions was the measure of the crude fibre content of the samples, after correcting for the ash contents, (AOAC, 1980).

2.8.1.4 Crude protein.

Crude protein content was determined by the microkjeldahl method using a Tecator Kjeltec distillation unit (model 1003). The conversion factor used for wheat was 5.70 (Osborne and Voogt, 1978, quoted by Jobling, 1983) whereas a value of 6.25 was used for calculating the protein in fishmeal and test diets.

2.8.1.5 Crude lipid.

Korn and Macedo's (1973) technique was used to determine the lipid content of all the samples since the method is both rapid and requires smaller quantities of samples. A finely ground sample was mixed with anhydrous sodium sulphate and placed in a column packed above and below the sample mixture with anhydrous sodium sulphate. The lipid from the sample was then extracted using 99.9% trichlorofluoro-methane (BDH, laboratory reagent) at room

temperature, and collected in preweighed aluminium foil cups. The weight of lipid after evaporation of the solvent was then recorded, and the lipid content was expressed as a percentage of the initial sample weight.

2.8.1.6 Chromic oxide.

Chromic oxide content of diets was determined by the wet oxidation method of Furukawa and Tsukahara (1966).

2.8.1.7 Carbohydrates.

Southgate (1969) has defined 'unavailable carbohydrate' as that carbohydrate which does not yield sugars as the end product of digestion. Therefore that proportion of carbohydrates in a diet which is actually available for hydrolysis and utilisation by a animal can be defined as the 'available' or 'hydrolysable' carbohydrate.

'Soluble carbohydrate' or nitrogen free extractives in the diet, includes all the components in the diet other than moisture, protein, lipid, ash and fibre (Castell and Tiews, 1980). Not all of the soluble carbohydrates in the diet are available to the fish for utilisation.

Hydrolysable carbohydrate.

Triplicate samples were initially solubilized in a

mixture of dimethylsulphoxide (Sigma) and 8M hydrochloric acid at 60°C for 30 - 60 minutes in accordance with the procedures recommended by Boehringer Mannheim (1984) for foodstuffs. The starch in the solubilised samples was then hydrolysed by slightly modifying the procedure of Keppler and Decker (1974), using 28 U of amyloglucosidase (Boehringer) in acetate buffer (0.2M, pH 4.8) at 40°C.

The liberated glucose in the cooled and neutralised samples was determined by the procedures of Hugget and Nixon (1957) and Werner et al. (1970) as modified by Bergmeyer and Brent (1974). Aliquots of the hydrolysate were treated with glucose oxidase (Aspergillus niger, 9 U/ml, Sigma), peroxidase (horseradish, 1.5 U/ml, Sigma) and O-dianisidine (50 ug/ml, Sigma) in phosphate buffer (0.12M, pH 7.0), to develop a brown colour. A series of glucose standards in the range of 0 to 0.10 mg were also similarly treated with each set of samples. The brown colour intensity was measured at 436 nm using a Kontron spectrophotometer (model UVIKON 810). The concentration of glucose was read from the standard curve (reaction volume 5.2 ml, Absorbance = 0.0211 + 9.43 mg glucose).

The available carbohydrates in the sample was finally calculated by the formula:

$$\text{Percent available carbohydrate} = \frac{C \times V_3 \times V_1}{W \times V_4 \times V_2} \times 100$$

where,

C = mg glucose from standard curve,
W = weight of dry sample in mg,
V_1 = total volume of solubilized sample,
V_2 = volume of solubilized sample taken for hydrolysis by amyloglucosidase,
V_3 = total volume of hydrolysate after amyloglucosidase hydrolysis,
V_4 = volume of hydrolysate taken for glucose assay.

Soluble carbohydrate.

The soluble carbohydrate fraction on a moisture free (MF) basis in the diet was calculated by difference (Castell and Tiews, 1980),

Soluble carbohydrate (MF %) = 100 - (crude protein % + crude lipid % + ash % + crude fibre %)

2.8.2 Faecal matter collection.

During the last two weeks of the experiments, samples of fish faecal matter were collected to estimate nutrient digestibilities. Daily collections were made before the second instalments of diets were given to the fish. In carp the diet takes about 4 to 5 hours to pass through the entire alimentary canal at 26°C (Maltzan cited by Hickling, 1970) while in tilapias the vent arrival time is estimated to be 4.9 and 6.8 hours after feeding, at temperatures of 25 and 30°C, respectively

(Ross and Jauncey, 1981). To facilitate easy collection of the faecal strings, the central stand pipes in the tanks were removed two hours before the evening feed, thus making the water flow out from the surface. Each tank was opened and fish observed for the release of faecal strings which were quickly removed using a pipette fitted with a rubber bulb. Such daily collections were dried overnight at 60°C and the pooled samples for each treatment were stored in airtight containers.

2.8.2.1 Analysis of faecal samples.

Triplicate samples of the fish faeces collected during the experiments were analysed for crude protein, hydrolysable carbohydrates and chromic oxide contents by the methods described above but using smaller quantities of sample (50-100 mg).

Apparent nutrient digestibilities were calculated using the formula of Maynard and Loosli (1969),

$$\text{Apparent nutrient digestibility (\%)} = 100 - \frac{(100 \text{ X CD X NF})}{(\text{CF X ND})}$$

where,

CD = mg chromic oxide per gram diet,
CF = mg chromic oxide per gram faecal sample,
NF = % nutrient in faecal sample and
ND = % nutrient in diet.

2.8.3 Blood sampling and analysis.

Blood was sampled using disposable syringes (1 ml) and hypodermic needles (21G) and vials which were pre-rinsed in heparin (sodium salt, Sigma) solution consisting of 500 units/ml and then dried at 30°C . The drop of heparin adhering onto the needle and syringe after drying was sufficient to prevent blood clotting without diluting the sample. About 500 ul of blood was drawn from each fish by puncturing the caudal vein. The blood sample was centrifuged at 2500 rpm and the resulting plasma was transferred immediately into another untreated vial for analysis.

2.8.3.1 Plasma glucose.

The modified procedures of Bergmeyer and Brent (1974) with glucose oxidase and peroxidase enzymes was adapted to determine the glucose content (for details see hydrolysable carbohydrate). Plasma samples were diluted ten times with distilled water and triplicate aliquots of 200 ul were used for the assay. The concentrations were expressed as mg glucose/dl of undiluted plasma.

2.8.3.2 Plasma protein.

Triplicate aliquots of diluted (1:100) plasma

samples were analysed for protein content by the standard Lowry's method (1951). Folin & Ciocalteu's phenol reagent was obtained from BDH (LR grade) and bovine serum albumin (400 ug/ml) from Sigma was used as the standard.

2.8.4 Hepatosomatic index.

The sampled experimental fish were killed by an overdose of benzocaine and their weights recorded after blotting away the excess water using paper towels. The viscera were dissected out and the gall bladder was exposed and transferred into another vial for collection of bile. The hepatopancreatic tissue was then removed and its total weight recorded to the nearest tenth of a mg using a Mettler AC100 balance. The hepatosomatic index (HSI) of each fish was then calculated by the formula:

$$\text{HSI (\%)} = \frac{\text{Total liver weight minus gall bladder (g)}}{\text{Whole fish wet weight (g)}} \times 100$$

2.8.5 Bile volume.

The gall bladder was placed in a vial, punctured with a needle and then centrifuged at 2000 rpm to separate the bile juice. The bile volume was measured using a micropippette (5-40 ul) and expressed as a percentage of

fish wet weight, by using the following formula:

$$\text{Bile volume (ul/100g fish)} = \frac{\text{Bile vol. (ul)}}{\text{Fish wet weight (g)}} \times 100$$

2.8.6 Gut fluid volume and gut solid weight.

The gut contents from the uncoiled intestine were carefully stripped with minimum loss into a clean centrifuge tube. The gut fluids were then separated from the solids by centrifuging at 11,600 xg (13,000 rpm) for 10 minutes in a Microcentaur centrifuge placed inside a refrigerator. The volume of gut fluid was measured using automatic pipettes (40-200 ul and 200-1000 ul), the weight of the solids was recorded in a Mettler AC100 balance (0.0001g accuracy) and then frozen.

The volume of gut fluid and gut solid weight thus obtained were then expressed as percentages of fish body weight using the formulae given below:

$$\text{Gut fluid volume (\%)} = \frac{\text{Gut fluid vol. (ml)}}{\text{Fish wet weight (g)}} \times 100$$

$$\text{Gut solid weight (\%)} = \frac{\text{Gut solid wt. (g)}}{\text{Fish wet weight (g)}} \times 100$$

2.8.7 Gut-somatic index.

After collecting the gut contents from the uncoiled intestine, the intestinal tissue was cleared of the adhering adipose tissues as far as possible. The intestine was then rinsed clean in cold fish ringer's solution (Ginsburg, 1963). Any excess solution adhering to the tissue sample was removed by blotting gently on a clean paper towel before recording its total weight using a Mettler AC100 (0.0001 g accuracy) balance. From the weight of the cleaned intestinal tissue the gut-somatic index was calculated by the formula:

$$\text{GSI (\%)} = \frac{\text{Weight of cleaned intestine (g)}}{\text{Fish wet weight (g)}} \times 100$$

2.8.8 Determination of enzyme activities.

2.8.8.1 Alpha-amylase.

Alpha-amylase (1,4-alpha-D-glucan gluconohydrolase, EC 3.2.11) activity levels in gut contents, bile and

tissue samples were assayed by the saccharogenic method of Bernfeld (1955) as modified by Rick and Stegbauer (1974). Starch substrate is hydrolysed by alpha-amylase to fragments whose hemiacetal groups can be determined with 3,5-dinitrosalicylic acid (DNSA). The concentration of the nitroaminosalicylic acid formed is measured colorimetrically, which corresponds to the concentration of newly formed terminal groups and therefore directly to the enzyme activity.

Soluble potato starch (Lintner) obtained from Sigma was used for all assays.

Sample preparation.

Samples of gut contents, bile or tissues from the experimental fish were removed from the storage freezer, thawed in a refrigerator (+5°C) and enzyme assays were carried out as quickly as possible thereafter.

Gut fluids were diluted 500 times with 20 mM phosphate buffer (pH 6.9) containing 10 mM sodium chloride. Triplicate aliquots of 50 ul of diluted samples, were used for the assay. Bile was diluted 1000 times. Dilutions were adjusted in such a manner that the extinctions on the spectrophotometer did not exceed 1.00.

Intestinal solids were eluted with four 1 ml instalments of buffer, shaken thoroughly in a vortex mixer and centrifuged after each addition of buffer at 2000 rpm for 10 minutes. The supernatants were pooled,

diluted a further 100 or 200 times with the same buffer and 50 ul aliquots were taken immediately for assay.

Tissue samples of hepatopancreas and intestines were weighed and homogenized using a high speed ultra turrex for 1 minute in 5 or 10 ml of cold phosphate buffer. The homogenate was then centrifuged at 8000 xg (9000 rpm) in a refrigerated centrifuge (MSC high speed 18, 0-5°C) for 30 minutes. The supernatant was diluted 100 times and 50 ul aliquots were used for assay.

Assay.

Triplicate samples were incubated with 1 ml of freshly prepared 1% w/v buffered starch substrate solution for ten minutes in a constant temperature water bath (Laboratory Thermal Equipment). Samples from the first experiment (Chapter 3) were assayed at a reaction temperature of 37°C, but in all subsequent trials this was reduced to a temperature of 25°C which is much closer to the temperature at which the fish were maintained (27-28°C).

After the 10 minute incubation period, the enzyme reaction was arrested with 2 ml portions of dinitrosalicylic acid reagent (1% w/v 3,5 dinitrosalicylic acid Sigma, 30% w/v potassium sodium tartrate BDH). Since several assays were carried out simultaneously, the substrate was added to each tube at 30 second intervals. Ten minutes after the addition of substrate, the reaction

was stopped in the same order and intervals.

The colour was developed by placing the reaction tubes in boiling water at 100°C for exactly five minutes, followed by rapid cooling in an ice bath. Extinctions were read after 30 minutes at 546 nm on a Kontron spectrophotometer (UVIKON 810). A sample blank was run simultaneously with each sample and a series of maltose (BDH, extra pure) standards in the range of 0 to 3.28 uMols were prepared for each set of 30 tubes. The concentration of maltose in uMol in the reaction mixture was obtained from this maltose standard curve (Absorbance = -0.00099 + 0.328 uMol maltose,

reaction volume 4.05 ml). Enzyme activity was expressed as uMol maltose produced per minute per ml of gut fluid or bile or per gram moist tissue sample or intestinal solids, as appropriate. One amylase unit (U) is the enzyme activity that releases 1 uMol maltose from starch in 1 minute at a specified temperature.

The specific activities were also calculated and expressed as uMol maltose/min/mg protein in the sample.

For the calculation of tissue enzyme activities, the tissue fluid content was assumed to be 75% of the sample taken (Bergmeyer, 1974).

2.8.8.2 Total proteolytic activity.

The total proteolytic activity (trypsin and chymotrypsin) in the intestinal fluids and solids were assayed by the modified procedures of Rick (1974). In this method proteases hydrolyse a protein substrate for a specified period to form peptides and amino acids which are soluble in trichloroacetic acid. These hydrolysis products are then quantified with the phenol reagent of Folin and Ciocalteu, which gives a measure of the activity. Denatured proteins such as haemoglobin or casein can be used as substrates. One percent w/v casein (hammersten, BDH) solution was used as the substrate in the present study.

Sample aliquots (100 ul) of diluted gut fluid (1:500) or gut solid eluates (1:200) in phosphate buffer (0.1 M, pH 7.6) were incubated with 1 ml of buffered substrate solution for 20 minutes at 25°C. The hydrolysis was stopped with 3 ml of cold trichloroacetic acid (5%) which dissolved all the products of hydrolysis and precipitated the excess substrate. After 30 minutes standing at room temperature (+20°C) the reaction mixture was filtered through Whatman no.4 filter paper. Five millilitres of 0.5 N sodium hydroxide solution was

added to 2.5 ml of the filtrate followed by 1.5 ml of three folds diluted Folin and Ciocalteu's (BDH) phenol reagent. A sample blank was run simultaneously but in this case the sample was added after the addition of TCA. A series of tyrosine standards (L-tyrosine Sigma) in the range of 0 to 0.3 uMols in 0.2N hydrochloric acid were also prepared and colour developed as for the sample. The extinctions of standards and samples were measured at a wavelength of 691 nm using a Kontron spectrophotometer (UVIKON 810) and the concentration of the casein hydrolysis products in the samples were read from the standard curve (Absorbance = 0.00065 + 1.36 uMol tyrosine, reaction volume 9 ml). Proteolytic activity was expressed as uMol tyrosine per minute per ml gut fluid or per gram intestinal solids. One unit of protease is that quantity of enzyme which releases 1 uMol tyrosine from casein in 1 minute at 25°C.

The protein content of the samples was determined by the standard Lowry's method (BSA standard 400 ug/ml, Sigma) and the specific activities (uMol tyrosine/min/mg protein) were also calculated.

2.9 Histopathology.

Samples of hepatopancreas fixed in 10% buffered formalin were processed in a tissue processor and embedded in paraffin wax. Five micron thick sections were cut

using a rotary microtome and sections were stained by the standard histochemical procedure for haematoxylin-eosin staining as detailed by Drury and Wallington (1980).

2.10 Statistical analysis of data.

The data collected during the experiments were processed and analysed using the Minitab (Penn State, University of Wisconsin) statistical package (Ryan et al., 1976). Simple and multiple regression equations were calculated, using transformations where necessary. The suitability of the regression equations were evaluated by plotting the standard residuals against the data and checking for any pattern. Equations that produced a trend were rejected. Pearsons product moment correlation coefficients were calculated to measure association between some of the parameters observed. Data on feeding trials were evaluated and compared between treatments by one way analysis of variance (ANOVA) at the 95% confidence interval.

The histograms and graphs were drawn using a Hewlett Packard (HP7440) plotter.

CHAPTER 3

STUDIES ON THE INACTIVATION OF CARP AND TILAPIA ALPHA-AMYLASES BY PURIFIED WHEAT AMYLASE INHIBITOR.

CHAPTER 3

STUDIES ON THE INACTIVATION OF CARP AND TILAPIA ALPHA-AMYLASES BY PURIFIED WHEAT AMYLASE INHIBITOR.

3.1 INTRODUCTION.

Alpha-amylases from different origins show differences in susceptibility to inhibition by wheat alpha-amylase inhibitor (Silano et al., 1975). Furthermore, inhibitor fractions isolated by different workers have been reported to have different specificities to amylases. Kneen and Sandstedt (1946) reported that the wheat inhibitor was active towards salivary and pancreatic alpha-amylases and also certain bacterial amylases but not plant amylases. Shainkin and Birk (1970) separated two fractions from wheat. One of these (AmI_2) had a specificity similar to that reported by Kneen and Sandstedt (1946) and was also found to inhibit the alpha-amylase from the midgut region of the yellow mealworm (Tenebrio molitor). The second form (AmI_1) inhibited only the insect alpha-amylase. Similar findings were reported by Silano et al. (1973, 1975) who tested the inhibitory activities of three albumin fractions with molecular weights of 60000, 24000 and 12500 daltons, towards 58 animal amylases and a number of plant amylases. Among the animal amylases tested were those from 23 aquatic species

such as squids, cuttlefish, octopus, several other molluscs, crustaceans such as prawn and crab and two marine fish species, scorpion fish (Scorpaena ustulata Rafinesque) and golden grey mullet (Mugil auratus Risso). An extract from their report is summarised in Table 3.1. From this table it can be seen that different amounts of wheat inhibitor are required to reduce by 30% the amylolytic activity of alpha-amylase extracted from different species. Furthermore the various fractions of the inhibitor protein have differing potencies for each of the alpha-amylases. None of the inhibitor fractions had any effect on the two fish alpha-amylase tested.

Silano et al., (1975) classified animal alpha-amylases as susceptible, partially susceptible and resistant varieties on the basis of the effectiveness of the wheat inhibitor fractions in reducing their amylolytic activity. Marine species were classified as possessing amylases that were susceptible to wheat inhibitor and the inhibitor fraction with molecular weight of 24000 daltons was found to be the most effective against alpha-amylases from marine animals. Silano et al., (1975) finally suggested that only amylases which have certain groupings in their structure are susceptible to the inhibitor from the wheat kernel.

Until recently the effect of wheat inhibitor on amylases from cultivable freshwater fishes had not been

Table 3.1 Selected values of Inhibition of amylases from some marine species by various fractions of inhibitor from wheat. (after Silano et al., 1973, 1975).

Amylase origin	Inhibitor fractions (ng) that give 30% inhibition of 1 U* amylase.				
	60000d	24000d	12500d	0.19 **	0.28 **
Loligo vulgaris	314	80	237	---	---
Sepia officinalis	318	25	48	139	88
Octopus vulgaris	513	12	73	50	NI
Murex trunculus	1500	90	NI	101	NT
Leander serratus	NI	NI	NI	---	---
Maja verrucosa	NI	31	NI	72	NT
Scorpaena ustulata	NI	NI	NI	---	---
Mugil auratus	NI	NI	NI	---	---

NI= no inhibition, NT= not tested, --- = no information,

d = daltons

* = One amylase unit is the amount of enzyme that gives 50% hydrolysis of the added starch under the experimental conditions of Silano et al., (1973, 1975).

** = Code No. of fractions based on electrophoretic mobility.

investigated. Hofer and Sturmbauer (1985) suggested that inhibitors occurring in wheat could affect starch digestion in carp and trout, since the amylase activity in the gut fluids collected from these fishes reduced on incubation with wheat flour. However, it was not conclusively demonstrated that this reduction was due to the effect of inhibitor in the wheat flour. Therefore, the aim of the present study was to test the effect of purified wheat amylase inhibitor on alpha-amylases from two distinct commercially important cultivable species of fish, mirror carp (Cyprinus carpio) and the Nile tilapia (Oreochromis niloticus).

3.2 MATERIALS AND METHODS.

3.2.1 Experimental protocol.

Five large mirror carp weighing between 92 and 122g and three male tilapia weighing from 45 to 56g were obtained from stock maintained by the Institute of Aquaculture and transferred into two separate tanks in the experimental system (Chapter 2). The fish were allowed to acclimatize for a period of seven days during which period they were fed holding ration (Appendix 1) at a rate of 3% of their body weight daily in two equal instalments. On the eighth day the fish were fed with the same diet and four hours later they were sacrificed by placing them in water containing 500 mg/litre benzocaine.

The fish were quickly dissected and the intestine and hepatopancreas were excised from the fish. The tilapia stomach tissue and contents, which have negligible amylase activity, were excluded. The hepatopancreas, excluding the gallbladder, was separated and weighed on a Mettler AC100 balance before transferring to a vial. The intestine was carefully uncoiled and stripped of its contents, then slit open and rinsed in cold fish ringer's solution (Ginsburg, 1963). The hind gut region was discarded and the rest of the intestine weighed and tranferred into a vial for storage.

The samples of hepatopancreas and intestine were frozen in liquid nitrogen and then stored at -20°C until required for analyses.

The intestinal contents were collected, pooled in a centrifuge tube and the fluid was separated by centrifuging at 8000 xg for 30 minutes in a refrigerated centrifuge (MSC high speed 18). The solid portion was discarded while the fluid was transferred into a cryotube and frozen rapidly in liquid nitrogen and then stored at -20°C.

Thus, a total of three samples (hepatopancreas, intestine and gut fluid) from both carp and tilapia were collected for subsequent assays.

Gut fluid samples were thawed and diluted up to 2000 times with phosphate buffer (50 mM, pH 6.9) containing 50 mM NaCl and 0.5 mM $CaCl_2$. The dilution rate depended upon the amylase activity of the samples. Dilutions were carried out in such a way that 50 ul of the diluted sample did not produce a final colour which exceeded 1.00 optical density unit on the spectrophotometer scale. Carp gut fluid amylase activity levels ranging from 74 to 206 mUnits were tested against inhibitor levels of up to 2 ug. Tilapia gut fluid was also similarly diluted and activity levels ranging from 51 to 223 mUnits were tested against 0.4 to 2.0 ug of inhibitor protein.

The inhibitor used was a commercial Sigma product

(Type I) obtained as lyophilised powder containing 1640 Sigma Inhibitor Units per mg of protein for human salivary amylase and 410 inhibitor units per mg for porcine pancreatic alpha-amylase. It should be noted that one Sigma Inhibitor Unit is that quantity of inhibitor which, after preincubation at 25°C, will reduce the activity of two units of alpha-amylase by 50%. One Unit of alpha-amylase is defined by Sigma as that activity that will liberate 1 mg of maltose from starch in three minutes at 37°C and at pH 6.9. This powder was dissolved in 1 ml of cold phosphate buffer (50 mM, pH 6.9) and small aliquots were diluted further to produce a solution containing 100 ug protein per ml and used for all assays.

Samples of around 100 mg of hepatopancreas were accurately weighed out and homogenized in 5 ml of cold phosphate buffer for 1 minute using a Ultra turrex homogenizer. The homogenates were then centrifuged at 8000 xg for 30 minutes (0-5°C) in a refrigerated high speed centrifuge (MSC high speed 18) and the supernatants were diluted up to 100 times. Activity levels ranging from 35 to 235 mUnits for carp or 42 to 195 mUnits for tilapia were tested with 0.2, 0.4, 0.6, 0.8 and 1.0 ug of inhibitor protein. Samples of intestinal tissue were treated in a similar manner. In this case 46 - 191 mUnits of alpha-amylase from carp gut tissue and 51 - 149 mUnits of amylase from tilapia gut tissue were used.

3.2.2 Determination of alpha-amylase activity.

Alpha-amylase activity levels in the test solutions were determined by the method of Rick and Stegbauer (1974) using Lintner's starch (Potato, Sigma) as the substrate at a temperature of 37°C. The pH of carp gut fluid was found to be 7.4 and that of tilapia 6.5, thus a phosphate buffer of pH 6.9 was selected for all assays. The assay method was described in detail in Chapter 2. However the concentration of phosphate buffer used in this instance was 50 mM and it contained 50 mM Nacl and 0.5 mM $CaCl_2$ as recommended in the procedures for inhibition studies by O'Donnell and McGeeney (1976).

The initial alpha-amylase activity levels tested with inhibitors in this study are summarised in Table 3.2.

3.2.3 Inhibition assay.

The method of O'Donnell and McGeeney (1976) was followed with some modifications to investigate the inhibitory activity of wheat amylase inhibitor on fish amylases.

The inhibitor solution was added to three tubes of buffer containing 1 mg of bovine serum albumin (BSA, Sigma), followed by diluted amylase solution. BSA was added to the reaction tubes to protect alpha-amylase against spontaneous inactivation. These mixtures were

Table 3.2 Initial alpha-amylase activity levels (mU/min) tested for inhibition by purified wheat amylase inhibitor.

	Alpha-amylase activity levels		
Amylase source	High	Medium	Low
Carp gut fluid	206	122	74
Carp gut tissue	191	139	46
Carp hepatopancreas	235	105	35
Tilapia gut fluid	223	123	51
Tilapia gut tissue	149	121	51
Tilapia hepatopancreas	195	89	42

incubated at 25°C for 30 minutes with occasional shaking in a water bath. The duration of this incubation period was determined in a separate experiment (see Section 3.2.4). Three control tubes containing buffer, albumin and amylase solution only, without inhibitor, were set up simultaneously for each level of amylase activity. After the 30 minute preincubation period, 1 ml of 1% w/v buffered starch substrate (Sigma) was added to each tube to start the amylolytic reaction and the tubes were incubated for exactly 10 minutes at 37°C. The hydrolysis was then stopped with dinitrosalicylic acid (see Chapter 2) and the maltose produced by amylase both in the presence and the absence of inhibitors was measured.

The amylase activity in the tubes without the inhibitor is the 'initial' activity and the activity measured after the action of the inhibitor on amylases is the 'residual' activity. The difference between the two is a measure of the inhibitory activity of the inhibitor protein under the conditions of the assay. This is then expressed as a percent of initial amylase activity reduced or 'percent inhibition'. The percent inhibition in each test was calculated by the formula,

$$\text{Percent inhibition} = \frac{\text{Activity without inhibitor} - \text{Activity with Inhibitor}}{\text{Activity without inhibitor}} \times 100$$

3.2.4 Determination of preincubation time.

The duration of preincubation of the inhibitor and enzyme mixture to realize maximum inhibition of alpha-amylases is reported to be up to 60 minutes, depending on the origin of the enzyme (Buonocore et al., 1977; O'Connor and McGeeney, 1981). Since no reports are available for fish amylases, an experiment was conducted to determine the optimum preincubation time.

This was achieved using a diluted carp gut fluid sample which had an initial amylase activity of 199 mU/min. Triplicate sample tubes containing amylase sample in phosphate buffer solution (50 mM, pH 6.9) containing 1 mg BSA were incubated with 1 ug of inhibitor (Sigma) protein at 25°C in a constant temperature water bath. Three control tubes were also set up without the inhibitor to determine the initial activity. Eight such groups, of six tubes each, were prepared. At 10 minute intervals, for up to 80 minutes, one set of six tubes (3 control + 3 test) were taken for amylase activity measurement in accordance with the method described in Section 3.2.2, above. The percent inhibition at the end of each incubation interval was then calculated.

The initial amylase activity of 199 mU/min was reduced to 84 mU/min within 10 minutes and further incubation for another 10 minute period reduced the activity to only 68 mU/min (Fig. 3.1). Between 20 and 80 minutes the residual activity remained stable at between 65 and 75 mU/min representing 60 to 69% inhibition.

Thus most of the inhibition occurred within the first 10 minutes and, by the end of 20 minutes, the maximum level of inhibition had been attained. Thereafter even up to 80 minutes of incubation caused no significant ($p > 0.05$) increase in the level of inhibition.

Based on these results, a 30 minute preincubation period was adopted in all assays.

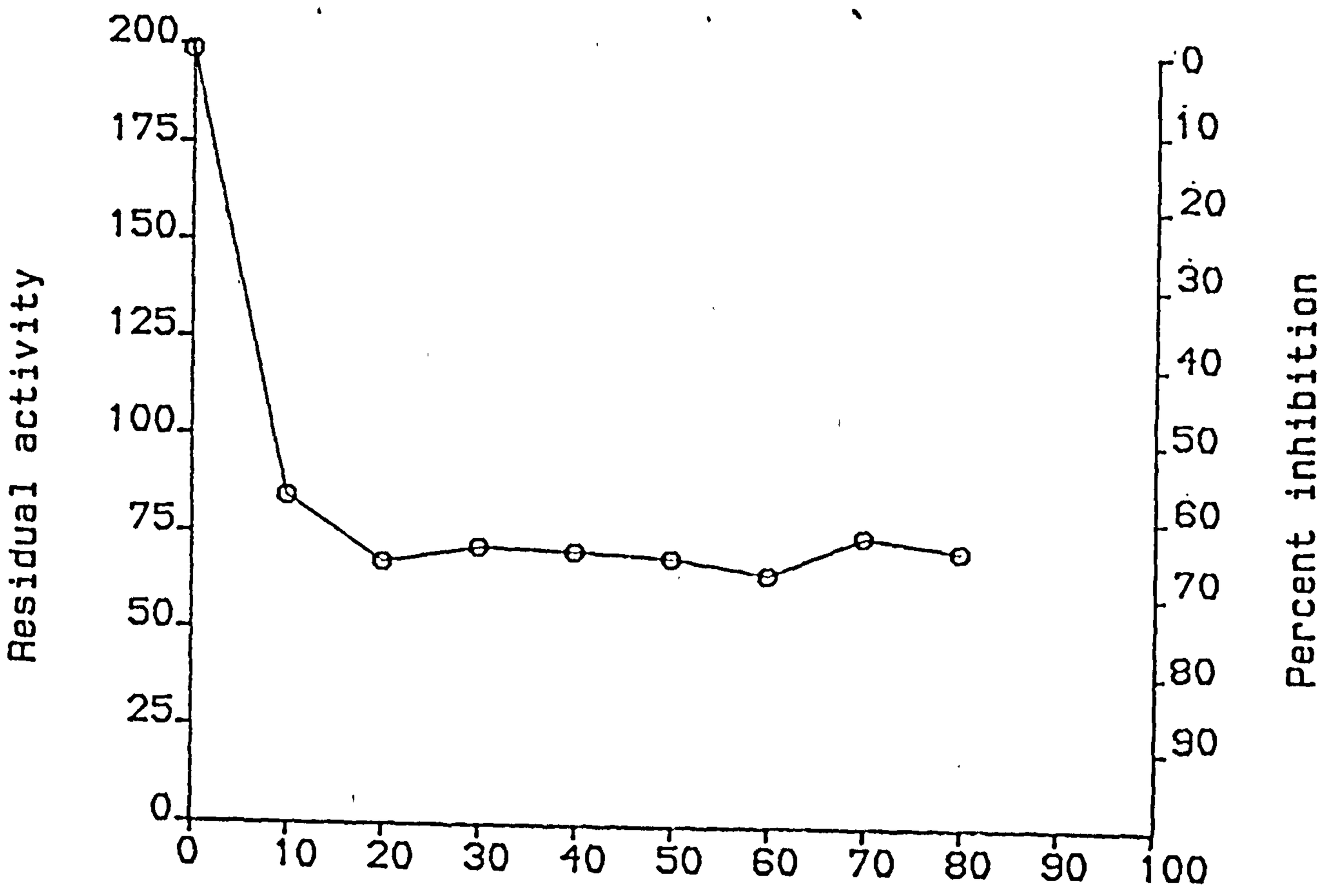

FIGURE 3.1 Effect of the duration of pre-incubation at 25°C on the inhibition of carp gut fluid alpha-amylase (initial activity 198.57 ± 6.31 mU/min) by 1 ug wheat amylase inhibitor protein.

3.3 RESULTS.

3.3.1 Inhibition of carp alpha-amylases.

When carp gut fluid alpha-amylase was mixed with 0.2 to 2.0 ug of inhibitor, the amylolytic activity was effectively reduced at the three initial activity levels tested (Figs. 3.2, 3.3 and Tables 3.3a, b). Even the lowest concentration of inhibitor protein could inhibit 14% of the amylase at the high level. At the other extreme, a maximum of 86% of the initial activity was reduced by 2.0 ug of inhibitor protein. Total inhibition (100%) was however never achieved at any of the inhibitor concentrations or initial activity levels tested. Even a low initial activity of 74 mU/min, when incubated with the highest concentration of 2.0 ug of inhibitor, resulted in a residual activity of 11 mU/min at the end of the test.

As the inhibitor concentration increased, the magnitude of residual activity at the end of the 30 minute incubation period decreased, or conversely the percentage of inhibition increased. The amylolytic activity reduced sharply at inhibitor concentrations from 0.2 ug up to 1 ug but thereafter very little further reductions were obtained (Figs. 3.2, 3.3). The incremental degree of inhibition became successively smaller at higher levels of inhibitor protein. For instance, at the fixed initial amylase activity of 122

Table 3.3a Residual activity of carp gut fluid alpha-amylase after incubation (30 min., 25°C) with and without various concentrations of purified wheat amylase inhibitor at three levels of initial enzyme activity.

ug inhibitor	Residual amylase activity** (mUnits/min) at 37°C		
	High level	Medium level	Low level
0.0	205.54 (3.90)	121.69 (5.60)	73.74 (2.83)
0.2	175.94 (3.27)	88.78 (3.46)	49.85 (6.60)
0.4	140.85 (7.45)	75.65 (2.73)	34.17 (4.03)
0.6	120.24 (4.79)	59.52 (0.43)	26.46 (0.65)
0.8	109.12 (4.54)	51.96 (2.36)	18.07 (1.32)
1.0	92.93 (2.71)	42.49 (1.12)	15.67 (3.49)
2.0	47.38 (2.74)	20.38 (4.08)	10.62 (3.74)

Table 3.3b Percent inhibition* of carp gut fluid alpha amylase produced by increasing concentrations of purified wheat amylase inhibitor protein (preincubation 30 min., 25°C).

ug inhibitor	% inhibition of 205.54 mU of amylase	% inhibition of 121.69 mU of amylase	% inhibition of 73.74 mU of amylase
0.2	14.40 (1.59)	27.04 (2.84)	32.41 (8.95)
0.4	31.47 (3.62)	37.84 (2.24)	53.66 (5.47)
0.6	41.50 (2.33)	51.08 (0.35)	64.11 (0.88)
0.8	46.91 (2.21)	57.30 (1.94)	75.50 (1.79)
1.0	54.79 (1.32)	65.09 (0.92)	78.75 (4.73)
2.0	76.95 (1.33)	83.25 (3.35)	85.60 (5.08)

$$\text{*Percent inhibition} = \frac{(\text{Initial activity} - \text{Residual activity})}{\text{Initial activity}} \times 100$$

** values in parenthesis show the SD (n = 3).

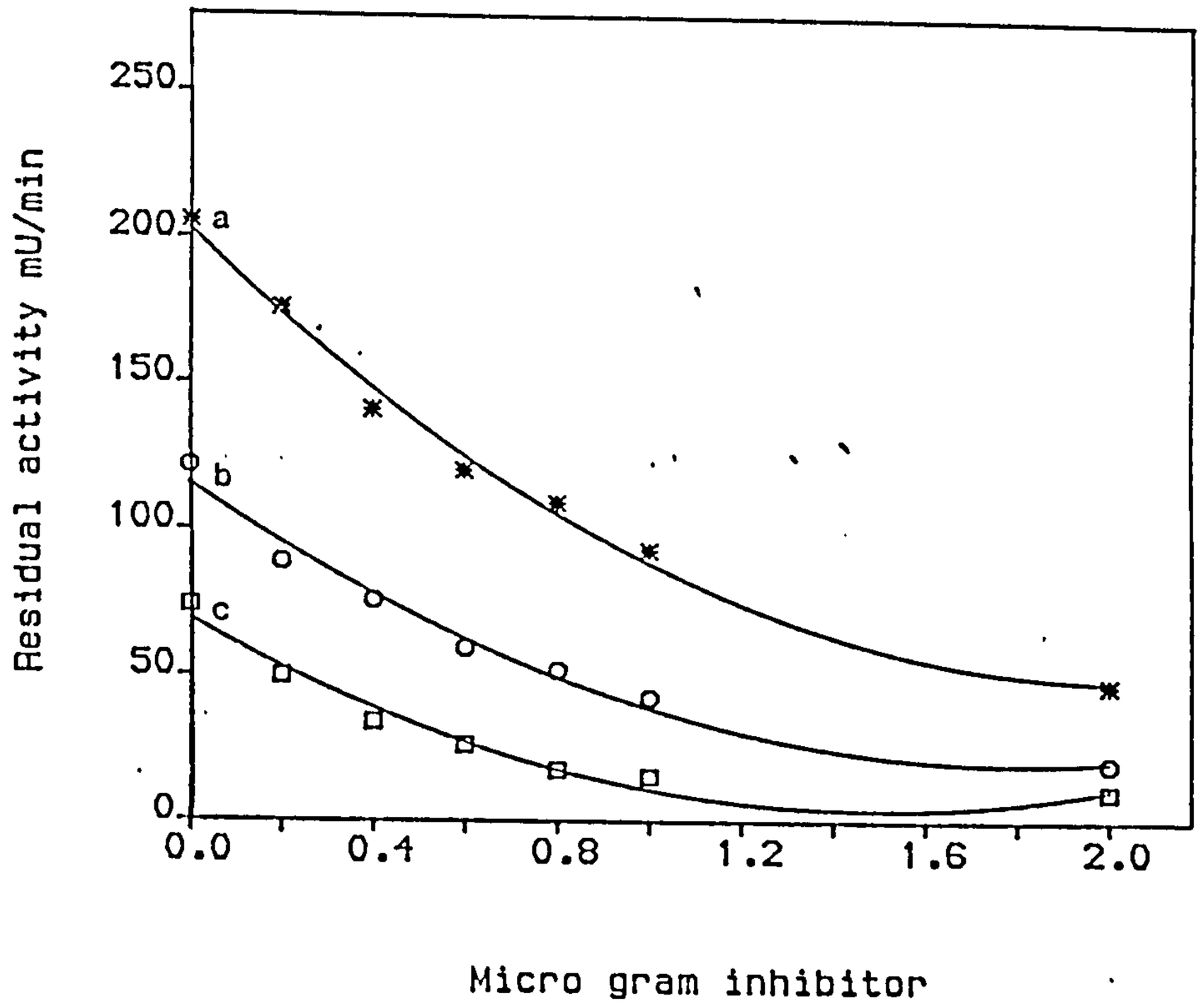

FIGURE 3.2 Residual activity of carp gut fluid alpha-amylase after incubation with various concentrations of purified wheat amylase inhibitor. (a) Initial activity 205.54 mU/min, (b) Initial activity 121.69 mU/min and (c) Initial activity 73.74 mU/min.

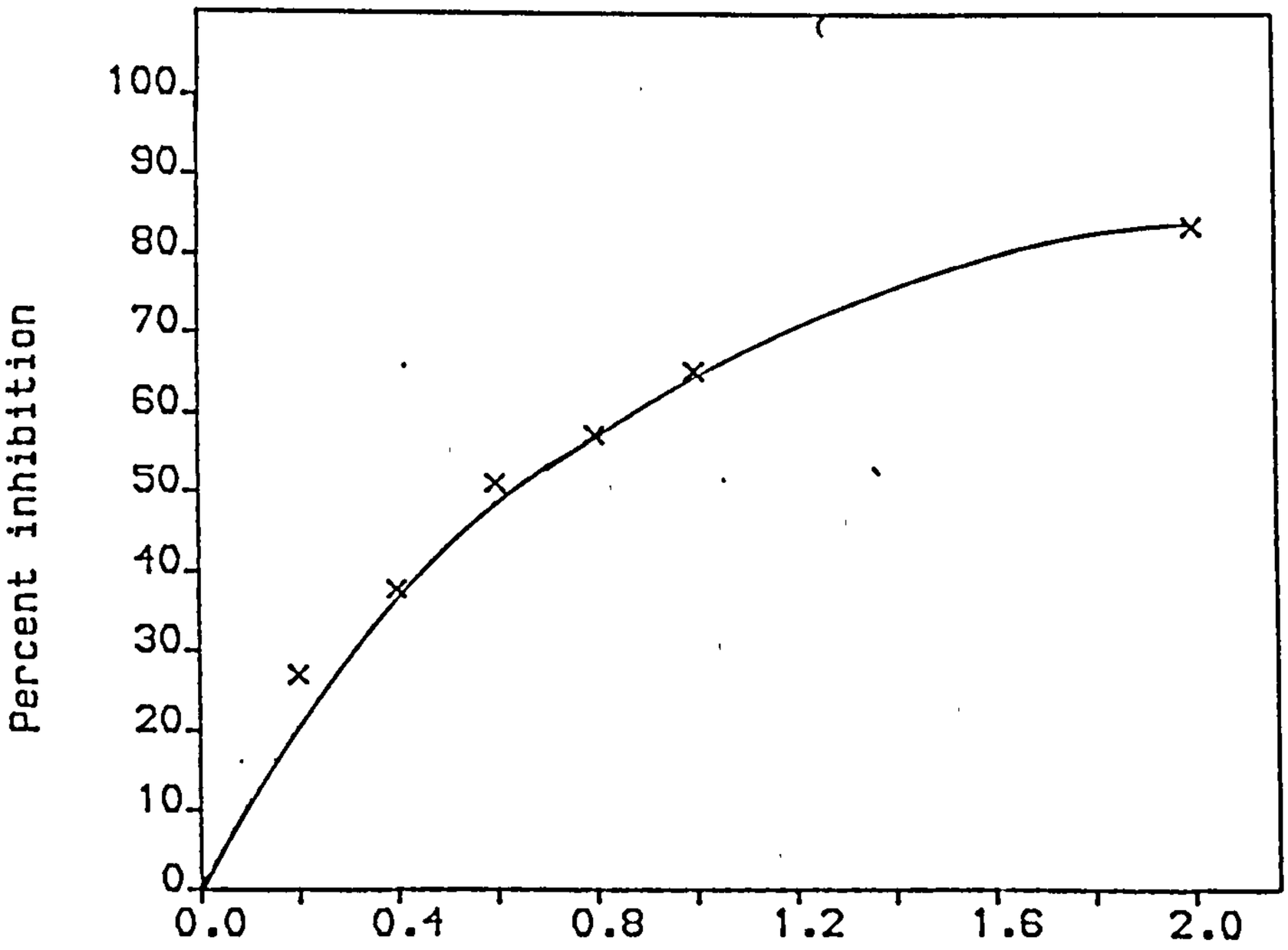

FIGURE 3.3 Percent inhibition of alpha-amylase from carp gut fluid by 0.2 to 2.0 ug purified wheat amylase inhibitor. Initial amylase activity 121.69 $\pm$ 5.60 mU/min at 37°C.

mU/min, by increasing the inhibitor concentration from 0.2 to 1.0 ug, the percent inhibition was raised from 27 to 65%. However, a further doubling of the inhibitor concentration did not increase the inhibition by a similar magnitude, since it only increased to 83% from 65% (Table 3.3b).

At any fixed inhibitor concentration, an increase in the level of initial enzyme activity resulted in an increase in the residual activity, that is the percentage of inhibition decreased (Table 3.3a).

Similar trends were seen in the inhibition of alpha-amylases in the extracts from carp intestinal and hepatopancreatic tissues, at all the three levels of initial activities tested. 18 to 85% of the initial amylase activity from gut tissue was reduced by 0.2 to 2.0 ug of inhibitor (Figs.3.4, 3.5 and Tables 3.4a, b) and the corresponding inhibition of hepatopancreatic amylase was from 20 to 88%, at the high, medium and low levels of initial activity (Figs.3.6, 3.7 and Tables 3.5a, b) tested in this experiment.

The results clearly indicate that carp alpha-amylase is highly susceptible to inhibition by wheat amylase inhibitors and that the residual amylase activity obtained after the action of the inhibitor is positively correlated with initial enzyme activity levels but negatively correlated with the concentration of the inhibitor.

Table 3.4a Residual activity of carp gut tissue alpha-amylase after incubation (30 min., 25°C) with and without various concentrations of purified wheat amylase inhibitor at three levels of initial amylase activity.

ug inhibitor	Residual amylase activity** (mUnits/min) at 37°C		
	High level	Medium level	Low level
0.0	190.96 (1.06)	138.82 (6.04)	45.62 (2.35)
0.2	152.36(10.11)	114.10 (3.04)	28.09 (1.65)
0.4	118.70 (6.45)	89.71 (4.66)	17.93 (1.91)
0.6	96.97 (5.81)	69.44 (5.68)	15.14 (1.87)
0.8	81.98 (2.46)	58.14 (2.82)	14.80 (3.08)
1.0	71.41 (4.61)	42.66 (0.39)	12.77 (2.54)
2.0	31.96 (2.66)	24.86 (2.04)	6.84 (1.28)

Table 3.4b Percent inhibition* of carp gut tissue alpha amylase produced by increasing concentrations of purified wheat amylase inhibitor protein (preincubation 30 min., 25°C).

ug inhibitor	% inhibition of 190.96 mU of amylase	% inhibition of 138.82 mU of amylase	% inhibition of 45.62 mU of amylase
0.2	20.21 (5.29)**	17.81 (2.21)	38.42 (3.62)
0.4	37.84 (3.38)	35.37 (3.36)	60.69 (4.18)
0.6	49.22 (3.04)	49.98 (4.09)	66.81 (4.10)
0.8	57.07 (1.29)	58.12 (2.03)	67.55 (6.75)
1.0	62.61 (2.41)	69.27 (0.28)	72.01 (5.57)
2.0	83.27 (1.39)	82.09 (1.47)	85.00 (2.80)

$$\text{*Percent inhibition} = \frac{(\text{Initial activity} - \text{Residual activity})}{\text{Initial activity}} \times 100$$

** values in parenthesis show SD (n = 3).

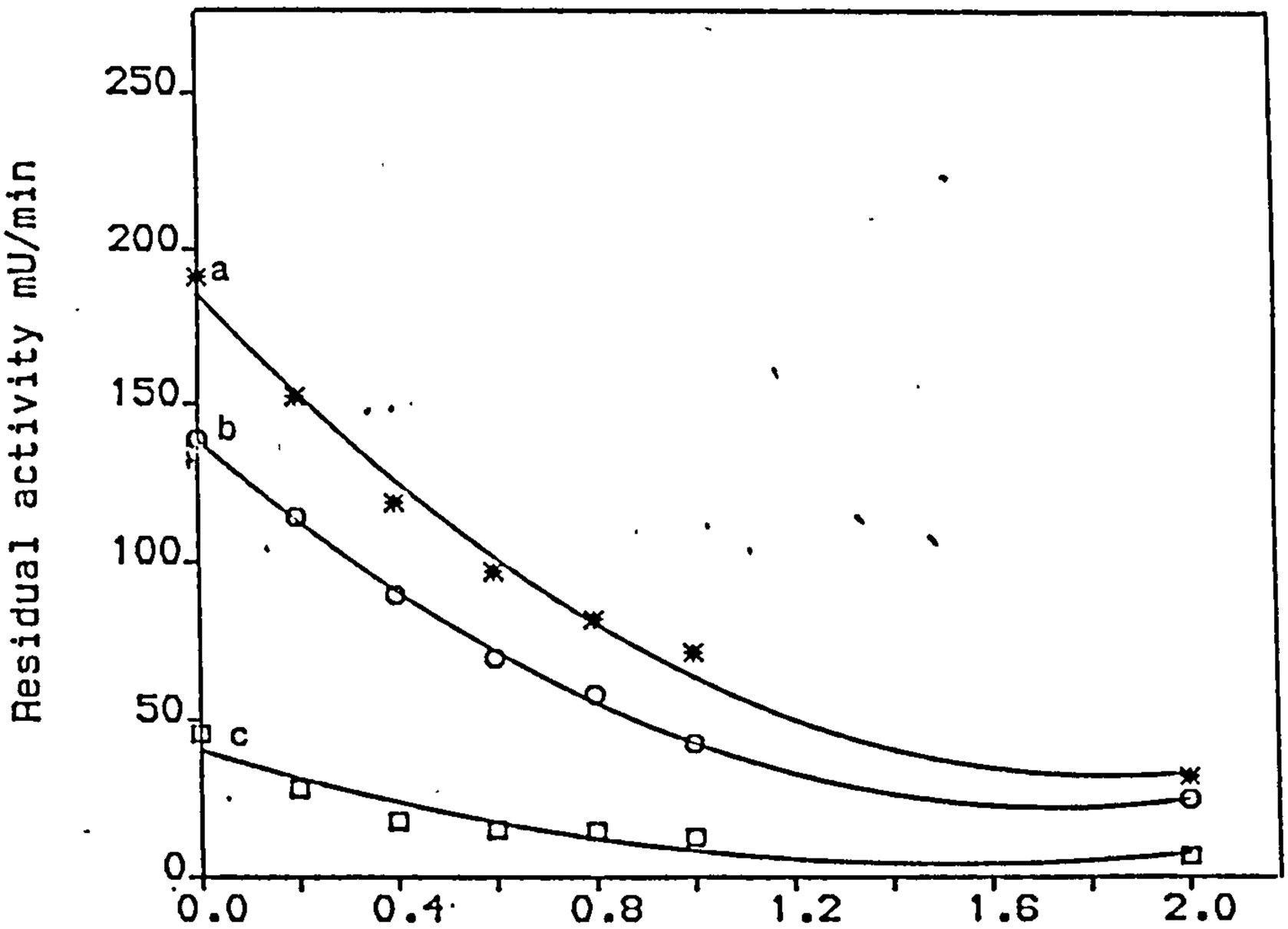

FIGURE 3.4 Residual activity of carp gut tissue alpha-amylase after incubation with various concentrations of purified wheat amylase inhibitor. (a) Initial activity 190.96 mU/min, (b) Initial activity 138.82 mU/min and (c) Initial activity 45.62 mU/min.

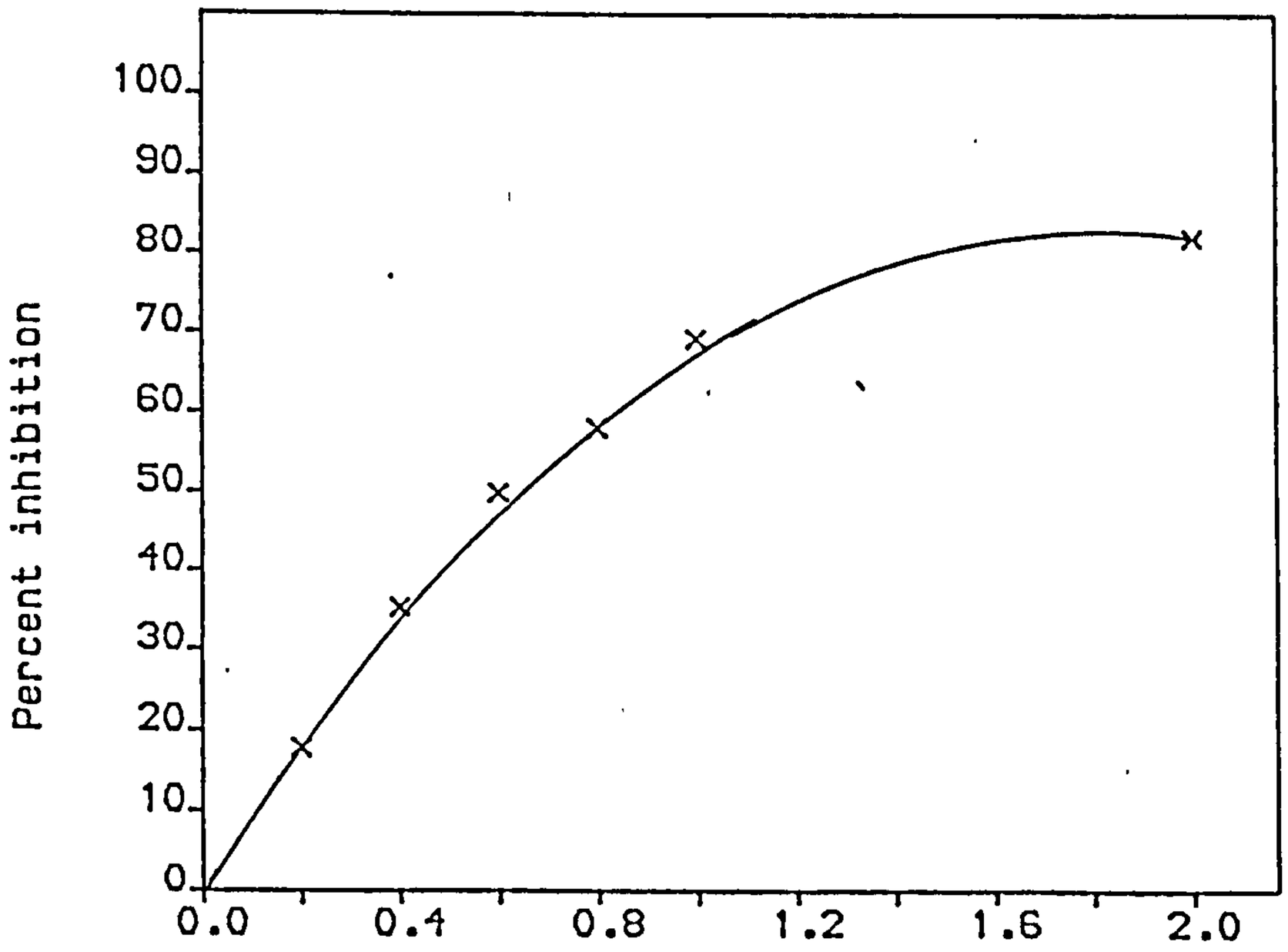

FIGURE 3.5 Percent inhibition of alpha-amylase from carp gut tissue by 0.2 to 2.0 ug purified wheat amylase inhibitor. Initial amylase activity 138.82 $\pm$ 6.04 mU/min at 37°C.

Table 3.5a Residual activity of carp hepatopancreatic tissue alpha-amylase after incubation (30 min., 25°C) with and without various concentrations of purified wheat amylase inhibitor at three levels of initial enzyme activity.

ug inhibitor	Residual amylase activity** (mUnits/min) at 37°C		
	High level	Medium level	Low level
0.0	234.87 (4.30)	104.97 (7.28)	34.55 (3.67)
0.2	187.88 (3.36)	75.93 (2.46)	23.08 (2.15)
0.4	148.76 (8.65)	54.36 (8.25)	12.27 (1.27)
0.6	136.57 (4.06)	47.12 (2.23)	8.48 (0.77)
0.8	120.15 (3.94)	35.85 (2.69)	11.26 (1.30)
1.0	101.30 (0.97)	27.52 (2.42)	8.73 (0.58)
2.0	56.55 (4.73)	12.48 (1.94)	5.35 (1.97)

Table 3.5b Percent inhibition* of carp hepatopancreatic alpha amylase produced by increasing concentrations of purified wheat amylase inhibitor protein (preincubation 30 min., 25°C).

ug inhibitor	% inhibition of 234.87 mU of amylase	% inhibition of 104.97 mU of amylase	% inhibition of 34.55 mU of amylase
0.2	20.01 (1.43)**	27.67 (2.34)	33.22 (6.23)
0.4	36.66 (3.68)	48.21 (7.86)	64.48 (3.69)
0.6	41.85 (1.73)	55.11 (2.13)	75.47 (2.24)
0.8	48.84 (1.68)	65.85 (2.56)	67.41 (3.76)
1.0	56.87 (0.42)	73.78 (2.31)	74.74 (1.69)
2.0	75.92 (2.02)	88.01 (1.84)	84.51 (5.69)

$$\text{*Percent inhibition} = \frac{(\text{Initial activity} - \text{Residual activity})}{\text{Initial activity}} \times 100$$

** values in parenthesis show SD (n = 3).

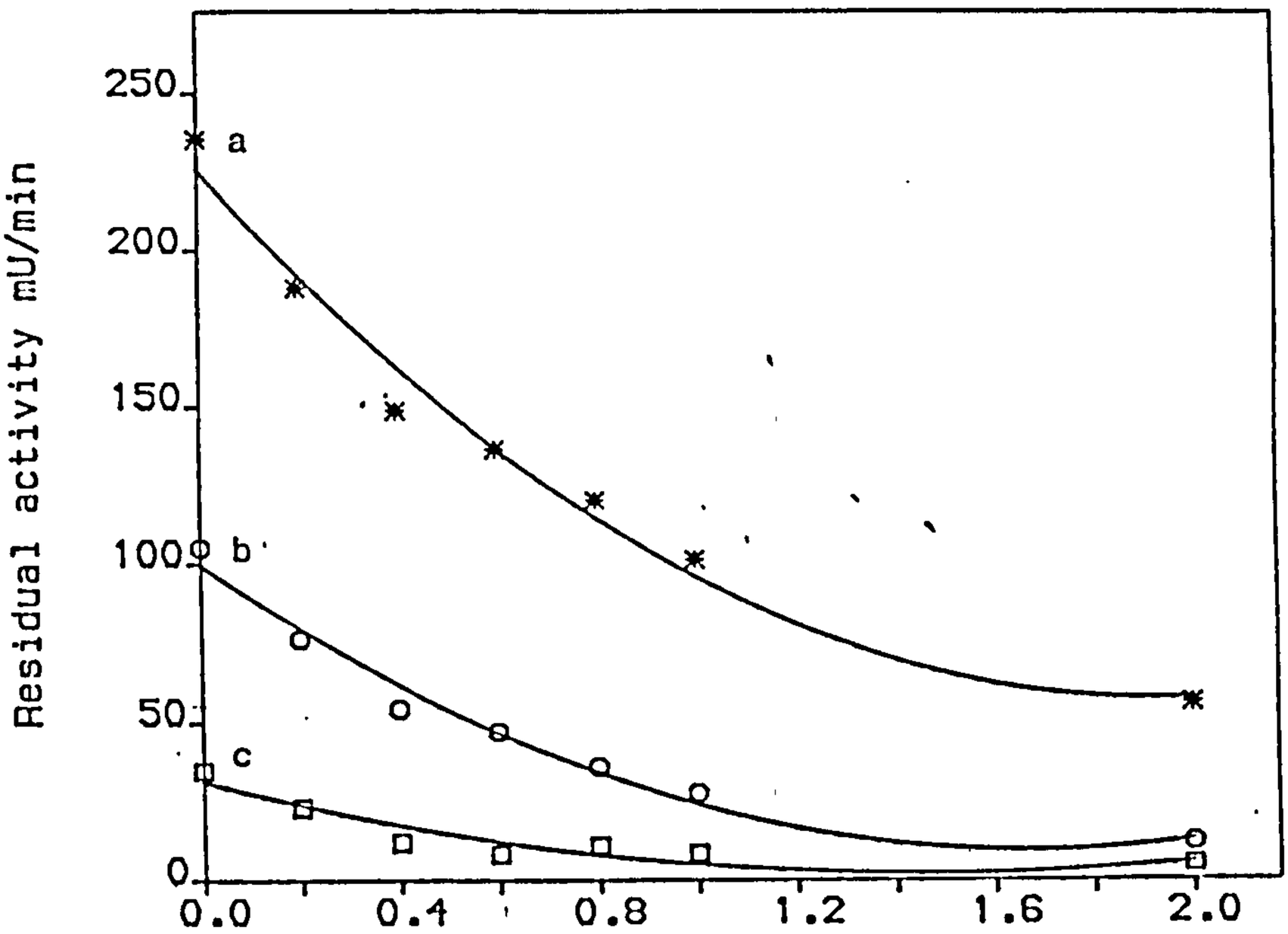

FIGURE 3.6 Residual activity of carp hepatopancreatic alpha-amylase after incubation with various concentrations of purified wheat amylase inhibitor. (a) Initial activity 234.87 mU/min, (b) Initial activity 104.97 mU/min and (c) Initial activity 34.55 mU/min.

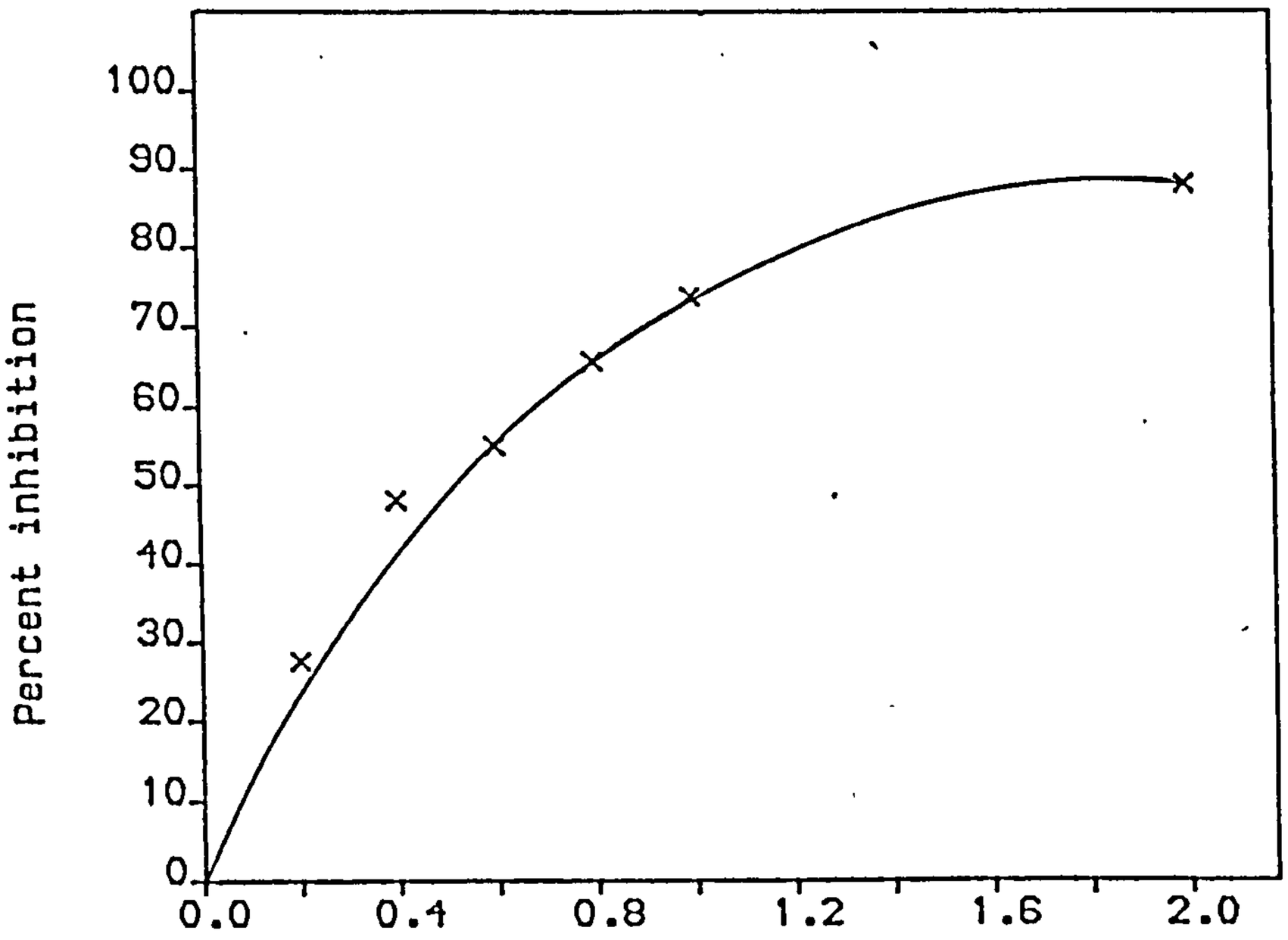

FIGURE 3.7 Percent inhibition of alpha-amylase from carp hepatopancreas by 0.2 to 2.0 ug purified wheat amylase inhibitor. Initial amylase activity 104.97 ± 7.28 mU/min at 37°C.

3.3.2 Inhibition of tilapia alpha-amylases.

In the case of tilapia gut fluid amylases, 0.4 ug of inhibitor protein reduced 223 mU of amylase activity to a final level of 174 mU/min, representing an inhibition of 22%. Two micrograms of inhibitor at this initial enzyme level produced 86% inhibition (Figs. 3.8, 3.9 and Tables 3.6a, b). Further tests with a similar range of inhibitor concentrations but at lower enzyme levels, resulted in increased inhibition of the amylolytic activity of the enzyme. Total inhibition was obtained in the combination of 2 ug inhibitor with 51 mU of enzyme (Tables 3.6a, b). The activity of enzyme from gut fluid reduced sharply up to an inhibitor concentration of about 1.0 ug and thereafter the magnitude of inhibition was not proportional to the inhibitor concentration, as in the case of carp gut fluid amylase. This can be seen from Fig.3.9, where up to 0.8 ug the line is almost linear and then the curve flattens out.

Wheat amylase inhibitor protein had a similar effect on the alpha-amylases from tilapia gut tissue and also hepatopancreas (Figs.3.10, 3.11, 3.12 & 3.13 and Tables 3.7a, b & 3.8a, b). 39 to 96% inhibition was obtained when 51 to 149 mU of gut tissue alpha-amylase was incubated with 0.4 to 2.0 ug of inhibitor (Figs.3.10, 3.11 and Tables 3.7a, b). Similarly 36 to 86% of

Table 3.6a Residual activity of tilapia gut fluid alpha-amylase after incubation (30 min., 25°C) with and without various concentrations of purified wheat amylase inhibitor at three levels of initial enzyme activity.

ug inhibitor	Residual amylase activity** (mUnits/min) at 37°C		
	High level	Medium level	Low level
0.0	223.16 (6.75)	123.08 (3.28)	50.83 (2.88)
0.4	174.19 (5.87)	69.50 (0.42)	21.15 (1.11)
0.6	137.83 (3.76)	43.80 (3.18)	9.23 (0.88)
0.8	109.54 (1.31)	35.15 (3.26)	6.01 (0.82)
1.0	81.68 (8.69)	21.01 (1.26)	4.15 (2.01)
2.0	31.83 (1.54)	10.83 (3.57)	0.00 (0.00)

Table 3.6b Percent inhibition* of tilapia gut fluid alpha amylase produced by increasing concentrations of purified wheat amylase inhibitor protein (preincubation 30 min., 25°C).

ug inhibitor	% inhibition of 223.16 mU of amylase	% inhibition of 123.08 mU of amylase	% inhibition of 50.83 mU of amylase
0.4	21.94 (2.63)**	43.53 (0.34)	58.39 (2.18)
0.6	38.07 (1.76)	64.41 (2.58)	81.84 (1.73)
0.8	50.92 (0.59)	71.44 (2.65)	88.17 (1.60)
1.0	63.40 (3.89)	82.93 (1.02)	91.83 (3.96)
2.0	85.74 (0.69)	91.20 (2.90)	100.00 (0.00)

$$\text{*Percent inhibition} = \frac{(\text{Initial activity} - \text{Residual activity})}{\text{Initial activity}} \times 100$$

** values in parenthesis show SD (n = 3).

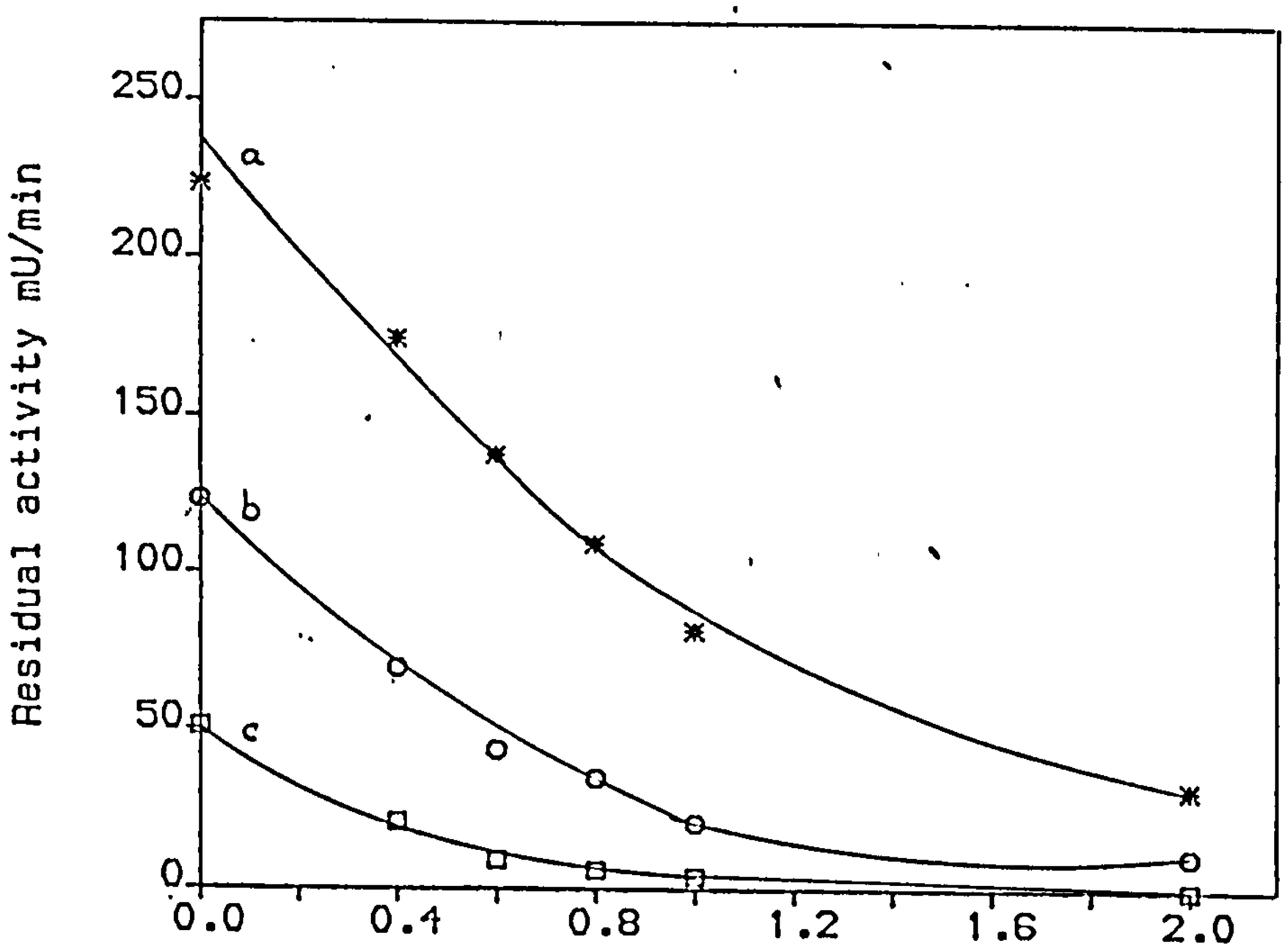

FIGURE 3.8 Residual activity of tilapia gut fluid alpha-amylase after incubation with increasing concentrations of purified wheat amylase inhibitor (a)Initial activity 223.16 mU/min, (b) Initial activity 123.08 mU/min and (c) Initial activity 50.83 mU/min.

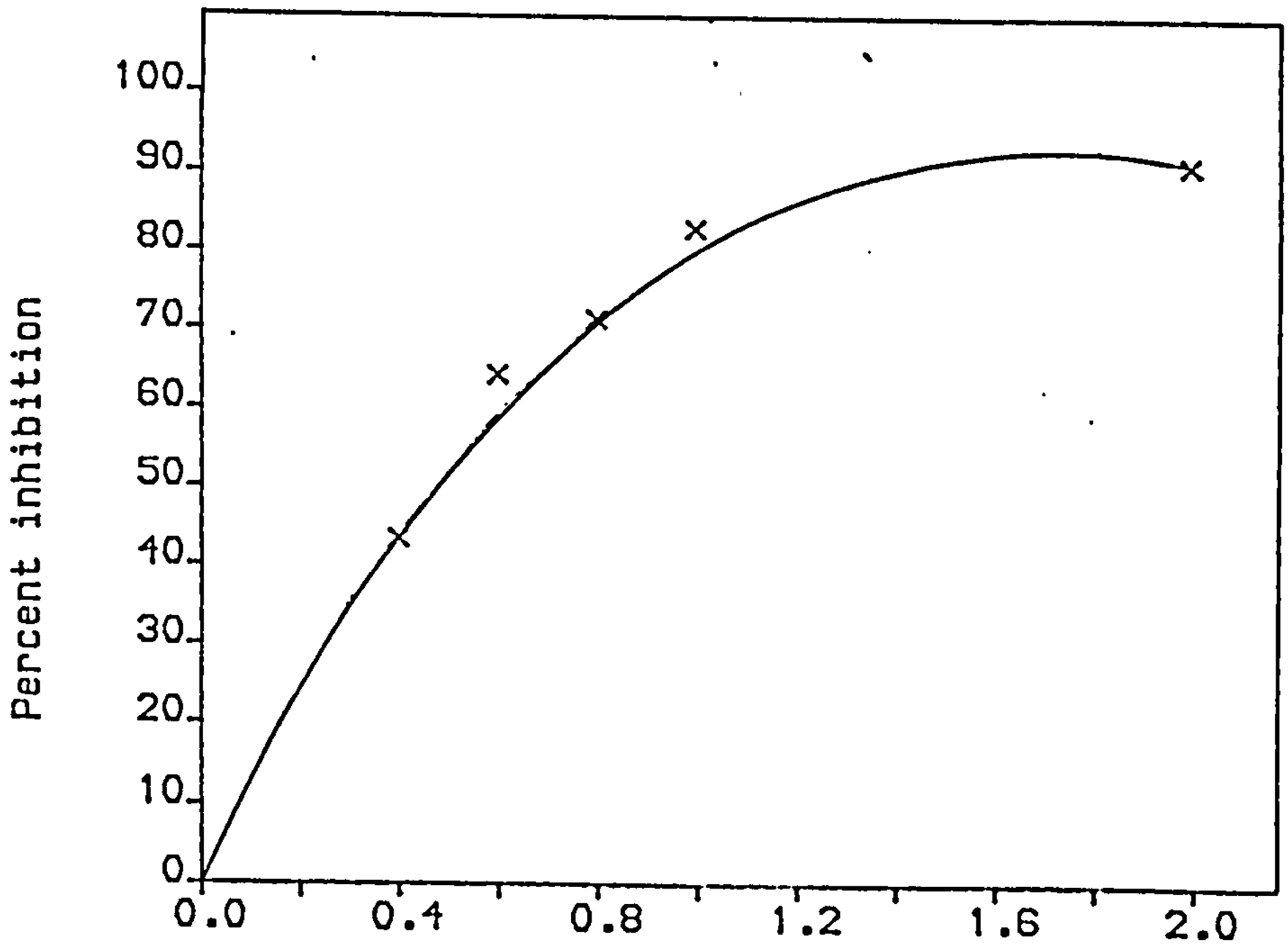

FIGURE 3.9 Percent inhibition of alpha-amylase from tilapia gut fluid by 0.4 to 2.0 ug purified wheat amylase inhibitor. Initial amylase activity 123.08 $\pm$ 3.28 mU/min at 37°C.

Table 3.7a Residual activity of tilapia gut tissue alpha-amylase after incubation (30 min., 25°C) with and without various concentrations of purified wheat amylase inhibitor at three levels of initial enzyme activity.

ug inhibitor	Residual amylase activity** (mUnits/min) at 37°C		
	High level	Medium level	Low level
0.0	148.77 (3.97)	121.16 (2.69)	51.11 (3.77)
0.4	91.15 (5.70)	60.57 (2.68)	13.84 (4.00)
0.6	64.11 (5.51)	35.20 (1.94)	7.95 (3.46)
0.8	47.46 (4.78)	25.01 (4.38)	7.61 (0.53)
1.0	32.46 (1.00)	20.35 (1.55)	4.54 (2.76)
2.0	14.24 (1.17)	11.35 (5.40)	2.24 (0.64)

Table 3.7b Percent inhibition* of tilapia gut tissue alpha amylase produced by increasing concentrations of purified wheat amylase inhibitor protein (preincubation 30 min., 25°C).

ug inhibitor	% inhibition of 148.77 mU of amylase	% inhibition of 121.16 mU of amylase	% inhibition of 51.11 mU of amylase
0.4	38.66 (3.93)**	50.01 (2.21)	72.93 (7.83)
0.6	56.91 (3.70)	70.95 (1.60)	84.44 (6.76)
0.8	68.10 (3.21)	79.36 (3.62)	85.11 (1.04)
1.0	78.18 (0.67)	83.21 (1.29)	91.12 (5.40)
2.0	90.43 (0.79)	90.63 (4.46)	95.62 (1.26)

$$\text{*Percent inhibition} = \frac{(\text{Initial activity} - \text{Residual activity})}{\text{Initial activity}} \times 100$$

** values in parenthesis show SD (n = 3).

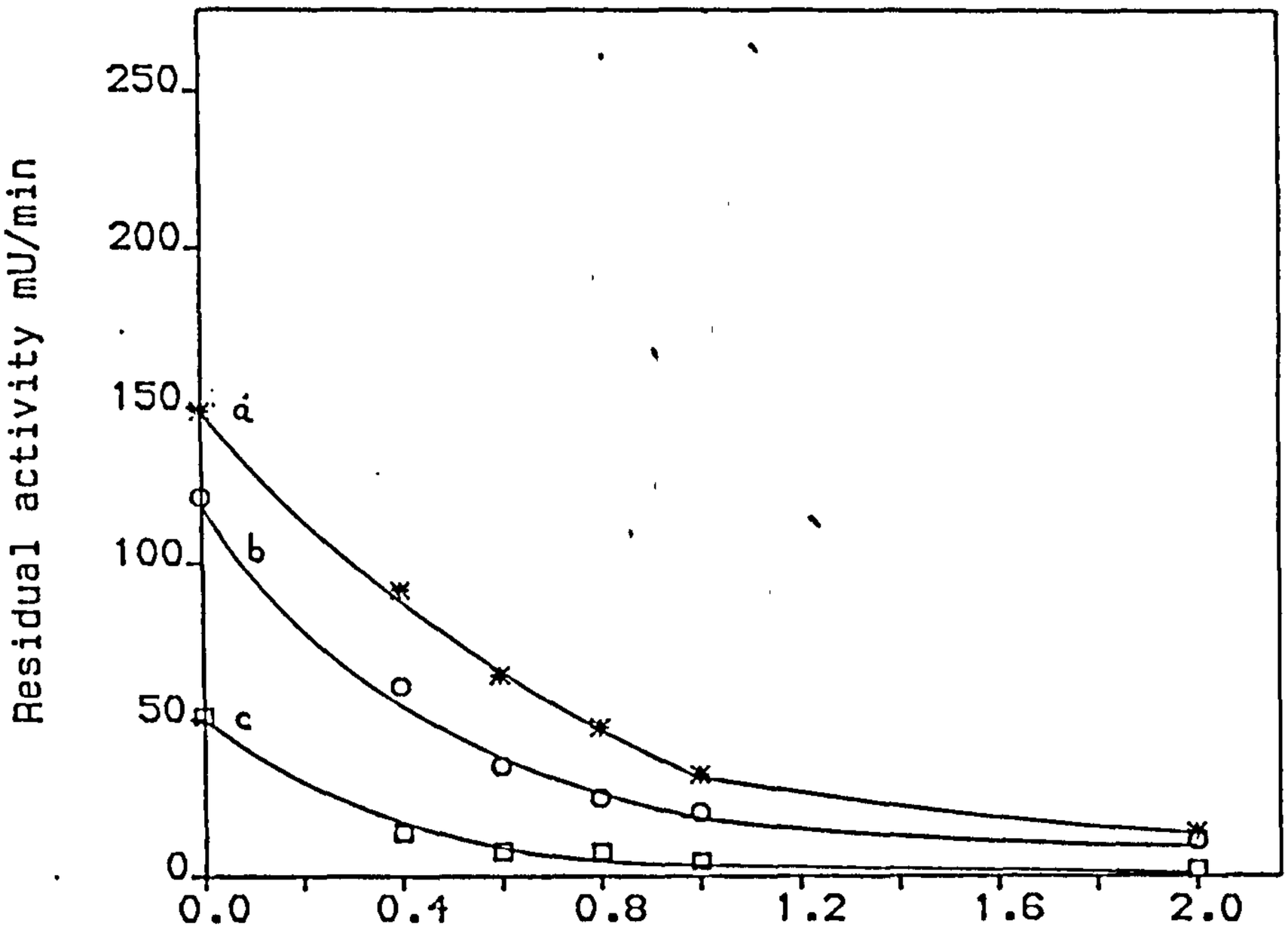

FIGURE 3.10 Residual activity of tilapia gut tissue alpha-amylase after incubation with increasing concentrations of purified wheat amylase inhibitor (a)Initial activity 148.77 mU/min, (b) Initial activity 121.16 mU/min and (c) Initial activity 51.11 mU/min.

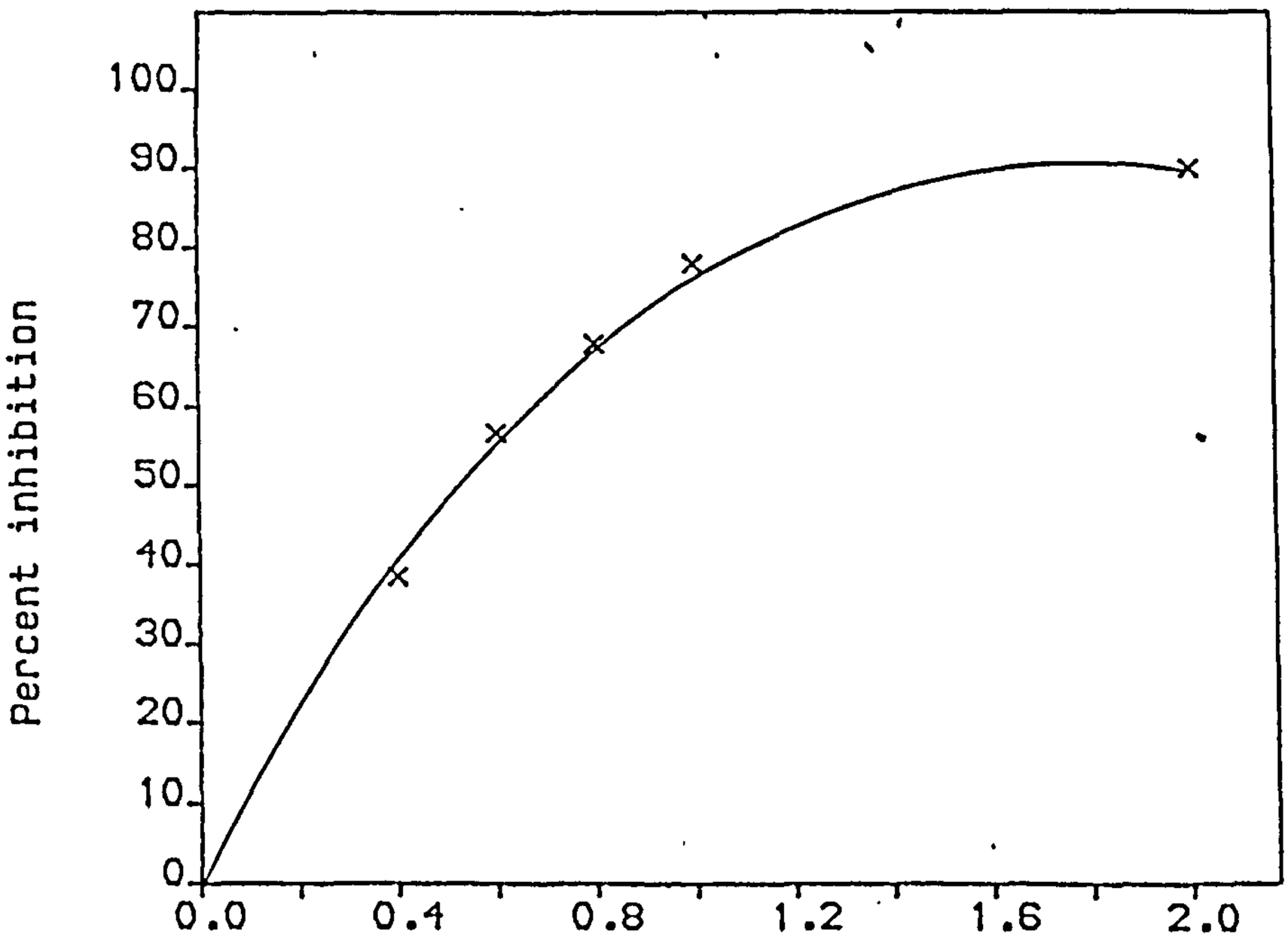

FIGURE 3.11 Percent inhibition of alpha-amylase from tilapia gut tissue by 0.4 to 2.0 ug purified wheat amylase inhibitor. Initial amylase activity 148.77 $\pm$ 3.97 mU/min at 37°C.

Table 3.8a Residual activity of tilapia hepatopancreatic tissue alpha-amylase after incubation (30 min., 25°C) with and without various concentrations of purified wheat amylase inhibitor at three levels of initial enzyme activity.

ug inhibitor	Residual amylase activity** (mUnits/min) at 37°C		
	High level	Medium level	Low level
0.0	195.32 (0.68)	89.34 (1.99)	41.98 (3.17)
0.4	125.20 (4.93)	46.94 (0.74)	15.47 (1.74)
0.6	94.83 (9.83)	33.07 (3.77)	12.93 (2.14)
0.8	72.38 (4.94)	20.10 (0.74)	9.33 (3.07)
1.0	50.01 (1.44)	15.88 (1.70)	8.18 (2.48)
2.0	26.44 (0.60)	12.40 (1.10)	5.97 (0.79)

Table 3.8b Percent inhibition* of tilapia hepatopancreatic tissue alpha amylase produced by increasing concentrations of purified wheat amylase inhibitor protein (preincubation 30 min., 25°C).

ug inhibitor	% inhibition of 195.32 mU of amylase	% inhibition of 89.34 mU of amylase	% inhibition of 41.98 mU of amylase
0.4	35.90 (2.52)**	47.46 (0.83)	63.16 (4.15)
0.6	51.45 (5.03)	62.98 (4.22)	69.20 (5.10)
0.8	62.94 (2.53)	77.50 (0.83)	77.78 (7.31)
1.0	74.40 (0.74)	82.22 (1.91)	80.51 (5.92)
2.0	86.46 (0.31)	86.12 (1.23)	85.77 (1.88)

$$\text{*Percent inhibition} = \frac{(\text{Initial activity} - \text{Residual activity})}{\text{Initial activity}} \times 100$$

** values in parenthesis show SD (n = 3).

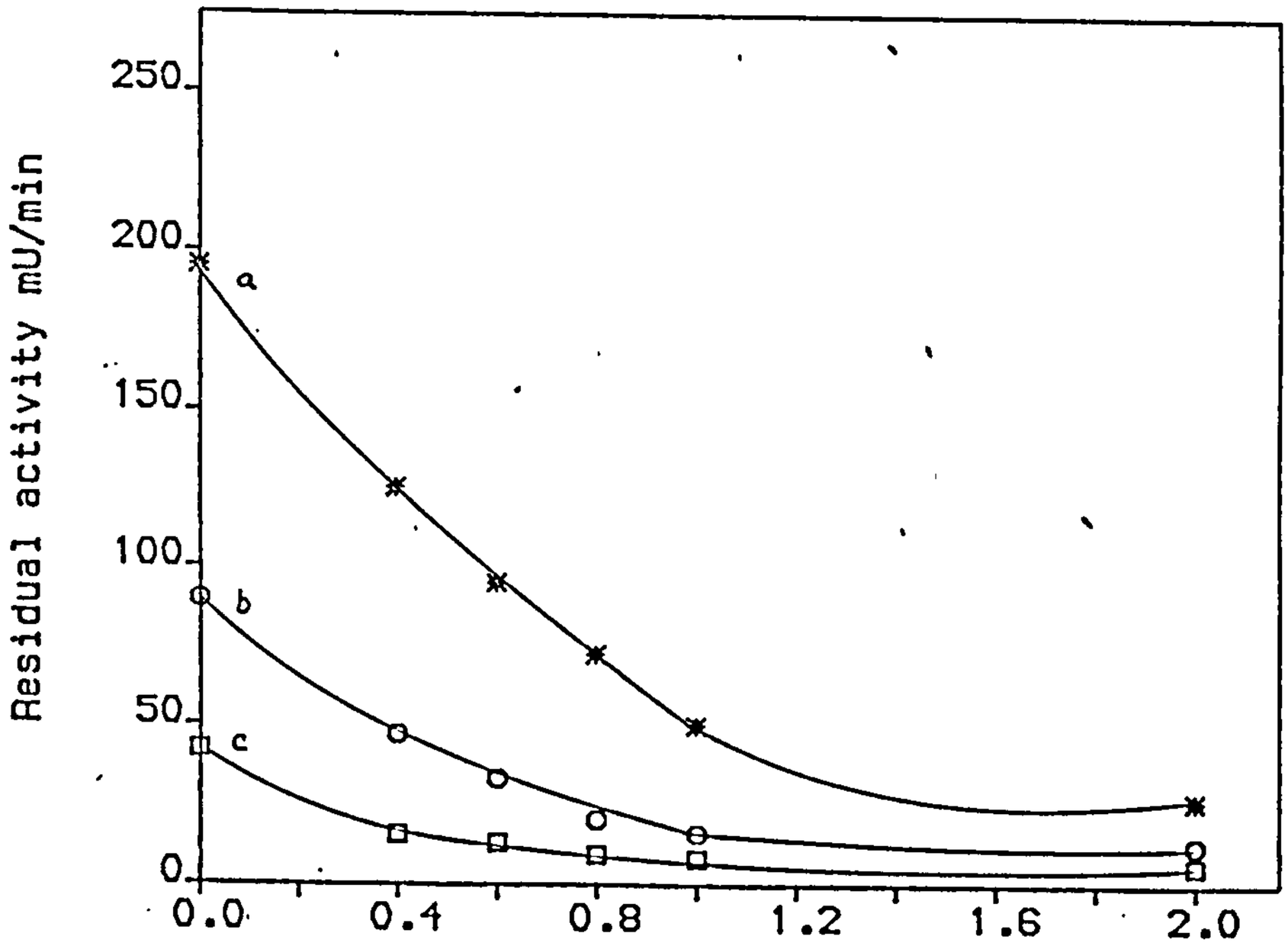

FIGURE 3.12 Residual activity of tilapia hepatopancreatic alpha-amylase after incubation with increasing concentrations of purified wheat amylase inhibitor (a)Initial activity 195.32 mU/min, (b) Initial activity 89.34 mU/min and (c) Initial activity 41.98 mU/min.

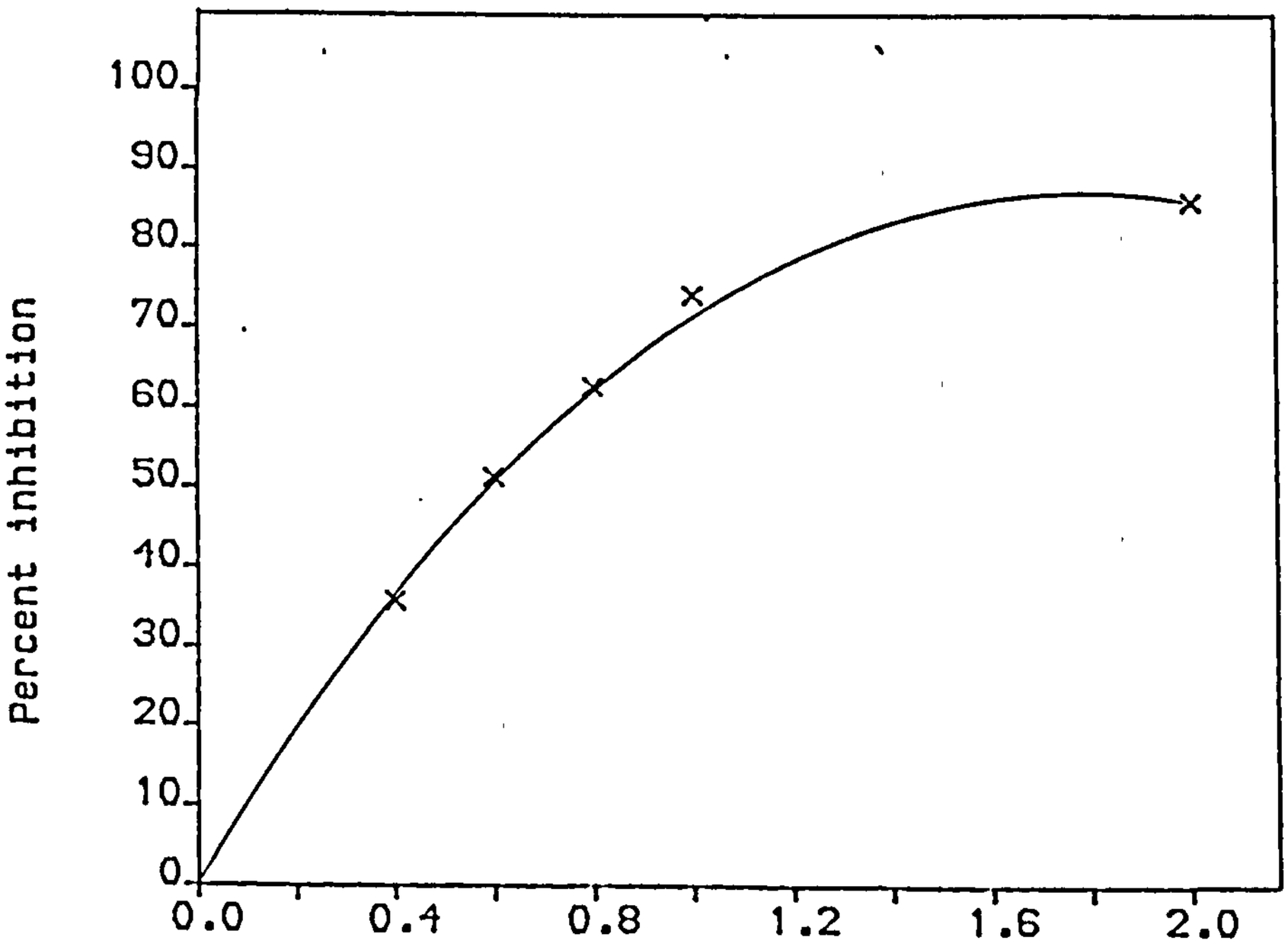

FIGURE 3.13 Percent inhibition of alpha-amylase from tilapia hepatopancreas by 0.4 to 2.0 ug purified wheat amylase inhibitor. Initial amylase activity 195.32 $\pm$ 0.68 mU/min at 37°C.

hepatopancreatic alpha-amylase (42 to 195 mU) was inactivated by 0.4 to 2.0 ug of inhibitor (Figs.3.12, 3.13 and Tables 3.8a, b).

From the above results it was clearly seen that tilapia alpha-amylase from all the three sources was strongly inhibited by the inhibitor from wheat grain. The residual tilapia amylase activity is positively correlated with the initial activity levels and negatively correlated with inhibitor concentrations as was seen in the case of carp amylases.

3.4 DISCUSSION.

From the results it is apparent that the enzyme activity after inhibition is a function of both the concentration of the inhibitor and of the initial amylase activity levels (Figs. 3.2, 3.4, 3.6, 3.8, 3.10 and 3.12). Furthermore, the relationships between residual activity and inhibitor concentration at all initial activity levels were found to be curvilinear for all amylase sources tested from both carp and tilapia.

Comparisons of the potency of the inhibitor against amylase from the three sources of the same species of fish, and also between carp and tilapia were not possible directly since the initial activity levels tested were not the same. Therefore second degree polynomial equations were derived using the data from these inhibition experiments to allow these comparisons to be made.

Three multiple regression equations for both carp and for tilapia were derived to describe the inhibition of gut fluid amylases, gut tissue amylases and hepatopancreatic tissue amylases. The equations are of the type:

$$A_2 = a + b A_1 - c I + d I^2$$

where,

a, b, c and d are constants,

A_1 = Initial amylase activity (mU/min),

A_2 = Residual amylase activity (mU/min),

I = Inhibitor concentration as ug protein

The equations for both carp and tilapia are presented in Table 3.9. The 't' ratio of all the predictor constants were highly significant ($p < 0.05$). If a fixed value for one of the two independent variables (A_1 and I) is assumed, then the residual activity (A_2) at different values of the other independent variable, can be calculated using these equations. For example, at a fixed inhibitor (I) concentration of 1 ug, the residual activity (A_2) in mU/min at various initial activity levels (A_1) can be estimated for carp and tilapia and these are tabulated in Table 3.10.

From Table 3.10 it can be seen that at any particular initial activity level, the residual activity is highest in gut fluid, followed by hepatopancreas and finally gut tissue, for both carp and tilapia amylases. Thus inhibition was greatest in the gut tissue, since low residual activity indicates high inhibition, and lowest in gut fluid. For example, if 250 mU of carp amylase from gut

Table 3.9 Multiple regression equations derived to explain the inhibition of carp and tilapia amylases by wheat amylase inhibitor.

	REGRESSION EQUATION	CORR.	SD	N
CARP				
Gut fluid	$A_2 = 23.68 + 0.769\ A_1 - 104.79\ I + 26.98\ I^2$	0.973	11.67	42
Gut tissue	$A_2 = 42.69 + 0.619\ A_1 - 114.00\ I + 32.49\ I^2$	0.959	14.34	29
Heptopancreas	$A_2 = 40.89 + 0.633\ A_1 - 108.53\ I + 30.98\ I^2$	0.954	18.18	35
TILAPIA				
Gut fluid	$A_2 = 46.89 + 0.652\ A_1 - 134.51\ I + 37.48\ I^2$	0.947	23.11	18
Gut tissue	$A_2 = 49.50 + 0.514\ A_1 - 132.76\ I + 42.79\ I^2$	0.947	14.89	18
Hepatopancreas	$A_2 = 51.45 + 0.517\ A_1 - 123.27\ I + 38.52\ I^2$	0.926	20.89	18

Table 3.9 Multiple regression equations derived to explain the inhibition of carp and tilapia amylases by wheat amylase inhibitor.

	REGRESSION EQUATION	CORR.	SD	N
CARP				
Gut fluid	$A_2 = 23.68 + 0.769\ A_1 - 104.79\ I + 26.98\ I^2$	0.973	11.67	42
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Heptopancreas	$A_2 = 40.89 + 0.633\ A_1 - 108.53\ I + 30.98\ I^2$	0.954	18.18	35
TILAPIA				
Gut fluid	$A_2 = 46.89 + 0.652\ A_1 - 134.51\ I + 37.48\ I^2$	0.947	23.11	18
Gut tissue	$A_2 = 49.50 + 0.514\ A_1 - 132.76\ I + 42.79\ I^2$	0.947	14.89	18
Hepatopancreas	$A_2 = 51.45 + 0.517\ A_1 - 123.27\ I + 38.52\ I^2$	0.926	20.89	18

Table 3.10 Estimated residual activity (A_2) for carp and tilapia amylases at a fixed inhibitor concentration of 1 ug.

Initial activity (mU/min) A_1	I (ug)	Estimated residual activity (A_2) mU/min of					
		*C.FLUID	C.GUT	C.HP	*T.FLUID	T.GUT	T.HP
100	1	23	23	27	15	11	18
150	1	61	54	58	48	37	44
200	1	100	85	90	80	62	70
250	1	138	116	122	113	88	96
300	1	177	147	153	145	114	122

* C = carp, T = tilapia, Fluid = gut fluid, gut = gut tissue and HP = hepatopancreas tissue.

fluid, gut tissue and hepatopancreas is incubated with 1 ug inhibitor, then it would result in the inhibition of 134 mU (i.e. initial - residual activity, from Table 3.10) of gut tissue amylase, 128 mU of hepatopancreas amylase and only 112 mU of gut fluid amylase.

From a comparison of the residual activities of carp and tilapia amylases, it is apparent that the values for carp amylases are always higher than the corresponding values obtained for tilapia (Table 3.10). For instance if 250 mUnits of hepatopancreatic amylase from carp and tilapia were incubated with 1 ug of inhibitor, then the residual activity would be 122 mU in the case of carp (inhibition 128 mU) compared to only 96 mU in the case of tilapia (inhibition 154 mU). This therefore indicates that tilapia amylases are more susceptible to the action of this inhibitor than amylases derived from carp.

The curves relating the inhibitor concentrations with the resulting residual activity of alpha-amylases from carp and tilapia show that inhibition increases almost linearly up to the point where about 60% reduction in amylase activity is effected and then breaks sharply. Above this point, at which the inhibitor concentration is optimum for the quantity of amylase in the sample, any further increase in inhibitor concentration results in much less pronounced loss of amylase activity. Almost identically shaped curves have been published for many amylase-inhibitor systems including human (Kneen and

Sandstedt, 1946), chick (Saunders, 1975) and flour beetle (Buonocore et al., 1976).

The wheat amylase inhibitor, although effective in inactivating both carp and tilapia alpha-amylases, could not produce complete inhibition of the amylolytic activity in the samples even when used at very high concentrations. Out of many assays conducted only in one case when 2 ug of inhibitor was incubated with 51 mU of tilapia gut fluid amylase sample was there no residual activity detected (Table 3.6). This general inability to achieve 100% inhibition of amylase by wheat inhibitors has also been noted by previous workers (Buonocore et al., 1977; O'Connor and McGeeney, 1981) working with amylases of different origins. The explanation suggested by many authors for this sort of result is that the Inhibitor-Enzyme complex itself has some amylolytic activity, the hypothesis being that the binding site of the inhibitor to the enzyme molecule is different from that of the binding site for the substrate. This is why the inhibitor is only effective against certain amylases which probably have groupings which can combine with the protein inhibitor from wheat (Buonocore et al., 1977). It is apparent from the results of this study that carp and tilapia alpha-amylases have binding sites for wheat amylase inhibitor proteins.

O'Donnell and McGeeney (1976) demonstrated that the specificity of wheat amylase inhibitor is 100

times greater towards human salivary amylase than towards pancreatic amylase. Alpha-amylase inhibitor proteins extracted from rye have also been shown to inhibit salivary enzyme 10 times faster than pancreatic enzyme (Louda and Marshall, 1974 quoted by Marshall, 1975). In the present study wheat inhibitor had the least effect on amylases from gut fluids of both carp and tilapia. This inhibitor is known to be easily destroyed by proteolytic digestion by proteases such as pepsin, trypsin and alpha-chymotrypsin (Kneen and Sandstedt, 1946, O'Connor and McGeeney, 1981) and the fish gut fluids certainly contain active proteases while the tissues have only the inactive zymogens of proteases. Furthermore the presence of carbohydrates in the diet and maltose from the hydrolytic digestion of starch by amylases in the gut lumen are also thought to counteract the effects of the wheat inhibitor (O'Connor and McGeeney, 1981). In both carp and tilapia, inhibition of gut tissue amylase was greater than that of hepatopancreatic amylases. Considerable amounts of glycogen are usually stored in the liver of fish. Since carbohydrate interferes with the activity of wheat amylase inhibitor it is probable that the presence of glycogen in the hepatopancreas samples used for the assays reduced the inhibitory activity.

The inhibitor unit (IU) is defined as the quantity of inhibitor required to reduce the activity of two units of amylase by 50% i.e. 1 unit of inhibitor inhibits 1.0

unit of enzyme under specified assay conditions (O'Donnell and McGeeney, 1976). Therefore the amounts of purified wheat amylase inhibitor (Sigma) required to reduce the activity of 250 mU of carp and tilapia amylases by half (50% inhibition) were calculated from the equations given in Table 3.9 (Table 3.11). The inhibitor units contained in 1 ug of protein were then computed. 1ug of purified wheat amylase inhibitor contains 95 IU for carp gut fluid amylase, 149 IU for carp gut tissue amylase and 134 IU for carp hepatopancreatic amylase. The corresponding values for tilapia gut fluid, gut tissue and hepatopancreas amylases were 152, 266 and 227 IU, respectively.

In order to determine the exact potency of wheat amylase inhibitor on fish amylases it is preferable to use purified enzymes if the interactions between the inhibitor protein and other contaminants in the crude preparations are to be avoided. Therefore it is probable that the estimates of inhibitor potency for gut tissue amylases obtained in the present study are close to the real values.

It is concluded from this experiment that under in vitro conditions carp and tilapia alpha-amylases are highly susceptible to the action of purified wheat amylase inhibitor protein. Tilapia amylase is inhibited to a greater extent than carp amylases. In order to investigate the effect of this inhibitor in vivo in mirror carp, the next experiment was undertaken.

Table 3.11 Estimated amounts of purified wheat amylase inhibitor (I) required for 50% inhibition of 250 mU (A_1) of carp and tilapia alpha-amylase.

AMYLASE SOURCE	CARP	TILAPIA
Gut fluid	1.31 ug	0.82 ug
Gut tissue	0.84 ug	0.47 ug
Hepatopancreas	0.93 ug	0.55 ug

CHAPTER 4

EFFECTS OF FEEDING MIRROR CARP WITH DIET CONTAINING SEMIPURIFIED WHEAT AMYLASE INHIBITOR.

CHAPTER 4

EFFECTS OF FEEDING MIRROR CARP WITH DIET CONTAINING SEMIPURIFIED WHEAT AMYLASE INHIBITOR.

4.1 INTRODUCTION.

Since the discovery of wheat amylase inhibitor by Kneen and Sandstedt (1943), few attempts have been made to investigate the *in vivo* effect of this protein on animal amylases. Appelbaum (1964) showed that the addition of a crude protein extract from wheat to a synthetic diet, adversely affected development and greatly increased mortality of the yellow mealworm (*Tenebrio molitor*) larvae. This author attributed such effects to the *in vivo* inhibitions of the insect amylase by wheat protein inhibitor.

The effects of alpha-amylase inhibitor in rats have been studied by Lang *et al*. (1974). Inclusion of wheat alpha-amylase inhibitor at 2, 4 and 8% levels in a starch-enriched diet for rats caused a large decrease in the availability of starch. When the inhibitor was inactivated by autoclaving however, starch availability was similar to that of the control. When sucrose replaced starch in the diet, there was no change in faecal carbohydrate content or in the daily weight gain in the presence of the inhibitor. It was thus concluded that the

biological activity of the inhibitor was due to its ability to interfere with starch metabolism.

Puls and Keup (1973) studied the influence of an amylase inhibitor preparation from wheat on blood glucose and insulin levels in human volunteers, rats and dogs. They showed that hyperglycaemia and hyperinsulinaemia resulting from ingesting raw starch, could be reduced dose-dependently by the addition of the inhibitor to the starch food.

The effects induced in chickens by intake of a starch-rich diet containing purified amylase inhibitor from wheat flour were studied by Marci et al. (1977). The inhibitor was enclosed in cellulose-coated microgranules to render them resistant to the action of acid and pepsin in the chicken gizzard. When such a preparation was fed to chickens from the first day after hatching at a rate of 0.4% of the diet, it significantly depressed growth rate. By contrast native wheat albumin at inclusion levels of up to 0.8% of the diet, did not show such an effect. After four weeks treatment, chickens showed a growth rate identical to that of the control, thus indicating that an adaptation to the presence of wheat albumin in the diet had occurred. Pancreatic amylase production was markedly increased in treated chickens. Treated chickens also showed pancreas hypertrophy and a number of histological changes in the pancreas indicating degeneration.

All of these studies have shown that, despite sensitivity of alpha-amylase inhibitor to proteases (Kneen and Sandstedt, 1946; Shainkin and Birk, 1970; O'Connor and McGeeney, 1981), inhibitor from wheat, when supplied in large amounts can withstand the digestion and cause deleterious effects in insects, birds and mammals including man.

In compounded diets for fish, wheat and wheat-based products are often included as a source of carbohydrate. The results of the <u>in vitro</u> experiments described in the previous chapter (Chapter 3) showed that mirror carp alpha-amylase is highly sensitive to wheat amylase inhibitor. Bearing in mind that fish in general are poor utilisers of carbohydrate compared to other animals (Cowey and Sargent, 1979), any factor that further decreases its utilization as a nutrient is of concern to the fish farmer. Therefore this experiment was undertaken to study the <u>in vivo</u> effect of this inhibitor on mirror carp and to investigate possible adaptations of the fish to the presence of inhibitor in its diet.

4.2 MATERIALS AND METHODS.

4.2.1 Experimental fish.

Thirty five mirror carp fingerlings in the size range 30 to 37g (mean 33.45 ± 1.79g) were selected from the stock system and distributed randomly into seven experimental tanks at a stocking density of five fish per tank. The fish were allowed to acclimatize to the new conditions for a period of 10 days during which time they were fed a high protein holding ration (Appendix I) at the rate of 3% body weight per day in two equal instalments.

4.2.2 Experimental diets.

4.2.2.1 Extraction of amylase inhibitor proteins from wheat.

The albumin fraction of wheat (Triticum sp.) seeds were extracted by the method of O'Donnell and McGeeney (1976) with some modifications.

Whole wheat grains (white variety) were milled in a hammer mill (AFM-Scotmec, Ayr) and then finely ground in a Tecator Cyclotec 1093 sample mill. The ground wheat was finally sieved through 1 mm mesh. The moisture content of this powder was determined and 900g (800g dry matter) of this flour was mixed thouroughly with 1 1/2 volumes of distilled water for one hour in a Hobart food mixer (A200

series) at room temperature (+20°C) and then centrifuged at 2000 rpm for 10 minutes. The residue was resuspended in one volume of water, stirred again for a further 60 minutes and centrifuged as before to obtain the second extract. The residue was discarded and the two extracts were pooled, filtered under negative pressure and then freeze dried.

The water soluble fraction from wheat produced a brown hygroscopic fluffy residue. This was then dissolved in a small quantity of distilled water and heated at 70°C for 30 minutes in a thermostatically controlled water bath to inactivate the plant alpha and beta amylases. Following rapid cooling the solution was centrifuged at 8000 xg for 10 minutes in a refrigerated (0-5°C) MSC high speed 18 centrifuge. The precipitate was discarded and the supernatant taken for alcohol fractionation.

The aqueous extract was mixed with absolute ethyl alcohol to obtain 60% alcohol saturation at room temperature (+20°C) which caused the carbohydrates to precipitate out. The precipitate was then separated by centrifuging at 2500 xg (MSC LR-6) for 30 minutes and discarded. The alcohol concentration in the supernatant was gradually increased to over 90% by slow addition of three volumes of absolute alcohol to precipitate the amylase inhibitory proteins, which were recovered by centrifuging again at 2500 xg for 30 minutes at

0-5°C. This alcohol fraction was then washed three times with 100% alcohol, transferred to a tared vial and allowed to dry overnight at 30°C in a vacuum desiccator. The weight of the dry precipitate obtained was 21g.

This 21g of dry precipitate was dissolved in a total of 270 ml of distilled water, and the resulting solution was divided into two equal portions. One portion was used directly to prepare the 'Active Diet' containing active amylase inhibitor. The other half was autoclaved (121°C, pressure 1.06 kg/cm^2) for 20 minutes, cooled and then used to prepare the 'Inactive Diet' which contained no potent amylase inhibitor.

4.2.2.2 Diet preparation.

The formulation of the basal mixture, which was to form the basis of the two experimental diets, is given in Table 4.1. All the dry ingredients were weighed out into the bowl of a Hobart food mixer (A200 series) and mixed thouroughly for about 10 minutes to obtain a homogeneous mix. The oils were then gradually added with continuous mixing.

This basal mixture was divided into two lots of 543g each (equivalent to 500g dry matter). One lot was returned to the mixing bowl and the previously prepared portion of the albumin solution from wheat containing the

Table 4.1. Diet formula of the basal mixture.

Ingredients	Moisture free %
Fishmeal (batch 2)	38.80
Wheat Starch	24.00
Alpha-Cellulose	23.95
Carboxymethyl Cellulose	1.00
Cod-liver oil	2.55
Corn oil	3.70
Mineral mix	4.00
Vitamin mix	2.00
Total	100.00

active inhibitor was added very slowly with continuous mixing. The mixer was allowed to operate for a further 10 minutes at slow speed. More water was added gradually to prepare a dough suitable for extruding. After the dough attained the required consistency, it was extruded and dried as described in Chapter 2. This dried diet contained active amylase inhibitor and was therefore named 'Active Diet'.

Similarly the portion of autoclaved and cooled albumin solution from wheat with deactivated inhibitor was mixed with the second portion of basal mixture and the strings of 'Inactive Diet' prepared and dried. The diets were stored at -20°C until required.

Samples of the final diets were ground and analysed for moisture, crude protein, crude lipid, crude fibre, hydrolysable carbohydrate and total ash by the standard AOAC (1980) methods detailed in Chapter 2. The composition is given in Table 4.2.

4.2.2.3 Inhibitor content in wheat and test diets.

Amylase inhibitory activity of the alcohol fraction extracted from wheat and used in the test diets was analysed by the modified procedures of O'Connor and McGeeney (1981). 46.3mg of wheat alcohol fraction were dissolved in 5 ml of phosphate buffer (pH 6.9, 50mM phosphate, 50mM NaCl, 0.5mM $CaCl_2$). The protein

Table 4.2. Proximate composition of test diets (mean ± SD).

Moisture free %	Active diet	Inactive diet
Moisture	6.51 ± 0.04	6.47 ± 0.01
Crude protein	29.13 ± 0.80	29.54 ± 0.10
Crude lipid	8.54 ± 0.09	8.18 ± 0.06
Total ash	9.43 ± 0.11	9.43 ± 0.23
Crude fibre	19.34 ± 0.10	19.64 ± 0.03
Hydrolysable carbohydrate	21.81 ± 0.73	23.92 ± 0.48
Soluble carbohydrate*	33.56	33.21

* calculated by difference (see Chapter 2, Section 2.9.1.7)

content in this solution was determined to be 1.33 mg/ml. Aliquots of this solution containing 1 ug protein were incubated for 30 minutes at 25°C with carp hepatopancreatic amylase, with initial activities of 55, 111 and 175 mU/min. Bovine serum albumin at a concentration of 1mg was used in all reaction media to protect the alpha-amylase against spontaneous inactivation. Control tubes without inhibitor were set up simultaneously. After incubation, the residual activities in the tubes were determined by the method of Rick & Stegbauer (1974) described in Chapter 2. The inhibitory potency of the wheat protein was calculated from the differences between the control tubes and the activity remaining in the tubes with inhibitor (see Chapter 3).

Based on the definition that one Unit (IU) of inhibitor reduces the activity of two amylase Units by 50% (see Chapter 3), a regression equation for the inhibitory activity of 1 ug of alcohol fraction protein from wheat on carp amylase was derived. From this equation, the initial activity level that will yield a residual activity which is 50% of the initial was extrapolated. Half of this initial activity value is the estimate of the Inhibitor Units in the protein. From the weight of alcohol fraction (21g) extracted from 900g of wheat and the inhibitory activity contained in 1 ug protein, the inhibitor content of the original wheat grain was estimated.

For comparison, the inhibitor fraction was also

tested on commercial porcine pancreatic amylase (Sigma).

The albumin in the test diets was extracted by the same procedure used for wheat and similarly tested for the presence of active inhibitor against carp hepatopancreatic alpha-amylase and porcine pancreatic amylase. Since the inhibitions obtained initially with 1 ug protein from diets were low, the assays with carp amylase were repeated using 5 ug of protein.

4.2.3 Experimental protocol.

On the day following the 10 day acclimatizion period to the experimental system, the individual weights of all fish were recorded. The Active and Inactive Diets were each randomly assigned to three tanks of fish. The remaining group of fish was sacrificed to determine initial levels of all parameters studied in this experiment (see below).

The experimental diets were fed for a period of three weeks during which time all the fish were individually weighed on a weekly basis in accordance with the procedure described in Chapter 2. The experimental groups of fish were fed on their respective diets at a daily rate of 3% body weight in two equal instalments and the feeding rate was adjusted every week to account for changes in biomass.

At weekly intervals for a total of three weeks, one

group of five fish from each treatment were killed with an overdose of benzocaine (Ross and Geddes, 1979) exactly four hours after their last meal. The viscera were dissected out and after collection of bile and hepatopancreas, the gut contents from the uncoiled intestine were stripped into a certrifuge tube for separation of gut fluid and solids (Chapter 2). Finally the intestinal tissues were rinsed clean in cold fish ringers solution (Ginsburg, 1963) and cut into three regions representing the foregut (up to the first 'V' fold), hindgut (the posterior quarter) and the intervening midgut regions. The volumes of gut fluid and bile in ul were measured using micropipettes and the weight of gut solids, hepatopancreas and the three regions of the intestine were recorded on a Mettler AC100 balance to the nearest tenth of a mg (Chapter 2). A small portion of hepatopancreatic tissue was then fixed in 10% buffered formalin for histopathological examination (for details see Chapter 2). The rest of the hepatopancreas was rapidly frozen in liquid nitrogen and then stored at -20°C until required for analyses. The samples of bile, gut fluids and solids, foregut, midgut and hindgut were preserved in the same manner.

Blood was sampled from the second week of the experiment to determine plasma glucose and plasma protein The detailed procedures are described in Chapter 2.

From the weights of hepatopancreas and total gut

tissue, the hepatosomatic and gut-somatic indices were calculated by the formulae given in Chapter 2. Gut fluid volume, gut solid weight and bile volume were expressed as percentages of fish body weight.

Alpha-amylase activity in the individual samples of gut fluid, gut solids, bile, hepatopancreas, foregut, midgut and hindgut tissues were determined at 25°C in accordance with the method of Rick and Stegbauer (1974) described in Chapter 2. The protein content of samples used for this enzyme assay were also determined by Lowry's method (1951) and the specific amylase activities were calculated.

4.3 RESULTS.

4.3.1 Inhibitor content in wheat grain and test diets.

The degree of inhibition and the residual amylase activity recorded after incubating aliquots of carp hepatopancreas homogenates with 1 ug of protein from wheat are shown in Table 4.3 and Fig. 4.1. The regression equation between initial activity and percent inhibition at this concentration of inhibitor was,

% Inhibition = 92.3 - 0.185 Initial activity in mU

(correlation = - 0.928)

From this equation, 1 ug of protein extracted from wheat would reduce the activity of 228.6 mU of carp amylase by 50% (Fig. 4.1). Therefore, 1 mg of protein had 114.3 IU of activity against carp hepatopancreas alpha-amylase or 100g wheat grains contained 38,306 Units of inhibitor.

The inhibitory activity of this protein on porcine pancreatic amylase was less effective. 1 ug of alcohol fraction of wheat could reduce only 136 mUnits of amylase by 50%, thus giving a value of 68 IU/mg. Therefore 100g of wheat was estimated to contain about 22,789 IU for porcine pancreatic amylase.

The Active Diet was prepared by mixing 500g (dry

Table 4.3 Residual activity (mean ± SD) and percent inhibition of carp hepatopancreatic alpha-amylase when incubated with 1 ug of protein extracted from wheat for 30 min. at 25°C.

Initial activity (mU/min)	Residual activity (mU/min)	% Inhibiton*
55.23 ± 2.94	8.28 ± 2.34	85.00
111.01 ± 5.99	37.05 ± 4.38	66.62
175.00 ± 2.95	65.97 ± 1.70	62.30

$$* \% \text{ Inhibition} = \frac{(\text{Initial activity} - \text{Residual activity})}{\text{Initial activity}} \times 100$$

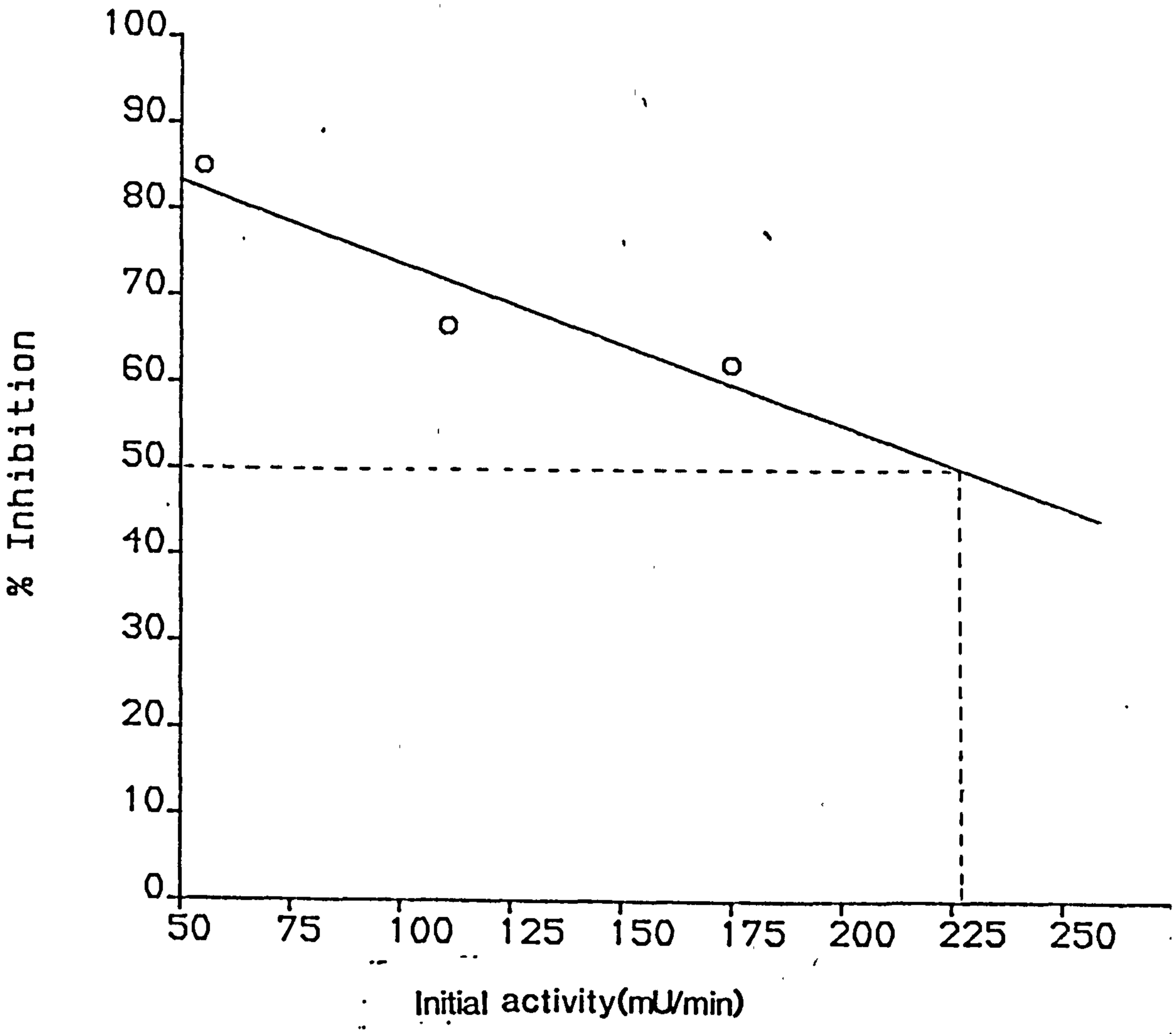

FIGURE 4.1 Percent inhibition of carp hepatopancreatic alpha-amylase by 1 ug protein extracted from wheat grains.

weight) of basal mixture with the albumin extracted from 400g (dry weight) of ground wheat powder. The alcohol fraction from 100g of wheat contained 38,306 Units of inhibitor. Therefore, the calculated inhibitory activity of this diet is at least 30,645 Inhibitor Units per 100g diet.

Incubation of 5 ug of the protein extracted from the Active Diet with carp hepatopancreatic alpha-amylase resulted in inhibition of between 40 - 45% of the initial activity levels of the enzyme samples (Table 4.4a). Inhibitory activity of 1 ug protein samples from Active Diet against porcine pancreatic amylase with initial activity levels of 14 to 64 mU varied between 8 and 41% (Table 4.4b). The alcohol fraction from Inactive Diet did not inhibit either of these amylases indicating complete inactivation of the inhibitor by the autoclaving process.

4.3.2 Fish growth.

During the 10 day acclimatization period the fish grew rapidly from an overall mean weight of 33.44 $\pm$ 1.79g, to a mean weight of 42.52 $\pm$ 3.01g, representing a specific growth rate of 2.40 %/day. At the start of the trial however, the initial fish weights between the two treatment groups did not vary significantly ($p > 0.05$). The two initial treatment means were 43.05 $\pm$ 2.71g and

Table 4.4a Residual activity (mean ± SD) and percent inhibition of carp hepatopancreatic alpha-amylase when incubated with 5 ug of protein from Active Diet for 30 min. at 25°C.

Initial activity (mU/min)	Residual activity (mU/min)	% Inhibiton*
55.23 ± 2.94	30.16 ± 2.05	45.39
111.01 ± 5.99	60.57 ± 2.45	45.44
175.00 ± 2.95	106.20 ± 5.39	39.31

Table 4.4b. Residual activity (mean ± SD) and percent inhibition of porcine pancreatic alpha-amylase when incubated with 1 ug of protein from Active diet for 30 min. at 25°C.

Initial activity (mU/min)	Residual activity (mU/min)	% Inhibition*
13.73 ± 2.09	8.06 ± 1.55	41.33
35.05 ± 2.51	29.07 ± 0.17	17.05
45.96 ± 2.48	42.61 ± 5.05	8.23
63.69 ± 2.49	54.47 ± 2.07	14.48

$$* \% \text{ Inhibition} = \frac{(\text{Initial activity} - \text{Residual activity})}{\text{Initial activity}} \times 100$$

42.81 ± 2.67g, for Active and Inactive treatment groups respectively (Fig.4.2). Specific growth rate during the first week in fish fed Active Diet was 0.53 %/day and the fish attained a mean weight of 44.68 ± 3.04g by the end of this period. The SGR reduced to 0.43 %/day during the second week (mean fish weight 46.04 ± 3.12g) and was only 0.16 %/day in the third week. Thus by the end of this trial the fish in this treatment almost stopped growing (Fig. 4.2) and had attained a final weight of only 46.56 ± 2.87g representing an overall SGR of only 0.37 %/day.

By contrast, the groups of fish fed Inactive Diet grew at specific growth rates of 1.23, 1.08 and 1.00 %/day during the first, second and third weeks, respectively and attained a final weight of 53.97 ± 5.28g. The overall SGR in this treatment for the three week period was 1.10 %/day.

The mean fish weights between treatments at the end of the first week were statistically different at the 10% confidence intervals but not different at the 5% level ($p = 0.076$). However, at the end of Week 2 and 3 the differences were highly significant at the 5% level of confidence ($p = 0.014$ and 0.025, respectively).

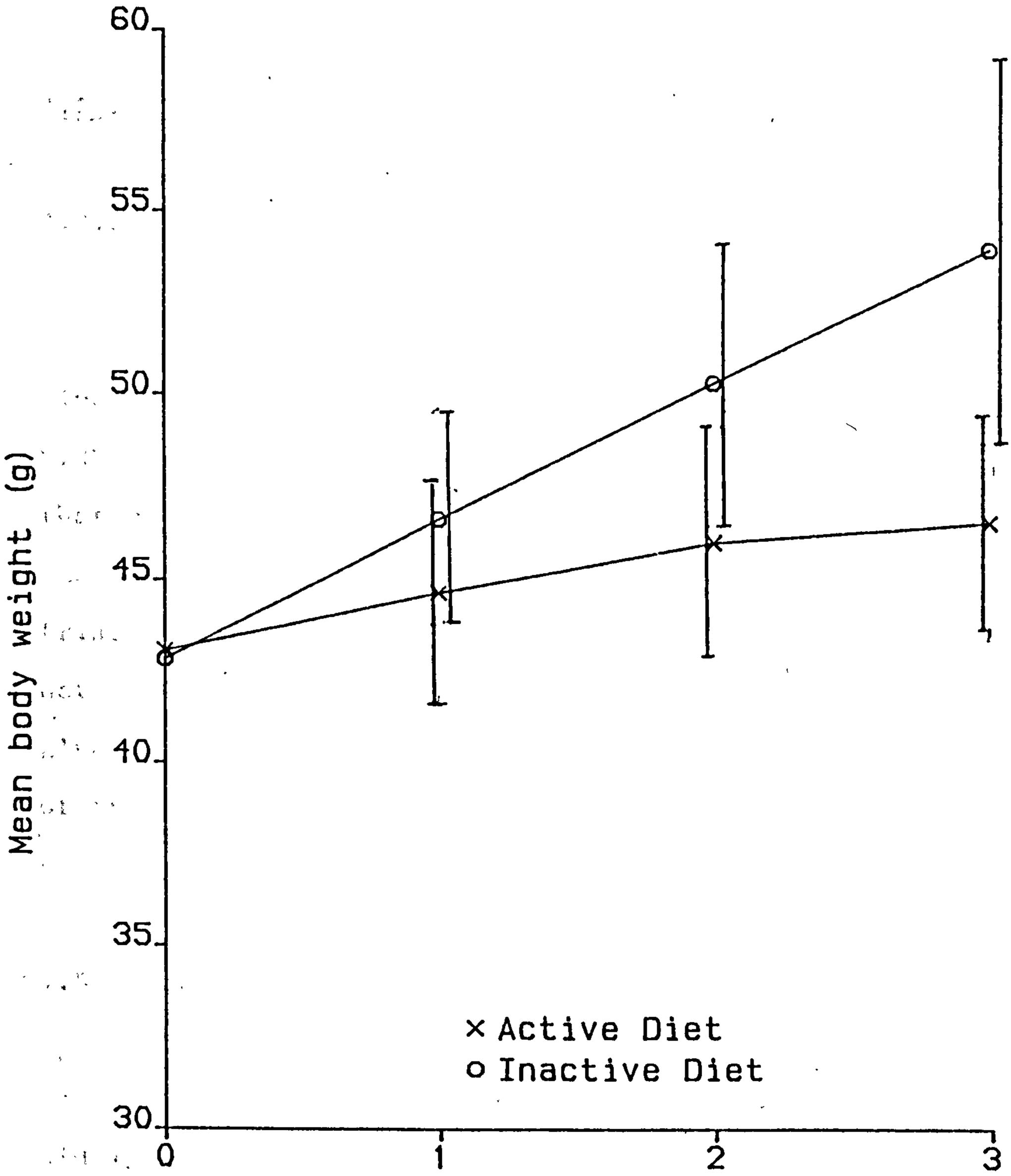

FIGURE 4.2 Increase in mean body weight of carp fed Active and Inactive Diets for three weeks. (vertical lines indicate SD)

4.3.3 Blood parameters.

4.3.3.1 Plasma glucose.

At the end of the second week the mean plasma glucose content of fish fed Active Diet was significantly higher ($p < 0.05$) with a mean of 120.26 $\pm$ 5.23 mg/dl than. that recorded from fish fed Inactive Diet, where the mean was 89.02 $\pm$ 4.91 mg/dl (Fig. 4.3). By the end of the trial the levels had decreased in both treatments and were not significantly different ($p > 0.05$). The mean plasma glucose in the Active and Inactive treatments at the end of the third week of the trial were 81.16 $\pm$ 5.16 and 80.63 $\pm$ 2.42 mg/dl, respectively.

4.3.3.2 Plasma protein.

The protein content of blood plasma varied less widely than glucose. In fish fed Active Diet the protein increased from 24.48 $\pm$ 0.43 mg/ml at the end of Week 2 to a mean of 28.28 $\pm$ 0.18 mg/ml by the third week of the experiment (Fig. 4.3). A similar trend was seen in the fish fed Inactive Diet, although the increase was of a lesser magnitude from 23.22 $\pm$ 1.02 mg/ml to 25.80 $\pm$ 0.25 mg/ml. Between the two treatments the difference in plasma protein levels was not significant at the end of the second week ($p > 0.05$), but had become highly significant

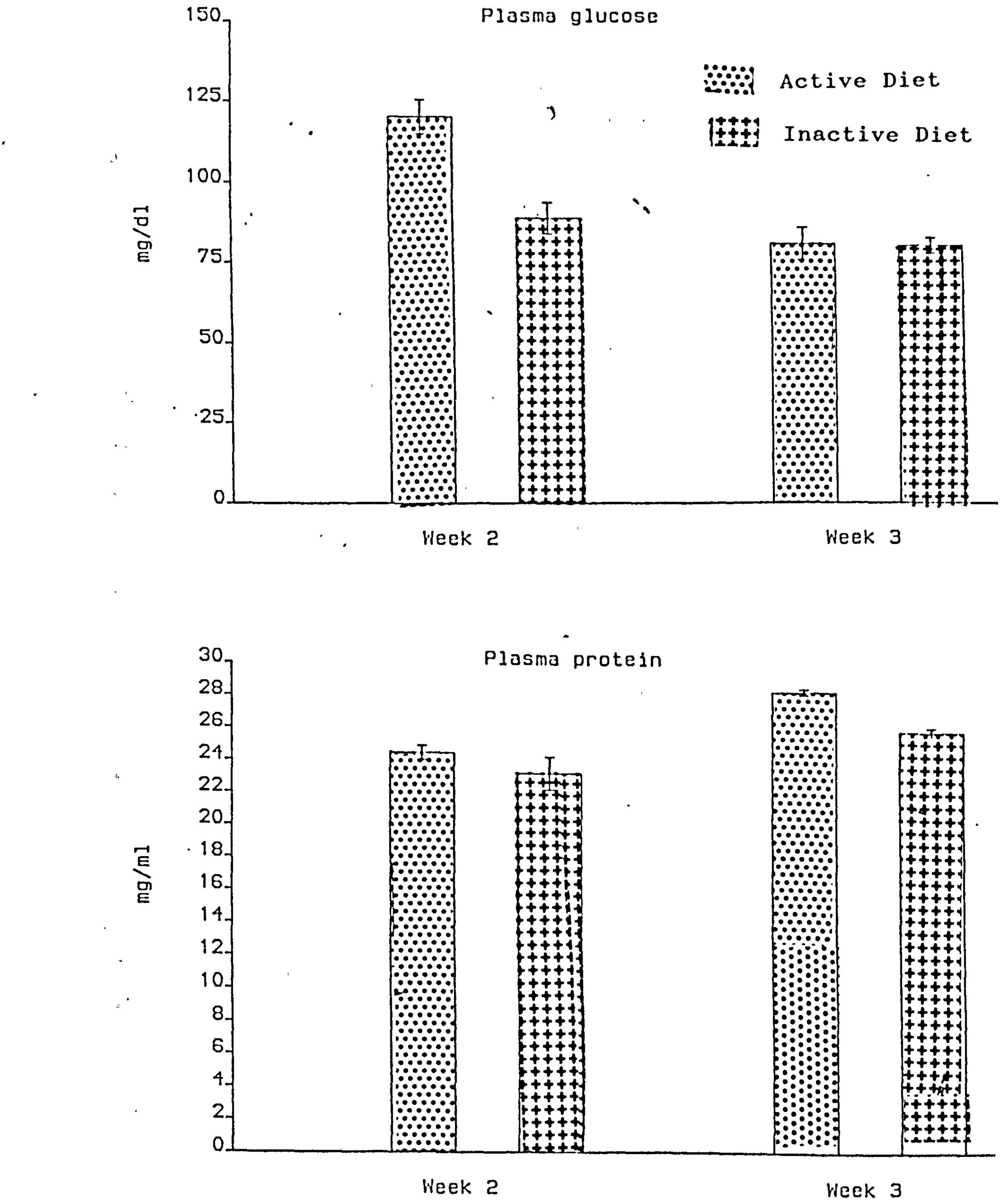

FIGURE 4.3 Plasma glucose and protein contents in carp fed Active Diet and Inactive Diet on the second and third weeks of the experiment.

by the end of the experiment ($p < 0.05$).

4.3.4 Hepatosomatic index.

The mean initial HSI was 1.84 $\pm$ 0.09, which remained more or less stable for the first two weeks of the experiment in both the treatments (Fig.4.4). During the last week of the trial the fish fed on Active Diet showed a sharp decrease in the HSI to 1.44 $\pm$ 0.10 which was significantly ($p<0.05$) lower than the initial value. By contrast, there was no significant change in HSI of fish fed Inactive Diet throughout the three week trial. However this difference between the two treatments was not statistically significant ($p > 0.05$).

4.3.5 Gut-somatic index.

The gut-somatic index reduced significantly ($p < 0.05$) in both the treatments on feeding the test diets during the three week experimental period (Table 4.5, Fig. 4.4) and the reduction was greater in fish fed on active inhibitor. From an initial mean value of 3.61 $\pm$ 0.22, gut-somatic index in fish fed Active Diet fell sharply to 2.51 $\pm$ 0.2 at the end of Week 1 and continued to decrease to a minimum value of 2.11 $\pm$ 0.09 at the end of the trial. By comparison, the decrease in Inactive treatment was not so steep, the gut-somatic index decreased to 2.98 $\pm$

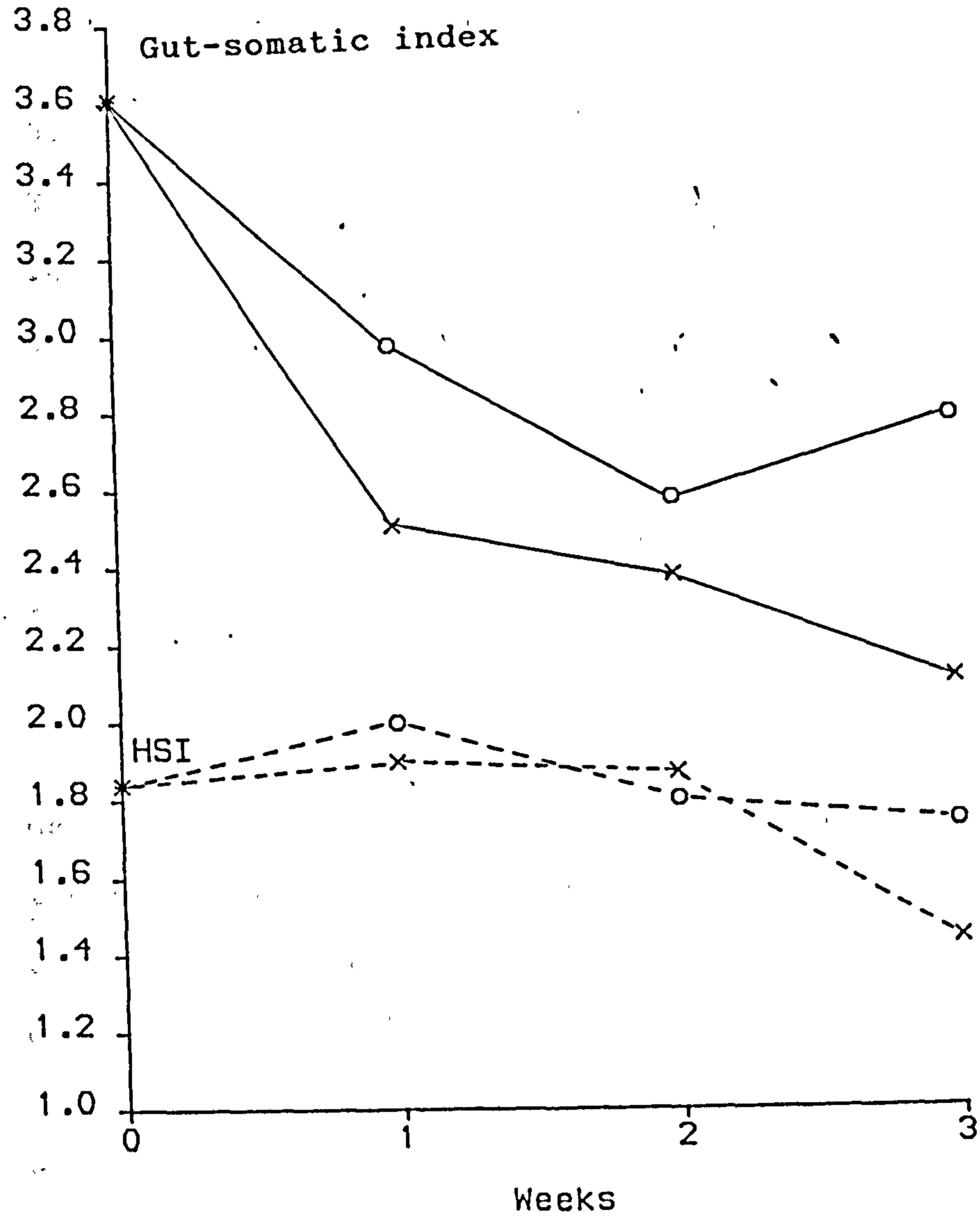

FIGURE 4.4 Gut-somatic index and hepatosomatic index of carp fed Active (×) and Inactive (o) Diets.

0.19 during the first week and to 2.59 ± 0.16 during Week 2. By the end of the trial there had been a small but insignificant rise in gut-somatic index of fish fed Inactive Diet. Comparing the weekly means of the two treatments, only the Week 3 values were slightly different ($p < 0.10$) and significant at the 10% level of confidence.

4.3.6 Bile volume.

The mean initial bile volume (106 ul/100g) decreased by about 60 to 70% on feeding test diets in both the treatments (Table 4.5). In the fish fed on Inactive Diet, the mean volumes remained stable throughout the experimental period of three weeks with a overall mean of 41 ± 7 ul/100g. Feeding Active Diet reduced the mean bile volume to only 30 ± 9 ul/100g at the end of the first week but at the end of the 2nd and 3rd weeks the volumes stabilised at around 40 ul/100g. However, differences in bile volumes between the initial volume and the two treatment groups as well as between treatments were not significant ($p > 0.05$) probably due to wide variations observed in individual fish (6 to 213 ul/100g).

Table 4.5 Gut fluid volumes, gut solid weights and bile volume in mirror carp fed Active and Inactive diets.

Diet	Week	G.Fluid Vol ml/100 g fish	G.Solid Wt g/100 g fish	Bile Vol ul/100 g fish
Initial		1.48(1.16)^{abcdef}	1.03(0.09)	106(47)
Active	1	0.80(0.06)^{a}	0.94(0.06)	30(9)
Inactive		0.79(0.14)^{b}	1.10(0.16)	40(12)
Active	2	0.95(0.07)^{cg}	1.07(0.06)	47(8)
Inactive		1.05(0.13)^{dh}	1.19(0.16)	42(10)
Active	3	0.72(0.08)^{eg}	0.87(0.12)	43(6)
Inactive		0.77(0.07)^{fh}	1.10(0.07)	42(20)

* values in parenthesis show the standard error.

** values in each column with same superscripts are significantly different ($p < 0.05$).

4.3.7 Gut fluid volume.

The volumes of fluid measured in the intestine of carp four hours after feeding on holding ration, ranged from 1.18 to 1.82 ml/100g with a mean of 1.48 $\pm$ 0.12 ml/100g. There was a significant ($p < 0.05$) decrease in gut fluid volume when the fish were fed both test diets (Table 4.5). Carp fed on diet containing active inhibitor for a week produced only 0.66 to 0.96 ml/100g of fluid in the gut (mean 0.80 $\pm$ 0.05 ml/100g). A similar reduction from the initial volume was seen in fish fed Inactive Diet where the mean volume recorded at the end of Week 1 was 0.79 $\pm$ 0.01 ml/100g (range 0.39 - 1.17). Comparisons of the weekly mean gut fluid volumes between the two treatments over the three week experimental period did not reveal any significant differences ($p > 0.05$).

4.3.8 Gut solid weights.

Feeding the experimental diets did not result in any significant ($p > 0.05$) change in the weight of solids in the intestine (Table 4.5). Furthermore statistical comparisons of the mean gut solid weight between weeks in each of the treatments and also between the two treatments over the three week experimental period did not reveal any significant differences ($p > 0.05$).

4.3.9 Alpha-amylase activity.

4.3.9.1 Alpha-amylase in gut fluids.

The mean initial alpha-amylase activity in gut fluids was 1077 ± 158 uMol/min/ml (range 711-1490). On feeding the experimental diets containing active and inactive inhibitor this enzyme activity increased to 1879 ± 394 and 1802 ± 174 uMol/min/ml, respectively at the end of the first week (Fig. 4.5). These increased levels were maintained for the duration of the three week trial and were significantly ($p < 0.05$) higher than the initial level of activity, with the exception of the sample taken from fish fed Inactive Diet ($p > 0.05$) at the end of the 3rd week where large variations were recorded. The weekly fluctuations within each treatment and also between the two treatments were not statistically significant ($p > 0.05$).

A similar pattern was seen in amylase specific activities where values doubled ($p < 0.05$) on feeding both dietary treatments (Table 4.6). Compared to the initial mean amylase specific activity of 25.5 ± 3.2 uMol/min/mg protein (range 17.9 - 33.3), the mean values recorded in fish fed Active Diet were between 48 and 56 uMol/min/mg during the three weeks of this experiment. The corresponding mean specific activity in the gut fluids of fish fed Inactive Diet were between 52 and 61 uMol/min/mg.

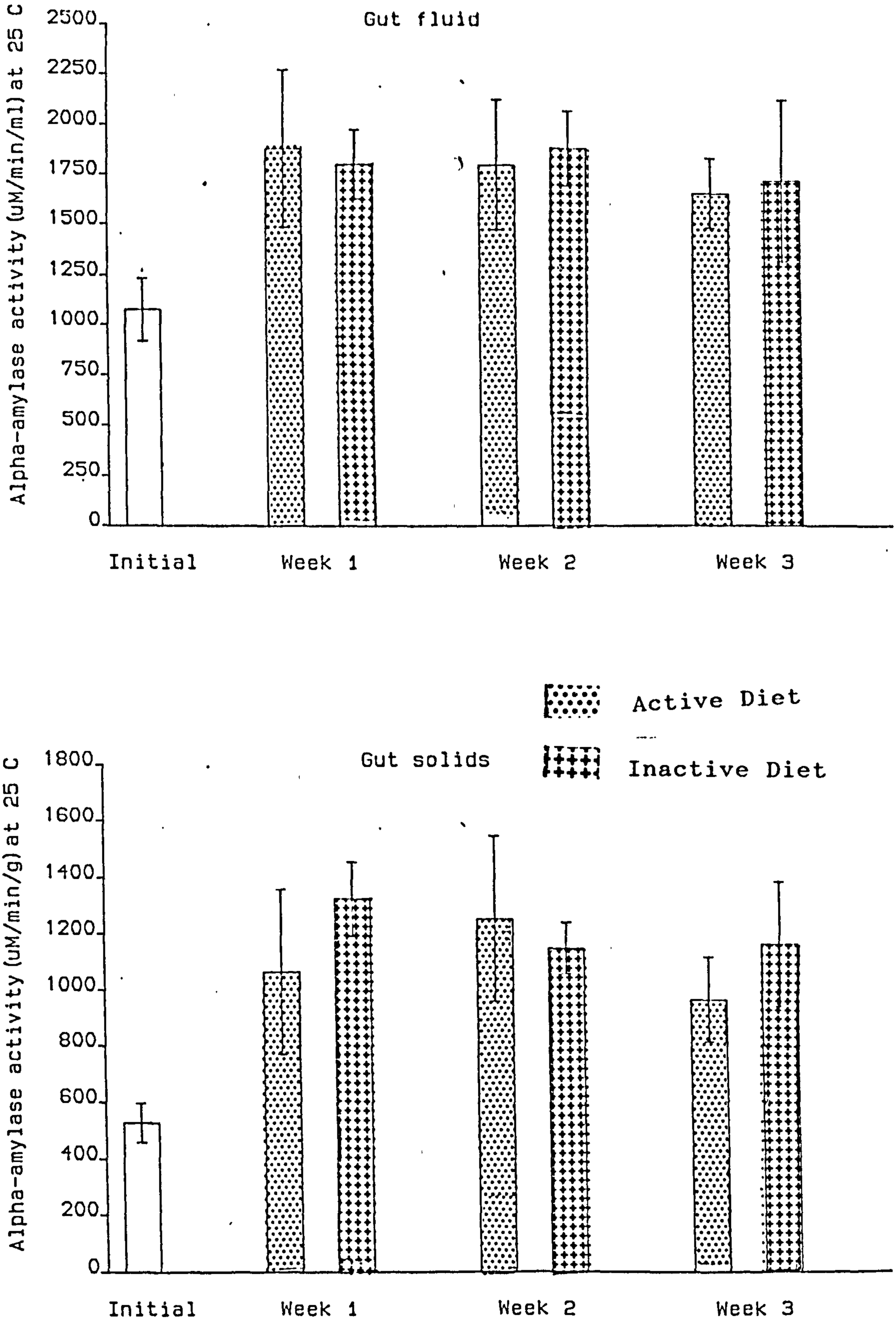

FIGURE 4.5 Alpha-amylase activity in gut fluid and gut solid of carp, four hours after feeding on Active and Inactive Diets.

Table 4.6 Alpha-amylase specific activity (uMol/min/mg protein) at 25 °C in mirror carp four hours after ingestion of Active and Inactive diets.

Diet	Week	Gut fluid	Gut solids	Bile	Hepato-pancreas	Foregut	Midgut	Hindgut
Initial		25.46(3.17)abc def	10.61(1.51)abc def	11.52(2.59)	5.56(0.99)abcd	4.80(0.77)	6.25(1.71)	2.35(1.25)ab
Active	1	55.97(8.74)a	28.58(7.25)a	19.92(3.78)	14.49(2.21)a	12.80(4.70)	4.84(1.06)a	5.42(1.10)b
Inactive		61.15(6.30)d	40.10(3.95)d	12.71(3.33)	11.99(1.84)c	7.16(1.96)	5.12(1.57)	3.99(0.59)c
Active	2	50.03(7.84)b	34.25(6.82)b	16.53(1.91)	12.68(1.18)b	4.63(0.49)	6.32(1.48)	4.91(0.79)
Inactive		53.31(4.63)e	38.94(5.03)e	15.71(2.89)	8.44(1.55)	5.31(0.47)	5.94(1.27)	4.45(0.66)d
Active	3	48.11(1.84)c	34.35(4.22)c	13.70(4.71)	9.09(1.58)	6.84(0.93)	8.39(1.00)a	7.80(1.18)a
Inactive		52.40(8.42)f	43.70(7.73)f	12.17(1.61)	13.46(2.49)d	9.04(2.38)	9.93(2.57)	8.23(1.50)bcd

* values in parenthesis show the standard error.
** values in each column with same superscripts are significantly different ($p < 0.05$).

Variations both within each treatment and also between the two treatments did not differ significantly ($p > 0.05$). However it can be seen from Table 4.6 that the specific activities in the Active Diet treatment were consistently lower ($p > 0.05$) than the corresponding values in the Inactive Diet treatment.

4.3.9.2 Alpha-amylase in gut solids.

The trend in the alpha-amylase activity recorded in the gut solids was similar to that in the gut fluids, but of a lower magnitude (Fig.4.5). Initially the mean activity was 529 $\pm$ 69 uMol/min/g solid (range 318 - 692) but on feeding the test diets the activity levels increased two fold and remained high throughout the three week trial. The range of activity recorded over this period in the Active Diet groups was 474 to 2192 uMol/min/g and in the groups fed denatured inhibitor the variations were between 749 and 1986 uMol/min/g.

The higher activity levels recorded in the experimental groups were all significantly different ($p < 0.05$) from the initial level, with the exception of the level at the end of the first week in fish fed the Active Diet, where wide variations were recorded between individual fish. The variations in weekly mean activity levels both within each treatment and also between the two treatments were not significantly different ($p > 0.05$).

The alpha-amylase specific activity (Table 4.6) was low initially with a mean value of only 10.6 ± 1.5 uMol/min/mg protein (range 5.6 - 14.0) but this increased by between 2 and 4 fold to 28.6 ± 7.25 (range 15.4 - 53.5) and 40.1 ± 4.0 (range 30.5 - 53.5) uMol/min/mg in the active and denatured inhibitor treatments, respectively, at the end of the first week. Specific activity levels then remained high for the duration of the 3 week trial.

The difference between the initial value and the weekly mean specific activity values were significant ($p < 0.05$), but between the two treatment groups and also within each treatment the variations were not significantly different ($p > 0.05$). As had been the case in gut fluid activity levels, the fish on Active Diet consistently had a lower specific activity compared to the corresponding activity in the other treatment (Table 4.6).

4.3.9.3 Alpha-amylase in bile.

Before feeding test diets the mean alpha-amylase activity in bile was 306 ± 64 uMol/min/ml (range 132-493) (Fig. 4.6). On feeding the test diets the activity level of the fish fed active inhibitor had more than doubled to 621 ± 55 uMol/min/ml (range 521-711) at the end of the first week ($p < 0.05$). By contrast, the activity level in fish fed denatured inhibitor had only

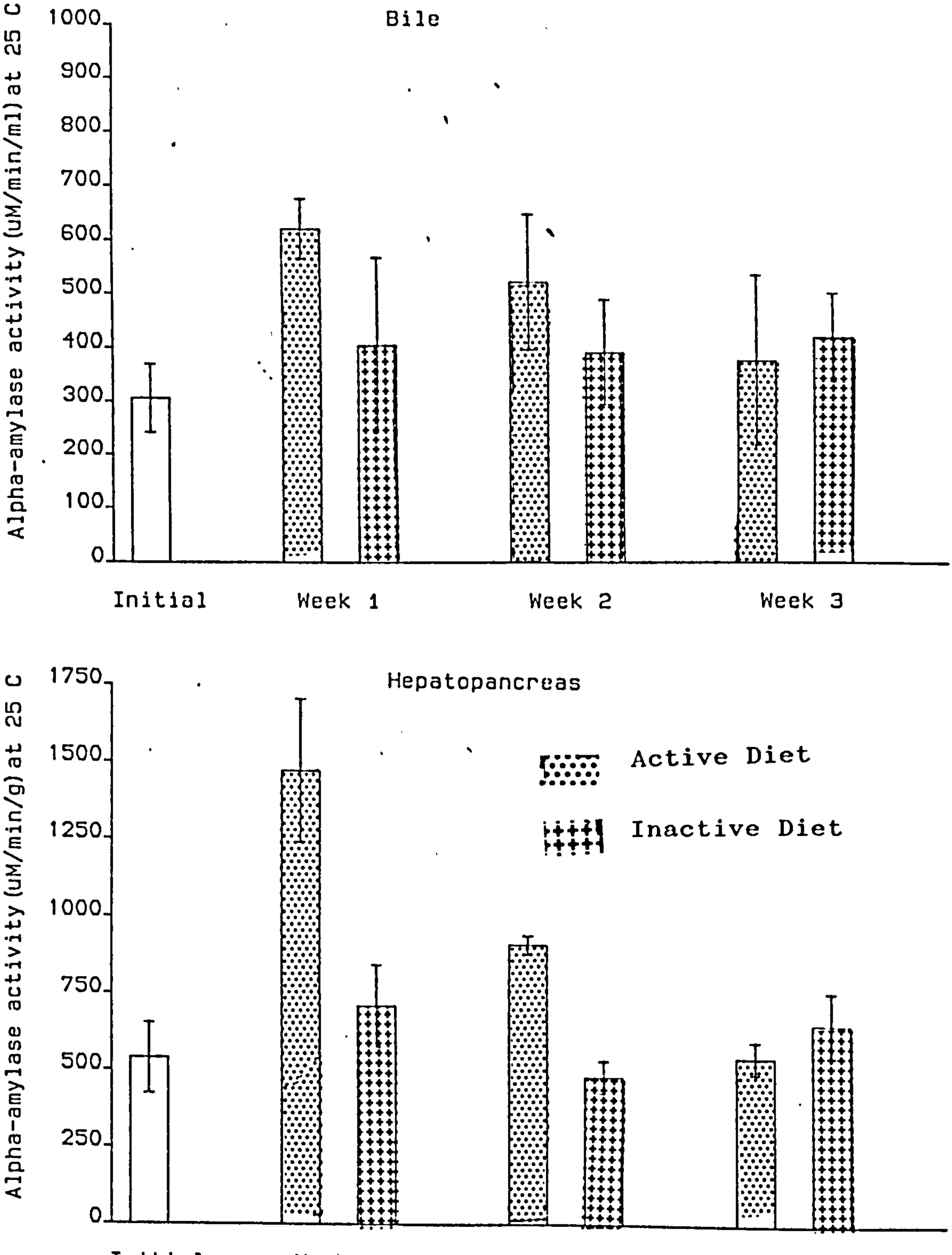

FIGURE 4.6 Alpha-amylase activity in bile and hepatopancreas in carp, four hours after feeding on Active and Inactive Diets.

increased by 30% to 405 ± 163 uMol/min/ml (range 104-867), and it remained around this level for the duration of the 3 week trial ($p > 0.05$). Levels in the Active Diet treatment however, decreased after the first week and by the end of the 3 week trial there was no significant difference ($p > 0.05$) between the two treatments and the levels were only slightly higher than the initial value ($p > 0.05$).

Alpha-amylase specific activity levels in bile fluctuated initially between 4.3 and 17.4 uMol/min/mg protein (mean 11.5 ± 2.6) but had increased to 19.9 ± 3.8 uMol/min/mg by the end of the first week in fish fed the diet containing active inhibitor (Table 4.6). Thereafter activity levels in this treatment decreased over the three week period to a level comparable to that seen in the Inactive Diet treatment. Statistical analysis of the data revealed a significant difference only between the initial level and the level at the end of the first week in the Active Diet treatment ($p = 0.104$).

4.3.9.4 Alpha-amylase in hepatopancreas.

The trend in alpha-amylase activity in the hepatopancreatic tissue was similar to that seen in bile (Fig. 4.6). From a mean initial activity of 540 ± 114 uMol/min/g tissue, the activity increased sharply and significantly ($p < 0.05$) to 1475 ± 232 uMol/min/g after

feeding Active Diet for a week. By contrast, the smaller increase to 711 ± 136 uMol/g seen in the fish fed Inactive Diet was not statistically significant. In the Active Diet treatment there was a sharp and significant ($p < 0.05$) decrease in activity at the end of the second week and by the end of the third week there was no significant difference between the two treatments. In the fish fed the diets containing denatured inhibitor, there were only small and insignificant fluctuations in amylase activity.

The trend in amylase specific activity was similar to that described above (Table 4.6). The specific activities were significantly higher ($p < 0.05$) in both dietary treatments compared to the initial value. Between the two treatments the weekly mean value in the Active Diet treatment were higher ($p < 0.05$) than the corresponding activities in the fish fed Inactive Diet at the end of both the second and third weeks.

4.3.9.5 Alpha-amylase in intestinal tissue.

In general the foregut and midgut regions exhibited higher amylase activities compared to the hindgut region. The initial mean alpha-amylase activity in the foregut was 377 ± 57 uMol/min/g which increased significantly ($p < 0.05$) to 785 ± 106 uMol/min/g at the end of the first week in the fish fed Active Diet (Fig.4.7). By contrast,

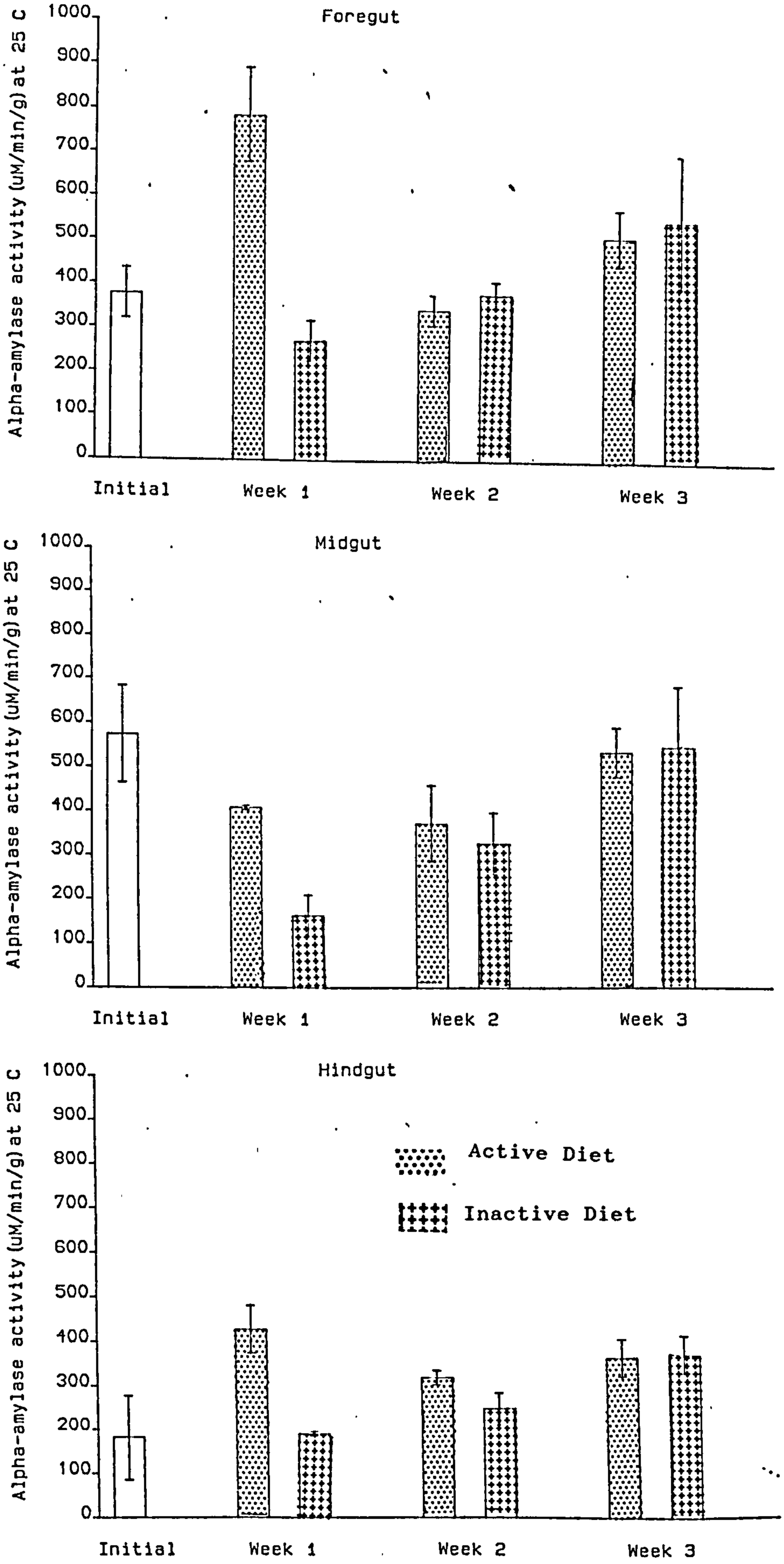

FIGURE 4.7 Alpha-amylase activity in the intestinal tissues of carp, four hours after feeding on Active and Inactive diets.

a slight but insignificant decrease was seen in fish fed on inactive inhibitor. Thereafter the activity increased in the Inactive Diet treatment while it decreased in the Active Diet treatment and by the end of the experimental period there was no significant difference between the two treatments. Furthermore, at the end of the trial, although activity levels in both treatments were slightly higher than the initial value, the differences were not significant.

The alpha-amylase activity recorded throughout the three week trial from the midgut portions of fish fed active inhibitor did not differ significantly from the initial level of 574 $\pm$ 109 uMol/min/g (Fig. 4.7). In fish fed inactive inhibitor however, there was a significant ($p < 0.05$) decrease at the end of the first week to only 163 $\pm$ 46 uMol/min/g. Thereafter the activity level increased back to the initial level when there were no significant differences either between treatments or between the initial and final activity levels.

The amylase activity levels in the hindgut exhibited a trend similar to that in the foregut and midgut regions of the intestine (Fig. 4.7). Between the two treatments the level of activity recorded at the end of the first week in fish fed Active Diet was significantly ($p < 0.05$) higher than in the Inactive Diet treatment. The mean values recorded at the end of Weeks 2

and 3 did not differ significantly between the two treatments ($p > 0.05$).

At the end of the first week, the amylase specific activities in the foregut and hindgut regions of fish fed Active Diet were higher than the corresponding initial levels. The activity in this treatment was also higher than the corresponding activity in fish fed Inactive Diet for one week (Table 4.6), however statistically this difference was insignificant ($p > 0.05$). The midgut region exhibited a general increasing trend in the specific amylase activity irrespective of the dietary treatment. By contrast, only the fish fed Inactive Diet exhibited a trend of increasing activities in the hindgut region, however in both treatments the Week 3 values were significantly higher than the initial level ($p < 0.05$).

4.3.10 Histological changes in hepatopancreas.

Histological examination of stained sections of hepatopancreas from carp fed on test diets for 2 and 3 weeks did not reveal any conclusive evidence of changes due to the presence of wheat amylase inhibitor in the diet (Plates 5 and 6). The liver and pancreatic cells appeared normal and active in fish from both treatments and normal levels of steroid deposits were seen in liver cells. In most of the samples examined, irrespective of the treatment, many zymogen granules were present in

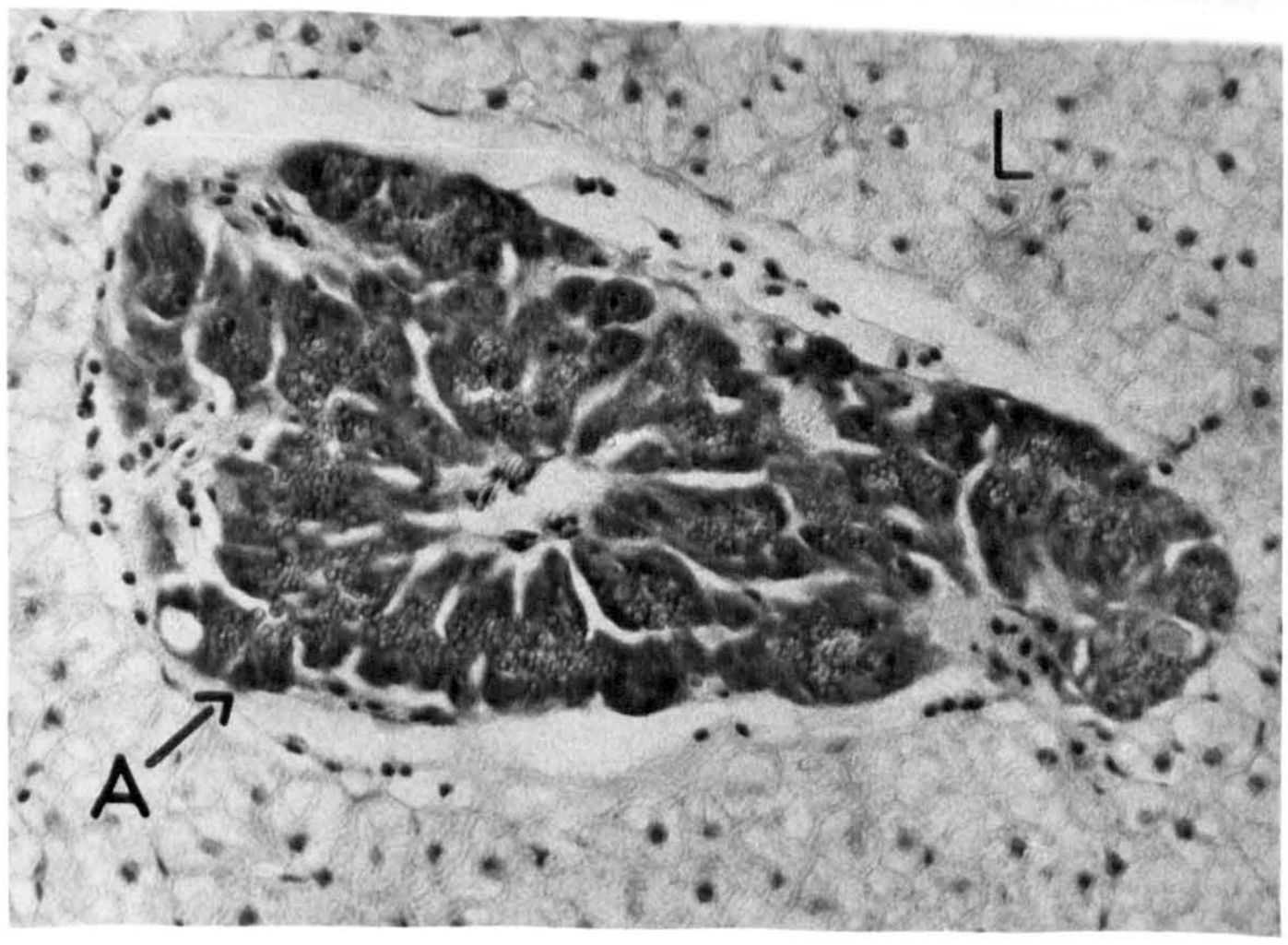

PLATE 5: Histology of hepatopancreas of carp fed Active Diet for three weeks. A = Acinar cells, L = Liver cells. X 375.

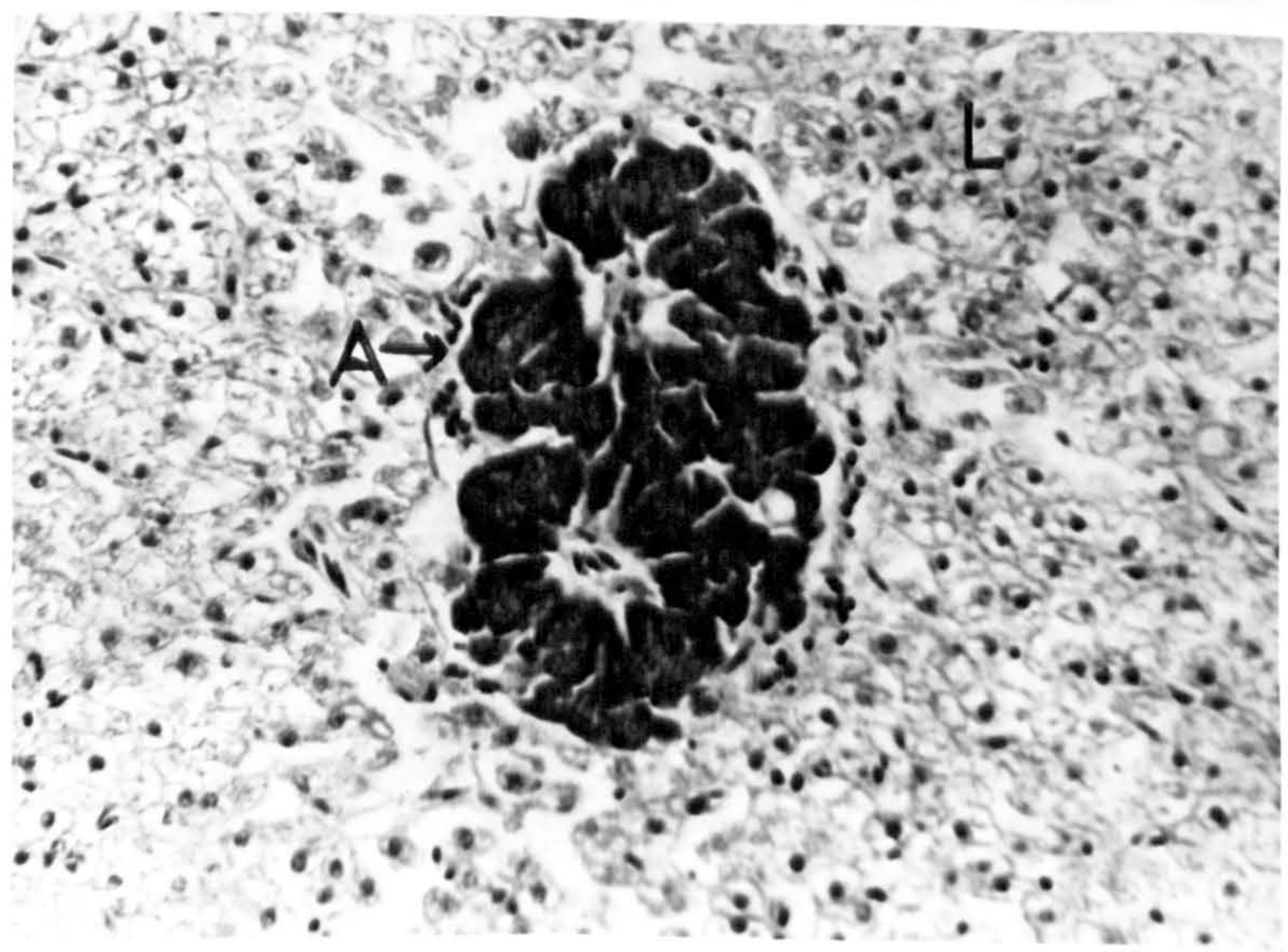

PLATE 6: Histology of hepatopancreas of carp fed Inactive Diet for three weeks. A = Acinar cells, L = Liver cells. X 375.

pancreatic cells suggesting active enzyme production. Varying amounts of fat deposits were noted, but these could not be correlated with dietary treatment. There were however increases in fat deposits between the second and third weeks in both treatments.

4.4 DISCUSSION.

When the food of mirror carp was changed from the holding ration to the test diets, in both dietary treatments the specific growth rate reduced compared to the initial rate (Fig. 4.2). This decrease in SGR is probably because of the lower protein content of the two test diets (29% crude protein) compared to the holding ration which contained 52 - 53% protein (Appendix I). The reported protein requirments for carp is 31 - 38% (Ogino and Saito, 1970; Takeuchi et al., 1979).

When active wheat amylase inhibitor was fed to mirror carp for three weeks, it caused a significant retarding effect upon fish growth. Autoclaving the inhibitor prior to feeding did not have such an effect on the fish (Fig.4.2). The contribution of protein to the diet by the added wheat albumin was negligible but the wheat albumin has an inhibitory effect on carp amylase (Table 4.4a,b) and therefore its presence in the active form appears to have had a retarding effect on the growth of carp. The sharp and significant decrease in hepatosomatic index (Furuichi and Yone, 1971a, b; Onwuka, 1980) and in gut-somatic index is also indicative of poor growth. Marci et al. (1977) fed chickens with purified wheat amylase inhibitor in the form of gastro-resistant pellets and showed a marked decrease in growth rate, however after 4 weeks the chickens in their trial were

able to totally adapt to the diet and grow at a rate similar to the controls. Mirror carp in the present experiment did not improve their growth rate at the end of three weeks.

The plasma glucose in fish fed Active Diet was significantly higher than that of those fed Inactive Diet at the end of the second week of the experiment (Fig. 4.3). However, by the third week both treatment groups had lower levels of plasma glucose and no difference was seen between the treatments. Puls and Keup (1973) reported that feeding starch-rich diet containing amylase inhibitor to rats caused a smaller increase in blood glucose compared to the increase in rats fed similar diet without inhibitor. Hyperglycaemia in blood is the most characteristic general response to any stress (Love, 1980). The rise in blood glucose due to stress has been documented in a large number of species, including carp (Mazeaud, 1969, 1973; Grigo, 1975; Murat, 1976a). Anaesthesia itself induces stress reactions (Ross and Ross, 1984). There is no specific study correlating the nature of the diet to the hyperglycaemic stress responses of fish with the exception of the recent work of Barton et al. (1988) who reported that salmon fed high-lipid diet exhibited higher levels of blood glucose when subjected to handling stress compared to fish fed low-lipid diet. Unbalanced diets or diets containing anti-nutritional factors are difficult to digest and utilise by the fish

(Jauncey, pers. comm.). Therefore it is thought that the presence of amylase inhibitor in the Active Diet could have added to the stress response of carp to handling or to the anaesthetic.

In the present experiment carp had 60 - 70% lower bile volumes than the initial level and throughout the experimental period the volumes were maintained at around 40 ul per 100g fish, in both dietary treatments (Table 4.5). It is well known that bile juice is stored in the gall bladder until released into the gut lumen on the stimulus provided by the presence of food materials in the intestine (Davenport, 1982). In fishes such as carp which do not have a discrete pancreas, the pancreatic secretions of digestive enzymes are also stored in the gallbladder and released into the intestine along with the bile. Love (1970, 1980) observed that in cod, Gadus morhua, caught from the wild, the bile volume increases with darkening of colour in starving fishes. By contrast, actively feeding fish had small volumes of pale, straw coloured bile. Similar findings were reported by Nawwab (1987) in Oreochromis niloticus. No other reports are available linking the nature of diet to the bile volumes in fishes. In the present study the presence of active amylase inhibitor proteins in the diet appears to have had no effect on the bile volume in the fish when measured four hours after the meal.

Mirror carp fed experimental diets containing

starch, produced 45% lower volumes of intestinal juices when compared to the initial volumes in fish at the end of the acclimatization period when they had been fed a protein-rich holding ration (Table 4.5). Since similar volumes were recorded in both the treatments, it appears that the presence of active and inactive amylase inhibitor albumin did not have any effect. Spannhof and Plantikow (1983) reported that diets rich in potato starch induced increased secretions of intestinal juice in rainbow trout. In these trout, the secretions of intestinal juice reached a maximum 6 hours after feeding and the volumes measured in fish fed on a protein-rich diet were less than half of that obtained from fish fed a starch-rich diet (Spannhof and Plantikow, 1983).

In carp, alpha-amylase is synthesised by the pancreatic acinar cells embedded in the liver tissue and the intestinal mucosal cells. The alpha-amylase secreted by the pancreas along with other digestive enzymes such as proteases and lipases, are released into the intestinal lumen via the common bile duct, while the enzymes contained in the intestinal brush border cells are released into the gut lumen when the cells are desquamated (ADCP, 1980; Davenport, 1982).

In this experiment, the alpha-amylase activity in the gut contents of carp increased sharply when they were fed the two test diets (Fig. 4.5). These increased amylolytic activities were seen by the end of the first

week of the trial and the increased levels were maintained for the rest of the experimental period in both the dietary treatments. Feeding carbohydrate-rich diet induces increased secretions of starch hydrolysing alpha-amylase in fish (Fish, 1960; Nagase, 1964; Agrawal et al., 1975; Hofer, 1979a, 1979b; Hofer and Schiemer, 1981; Reimer, 1982). Similar adaptation of digestive enzymes to feeding habits and diet compositions has already been well established in many types of animals (Marchis-Mouren et al., 1963; Reboud et al., 1966; Corring, 1980). From Appendix I it can be seen that the holding ration contained only about 5% hydrolysable carbohydrate. Therefore the two fold increase in alpha-amylase activity in the gut contents of carp fed on the two test diets, which both contain 24% wheat starch, is in accordance with the increase in dietary carbohydrate level.

Comparison of the alpha-amylase activity levels in the gut contents between the two treatment groups did not reveal any difference. Despite the presence of active amylase inhibitor in the Active Diet, the fish were able to maintain alpha-amylase activity levels similar to that in fish fed Inactive Diet (Fig. 4.5). Sturmbauer and Hofer (1986) stated that the actual amount of amylase enzyme in the gut fluid of carp fed raw wheat could be higher than that in gut fluid from fish fed extruded wheat, but apparently a major portion of the enzyme

secreted by the fish fed raw wheat is bound by the wheat amylase inhibitor and cannot take part in digesting carbohydrate. Thus the assay of samples for amylase activity levels reveals only the activity of the free uninhibited enzyme and the amylase molecules already bound to the inhibitor proteins are not estimated. Further evidence that the fish may be producing more amylase enzyme when fed inhibitor containing Active Diet was seen in the higher alpha-amylase activity levels of the hepatopancreas, intestine and bile (Fig.4.6, Fig.4.7).

Fish fed active amylase inhibitor in the diet responded by a more than two fold increase in the pancreatic alpha-amylase production (Fig. 4.6). The alpha-amylase activity in the bile juice and the hepatopancreatic tissue collected at the end of the first week of the experiment revealed this hyperactivity. By contrast, the level of alpha-amylase activity in the bile and hepatopancreas of fish fed the denatured inhibitor was only 30% higher than the initial level. The intestinal tissue, which was in close contact with the inhibitor in the diet, also showed a similar sharp increase in alpha-amylase production at the end of Week 1 (Fig.4.7).

A similar effect was reported by Marci _et al_. (1977) in chickens fed gastro-resistant wheat inhibitor preparations. They reported a 71% increase (335 amylase units/mg tissue) in the pancreatic alpha-amylase production in chickens fed this inhibitor compared

to the control group of birds (196 units/mg). At the end of the first week in the present study, hepatopancreas samples taken from carp fed Active Diet had 107% more alpha-amylase activity than the fish fed Inactive Diet. However, by the end of the third week, there were no significant difference between the alpha-amylase activities in both treatments.

Thus, the alpha-amylase activity in the gut contents of carp fed diet containing inhibitor was at a similar level to that in fish fed denatured inhibitor. This was probably achieved by the increased synthesis and secretion of alpha-amylase in the secretory tissues of the fish fed Active Diet. It is also possible that the slight increase in activity seen in the hepatopancreas from fish fed denatured inhibitor, is the level of increase required to hydrolyse the higher carbohydrate content of the ingested diet. An additional increase in enzyme synthesis was necessary in the case of fish fed Active Diet, due to the enzyme inactivations in the gut lumen.

The difference in alpha-amylase activity between the two dietary treatments was observed only at the end of the first week of the trial (Fig.4.6 and Fig. 4.7). By the third week the activity levels in the hepatopancreas, intestine and bile were not significantly different ($p > 0.05$) in both groups of fish.

Considering the similar alpha-amylase activity

levels in all parts of the fish from both dietary treatments at the end of the third week, it appears that either the fish fed Active Diet had adapted their enzyme system to the presence of wheat amylase inhibitor in their diet, or the pancreas has reduced its enzyme synthesis for some other reason. Histology of the hepatopancreas did not reveal any degeneration (Plate 5). The carp fed Active Diet exhibited reduced growth rate even at the end of the three week observation period. Marci et al (1977) noted adaptations to the inhibitor in chickens after four weeks. Hofer and Sturmbauer (1985) suggested that the presence of inhibitor in the diet could cause excessive loss of enzymes in the faeces of fish and may negatively influence the overall protein balance. Lyman and Lepkovsky (1957) considered that growth depression in animals fed trypsin inhibitor may be a consequence of an endogenous loss of essential aminoacids derived from the hyperactivated pancreas. Therefore by the end of the present study the carp fed Active Diet were probably utilising body proteins for synthesising enzymes. Increased plasma proteins and the decreasing trend in hepatosomatic index both indicate some mobilisation of body proteins. Even the presence of fishmeal protein at optimum levels in the diet could not curtail this trend.

From this experiment it was concluded that wheat amylase inhibitor, when supplied in large amounts does affect the digestive processes in mirror carp and can

drastically retard growth. In order to see whether a similar effect on growth is elicited in tilapia the next set of experiment was conducted.

CHAPTER 5

EFFECTS OF FEEDING NILE TILAPIA WITH DIET CONTAINING SEMIPURIFIED WHEAT AMYLASE INHIBITOR.

CHAPTER 5

EFFECTS OF FEEDING NILE TILAPIA WITH DIET CONTAINING SEMIPURIFIED WHEAT AMYLASE INHIBITOR.

5.1 INTRODUCTION.

In the previous chapter it was seen that wheat amylase inhibitor when incorporated in the diet of mirror carp retards growth and hyperactivates the enzyme secretory tissues to compensate for the inhibitions of alpha-amylase in the gut lumen (Chapter 4). Carp and tilapia are both omnivores. However, the digestive system in the Nile tilapia differs from that of carp by having a well developed stomach with distinctly acidic gastric secretions. The pH in the stomach of this fish can even be as low as 1.0 after a meal (Moriarty, 1973; Bowen, 1976; Coulton, 1976; Payne, 1978) due to copious secretions of hydrochloric acid. This acid helps in the initial breakdown of the food so as to facilitate subsequent complete digestion in the intestine by the pancreatic enzymes acting at neutral to alkaline pH (Fish, 1960; Nagase, 1964).

Another major distinction between these two freshwater cultivable species of fish is that mirror carp do not secrete pepsin (Ash, 1985) whereas tilapia gastric juice has high peptic activities. However, tilapia intestines and pancreas secrete appreciable amounts of

trypsin and other alkaline proteases (Fish, 1960; Nagase, 1964) as is the case in mirror carp.

Considerable alpha-amylase activity has been detected throughout the alimentary canal of tilapia (Fish, 1960; Nagase, 1964). Amylases of tilapia are mainly produced in intestinal and pancreatic tissues, as in carp. The activity of amylase detected in the stomach of this fish is minimal and does not play a major role in digesting the ingested carbohydrates (Nagase, 1964) even though it is active over the wide pH range of 5 to 8 (Fish, 1960). Hence, all carbohydrates ingested by tilapia are hydrolysed in the intestinal lumen. Tilapia can utilise carbohydrates as well as carp. Recently, Anderson et al. (1984) showed that juvenile O.niloticus can utilise simple and complex carbohydrates up to levels of 25% or more in the diet resulting in good growth, protein retention and feed conversions.

Tilapia alpha-amylase can be easily inactivated by wheat amylase inhibitor. In this thesis, the in vitro experiments demonstrated that the amylase from this fish is more susceptible to the action of the wheat inhibitor than carp alpha-amylase under similar assay conditions (Chapter 3).

It was further shown that this inhibitor can overcome the digestive processes in the intestine of 'agastric' carp and can affect the fish in several ways (Chapter 4). Thus, since the capacity to digest and

utilise carbohydrates by carp and tilapia is more or less equal, the major difference between these two species that could affect the <u>in vivo</u> action of the wheat amylase inhibitor, may possibly be the activity of pepsin present in the stomach of tilapia. This chapter describes an experiment designed to investigate this hypothesis and to study how these anatomical and physiological differences influence the nutrition of juvenile Nile tilapia when fed diets incorporating amylase inhibitor albumin extracted from wheat grain.

5.2 MATERIALS AND METHODS.

5.2.1 Experimental fish.

Sixty five tilapia fingerlings (O.niloticus) of mean weight 12.22 ± 1.35g were randomly divided into 13 groups of five fish. The fish were held in 50 litre tanks in the experimental recirculatory system for an acclimatization period of 12 days prior to the experiment. During this period the fish were fed twice daily on a high protein holding ration (Appendix I) at a rate of 3% body weight per day.

5.2.2 Experimental diets.

A basal mixture was prepared containing 38.8% herring meal as the protein source and 24% unmodified wheat starch to provide the carbohydrates. Alpha-cellulose formed another major portion constituting nearly 23.5% of the mixture. The full dietary formulation is presented in Table 5.1. All the ingredients used to prepare the experimental diets for this experiment were from the same batches as used in the previous experiment on carp (Chapter 4, see also Table 2.5 in Chapter 2).

Albumin from 900g of wheat powder was extracted in accordance with the method of O'Donnell and McGeeney

Table 5.1 Proportions of ingredients used for preparation of basal mixture.

Ingredients	Moisture free %
Herring meal (batch 2)	38.80
Wheat Starch	24.00
Alpha-Cellulose	23.45
Cod-liver oil	2.55
Corn oil	3.70
Mineral mix	4.00
Vitamin mix	2.00
Carboxymethyl Cellulose	1.00
Chromic oxide	0.50
Total	100.00

(1976) described in Chapter 4. One half of the extracted albumin was mixed with 546g of basal mixture (500g, dry weight) and pelleted to prepare the 'Active' diet (Diet A) containing potent amylase inhibitor. The other half portion was autoclaved (121°C, 20 min), cooled and mixed with 546g of basal mixture to prepare the 'Inactive' diet (Diet B) containing denatured amylase inhibitor protein. The general details of preparation of test diets were given in Chapter 2. The two diets were isonitrogenous, containing about 29% crude protein and 20 to 21% hydrolysable carbohydrates. The proximate compositions of the two diets are given in Table 5.2. The presence or absence of active amylase inhibitor in the diets was tested according to the procedure of O'Connor and McGeeney (1981) described in Chapter 4, but in this case 54, 115, and 248 mU of alpha-amylase from tilapia hepatopancreas were incubated at 25°C for 30 minutes with 5 ug of protein extracted from the diets. In addition, 1 ug aliquots of protein were tested against four activity levels of porcine pancreatic amylase (14, 35, 46 and 64 mU) under identical assay conditions.

5.2.3 Experimental protocol.

At the end of the acclimatization period the fish were individually weighed to the nearest 0.01g. The Active and Inactive diets (Diets A and B, respectively) were each

Table 5.2 Proximate composition of test diets (Mean ± SD).

Moisture free %	Diet A	Diet B
Moisture	7.63 ± 0.02	6.00 ± 0.01
Crude protein	29.29 ± 0.50	29.35 ± 0.19
Crude lipids	9.00 ± 0.03	9.06 ± 0.17
Total ash	9.58 ± 0.25	10.01 ± 0.16
Crude fibre	19.78 ± 0.07	19.56 ± 0.25
Hydrolysable carbohydrate	20.24 ± 0.28	21.06 ± 0.29
Soluble carbohydrate*	32.35	32.02
Chromic oxide	0.39	0.36

* calculated by difference (see Chapter 2, Section 2.9.1.7)

randomly assigned to six tanks of fish. All the fish in the remaining group were sacrificed to determine the initial levels of the parameters studied in this experiment (see below). The fish were fed on the experimental diets at a daily feeding rate of 3% body weight in two equal instalments. The feeding rate was adjusted on a weekly basis for the duration of this six week trial.

Individual fish weights were recorded at weekly intervals and one replicate group of five fish from each treatment was sampled at the end of each of the first three weeks. At the beginning of the fourth week of the trial it was found necessary to pool the remaining three groups of fish from each treatment into a single tank in order to suppress the increasing aggressiveness and territorial behavior of the fish observed during the early part of the experiment. The fish were continued to be weighed at weekly intervals for the remaining three weeks of the trial.

At the end of each of the first three weeks of this trial, fish were sampled four hours after feeding and sacrificed by an overdose of benzocaine (Ross and Geddes, 1979). The alimentary tract was dissected out and stomach and intestinal contents were collected. The fluid portions were separated by centrifugation and their volumes were determined to the nearest ul. The stomach and intestinal solids were weighed to the nearest tenth of

a milligram. The volume of bile in the gallbladder was recorded and the weight of the hepatopancreas, stomach and intestine were noted. The HSI and gut-somatic index were calculated using the formulae given in Chapter 2. The volumes of bile, stomach and intestinal fluids and the weight of solids in the stomach and intestine were all expressed as percentages of fish body weight (Chapter 2).

At the end of the sixth week, the contents of the stomach, foregut, midgut and hindgut regions of the fish fed on Active Diet (Diet A) only were collected separately, pooled and stored frozen until required for subsequent extraction and assays for the presence of amylase inhibitor. The fish fed on Inactive Diet (Diet B) were not sampled since the diet did not contain any active inhibitor. The protein in these samples of gut contents was extracted by the procedure of O'Donnell and McGeeney (1976). Triplicate subsamples of 5 ug protein from each of the extracts, that is from stomach, foregut, midgut and hindgut contents, were incubated with tilapia hepatopancreas alpha-amylase (8, 13, 31 and 42 mU) to test for the presence of inhibitory activity at 25°C according to the method of O'Connor and McGeeney (1981) (see Chapters 3 and 4).

The faeces produced by the experimental fish were collected daily from the second week of the experiment and dried at 60°C overnight before analysing for chromic oxide, crude protein and hydrolysable carbohydrates using the methods described in Chapter 2.

5.3 RESULTS.

5.3.1 Inhibitor content in diets.

Five micrograms of protein extracted from Diet A reduced the tilapia hepatopancreatic alpha-amylase activity of 54 mU to 248 mU, by 65% to 30% in 30 minutes at 25°C (Table 5.3a). When 1 ug of the extracted protein was incubated with 14 mU to 64 mU of porcine pancreatic amylase, 16% to 9% of the initial activity was inhibited (Table 5.3b).

The protein extracted from Diet B had no inhibitory activity at all the levels of amylase activities tested.

5.3.2 Fish growth performance.

Tilapia fed on the two test diets more than doubled in body weight over the 42 day period of this trial (Fig. 5.1). The body weight of fish fed Diet B increased from 16.14 $\pm$ 2.04g to a mean final weight of 34.53 $\pm$ 5.54g, while the increase in those fed Diet A was from 15.82 $\pm$ 2.05g to 30.64 $\pm$ 4.60g. The specific growth rate (SGR) which was 2.24 %/day before the experiment decreased in both the treatments to 1.57 %/day in Diet A treatment and 1.81 %/day in the Diet B treatment. Even though the Diet A produced a lower growth rate in the fish, statistical analysis of the final body weights attained by the

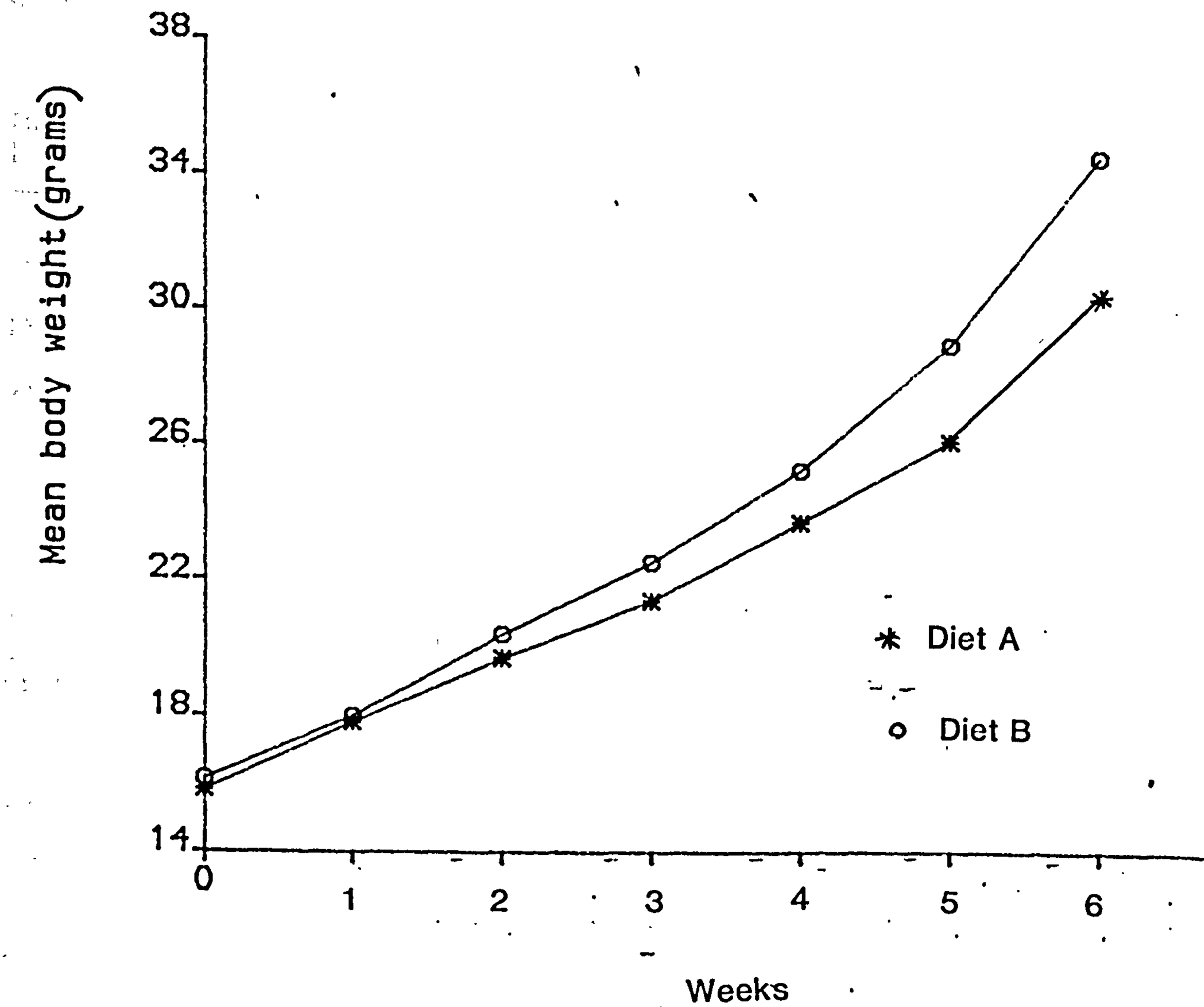

FIGURE 5.1 Increase in mean body weight of tilapia, fed Diet A and Diet B for six weeks.

Table 5.3a Residual activity (mean ± SD) and percent inhibition of tilapia hepatopancreatic alpha-amylase when incubated with 5 ug protein from Diet A for 30 min. at 25°C.

Initial activity (mU/min)	Residual activity (mU/min.)	% Inhibition*
54.33 ± 5.46	19.04 ± 2.95	64.96
115.01 ± 1.47	62.12 ± 0.70	45.99
247.65 ± 5.86	172.80 ± 3.20	30.22

Table 5.3b Residual activity (mean ± SD) and percent inhibition of porcine pancreatic alpha-amylase when incubated with 1 ug protein from Diet A for 30 min. at 25°C.

Initial activity (mU/min)	Residual activity (mU/min.)	% Inhibition*
13.73 ± 2.09	11.54 ± 0.96	15.95
35.05 ± 2.51	30.57 ± 1.83	12.79
45.96 ± 2.48	41.50 ± 1.61	9.70
63.69 ± 2.49	56.70 ± 2.11	10.98

$$* \% \text{ Inhibition} = \frac{(\text{Initial activity} - \text{Residual activity})}{(\text{Initial activity})} \times 100$$

fish revealed no significant difference ($p > 0.05$), between them.

5.3.3 Hepatosomatic index.

By the end of three weeks the HSI in both treatments had increased from an initial value of 1.69 to final values of 2.32 and 1.97 for fish fed Diets A and B, respectively (Table 5.4). However, the differences were not significant ($p > 0.05$), nor was there a consistent trend in values during the three weeks of observation.

5.3.4 Gut-somatic index.

The gut-somatic index of fish fed experimental diets did not change significantly ($p > 0.05$) from the initial value of 2.59 (Table 5.4). There were also no significant differences in gut-somatic index either with time or between dietary treatments ($p > 0.05$).

5.3.5 Bile volume.

There was a wide and erratic variation in bile volumes, both between sample periods and between individual fish in each sample group, despite samples having been consistently collected 4 hours after feeding (Table 5.4). There was no detectable trend with time in

Table 5.4 Bile volume, hepatosomatic index and gut-somatic index of tilapia fed Diet A and Diet B. (Mean ± SE)

Diet	Week	Bile vol ul/100 g	Hepato-somatic index	Gut-somatic index
Initial		142 ± 53[a]	1.69 ± 0.13	2.59 ± 0.24
A	1	212 ± 84	2.03 ± 0.20	2.76 ± 0.26
B	1	326 ± 136	2.19 ± 0.24	2.57 ± 0.13
A	2	61 ± 16[bc]	1.85 ± 0.19	2.16 ± 0.10
B	2	559 ± 154[ac]	1.94 ± 0.05	2.04 ± 0.18
A	3	199 ± 18[b]	2.32 ± 0.42	2.36 ± 0.10
B	3	117 ± 61	1.97	2.32 ± 0.10

* values in each column with same superscripts are significantly different ($p < 0.05$).

either treatment but there was a significant difference between the treatments during the second week because of the extreme values recorded.

5.3.6 Stomach and intestinal contents.

The stomach fluid volume in tilapia decreased after feeding the test diets whereas the solid contents weighed more than the initial level (Table 5.5). However, these differences were not statistically significant ($p > 0.05$).

The volumes of fluid in the intestine of fish fed Diet A were similar to the initial volume, but were significantly ($p < 0.05$) higher than in fish fed Diet B at the end of the first and second weeks of this experiment. Also, the intestinal solid weight was higher in treatment A than in treatment B, but this difference was only significant ($p < 0.05$) at the end of the second week.

Comparing the stomach solid weights and intestinal solid weights in the two treatments at the end of the first two weeks (Table 5.5), it was apparent that even though the fish were sampled at similar time intervals after feeding, the stomach solids weight in fish fed Diet A was lower than that in fish fed the Diet B. On the other hand, the fish fed Diet A had higher intestinal solid weights compared to those fed Diet B during the same period.

Table 5.5 Stomach and intestinal fluid volumes and solid weights in tilapia fed Diet A and Diet B.

		Stomach		Intestinal	
Diet	Week	fluid ml/100g	solid g/100g	fluid ml/100g	solid g/100g
Initial		0.24(0.09)	0.52(0.13)^{b}	1.34(0.21)^{a}	1.42(0.14)^{ab}
A	1	0.10(0.02)	0.75(0.07)	1.44(0.17)^{be}	1.11(0.14)^{c}
B	1	0.14(0.05)	0.80(0.20)	0.66(0.01)^{dea}	0.92(0.22)
A	2	0.19(0.05)	0.63(0.07)^{a}	1.45(0.05)^{cf}	1.37(0.15)^{de}
B	2	0.12(0.09)	1.20(0.10)^{ab}	0.95(0.08)^{df}	0.76(0.09)^{be}
A	3	0.15(0.04)	0.77(0.15)	0.63(0.10)^{bc}	0.40(0.17)^{acd}
B	3	0.23(0.12)	0.63(0.37)	0.85(0.19)	0.84(0.16)

* values in parenthesis show the SE
** values in each column with same superscripts are significantly different ($p < 0.05$).

5.3.7 Inhibitor content in stomach and intestinal contents.

The protein extracts from the contents of the stomach and the three regions of the intestine did not reduce the tilapia hepatopancreatic alpha-amylase activity at any of the enzyme activity levels tested by the *in vitro* method.

5.3.8 Nutrient Digestibility.

There were no significant differences in either the protein or available carbohydrate digestibilities of tilapia fed the two dietary treatments. Protein digestibilities were 91.27 and 91.78% for fish fed Diets A and B, respectively while available carbohydrate digestibilities were even higher with a value of 97.63% for fish fed Diet A and 97.48% for fish fed the Diet B.

5.4 DISCUSSION.

The growth of juvenile Nile tilapia was not significantly affected by the presence of large amounts of active amylase inhibitor in the diet (Fig. 5.1). Feeding Diet A to tilapia caused only a slight decrease in SGR ($p > 0.05$) when compared to the growth rates seen in tilapia fed the diet containing similar amounts of denatured extracted amylase inhibitor protein. As in the previous experiment with mirror carp (Chapter 4), there was a general decrease in SGR when the experimental groups of tilapia were changed over from maintenance ration to the test diet, but this was almost certainly due to the lower protein content of the latter. Unlike in the previous trial where a decreasing trend in hepatosomatic and gut somatic indices were recorded, the present experiment with tilapia showed an increase in HSI in both dietary treatments, whereas the gut-somatic index remained stable (Table 5.4).

Inhibition assays with water soluble albumin extracted from Diets A and B showed detectable inhibitory activity only in the protein from Diet A (Table 5.3 a and b). 5 ug of extracted albumin from Diet A inhibited up to 65% of the initial tilapia alpha-amylase activity tested in the assays. On the other hand, equal amounts of protein from Diet B had no inhibitory activity on either tilapia hepatopancreatic alpha-amylase and

porcine pancreatic amylase. Despite the presence of active inhibitor in Diet A, samples of stomach and intestinal contents collected from tilapia four hours after feeding on this diet, had no inhibitory activity. Ross and Jauncey (1981) reported that in a tilapia hybrid the stomach evacuation time is over 8 hours at 25 to 30°C. Because no inhibitor activity was detectable in the stomach contents, it appears that the inhibitor in the diet of tilapia is denatured in the stomach within half the time it takes for the complete diet to pass through the stomach into the intestine.

There is conclusive evidence that the wheat amylase inhibitor protein is not destroyed by extremes of pH. Buonocore et al. (1977) found that incubation of the 0.19 inhibitor fraction for 1 hour at highly alkaline (up to 11.7) or acidic (down to 3.0) pH values did not affect its inhibitory activity towards both human salivary and insect alpha-amylases. Other workers have also incubated this protein in 0.02 N hydrochloric acid and found no inactivation (Shainkin and Birk, 1970; O'Connor and McGeeney, 1981). Therefore the acidic pH conditions in the stomach of tilapia could not have destroyed the ingested inhibitor in Diet A.

By contrast, proteases, particularly pepsin, have the capacity to destroy the wheat amylase inhibitor protein (Kneen and Sandstedt, 1946; Militzer et al., 1946; Shainkin and Birk, 1970; Petrucci et al., 1976;

O'Connor and McGeeney, 1981). Other proteases affect various fractions of this inhibitor protein at differing levels. Shainkin and Birk (1970) found that of the two fractions, AmI_1 and AmI_2, isolated by them, both were inactivated by digestion with pepsin and pronase, but were not affected by carboxypeptidases A and B. AmI_1 lost its activity when submitted to digestion with trypsin and chymotrypsin, whereas AmI_2 was poorly affected by these enzymes. The 0.19 inhibitor fraction of Buonocore et al. (1977) is resistant to trypsin but is completely destroyed by incubation with pepsin. Similarly all the four fractions of O'Connor and McGeeney (1981) are totally inactivated by pepsin within 2 hours while only Inhibitor No.4 (63,000 dalton) is destroyed by trypsin and alpha-chymotrypsin.

Unlike carp which secretes only alkaline proteases (Ishida, 1936; Sarbahi, 1951; Jany, 1976, Ash, 1985), tilapia secretes both acid and alkaline proteases. Pepsin and pepsin-like proteases which have acidic pH optima have been detected in the gastric fluid of tilapia, in addition to the trypsin and other trypsin-like proteases which are secreted by the intestinal mucosal cells and pancreatic tissues (Fish, 1960; Nagase, 1964). Since pepsin is totally inactive at neutral pH (Davenport, 1982), all the protease activity detected in the intestine of tilapia is due to trypsin, chymotrypsin and other proteases. Hofer and Schiemer (1981) estimated that the

total proteolytic activity produced by 100g Mozambique tilapia over a 24 hour period is 643.5 X 10^3 Units. They detected a proteolytic activity of 15.8 X 10^3 U/ml in the total gut contents of this fish. Nawwab (1987) determined peptic activity in the stomach of O.niloticus, 4 - 6 hours after feeding carbohydrate-rich diet (33% carbohydrate, 35% crude protein), to be 3.45 uMol tyrosine/min/g tissue at 30°C (pH 2.0). The trypsin-like enzyme activity at pH 8.2 in the intestinal tissues of these fish was 5.99 uMol/min/g at 30°C. Protease activities in tilapia fed high protein diets were at higher levels. Taking this as a rough guideline, it is possible that about 40% of the total proteolytic activity in tilapia is due to pepsin and pepsin-like acid proteases.

Thus there is strong evidence to suggest that the high peptic activity in tilapia stomach caused the total inactivation of the inhibitor in Diet A before it could pass through into the intestinal region where it could bind with alpha-amylase. If any inhibitor was present in the intestine with its activity intact, then it would also be susceptible to the action of the other proteases present. It therefore appears highly unlikely that there was any intact amylase inhibitor left to react with and inhibit alpha-amylases in the intestine.

Further evidence that alpha-amylase activities were not affected in this experiment is provided by the

digestibility values for carbohydrate. The presence of large amounts of active amylase inhibitor in Diet A did not reduce the digestibility and utilisation of carbohydrate in the experimental fish. Tilapia fed both Diet A and Diet B had high carbohydrate digestibilities of around 97%.

Protein digestibility was also equally high with values of 91% for both diets. Fish proteases are sensitive to protease inhibitor such as soybean trypsin inhibitor (Sandholm et al., 1976; Croston, 1960; Overnell, 1973; Cohen et al., 1981; Krogdahl and Holm, 1983). Small amounts of trypsin inhibitor have been identified in wheat (Shyamala and Lyman, 1964) , but the negligible quantities present caused no detectable effect, and did not appear to influence the inactivation of amylase inhibitor by proteases in tilapia.

Reports indicate that chicken amylase, like tilapia alpha-amylase (Chapter 3), is also highly susceptible to wheat inhibitor when tested under in vitro conditions. When Marci et al. (1977) fed 0.8% native wheat albumin to chickens it did not produce any effect on the birds. However, feeding only half that amount in a form resistant to gastric digestion caused a significant reduction in growth for the first four weeks during which time it even affected the pancreas. Eventually, however, the birds totally adapted to this inhibitor and grew at a rate similar to the controls.

Overall the presence of inhibitor in Diet A did not cause any deleterious effect on juvenile Nile tilapia. There were no obvious nutritional or physiological effects which were attributable to the wheat amylase inhibitor albumin. Because of these observations, experimentation with tilapia was discontinued at this stage. However, since this inhibitor had significant effects on mirror carp (Chapter 4), its action when present in whole wheat, which is the form in which it is normally fed to fishes, was investigated further.

CHAPTER 6

EFFECTS OF FEEDING RAW AND AUTOCLAVED WHEAT ON THE ALPHA-AMYLASE ACTIVITY IN MIRROR CARP.

CHAPTER 6

EFFECTS OF FEEDING RAW AND AUTOCLAVED WHEAT ON THE ALPHA-AMYLASE ACTIVITY IN MIRROR CARP.

6.1 INTRODUCTION

In Chapter 4 it was demonstrated that albumin extracted from wheat can reduce the growth of mirror carp. By *in vitro* experiments it was also shown that carp alpha-amylase is highly susceptible to this inhibitor (Chapter 3). In terms of practical application in fish nutrition and the feed industry, it is essential to know the level of influence this inhibitor has when fed to fish in the form of whole native wheat.

The amylase inhibitor protein in wheat is located in the endosperm (Kneen and Sandstedt, 1946) and is part of the storage protein in the grain (Varner, 1965; Kasarda *et al.*, 1971). This observation has been confirmed by microscopic studies (Sandstedt and Beckord, 1946) and by the assay of inhibitor contents in different wheat milling fractions (Saunders, 1975). The level of inhibitor was markedly lower in wheat brans than in whole ground wheat. The linear relationship observed between inhibitor and starch content of different wheat milling fractions also indicated that the inhibitor is closely associated with starch granules.

It has been suggested that 66% - 80% of the albumins in mature wheat grains have amylase inhibitory activity (Petrucci et al., 1974; Marci et al., 1977). In addition, the inhibitor was present in similar amounts in all varieties of wheat tested by Kneen and Sandstedt (1946), namely hard red winter wheat, soft red winter wheat and hard red spring wheat. Bedetti et al. (1974) and Vitozzi and Silano (1976) reported that hybrid wheat of Triticum species exhibit the presence of protein similar to amylase inhibitor albumin.

Silano et al. (1975) suggested that this inhibitor acts as a natural defence mechanism against the pests and predators of wheat, since the inhibitor is more effective on amylases from insect species that normally feed on wheat. Therefore it appears that this inhibitor could be effective even when it is present in whole wheat grain.

Hofer and Sturmbauer (1985) showed that when incubated with wheat flour, carp gut fluid amylase activity was strongly inhibited while trout amylase was only moderately inactivated. The decreasing trend in inhibition during their four hour in vitro experiments was attributed to the action of proteolytic enzymes. Sturmbauer and Hofer, (1986) later suggested that carp are capable of compensating for the amylase inactivation by inhibitor in the diet by a 3 to 4 fold increase in the secretion of enzymes. The fish in their experiment were

fed solely on ground wheat which on average contains less than 15% protein (NRC, 1983). The optimum protein requirement of carp is at least double that amount (Ogino and Saito, 1970; Jauncey, 1979).

Therefore in the present investigation, the nature and extent of the effect of amylase inhibitor protein present in whole wheat in diets fed to mirror carp containing optimum levels of dietary protein, and the eventual adaptation of the fish to the presence of inhibitor in their diet is elaborated.

6.2 MATERIALS AND METHODS.

6.2.1 Experimental fish.

The 66 mirror carp fingerlings used for this experiment ranged in weight from 29.5 to 48.3 g (mean 37.75 $\pm$ 0.57g) and were selected from the stock system. The fish were randomly distributed in six experimental tanks in the freshly cleaned and prepared experimental system (Chapter 2) at a stocking density of 11 fish per tank. During the 10 day acclimatization period the fish were fed on a commercial high protein holding ration (Appendix I) at a rate of 3% of their total body weight in two equal instalments per day. The water quality conditions in the system were within the recommended range for the wellbeing of carp (see Chapter 2).

6.2.2 Experimental diets.

Two diets were prepared, one containing raw wheat with its inherent content of amylase inhibitor protein and the other with autoclaved wheat, where the inhibitor had been denatured by heat.

Whole wheat grain (white variety) was milled in a hammer mill (AFM-Scotmec, Ayr), then finely ground in a Tecator Cyclotec 1093 sample mill and finally sieved through a 1 mm mesh. This flour was used as the source of

carbohydrate and amylase inhibitor in the 'Raw wheat Diet'. To prepare the 'Autoclaved wheat Diet' without active inhibitor, the wheat flour was heated in steam under pressure (121°C, 1.06 kg/cm²) for 20 minutes and dried at 35°C for 12 hours and then incorporated in the diet.

The other main dietary ingredients were herring fish meal (batch I) and alpha-cellulose, which constituted 32.5% and 16.43%, respectively, of the final diets on a moisture free basis (Table 6.1). The test diets were prepared and analysed for moisture, crude protein, crude lipid, crude fibre, hydrolysable carbohydrate and total ash in accordance with the procedures described in Chapter 2. The compositions of the dietary ingredients and of the final prepared test diets are presented in Table 2.5 (Chapter 2) and Table 6.2, respectively.

The amylase inhibitor content of the wheat grains and also their potency in the prepared test diets were determined, after extracting the inhibitor protein as described in Chapter 4.

6.2.3 Experimental protocol.

The Raw wheat Diet was assigned to three randomly selected groups of fish and the remaining three groups were assigned the Autoclaved wheat Diet.

Table 6.1 Formulae of test diets (Moisture free %).

Ingredients	Raw wheat Diet	Autoclaved wheat Diet
Wheat powder	37.32	-----
Wheat powder autoclaved	-----	37.32
Fishmeal (batch I)	32.50	32.50
Alpha-Cellulose	16.43	16.43
Carboxymethyl Cellulose	1.00	1.00
Cod-liver oil	2.55	2.55
Corn oil	3.70	3.70
Mineral mix	4.00	4.00
Vitamin mix	2.00	2.00
Chromic oxide	0.50	0.50
Total	100.00	100.00

Table 6.2 Proximate composition of experimental diets (mean ± SD, n = 3).

Moisture free %	Raw wheat Diet	Autoclaved wheat Diet
Moisture	5.82 ± 0.14	4.82 ± 1.15
Crude protein	30.12 ± 0.22	30.11 ± 0.10
Crude lipid	9.56 ± 0.06	9.44 ± 0.19
Total ash	9.75 ± 0.16	9.77 ± 0.02
Crude fibre	14.16 ± 0.43	14.86 ± 0.43
Hydrolysable carbohydrate	21.99 ± 1.43	21.11 ± 0.52
Soluble carbohydrate*	36.41	35.82
Chromic oxide	0.47 ± 0.003	0.48 ± 0.004

* calculated by difference. (see Chapter 2, Section 2.9.1.7)

After the 10 day acclimatization period, the initial weights of all fish were recorded and one fish from each group was sacrificed for sampling blood, gut contents and tissues. The remaining ten fish in each group were returned to their respective tanks and were fed the test diets from the next day at 3% of their total body weight in two equal instalments. Thereafter at weekly intervals all fish were weighed to record their growth and to readjust the feeding rate according to the increased body weight.

Also at weekly intervals three fish from each tank (9 fish per treatment) were killed by placing them in water containing an overdose of benzocaine, exactly five hours after their morning instalment of feed. Samples of blood, gut contents (fluid and solid), bile, hepatopancreas and intestinal tissues were then taken as described in Chapter 2.

The volumes of gut fluid and bile were recorded using micropipettes, the gut fluid pH was recorded using a pH probe and gut solid, hepatopancreas and intestine weights were recorded on a Mettler AC100 balance prior to rapidly freezing all the samples in liquid nitrogen before storing at -20°C until needed for analyses.

From the weights of hepatopancreas and total gut tissue the hepatosomatic index and gut-somatic index were calculated using the formulae given in Chapter 2. Plasma glucose was determined by the procedures of Bergmeyer and

Brent (1974) as described in Chapter 2. The gut fluid, gut solid, bile, hepatopancreas and intestine collected from nine fish in each treatment were pooled into three composite samples, each containing samples from three fish, for determination of alpha-amylase activity at 25°C using the method of Rick and Stegbauer (1974) described in Chapter 2. Total protease activity in the three pooled samples of gut fluid and solid from each treatment were assayed by the modified procedures of Rick (1974) given in Chapter 2. Protein content of the samples used for enzyme assay, was determined by Lowry's (1951) method and the specific amylase and protease activities were calculated as before (Chapter 2).

During the last two weeks of this three week experiment, faeces were collected from both treatment groups and were analysed for protein and carbohydrate digestibilities as described in Chapter 2.

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6.3 RESULTS

6.3.1 Inhibitor content in Raw wheat Diet and Autoclaved wheat Di

The wheat used in the preparation of experimental diets had an inhibitor content of 38,306 Inhibitor Units per 100g (Chapter 4). Therefore the Raw wheat Diet contained at least 14,296 Units of inhibitor per 100g of the final diet. Furthermore, carp hepatopancreatic alpha-amylase with activity levels of 55, 111 and 175 mU/min was reduced by 85 to 59% (Table 6.3a) when incubated with 5 ug of protein extracted from Raw wheat Diet indicating that this diet contained active amylase inhibitor. Raw wheat Diet also contained proteins capable of inhibiting porcine pancreatic amylase (Table 6.3b). 1 ug of protein reduced 5 to 16% of the porcine pancreatic amylase activity.

The alcohol fraction extracted from Autoclaved wheat Diet did not contain any inhibitor since no reduction in amylase activity was recorded when tested in a similar manner.

6.3.2 Fish growth.

After the ten day acclimatization period the mean fish weight was 49.41 $\pm$ 0.77g and this initial weight did not differ significantly between the groups in the two

Table 6.3a Residual activity (mean ± SD) and percent inhibition of carp hepatopancreatic alpha-amylase when incubated with 5 ug protein from Raw wheat Diet for 30 min at 25°C.

Initial activity (mU/min)	Residual activity (mU/min)	% Inhibition*
55.23 ± 2.94	8.49 ± 2.79	84.63
111.01 ± 5.99	32.72 ± 2.80	70.72
175.00 ± 2.95	72.19 ± 2.37	58.75

Table 6.3b Residual activity (mean ± SD) and percent inhibition of porcine pancreatic amylase when incubated with 1 ug protein from Raw Wheat diet for 30 min. at 25°C.

Initial activity (mU/min)	Residual activity (mU/min)	% Inhibition*
133.53 ± 2.69	111.69 ± 8.55	16.35
253.02 ± 5.70	242.88 ± 4.52	4.01
376.83 ± 6.63	346.60 ± 4.19	8.02
486.83 ± 0.61	460.72 ± 5.01	5.36
646.96 ± 3.03	616.49 ± 8.67	4.56

$$* \% \text{ Inhibition} = \frac{(\text{Initial activity} - \text{Residual activity})}{(\text{Initial activity})} \times 100$$

treatments ($p > 0.05$). Carp fed on diets containing raw wheat grew slowly during the first week with a specific growth rate of only 0.43 %/day (Fig 6.1). At the end of Week 1 the fish in this treatment had attained a mean weight of 50.91 $\pm$ 1.02g while the fish on Autoclaved wheat Diet grew to a mean weight of 53.73 $\pm$ 1.29g. The specific growth rate in this treatment was 1.20%/day. The weights attained by the fish in the two treatments at the end of Week 1 were significantly different at the 90% level of significance ($p < 0.10$).

During the second week of the trial, the growth of fish fed on Raw wheat Diet improved to 1.42 %/day and during the 3rd week it was 1.36 %/day. During the corresponding periods the growth rates in fish fed on Autoclaved wheat Diet were 1.18 %/day and 1.48 %/day, respectively.

By the end of the three week trial, the fish in the two treatments attained mean final weights of 61.85 $\pm$ 2.36g and 64.72 $\pm$ 2.54g, in the Raw wheat Diet and Autoclaved wheat Diet treatments, respectively which were not statistically different ($p > 0.05$).

6.3.3 Plasma glucose.

The initial glucose content of plasma was 84.53 $\pm$ 9.38 mg/dl. On feeding the test diets the plasma glucose rose sharply and significantly ($p < 0.05$) in both the

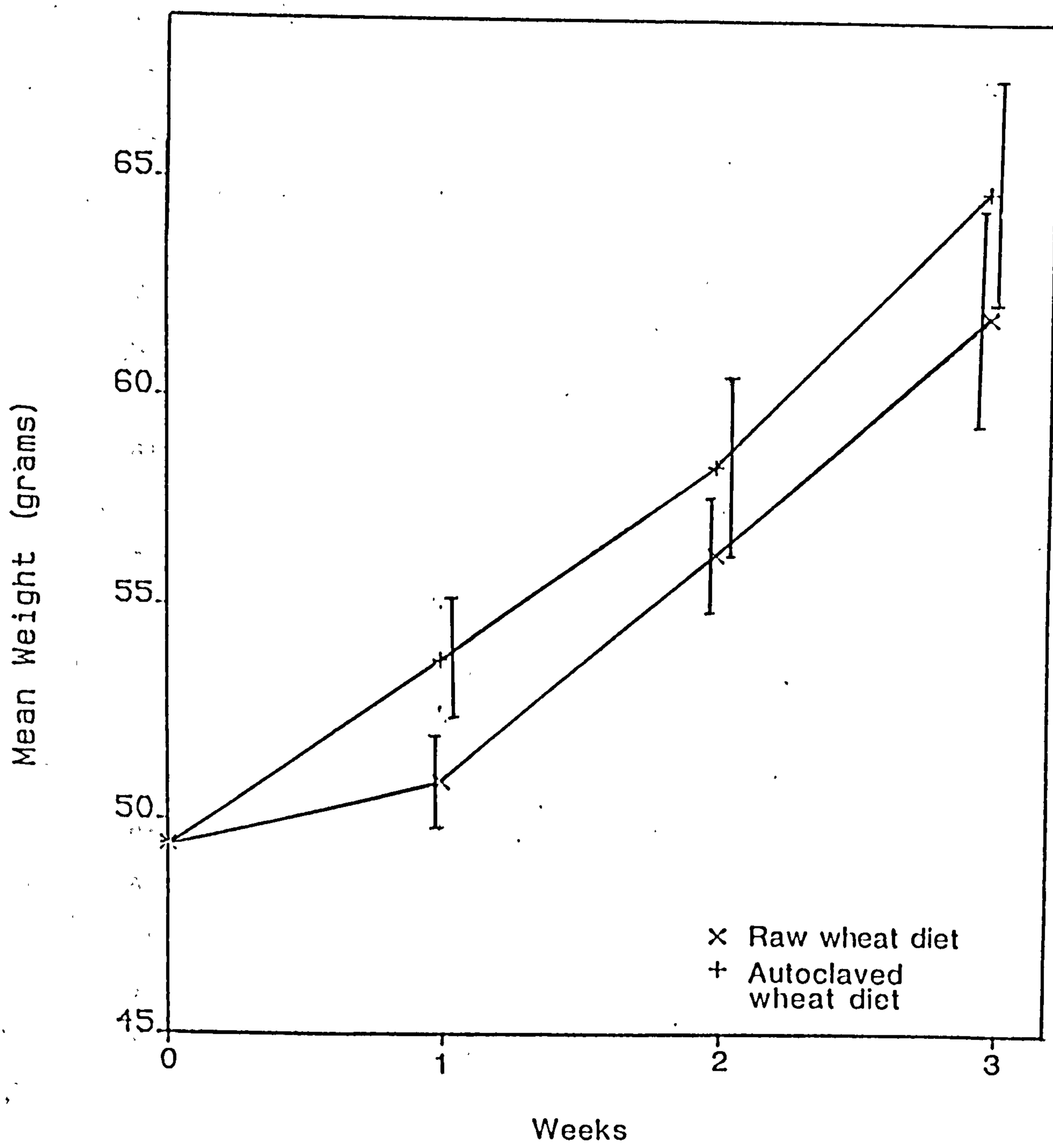


FIGURE 6.1 Increase in mean body weight of carp fed Raw wheat Diet and Autoclaved wheat Diet for three weeks. (vertical lines indicate SD)

treatments, and remained significantly higher throughout the three week trial (Fig.6.2). The maximum plasma glucose levels occurred at the end of the first week in both treatments (165 and 151 mg/dl in Raw wheat Diet and Autoclaved wheat Diet, respectively). Thereafter levels fell over the next two weeks. Comparison of the weekly mean plasma glucose levels between the two treatments however did not reveal any statistically significant differences ($p > 0.05$).

6.3.4 Hepatosomatic index.

Initially the HSI was 1.74 $\pm$ 0.09 (Table 6.4). During the entire experiment this index did not vary significantly ($p > 0.05$) from the initial value in the experimental fish fed on Raw wheat Diet. In the fish fed Autoclaved wheat Diet however, an increase was noticed after two weeks which was higher than the initial as well as the Week 1 values, but significant only at the 90% level of confidence ($p < 0.10$).

6.3.5 Gut-somatic index.

The gut-somatic index at the start of the experiment was 3.23 $\pm$ 0.18 which subsequently decreased significantly ($p < 0.05$) when the fish were fed either of the two test diets (Table 6.4). No difference was seen

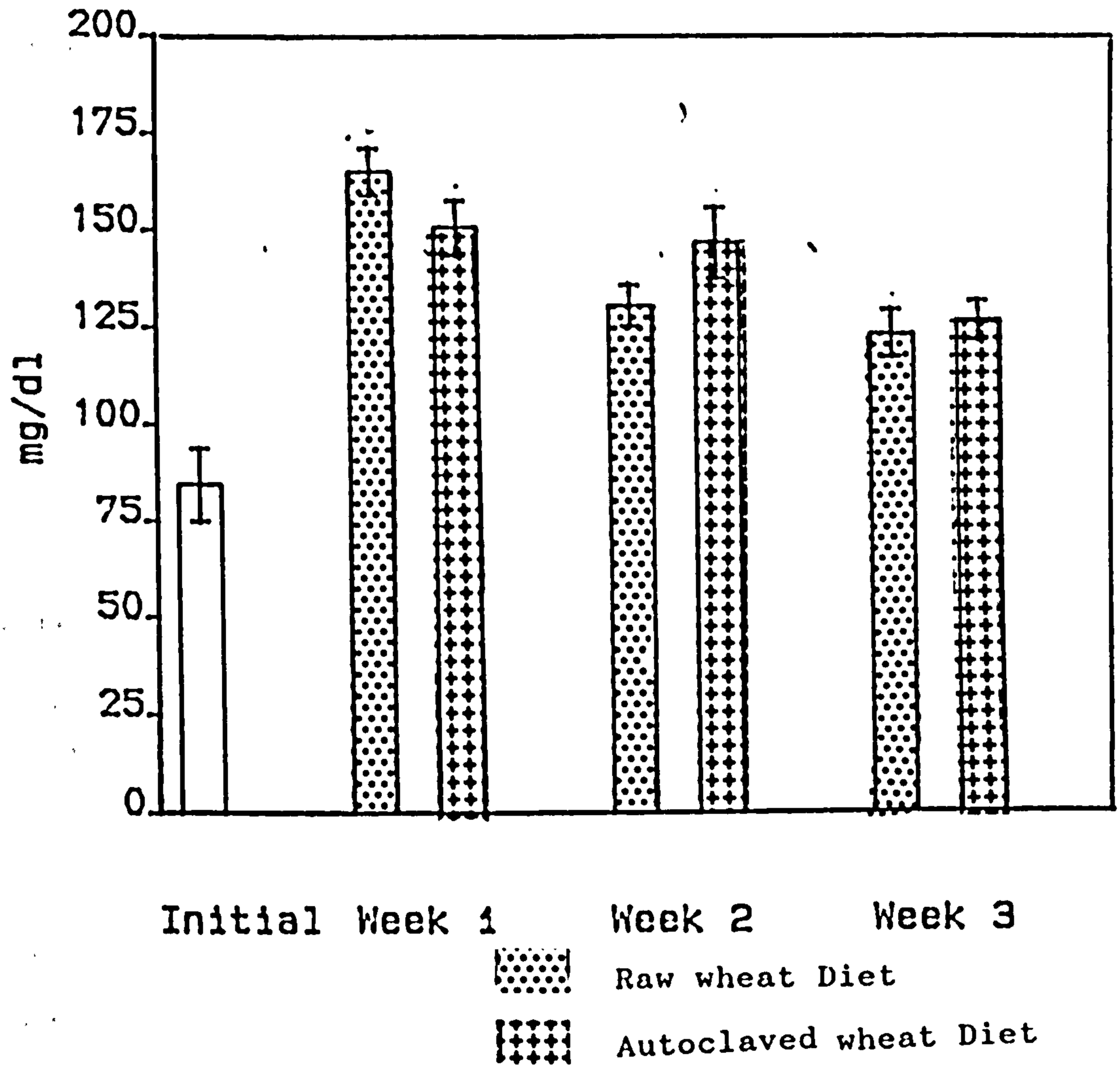


FIGURE 6.2 Plasma glucose levels in carp fed Raw wheat Diet and Autoclaved wheat Diet.

Table 6.4 Hepatosomatic index, gut-somatic index and gut fluid pH in mirror carp fed Raw wheat and Autoclaved wheat Diet.

Diet	Week	Hepatosomatic index	Gut-somatic index	Gut fluid pH
Initial		1.74(0.09)	3.23(0.18)^{abc}	6.85(0.10)
Raw Wh.	1	1.68(0.08)	2.80(0.13)	6.72(0.03)^{a}
Auto Wh.		1.71(0.11)	2.62(0.10)^{b}	6.68(0.06)
Raw Wh.	2	1.85(0.09)	2.94(0.19)	6.78(0.04)^{b}
Auto Wh.		1.97(0.08)	2.98(0.20)	6.68(0.06)
Raw Wh	3	1.78(0.11)	2.73(0.15)^{a}	7.02(0.08)^{ab}
Auto Wh.		1.97(0.12)	2.56(0.12)^{c}	6.80(0.10)

* values in parenthesis show the standard error.
** values in each column with same superscripts are significantly different ($p < 0.05$).

between the two dietary treatments ($p > 0.05$).

6.3.6 Gut fluid pH.

The pH of gut fluid in carp fluctuated over a narrow neutral range of 6.35 to 7.46 throughout the experiment in all the fish examined (Table 6.4). Initially the mean pH was 6.85 $\pm$ 0.10 which remained more or less stable in the fish fed Autoclaved wheat Diet but a gradual increase was seen in fish fed the Raw wheat Diet. The gut fluid pH at the end of Week 1 in the latter treatment was significantly ($p < 0.05$) lower than the pH at the end of Week 3.

6.3.7 Gut fluid volume.

The volume of fluid in the intestine of carp five hours after their meal of maintenance ration was 1.18 $\pm$ 0.08 ml per 100g fish body weight (Table 6.5). In fish fed Raw wheat Diet there was a small but insignificant increase in gut fluid volume at the end of both the 1st and 2nd weeks. By comparison the gut fluid volume of fish fed Autoclaved wheat Diet decreased slightly over the same period. However there were no significant ($p > 0.05$) differences between any of the mean values. The unusually low volumes at the end of the third week resulted from the samples having been centrifuged at a lower speed of 2000

Table 6.5 Gut fluid volume, gut solid weight and bile volume in mirrorcarp fed Raw wheat Diet and Autoclaved wheat Diet.

Diet	Week	G.Fluid vol ml/100 g fish	G.Solid wt g/100 g fish	Bile vol ul/100 g fish
Initial		1.18(0.08)	1.25(0.10)[a]	35.6(10.8)
Raw Wh.	1	1.22(0.04)	1.10(0.06)[b]	43.3(5.9)[a]
Auto Wh.		1.08(0.06)	0.98(0.07)[a]	28.7(4.4)
Raw Wh.	2	1.20(0.09)	1.33(0.07)[bc]	25.8(5.5)[a]
Auto Wh.		1.04(0.08)	1.08(0.09)[c]	41.7(7.4)
Raw Wh	3	0.70(0.05)	1.22(0.09)	38.9(7.2)
Auto Wh.		0.84(0.05)	1.19(0.07)	32.9(11.6)

* values in parenthesis show the standard error.
** values in each column with same superscripts are significantly different ($p < 0.05$).

rpm, due to a centrifuge malfunction, and these values cannot therefore be compared with those recorded in the previous two weeks. It can be seen however, that there was no significant difference between the two treatments at the end of the trial.

6.3.8 Gut solid weight.

The solid material in the gut contents of fish fed experimental test diets decreased from the initial weight of 1.25 $\pm$ 0.1 g/100g fish at the end of Week 1 in both the dietary treatments (Table 6.5). In general the gut solid weights in fish fed Raw wheat Diet were higher than those in the fish fed Autoclaved wheat Diet throughout the three week experimental period and this difference was significant ($p < 0.05$) at the end of the second week. The values recorded at the end of the trial were probably also affected by the improper separation of fluids resulting from the use of a lower centrifugation speed (see Section 6.3.7, above).

6.3.9 Bile volume.

The volumes of bile measured in the experimental fish, five hours after their meal, did not exhibit any consistent trend in both the treatments (Table 6.5) and the differences observed were also not significant (p

>0.05).

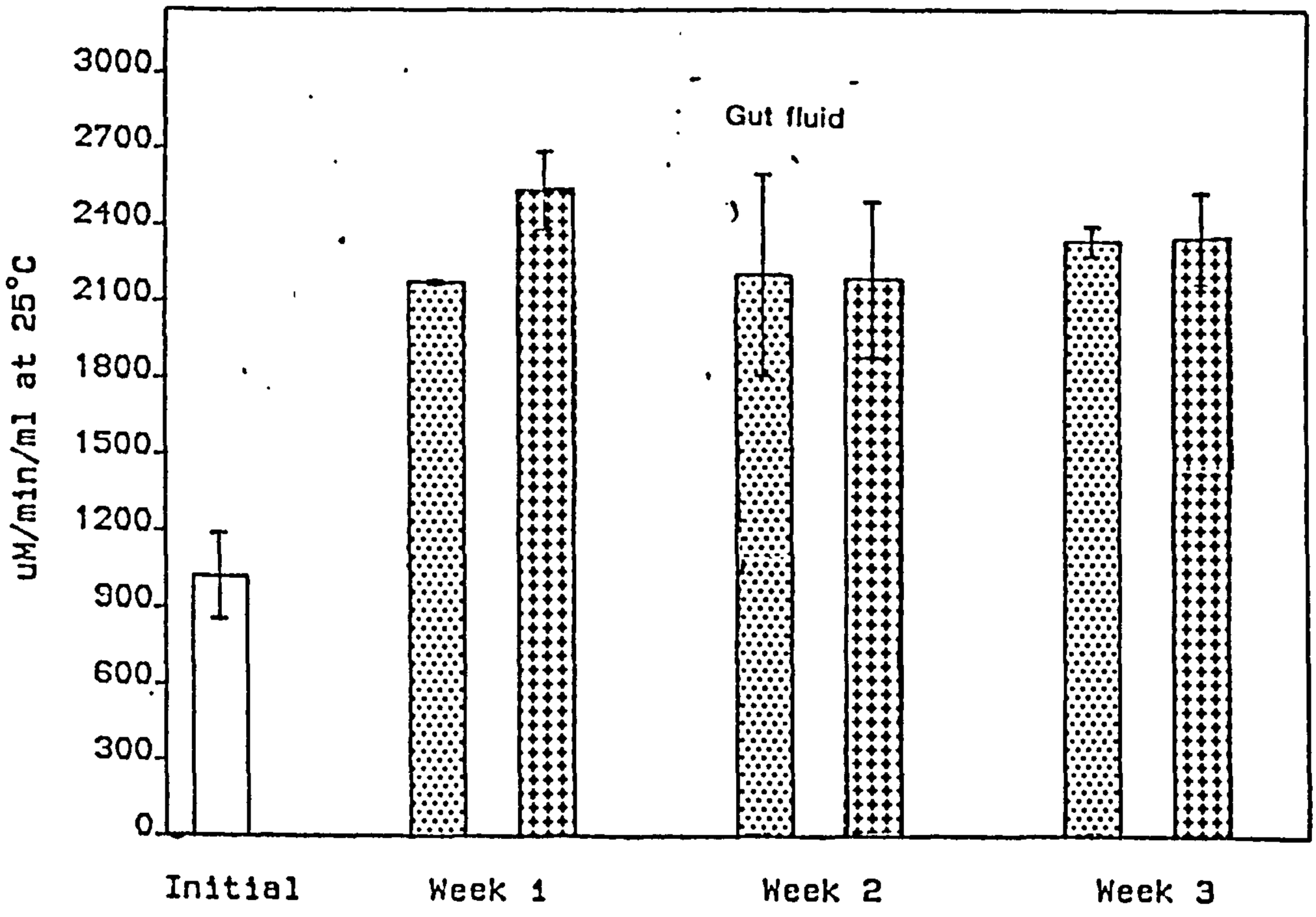
6.3.10 Enzyme activity.

6.3.10.1 Alpha-amylases.

(a) Alpha-amylase in gut fluids:

The mean initial alpha-amylase activity in the gut fluid of carp was 1024 ± 166 uM/ml/min. On feeding the test diets, the alpha-amylase activity more than doubled and remained around this level for the duration of the three week trial (Fig. 6.3). The difference between the initial level and the activities in the two treatment groups were highly significant ($p < 0.05$). The weekly mean activities in the two treatments over the three week trial period were not significantly different ($p > 0.05$). Nevertheless the activity in fish fed Raw wheat Diet at the end of Week 1 was slightly lower than the corresponding level in fish fed Autoclaved wheat Diet.

Comparison of specific activities of amylase in gut fluids from the two treatments (Table 6.6) showed a similar trend with significantly higher activities ($p < 0.05$) during the experimental period when compared to the initial value of 21.30 uM/min/mg. There were no significant differences ($p > 0.05$) between the weekly means from the two treatments. However in the groups fed Raw wheat Diet, the specific activity at the end of the first week (64.37 uM/mg) was significantly ($p < 0.05$) lower



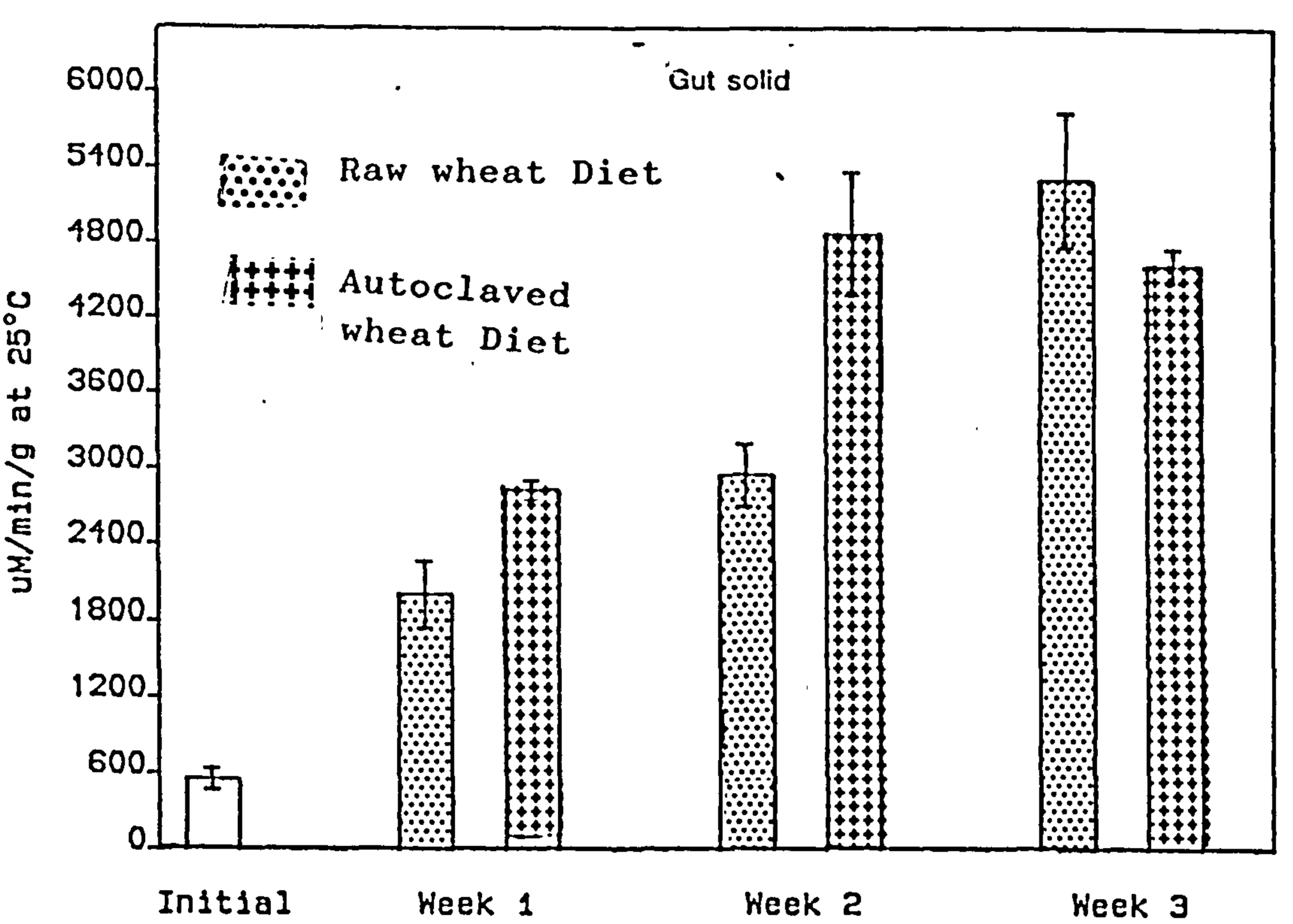


FIGURE 6.3 Alpha-amylase activity in the gut fluid and gut solid of carp, five hours after feeding on Raw wheat Diet and Autoclaved wheat Diet.

Table 6.6 Alpha-amylase specific activity (uMol/mg protein/min) at 25°C in carp fed Raw wheat Diet and Autoclaved wheat Diet.

Diet	Week	Gut fluid	Gut solid	Bile	Hepatopancreas	Gut tissue
Initial		21.30(3.31)abcdef	12.01(2.03)abcdef	9.27(1.35)abcdef	5.67(0.69)abcdef	2.54(0.25)abcdef
Raw wh.	1	65.93(0.13)ag	69.82(7.99)ag	17.91(0.61)aghi	17.07(1.25)ah	9.07(0.58)ag
Auto wh.		65.38(3.64)d	85.54(1.70)dij	26.43(2.93)di	11.28(0.98)dgh	8.14(0.13)dij
Raw wh.	2	66.74(8.50)b	95.21(3.46)bh	30.13(4.05)bg	17.96(2.71)b	8.95(0.20)bh
Auto wh.		60.22(4.83)e	137.68(18.00)ei	27.49(2.75)e	16.67(0.28)eg	9.85(0.48)ei
Raw wh.	3	73.70(2.09)cg	153.98(12.40)cgh	29.95(2.18)ch	25.03(5.01)c	11.86(0.56)cgh
Auto wh.		69.48(8.36)f	137.57(5.16)fj	26.79(3.11)f	24.82(0.96)fg	11.98(0.66)fd

* values in parenthesis show the standard error.
** values in each column with same superscripts are significantly different ($p < 0.05$).

than the activity level of 73.70 uM/mg recorded at the end of the trial.

(b) Alpha-amylase in gut solids:

Initially the alpha-amylase activities recorded in the gut solids were low giving a mean activity of only 554 ± 83 uM/g/min at 25°C (Fig. 6.3). After the fish had been fed the test diets for a week the values recorded in both treatments were significantly higher ($p < 0.05$) at about 4 to 5 times the initial level. Levels in fish fed raw wheat continued to increase significantly throughout the three week experiment whereas in those fed autoclaved wheat a maximum level appeared to have been reached after two weeks. At the end of both the first ($p < 0.05$) and second ($p < 0.10$) weeks, amylase activities in gut solids of fish fed autoclaved wheat were significantly higher than those in fish fed raw wheat. However by the end of the trial there was no significant difference in activity levels between treatments.

The amylase specific activity values also exhibited a similar trend. From an initial low activity level of 12 uM/mg protein, the activities had risen 6 to 7 fold at the end of Week 1 and were 8 to 10 times higher at the end of Week 3 after feeding test diets (Table 6.6). This increase was highly significant ($p < 0.05$) in the two treatments. Between the two treatments however the activity in fish fed Raw wheat Diet was lower than that in

fish fed Autoclaved wheat Diet but this difference was significant only at the 90% level of confidence ($p < 0.10$). At the end of the trial there was no difference between the two treatments.

When the total alpha-amylase activity in the gut contents (that is the activities in fluid plus solid) is considered (Fig. 6.4) it can be seen that this enzyme attained a maximum level of activity by the end of the second week in fish fed Autoclaved wheat Diet and that it stabilized thereafter. Fish fed Raw wheat Diet had relatively lower activities up to Week 2 but activity levels in the two treatments were similar at the end of the third week.

(c) Alpha-amylase in bile:

The initial alpha-amylase activity in bile was 1032 $\pm$ 259 uM/ml/min (Fig. 6.5). Feeding carp with raw wheat in the diet for one week did not significantly increase ($p > 0.05$) the amylase activity. However, after two weeks a significant ($p < 0.05$) increase to 2929 uM/ml/min was observed in the activity levels in this treatment and the activity remained significantly higher ($p < 0.05$) than the initial level to the end of the trial.

By contrast, in carp receiving Autoclaved wheat Diet, there was a significantly large ($p < 0.05$) increase in amylase activity to a mean of 2519 $\pm$ 866 uM/ml/min at the end of the first week which continued to be high

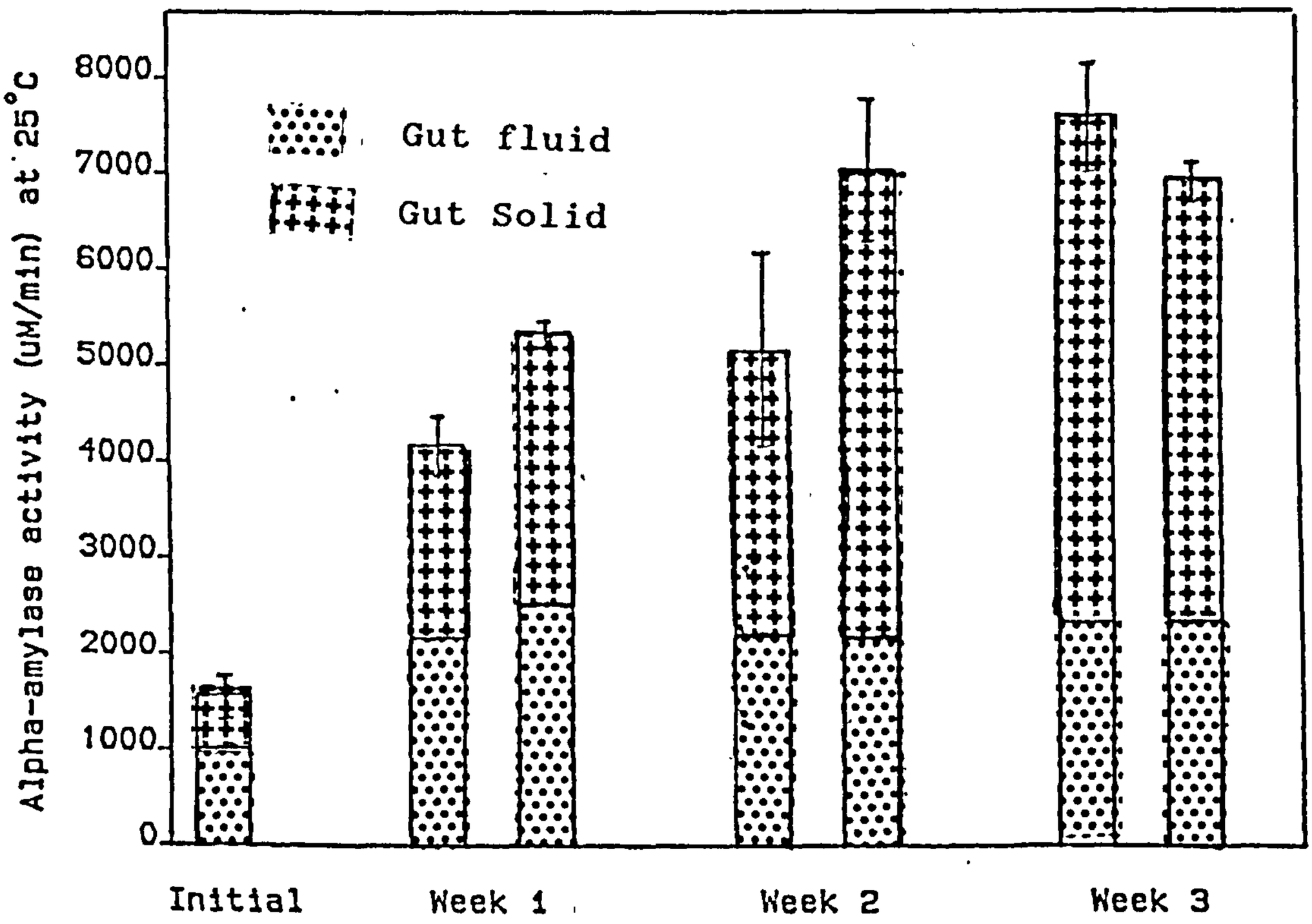
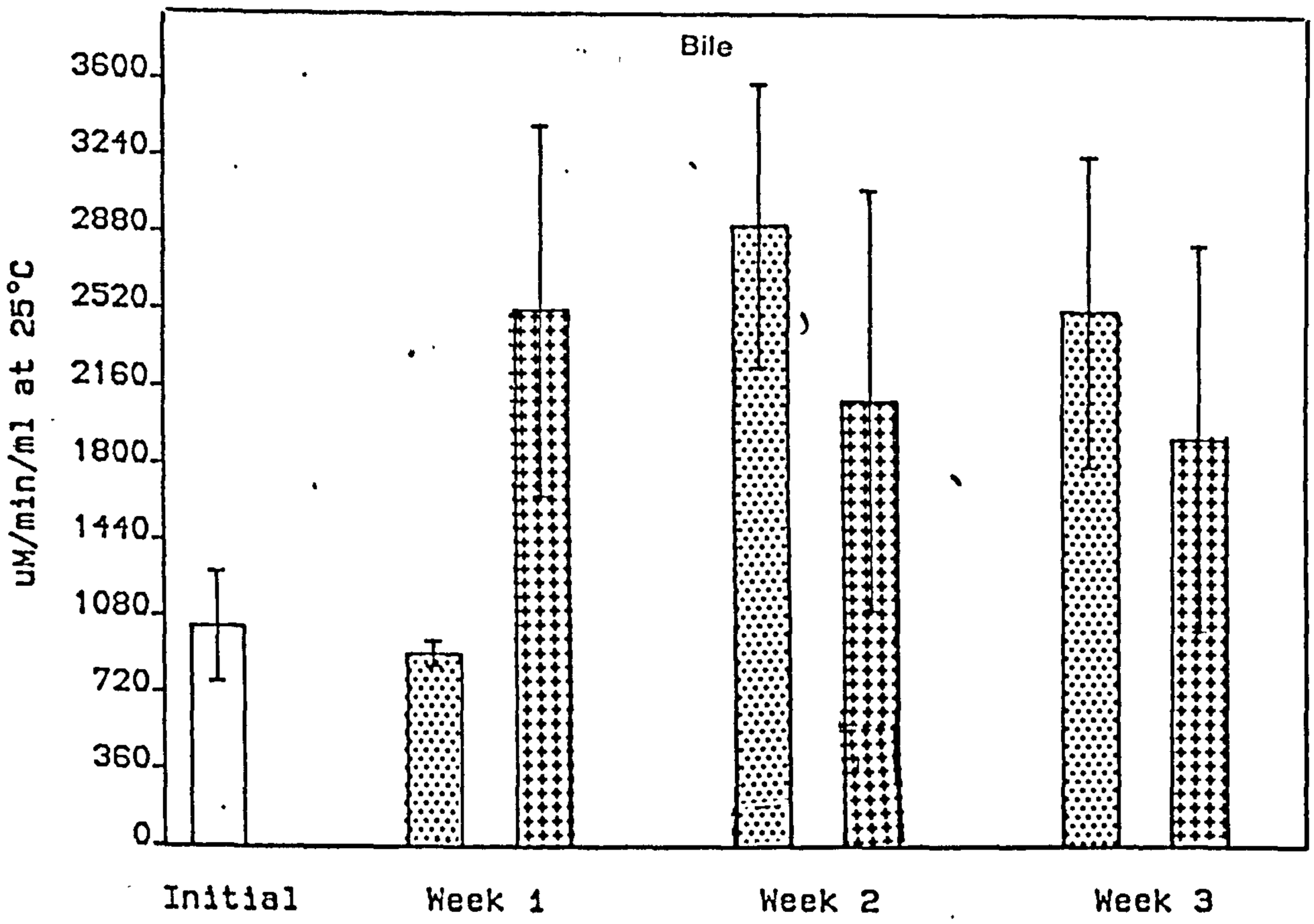


FIGURE 6.4 Total alpha-amylase activity in gut contents (fluid + solid) of carp, five hours after feeding on Raw wheat and Autoclaved wheat Diets.



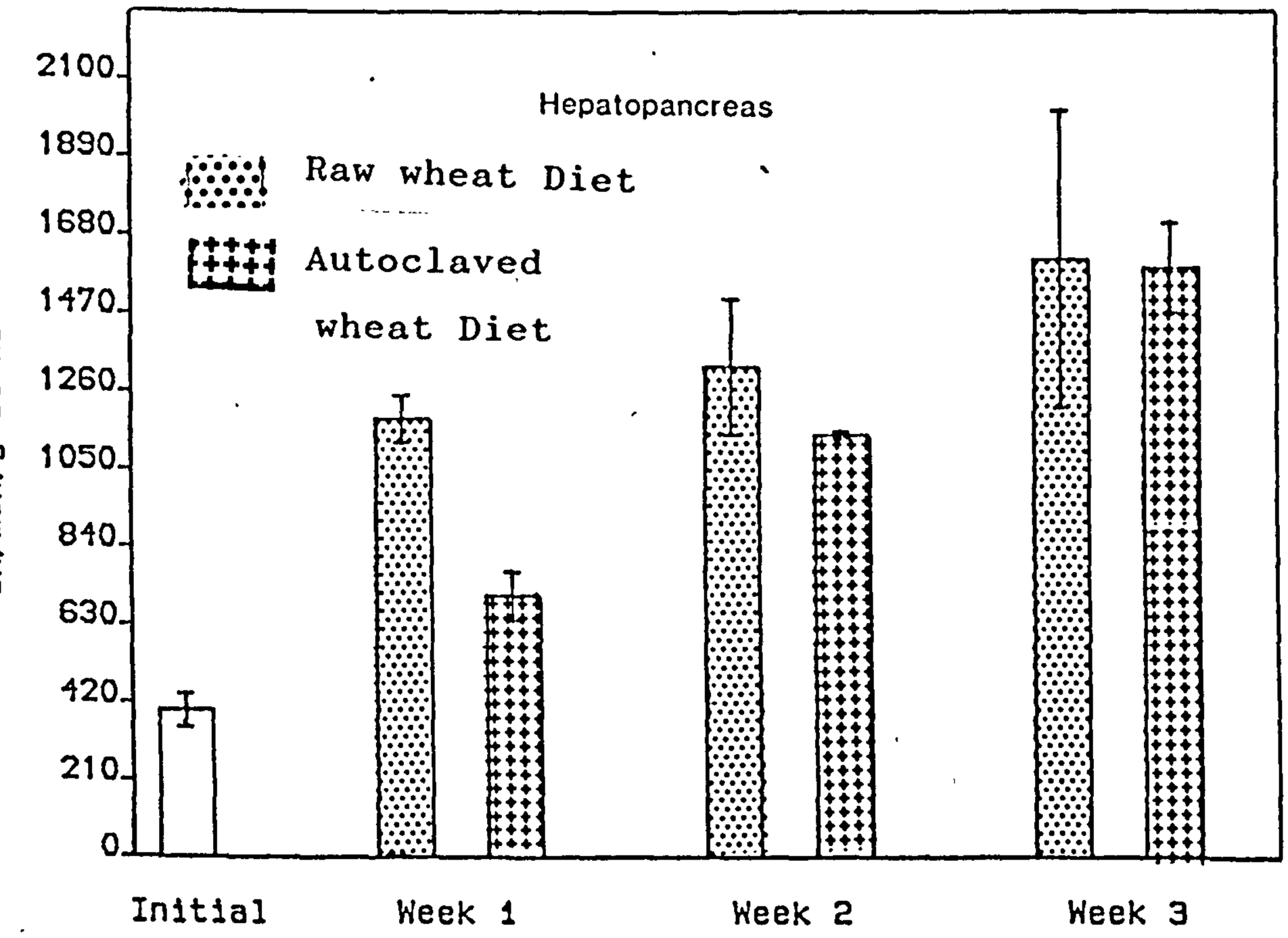


FIGURE 6.5 Alpha-amylase activity in bile and hepatopancreas of carp, five hours after feeding on Raw wheat Diet and Autoclaved wheat Diet.

throughout the three week experimental period. The difference in mean weekly alpha-amylase activities between the two treatments was significant only at the end of Week 1 ($p < 0.10$).

Comparing the weekly mean specific activities of alpha-amylase in bile (Table 6.6) between the two treatments (Table 6.6), it was seen that the activity at the end of Week 1 in fed raw wheat was very low ($p < 0.05$) compared to the corresponding activity in the other treatment. However, at the end of Weeks 2 and 3, the specific activities of fish fed raw wheat were higher than in those fed autoclaved wheat, although the difference was not significant ($p > 0.05$).

(d) Alpha-amylase in hepatopancreas:

The initial level of alpha-amylase activity recorded in hepatopancreas was 399 $\pm$ 45 uM/min/g (Fig. 6.5). When the fish were fed the test diets, the trend seen in activity levels was distinctly different from the trend in the gut contents or bile.

In fish fed raw wheat, the amylase activity in the hepatopancreas at the end of the first week had significantly increased ($p < 0.05$) to about three times the initial level. By contrast, in fish receiving autoclaved wheat the activity level at the end of Week 1 was only less than twice the initial level. Statistically this difference between the two treatments was significant (p

<0.05). However, by the end of Week 2, the amylase activity in the Autoclaved wheat Diet treatment had also increased, reducing this difference considerably and finally there was no difference between the two groups of fish after three weeks (p >0.05).

The trend in the amylase specific activity (Table 6.6) was similar to the trend described above. Statistically the mean values between the treatments were significant after Week 1 (p <0.05) but not after Weeks 2 and 3 (p >0.05).

(e) Alpha-amylase in gut tissue:

The amylase measured from samples of intestine had much lower levels of activity than that from the hepatopancreatic tissue (Fig.6.6), but the increasing trend noted in the hepatopancreas was again apparent. In both groups of fish, there was a gradual and significant (p <0.05) increase in the intestinal tissue amylase activity during the three week trial to a maximum level at the end of the third week. The difference in weekly means between the two treatments however, was only significant (p <0.05) at the end of Week 2 when the fish fed Raw wheat Diet groups exhibited lower activity levels than those fed Autoclaved wheat Diet.

The amylase specific activity followed a trend similar to that described above. ANOVA of the weekly means (Table 6.6) revealed that the differences between

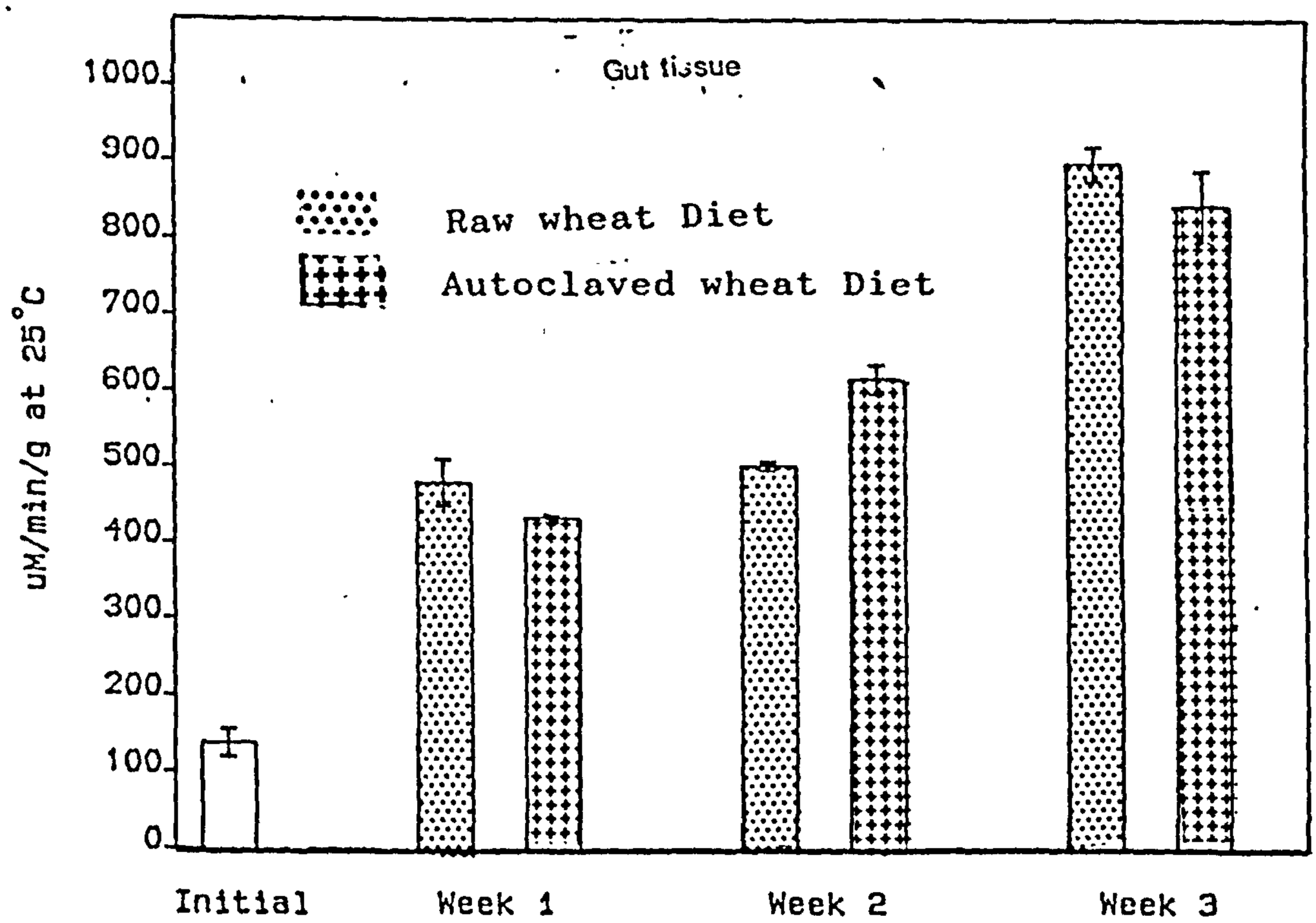


FIGURE 6.6 Alpha-amylase activity in the intestinal tissues of carp, five hours after feeding on Raw wheat Diet and Autoclaved wheat Diet.

the two treatments were not significant ($p > 0.05$). With the exception of the increase in specific activity in fish fed Raw wheat Diet between Weeks 1 and 2, increases in weekly means within each treatment were significant at the 95% level ($p < 0.05$).

6.3.10.2 Total protease activity in gut contents.

(a) Proteases in gut fluids:

The total protease activity assayed in samples of gut fluid from fish at the start of the experiment was 12.67 $\pm$ 1.75 uM/ml/min at 25°C. In the groups of fish fed with the Raw wheat Diet, there was a gradual increase in protease activity level over the 3 week trial (Fig. 6.7) and the maximum activity of 18.62 uM/min/ml was recorded at the end of the third week. In fish fed on autoclaved wheat however, the activity increased very sharply at the end of Week 1 to 18 uM/min/ml and thereafter fluctuated between 17.55 and 19.00 uM/min/ml during the next two weeks. The activity levels recorded in the two treatments after Week 1 were significantly ($p < 0.05$) different.

A similar trend was seen in the weekly mean specific protease activities (Table 6.7). In both treatments the increase at the end of the first week was significantly ($p < 0.05$) higher than the initial value but thereafter there was no significant increase. Furthermore there was no significant difference between the two

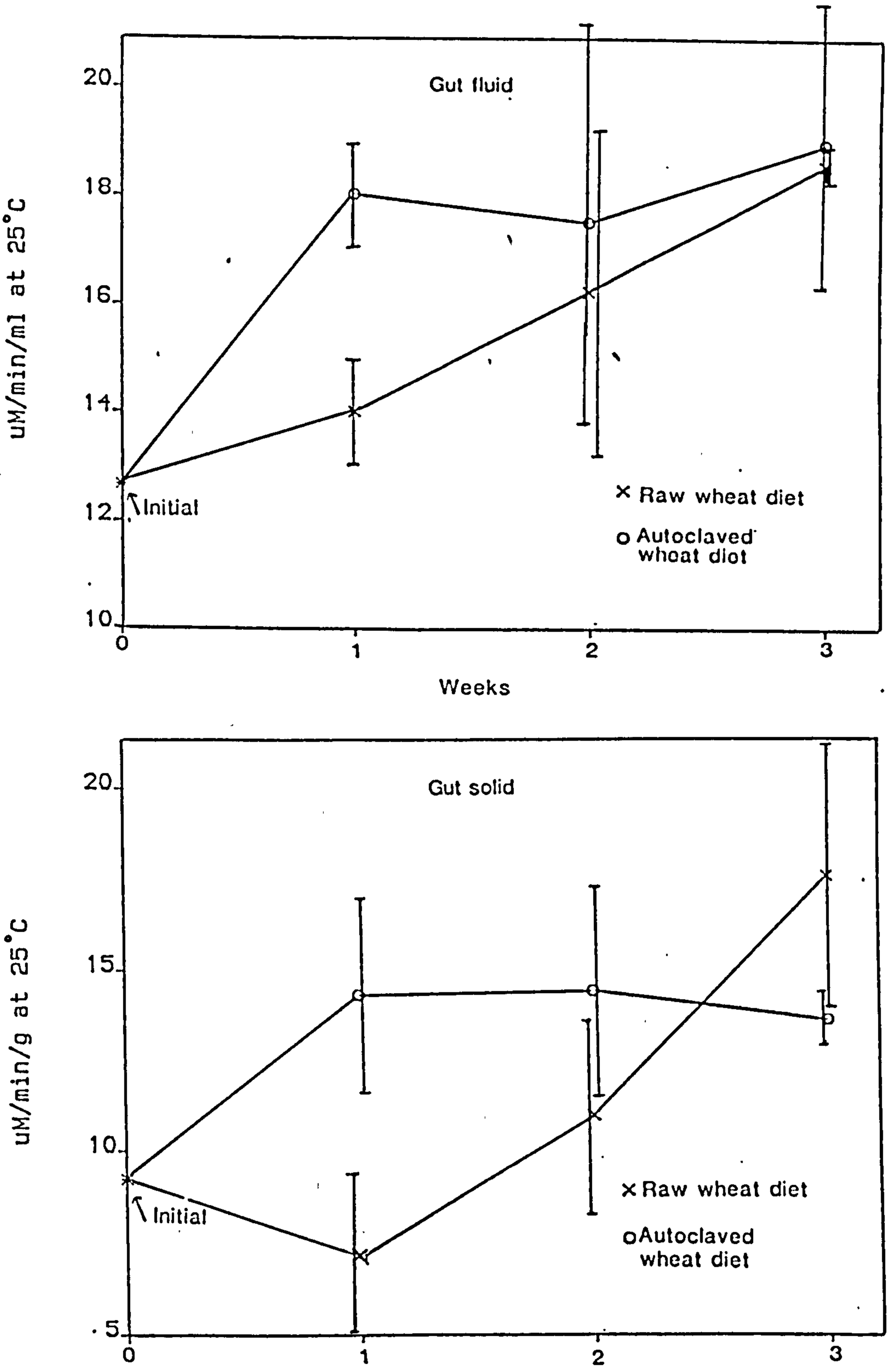


FIGURE 6.7 Total proteolytic activity in the gut fluid and gut solid of carp, five hours after feeding on Raw wheat Diet and Autoclaved wheat Diet. (vertical lines indicate SE)

Table 6.7 Protease specific activity in gut fluid and gut solid at 25°C in carp fed Raw wheat Diet and Autoclaved wheat Diet. (Mean ± SE).

Diet	Week	Gut fluid uM/mg/min	Gut solid uM/mg/min
Initial		0.26 ± 0.03^{abcdef}	0.19 ± 0.04^{abcd}
Raw wh.	1	0.42 ± 0.03^{a}	0.25 ± 0.07
Auto wh.		0.46 ± 0.02^{d}	0.43 ± 0.07^{b}
Raw wh.	2	0.49 ± 0.06^{b}	0.36 ± 0.09
Auto wh.		0.48 ± 0.08^{e}	0.41 ± 0.07^{c}
Raw wh.	3	0.58 ± 0.05^{c}	0.51 ± 0.11^{a}
Auto wh.		0.56 ± 0.03^{f}	0.41 ± 0.03^{d}

* values in each column with same superscript are significantly different ($p < 0.05$).

treatments.

(b) Protease in gut solids:

Initially the protease activity in gut solids was 9.22 uM/min/g (Fig. 6.7). In carp fed Raw wheat Diet, after an initial small and insignificant ($p > 0.05$) fall in activity at the end of the first week, the protease activities increased over the following two weeks to a maximum level of 17.62 uM/min/g at the end of the third week, which was significant ($p < 0.10$) at the 90% level of confidence when compared to the initial level. By contrast, in the fishes fed diet containing autoclaved wheat, the activity reached a maximum level of 14.35 uM/min/g after the first week and remained around this level for the duration of the trial. The difference between the two treatments at the end of each week however were not significant ($p > 0.05$).

The trend in protease specific activities in gut solid samples were also similar to the trend described above (Table 6.7). The initial activity was significantly ($p < 0.05$) lower than all the weekly mean activities recorded in fish from the Autoclaved wheat Diet treatment. However in the Raw wheat Diet treatment, only the final activity at the end of Week 3 was significantly ($p < 0.05$) different from the initial level. The Week 2 level in this treatment was significantly different from the initial value only at 90% level of confidence (p

<0.10). Again the weekly mean activity levels between the two treatments were not significantly different.

6.3.11 Nutrient digestibility.

Carp fed on diet containing raw wheat gave an apparent hydrolysable carbohydrate digestibility value of 88.4%, while the value from fish fed on the diet containing autoclaved wheat was 92.9%.

Unlike the carbohydrate digestibilities, the protein digestibility was lower in fish fed autoclaved wheat in the diet with a value of only 63.7% when compared to that in the fish fed on Raw wheat Diet where the apparent protein digestibility value was 77.3%.

6.4 DISCUSSION.

In this experiment mirror carp fed diets containing raw wheat grew at a rate of only 0.43 %/day during the first week of the trial (Fig. 6.3) but by the end of the second week, the growth rate had increased to a level similar to that of the fish fed Autoclaved wheat Diet. In the previous experiment with mirror carp (Chapter 4), there had been no improvement in growth rate in fish fed diet containing active inhibitor even after three weeks. This clearly indicates that the growth-retarding effect of the amylase inhibitor is more pronounced when incorporated in diet after extraction from the grains but markedly less effective when fish are fed whole wheat.

When ground wheat in the diet enters the fish lumen a process similar to that in germinating seeds might be expected to occur. Amylase inhibitor albumin in wheat grain is closely associated with the starch granules (Kneen and Sandstedt, 1946; Sandstedt and Beckord, 1946; Saunders, 1975). In addition to providing protection against insect pests of wheat (Silano et al., 1975), it also acts as a storage protein (Varner, 1965; Kasarda et al., 1971). During the process of seed germination these albumins absorb large amounts of water and swell up, which causes them to separate from the starch granules and exposes the starch to the activity of beta-amylase in the germinating seedling, while the albumins are themselves

broken up by plant proteases (Buonocore et al., 1977). This whole process is obviously slower than when semipurified inhibitors are present in the diet which would be able to act directly on, and bind, with the alpha-amylase present in the fish gut lumen.

Raw wheat diet contained amylase inhibitory activity when tested by the in vitro methods after extraction from the diet (Table 6.3a & b). However, the amount of inhibitor present at any one time in the gut may not have been large enough to significantly reduce the high alpha-amylase activities recorded in the gut contents (Fig. 6.6). On average the mirror carp (50g b.w) in this experiment were fed approximately 0.75g of Raw wheat Diet per instalment, which contained about 100 Units of inhibitor (see Section 6.3.1) capable of reducing only 200 amylase Units by 50%. By comparison, 0.75g of gut contents from carp in this experiment had almost 10 times (approximately 1500 - 2000 uM/min) more amylase activity.

Elevated plasma glucose was noted in both treatments with the maximum level being recorded at the end of the first week in both treatments. As the experiment progressed, the magnitude of this hyperglyceamia after each successsive sampling, reduced. Anaesthesia, handling and weighing can elicit the secondary stress response of elevated blood glucose in fish (Flos et al., 1988). The similarity of plasma glucose levels observed in both the dietary treatments

indicates that the effect of amylase inhibitor in the Raw wheat diet was minimal.

Other non-enzymatic parameters recorded in the experiment such as hepatosomatic index, gut-somatic index, volumes of gut fluid and bile, gut fluid pH or the weights of solids in the intestine did not differ significantly ($p > 0.05$) between the two treatments (Table 6.5).

The alpha-amylase activity in the gut fluids and solids increased when carp were fed the two test diets. This substrate-induced increase was more than double the initial level in gut fluid and 4 to 5 times the initial level in the gut solids (Fig. 6.5). In total, the alpha-amylase activity in the gut contents had increased almost three fold at the end of the first week of the experiment (Fig. 6.6). Between the two treatments there was no significant difference ($p > 0.05$) in the magnitude of amylase activity.

The activity of the alpha-amylase in hepatopancreas was enhanced during the first week of the experiment in fish fed raw wheat. The activity in this treatment was 68% higher than in the fish fed Autoclaved wheat Diet ($p < 0.05$; see Fig. 6.7). The activity in the intestinal mucosa was not significantly different from that of the fish fed Autoclaved wheat Diet (Fig. 6.8). The bile in fish fed raw wheat also exhibited low activity levels at the end of the first Week (Fig.6.7). This clearly indicates that all the amylase enzyme secreted by the

hepatopancreas was being released into the gut lumen in order to replenish and maintain sufficient activity levels necessary to digest the feed.

Feeding antitrypsin inhibitors from soyabean to animals also elicits hyperactivity of the pancreas and sometimes even results in enlargement and an increase in weight of this organ (Chernick et al., 1948; Booth et al., 1960; Konijn and Guggenheim, 1967; Schingoethe et al., 1970). In the present study no increase in hepatosomatic index was recorded, despite the hyperactivity of the hepatopancreas.

The results of this experiment agree very closely with the findings of Sturmbauer and Hofer (1986) who reported that there was no difference in the total amylase activity in the gut fluids from carp fed either solely on ground wheat or extruded wheat. However, by dissociation and estimation of the bound amylases in the gut fluid, they were able to state that the actual secretion of amylase in carp fed raw wheat is 3 to 4 times higher than that of fish fed extruded wheat. Unfortunately the period elapsed between the last meal of the experimental fish and the collection of samples was not stated. It is possible that the samples were collected soon after the fish were fed since the inhibitors are likely to be destroyed by the proteases which are active through out the gut lumen of carp. It has been shown that total inactivation of this inhibitor by proteases can

occur in less than 2 hours (Shainkin and Birk, 1970).

Determination of total proteolytic activity in the gut contents of carp fed the two test diets showed that the fish fed autoclaved wheat had attained a maximum level of activity of this enzyme by the end of the first week (Fig.6.9). By contrast, the level in those fed on Raw wheat Diet was about 35% lower at the end of the first week but this gradually increased to a comparable level by the 3rd week. About 1% of the proteins in raw wheat are reported to have anti-trypsin activity (Shyamala and Lyman, 1964). Channel catfish and trout proteases are sensitive to soybean trypsin inhibitors (Sandholm et al., 1976; Robinson et al., 1981), but there has been no work to date to determine whether wheat trypsin inhibitors are active against fish proteases. It is possible that carp proteases are sensitive to trypsin inhibitors in wheat, and if this were the case then some of the proteases in the gut content samples from carp fed Raw wheat Diet could have been in the inactive bound form and not detected in the assay. The wheat used for preparing the Autoclaved wheat Diet could not have contained any active trypsin inhibitors, since they are heat labile (Shyamala and Lyman, 1964) and thus would have been destroyed by autoclaving.

Heat treatment of wheat by autoclaving appears to have denatured the amylase inhibitor protein contained in it (Table 6.3a, b). This was confirmed when the proteins

extracted from the Autoclaved wheat diet did not exhibit any amylase inhibitory activity when tested by the in vitro method (see Section 6.3.1). This treatment also resulted in improving the digestibility of the starch present in the wheat. The carbohydrate digestibility of fish fed Autoclaved wheat Diet was 92.9% compared to only 88.4% in fish fed Raw wheat Diet. Lang et al. (1974) showed that the presence of amylase inhibitor albumin in the diet of rats reduced the availability of starch for digestion and the carbohydrate content of the faeces consequently increased.

Autoclaving starch using wet steam, results in gelatinization due to the swelling and hydration of the starch granules. Gelatinized starch is more easily digested and utilised by fish compared to raw starch (ADCP, 1980). Heat treatment of feed ingredients which contain heat-labile anti-nutritional compounds by a process similar to autoclaving, is recommended before feeding to animals and fish (NRC, 1983). It must be noted that gelatinization of starch in any feed ingredient such as wheat, is a function of the temperature, duration of heating, moisture conditions, particle size etc., and that these factors also determine the extent of inactivation of the anti-nutritional factors. For example, Bessho and Kurosawa (1967) reported the presence of amylase inhibitor in the centre of bread loaves, despite the high temperatures of the oven. Rackis (1965)

reported that 50% - 100% inactivation of the trypsin inhibitor in soybean can be achieved by heat processing. In most commercially toasted samples of soybean oil meal, 90% to 92% of the trysin inhibitor activity is destroyed (Kakade and Liener, 1973; Kakade et al., 1974). Many other authors have recommended heat processing to render the ingredient safe for inclusion in animal feeds, but in general, insufficient emphasis is placed on the fact that excessive heat, or even the level of heating required for total destruction of the antinutrient, may damage the nutritive value of the protein.

In the present experiment the autoclaving process was effective in inactivating all the inhibitors in wheat and also resulted in a slight improvement in carbohydrate digestibility (Section 6.3.11). However, the concurrent reduction in protein digestibility from 77.3% to 63.7% indicated that protein quality had deteriorated. It is possible that the growth of fish in this dietary treatment could have been improved had the nutritive value of the wheat protein not been damaged. Thus the absence of inhibitor activity is not always a guarantee that the product after heat treatment will be of optimal nutritional quality. Complete destruction of inhibitors may not be necessary to attain maximum growth or feed efficiency. Rackis et al. (1975) determined the biological threshold level in rats of trypsin inhibitory activity in soybean meal that had been heat processed to provide

varying degrees of destruction of the trypsin inhibitor. Maximum body weight and protein efficiency values were obtained with rats fed soybean in which about 80% of the inhibitor was inactivated. No pancreatic hypertrophy occurred in rats fed soy flour in which 50 - 60% of the inhibitor activity had been destroyed.

This experiment thus indicates that when raw wheat is fed to mirror carp, the amylase inhibitor albumin contained in it elicits a response in mirror carp in the form of hyperactivity of the pancreas and some reduction in growth rate for a short period. The digestibility of the carbohydrate in the diet containing raw wheat was lower than that attained by the fish fed the Autoclaved wheat Diet. The results also indicate that when mirror carp are fed diet containing 37% ground wheat, adaptations to the amylase inhibitor apparently occur within 7 to 14 days.

However the point that remains to be clarified, is whether the presence of raw starch itself in the fish diet could have influenced the increase in amylase activities. If this were the case, then the changes in amylolytic activity recorded in this experiment may not have been due solely to the presence of inhibitor proteins in the diet. Gelatinized starches are more easily digested by amylases because of the greater affinity between them. Hence it is not surprising to see reports (ADCP, 1980) that cooked maize and wheat starch are utilised much better by fish

such as channel catfish and rainbow trout than raw starches. In order to clarify this point, it would be necessary to record changes in alpha-amylase activity in carp fed isonitrogenous diets containing raw or autoclaved starch.

CHAPTER 7

EFFECTS OF FEEDING RAW AND AUTOCLAVED WHEAT STARCH ON THE ALPHA-AMYLASE ACTIVITY IN MIRROR CARP.

CHAPTER 7

EFFECTS OF FEEDING RAW AND AUTOCLAVED WHEAT STARCH ON THE ALPHA-AMYLASE ACTIVITY OF MIRROR CARP.

7.1 INTRODUCTION.

Hydrolysis of starch by alpha-amylase depends on the relative proportions of amylose and amylopectin in the starch granule (ADCP, 1980). The amylase activity in fish also depends on the solubility of starch as shown by Spannhof and Plantikow (1983). Partially hydrolysed polysaccharides such as dextrin are better utilised by fish than native starch (Singh and Nose, 1967; Anderson *et al.*, 1984).

The crystalline structure of starch also appears to influence its attack by amylase as demonstrated by the two-fold increase in metabolizable energy content of fully cooked and gelatinized maize in feeding trials with channel catfish (ADCP, 1980). The swelling and hydration of the starch granules when heated with water, loosens the hydrogen bonds of the starch molecule and it loses its crystalline nature. Such gelatinized starches have greater affinity for amylase enzyme compared to raw starch.

The positive effects of cooking on starch digestibility and utilisation have been well established and documented in salmonids (Phillips *et al.*, 1940,

1948; Inaba et al., 1963; Pieper, 1977; Bergot and Breque, 1983), carp (Chiou and Ogino, 1975), eel (Spannhof, 1976; Schmitz et al., 1982 cited by Degani and Viola, 1987) and yellowtail (Shimeno et al., 1977).

Thus there is ample evidence to demonstrate that gelatinized starch is preferable to raw starch for use in fish diets. However, there is relatively little work comparing the nature of starch with amylase secretion and activity in fish. A notable exception is the work of Spannhof and Plantikow (1983) who compared soluble and insoluble starch with reference to amylase activity in trout.

Bergot and Breque (1983), again working with trout, stated that alpha-amylase hydrolysis occurs more rapidly with gelatinized starch compared to native starch. They arrived at the conclusion that raw starch is difficult to hydrolyse by alpha-amylase because they found that the products of hydrolysis are released slowly in trout fed raw starch compared to gelatinized starch.

In the literature surveyed, there is no direct reference comparing amylase activity levels induced by gelatinized starch and raw starch, in any species of fish.

In Chapter 6 it was seen that heat processed wheat induced comparatively lower amylase activities than in mirror carp fed raw wheat in the diet for a period of a week. This was attributed to the response of the fish to

the presence of amylase inhibitor proteins in raw wheat. During heat processing of powdered wheat to inactivate the amylase inhibitor, the starch contained in it would have undergone the process of gelatinization. It is not known exactly how much of the differences recorded in the amylase activity levels in the two groups of fish in that experiment (Chapter 6) was due to the gelatinous or raw nature of the wheat starch. Therefore the objective of the present experiment was to differentiate the influence of autoclaved and raw starch on alpha-amylase activities in mirror carp.

7.2 MATERIALS AND METHODS.

7.2.1 Experimental fish.

Six experimental tanks were stocked with mirror carp selected from the stock system at a rate of 11 fish per tank. The fish used, ranged from 30 to 46 grams in weight (mean 37.46 $\pm$ 4.56g) and they were distributed randomly into the tanks to form groups of approximately uniform weight. The fish were acclimatized to this system for 10 days during which period they were fed the holding ration (Appendix I) at a rate of 3% body weight in two instalments.

7.2.2 Experimental diets.

Two diets were prepared using unmodified wheat starch (Sigma) as the sole carbohydrate source. One diet contained 25% raw wheat starch, while the other contained a similar proportion of autoclaved (121°C) and dried (35°C) starch. All the other ingredients used were the same as in the previous experiment which is detailed in Chapter 6. The proportions of the dietary ingredients used for preparation of the diets and their compositions can be found in Table 7.1 and Table 2.5 (Chapter 2), respectively. The two diets namely, 'Raw starch Diet' and 'Autoclaved starch Diet' were prepared using the

Table 7.1 Diet formulae of the test diets used in the experiment. (moisture free %).

Ingredients	Raw starch Diet	Autoclaved starch Diet
Fishmeal (batch 1)	39.80	39.80
Wheat starch (raw)	25.00	----
Wheat starch (autoclaved)	----	25.00
Alpha-Cellulose	21.45	21.45
Carboxymethyl Cellulose	1.00	1.00
Cod-liver oil	2.55	2.55
Corn oil	3.70	3.70
Mineral mix	4.00	4.00
Vitamin mix	2.00	2.00
Chromic oxide	0.50	0.50
Total	100.00	100.00

procedures followed for the earlier experiment in Chapter 6 (Section 6.2.2). The diets were analysed for their proximate composition but not tested for the presence of amylase inhibitors. The composition of the diets is tabulated in Table 7.2.

7.2.3 Experimental protocol.

The protocol detailed in Section 6.2.3 of Chapter 6 was repeated for this experiment using the two diets described above. Of the six groups of carp, three were fed Raw starch Diet and the other three were given the Autoclaved starch Diet at a rate 3% body weight, daily. The sampling schedules, number of fish sampled and procedures of sample collection were same as in Chapter 6. The parameters monitored in this experiment were plasma glucose, HSI, gut-somatic index, gut fluid pH and volume, gut solid weight, bile volume, alpha-amylase activities in samples of gut contents, hepatopancreas, bile and gut tissue, and protease activity in samples of gut contents. Carbohydrate and protein digestibilities were determined from the faecal samples collected during the last two weeks of the three week experiment. All the analytical procedures adopted for analysing the samples collected in this experiment are described in Chapter 2.

Table 7.2 Proximate compositions of the starch diets used in the experiment (mean ± SD, n=3).

Moisture free %	Raw starch Diet	Autoclaved starch Diet
Moisture	4.53 ± 0.11	4.64 ± 0.14
Crude protein	31.89 ± 0.61	31.19 ± 0.14
Crude lipid	8.37 ± 0.24	8.48 ± 0.33
Total ash	10.62 ± 0.11	10.50 ± 0.17
Crude fibre	17.99 ± 0.39	17.90 ± 0.53
Hydrolysable carbohydrate	23.83 ± 0.39	22.97 ± 0.46
Soluble carbohydrate*	31.12	31.93
Chromic oxide	0.47 ± 0.002	0.47 ± 0.007

* calculated by difference (see Chapter 2, Section 2.9.1.7)

7.3 RESULTS.

7.3.1 Fish weight.

After the 10 day acclimatization period there were no significant differences ($p > 0.05$) in the mean weights of the fishes in the six groups. During the experimental period the fish fed the two types of starch did not differ in their growth and the weekly fish weights in the two treatments were not significantly different when tested by ANOVA ($p > 0.05$) (Table 7.3). Carp fed Raw starch Diet attained a mean final weight of 54.9 $\pm$ 9.0g after three weeks while the final weights in fish fed autoclaved starch was slightly lower with a mean of 51.9 $\pm$ 7.0g, however this difference between the two treatments was not statistically significant ($p > 0.05$).

7.3.2 Plasma glucose.

On feeding both test diets there was a significant ($p < 0.05$) increase in plasma glucose concentration from the initial level of only 85 mg/dl (Fig. 7.1). In the groups fed raw starch the plasma glucose levels increased to 136 mg/dl by the end of the first week and thereafter fell to 119 mg/dl by the end of the trial. The trend in fish fed autoclaved starch was similar with a slightly higher maximum value of 149 mg/dl at the end of the first

Table 7.3 Mean weight (± SD) of mirror carp fed Raw starch Diet and Autoclaved starch Diet.

	Raw starch Diet	Auto. starch Diet	ANOVA 'p'
Initial	46.46 ± 6.11	47.72 ± 5.27	>0.05
Week 1	47.22 ± 6.03	48.27 ± 4.87	>0.05
Week 2	50.43 ± 7.34	50.54 ± 5.64	>0.05
Week 3	54.85 ± 9.00	51.87 ± 6.97	>0.05

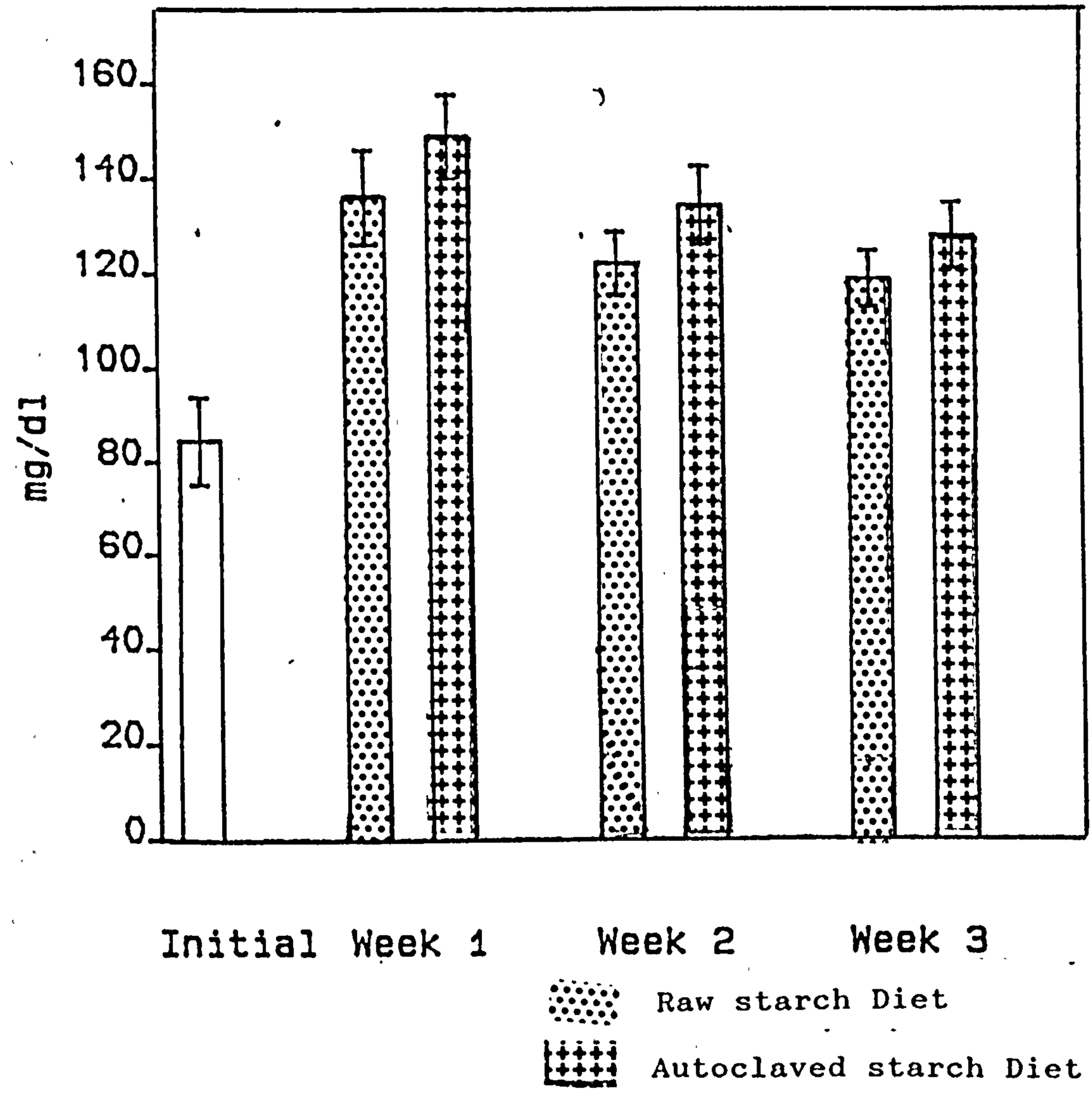


FIGURE 7.1 Plasma glucose levels in carp fed Raw starch Diet and Autoclaved starch Diet.

week followed by a gradual drop to 128 mg/dl by the end of the trial. At the end of each week, the glucose levels were slightly higher when the fish were fed autoclaved starch when compared to those fed on raw starch, however this difference between treatments was not significant (p >0.05).

7.3.3 Hepatosomatic index.

At the end of the first week there was an insignificant (p >0.05) drop in HSI of fish fed raw starch from 1.74 to 1.57 (Table 7.4). Thereafter the index remained very constant. When the fish were fed autoclaved starch, there was no significant change in HSI at the end of the first week but in subsequent weeks the index fell gradually to the significantly (p <0.05) lower value of 1.49 at the end the third week. The weekly mean HSI was not significantly different between the two treatments.

7.3.4 Gut-somatic index.

There was a significant (p <0.05) reduction in the gut-somatic index on feeding both test diets (Table 7.4). At the end of the first week the gut-somatic index had fallen from an initial value of 3.23 to 2.45 in those fed raw starch and to 2.53 in those fed autoclaved starch in the diet. There were no further significant changes in

Table 7.4 Hepatosomatic index, gut-somatic index and gut fluid pH in mirror carp fed Raw starch Diet and Autoclaved starch Diet.

Diet	Week	Hepatosomatic index	Gut-somatic index	Gut fluid pH
Initial		1.74(0.09)[a]	3.23(0.18)[abcdef]	6.85(0.10)
Raw starch	1	1.57(0.07)	2.45(0.11)[a]	6.79(0.07)
Auto starch		1.77(0.10)	2.53(0.11)[bg]	6.71(0.05)[ab]
Raw starch	2	1.57(0.07)	2.52(0.16)[c]	6.95(0.07)
Auto starch		1.64(0.80)	2.40(0.08)[dh]	7.01(0.05)[a]
Raw starch	3	1.58(0.09)	2.17(0.07)[e]	6.89(0.13)
Auto starch		1.49(0.07)[a]	2.06(0.07)[fgh]	7.06(0.11)[b]

* values in parenthesis show the standard error.

** values in each column with same superscripts are significantly different ($p < 0.05$).

this parameter at the end of the second week but by the end of the trial the gut-somatic index again fell sharply and significantly to 2.17 and 2.06 in fish fed Raw starch and Autoclaved starch Diets, respectively. There were however, no significant differences between the two treatments.

7.3.5 Gut fluid pH.

The gut fluid pH was within the normal range of values for carp and did not change significantly ($p > 0.05$) during the three week trial (Table 7.4).

7.3.6 Gut fluid volume.

The fluid content in the intestines of the test fishes were significantly lower ($p < 0.05$) than the initial volume, throughout the three weeks of observation (Table 7.5). Between the two treatments however, there were no significant differences ($p > 0.05$) in weekly means.

7.3.7 Gut solid weight.

At the end of the first week of feeding the two test diets, gut solid weight five hours after feeding had fallen significantly ($p < 0.05$) in both treatments (Table 7.5). This level was maintained in fish fed raw starch,

Table 7.5 Gut fluid volume, gut solid weight and bile volume in mirror carp fed Raw starch Diet and Autoclaved starch Diet.

Diet	Week	G.fluid vol ml/100 g fish	G.solid wt g/100 g fish	Bile vol ul/100 g fish
Initial		1.18(0.08)^{abcd}	1.25(0.10)^{abcd}	35.6(10.8)
Raw starch	1	0.63(0.03)^{a}	0.78(0.04)^{ae}	57.5(10.2)
Auto starch		0.72(0.05)^{c}	0.95(0.06)^{ce}	51.5(7.7)
Raw starch	2	0.68(0.06)^{b}	0.78(0.04)^{b}	39.9(6.8)
Auto starch		0.75(0.05)^{d}	0.75(0.05)^{d}	36.3(6.3)
Raw starch	3	0.40(0.05)	1.01(0.07)	43.0(8.0)
Auto starch		0.44(0.02)	0.97(0.07)	42.8(5.0)

* values in parenthesis show the standard error.
** values in each column with same superscripts are significantly different ($p < 0.05$).

while in those fed autoclaved starch the gut solid weight continued to decrease significantly ($p < 0.05$) to a minimum level at the end of second week. Between the two treatments, the gut solid weights were significantly different ($p < 0.05$) at the end of the first week, but not at the end of the second week. Unfortunately due to the lower centrifugation speed (2000 rpm) used for separating the gut fluids from the solids at the end of the trial, the values obtained cannot be compared with those of the earlier weeks. However the values do show that there was no significant differences between the two treatments.

7.3.8 Bile volume.

The volumes of bile measured from individual fish varied widely. Consequently, despite increases from an initial value to 57.5 and 51.5 ul in fish fed Raw starch Diet and Autoclaved starch Diet (Table 7.5) at the end of first week, the differences were not significant ($p > 0.05$). Between the two dietary treatments there were also no significant differences in weekly mean values ($p > 0.05$).

7.3.9 Enzyme activity.

7.3.9.1 Alpha-amylases.

(a) Alpha-amylase activity in gut contents:

The alpha-amylase activity in the gut fluids was increased significantly ($p < 0.05$) by feeding starch to the fishes (Fig. 7.2). Both raw and autoclaved starch induced similar amounts of amylase activity in the gut fluids and comparison of the weekly mean activity did not show any significant ($p > 0.05$) differences between them.

The activities of enzymes eluted from the solid portions of the gut contents increased significantly ($p < 0.05$) from the initial mean activity level of 554 uM/g to 1283 uM/g and 1773 uM/g in fish fed raw and autoclaved starch, respectively at the end of the first week. Between the two treatments, the amylase activity was significantly higher ($p < 0.05$) at the end of the first week in fish fed autoclaved starch, although no significant difference was observed during the rest of the experimental period. Levels continued to increase significantly ($p < 0.05$) in both treatments over the three week trial and by the end of the third week they were six times the initial value.

The specific amylase activity in the fluid and solid portion of the gut contents exhibited a similar trend to that described above (Table 7.6).

In order to show the trend in the amylase activity

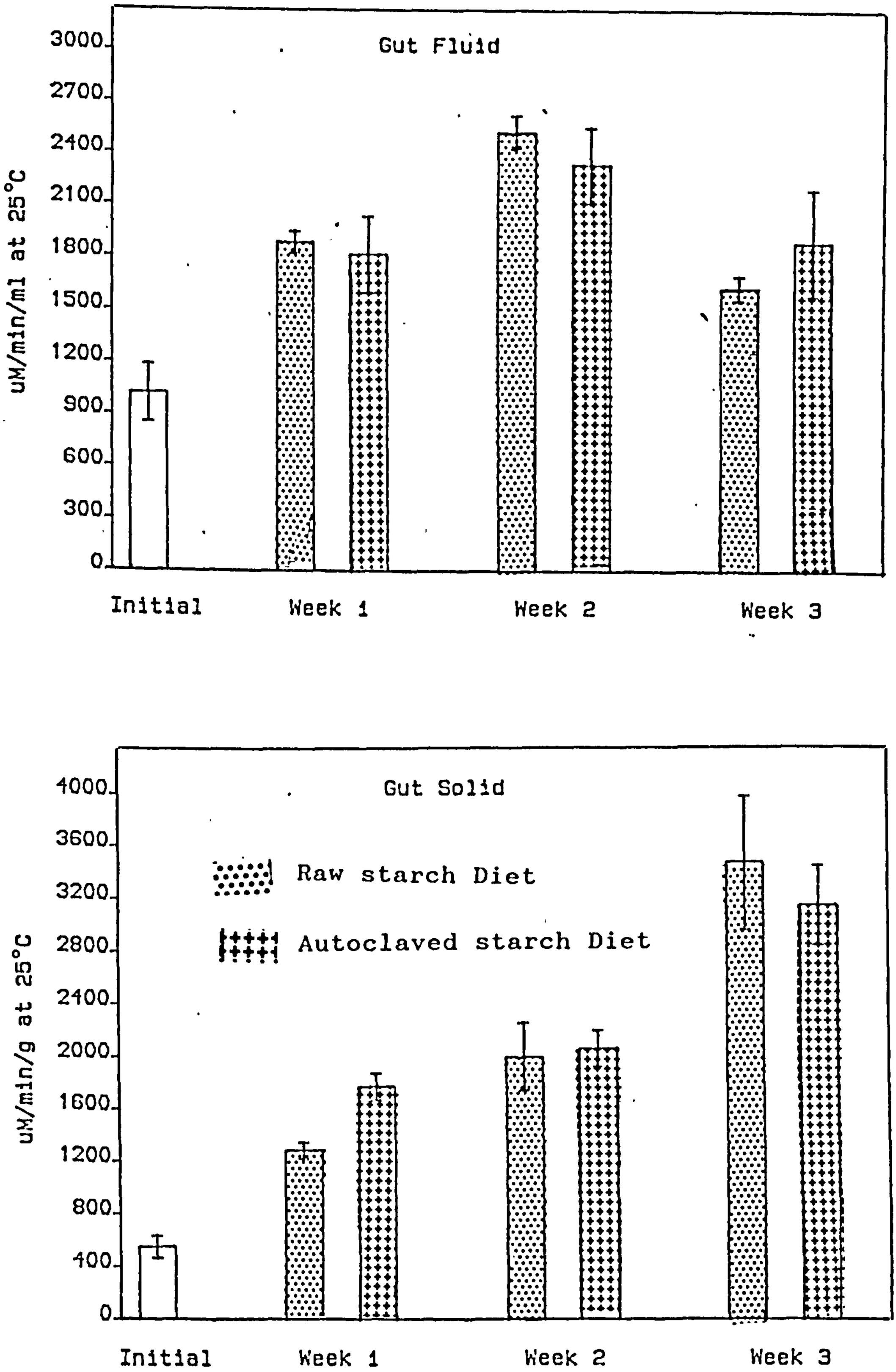


FIGURE 7.2 Alpha-amylase activity in the gut fluid and gut solid of carp, five hours after feeding on Raw starch Diet and Autoclaved starch Diet.

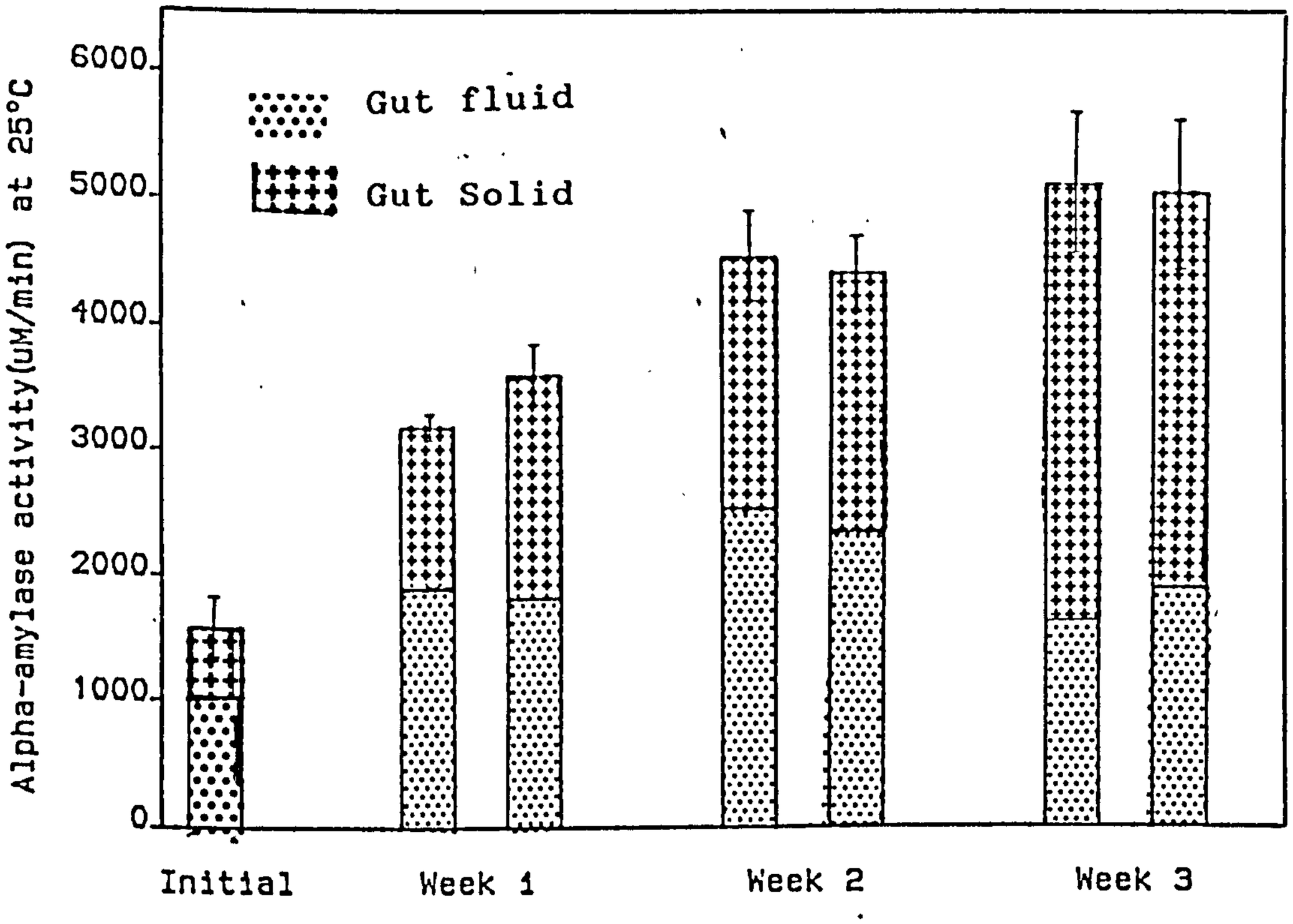


FIGURE 7.3 Total alpha-amylase activity in the gut contents (fluid + solid) of carp, five hours after feeding on Raw starch Diet and Autoclaved starch Diet.

Table 7.6 Alpha-amylase specific activity (uMol/mg/min) at 25°C in mirror carp fed Raw starch Diet and Autoclaved starch Diet.

Diet	Week	Gut fluid	Gut solid	Bile	Hepatopancreas	Gut tissue
Initial		21.30(3.31)abcdef	12.01(2.03)abcdef	9.27(1.35)abcde	5.67(0.69)abcdef	2.54(0.25)abcdef
Raw starch	1	50.21(1.19)ag	23.59(0.88)agh	12.53(1.16)	8.51(0.26)ag	8.92(1.03)a
Auto starch		47.22(4.15)d	41.74(6.06)di	15.15(1.09)cf	15.53(0.78)dh	7.18(0.36)d
Raw starch	2	62.00(3.80)bgh	50.56(8.50)bg	16>59(3.49)a	11.41(0.70)bg	9.44(1.29)b
Auto starch		61.77(5.48)e	50.40(5.54)e	16.26(0.95)d	11.17(1.06)eh	7.85(0.36)e
Raw starch	3	45.64(3.43)ch	70.70(12.40)ch	21.22(3.32)b	13.03(2.64)c	10.55(0.83)c
Auto starch		49.89(6.68)f	69.22(7.25)fi	20.81(2.59)ef	12.49(0.93)f	10.05(1.18)f

* values in parenthesis show the standard error.
** values in each column with same superscripts are significantly different ($p < 0.05$).

in the gut contents as a whole, the levels recorded in the fluid and solid portions were combined (Fig. 7.3). This demonstrated that the total amylase activity in the gut contents of fish from both dietary treatments doubled at the end of the first week and continued to increase over the three week experimental period to attain final levels which were approximately three times the initial value. Between the treatments, the total amylase activity of gut contents did not differ significantly ($p > 0.05$) at the end of each week.

(b) Alpha-amylase in bile:

In contrast to the trend seen in the gut contents, the amylase activity in the bile juice had decreased ($p > 0.05$) at the end of the first week in both treatments (Fig. 7.4). Thereafter activities increased and Week 1 and Week 3 values were significantly different ($p < 0.05$) in both the treatments. Between the two treatments the activity levels recorded at weekly intervals did not vary significantly ($p > 0.05$). The specific bile amylase activity values were always higher ($p < 0.05$) than the initial value, but did not vary significantly between the two starch diet treatments.

(c) Alpha-amylase in hepatopancreas:

The amylase activity in hepatopancreas increased significantly ($p < 0.05$) at the end of the first week when

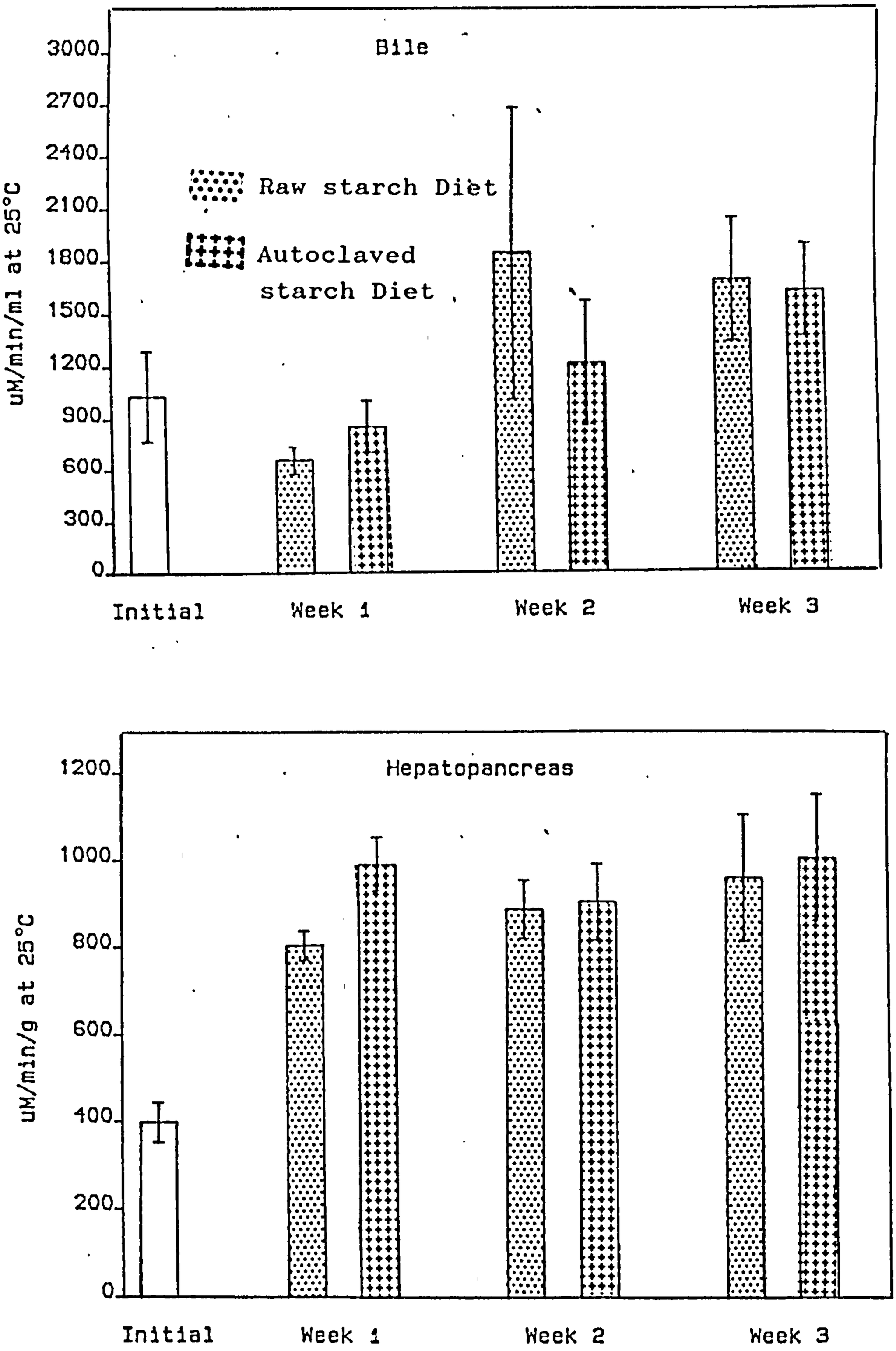


FIGURE 7.4 Alpha-amylase activity in the bile and hepatopancreas of carp, five hours after feeding on Raw starch Diet and Autoclaved starch Diet.

the fish were fed the two test diets (Fig. 7.4). Thereafter the activity remained at an high level in both treatments with little variation. Between Weeks 1 and 3, the differences in alpha-amylase activity in each treatment and also between the two treatments were insignificant ($p > 0.05$).

The specific amylase activity in the hepatopancreas followed a similar trend with higher values being recorded throughout the three week experimental period in both treatments compared to the initial level ($p < 0.05$) of 5.7 uM/mg protein (Table 7.6). In fish fed raw starch, the specific activity at the end of the first week was significantly ($p < 0.05$) lower than that at the end of the second week whereas in the case of fish fed autoclaved starch, the converse was recorded.

(d) Alpha-amylase in gut tissues:

The increasing trend seen in amylase activity of the gut contents and hepatopancreas was also recorded in the gut tissue samples (Fig. 7.5). Compared to the initial activity level, there was a significant ($p < 0.05$) three fold increase in both treatments. In addition within each treatment, the activity at the end of the third week was considerably higher than the levels at the end of Week 1. However, differences between the weekly means of the two treatment groups were again not statistically different ($p > 0.05$). The alpha-amylase specific activities in gut

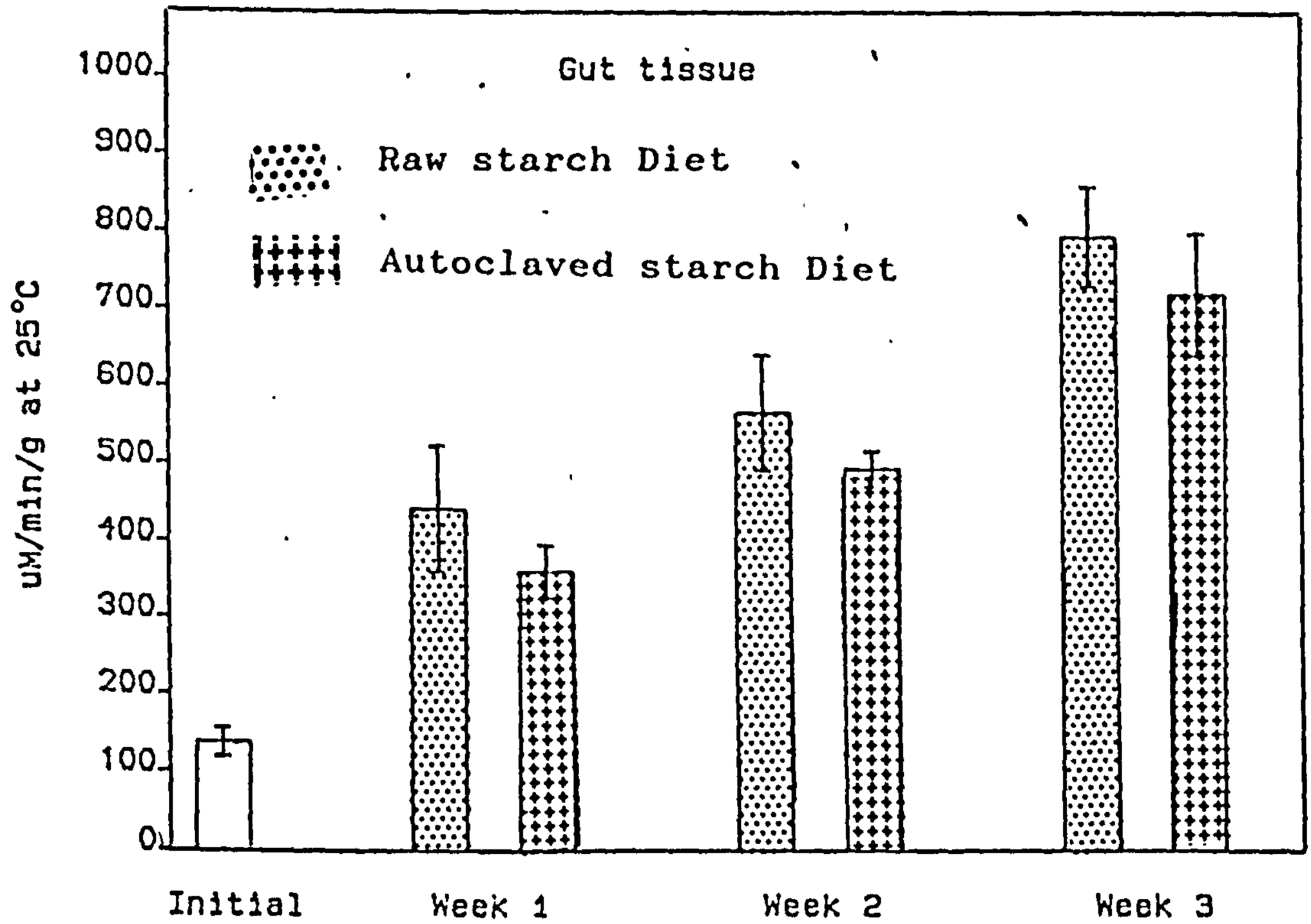


FIGURE 7.5 Alpha-amylase activity in the intestinal tissues of carp, five hours after feeding on Raw starch Diet and Autoclaved starch Diet.

tissues were also significantly higher than the initial level ($p < 0.05$), but when the weekly means are compared in each treatment the differences were not significant ($p > 0.05$).

7.3.9.2 Total protease activity in gut contents.

The activities of proteolytic enzymes in both the gut fluid and solid samples from fish fed raw and autoclaved starch had increased significantly ($p < 0.05$) on feeding the test diets by the end of the first week and remained high throughout the three week experimental period (Fig.7.6 and Table 7.7). The fluctuations seen during the three week period in each treatment were not statistically significant ($p > 0.05$). In addition, activities between the two dietary treatments did not differ significantly ($p > 0.05$).

7.3.10 Nutrient digestibility.

The apparent carbohydrate digestibility in the two treatment groups receiving Raw and Autoclaved starch Diets did not vary. The fish fed with raw starch had a carbohydrate digestibility of 97.15% compared to 95.65% in those fed Autoclaved starch Diet. Protein digestibility of fish fed Raw starch Diet was 82.19%, while in those fed Autoclaved starch Diet it was lower at only 74.59%.

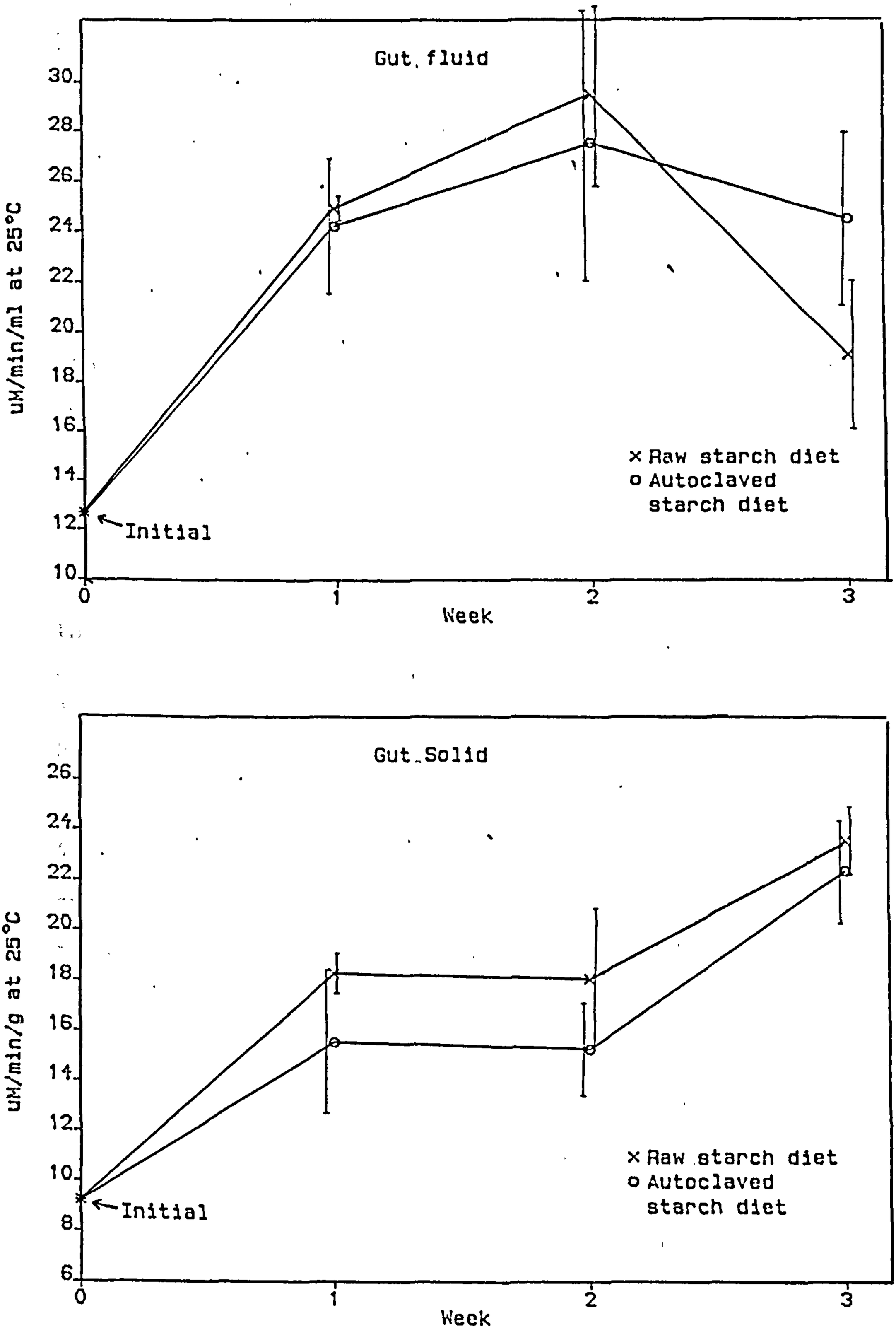


FIGURE 7.6 Total protease activity in the gut fluid and gut solid of carp, five hours after feeding on Raw starch Diet and Autoclaved starch Diet. (vertical lines indicate SE)

Table 7.7 Protease specific activity in gut fluids and gut solids at 25°C in mirror carp fed Raw starch Diet and Autoclaved starch Diet (mean ± SE).

Diet	Week	Gut fluid uM/mg/min	Gut solid uM/mg/min
Initial		0.26 ± 0.03^{abcdef}	0.19 ± 0.04^{abcdef}
Raw starch	1	0.67 ± 0.02^{a}	0.34 ± 0.01^{a}
Auto starch		0.64 ± 0.07^{b}	0.34 ± 0.05^{b}
Raw starch	2	0.72 ± 0.05^{c}	0.45 ± 0.08^{c}
Auto starch		0.72 ± 0.11^{d}	0.37 ± 0.05^{d}
Raw starch	3	0.54 ± 0.10^{e}	0.47 ± 0.01^{e}
Auto starch		0.64 ± 0.05^{f}	0.49 ± 0.04^{f}

* values in each column with same superscript are significantly different ($p < 0.05$).

7.4 DISCUSSION.

Both the diets induced hyperglycaemia in blood. The initial increase and the subsequent gradual reduction in plasma glucose levels were similar in both dietary treatments (Fig. 7.1). In contrast to the higher levels of blood glucose recorded in fish fed diets containing active amylase inhibitor (Chapter 6 and 4), in the present experiment both raw and autoclaved starch diets increased blood glucose to similar levels.

The response of fish fed either raw starch or autoclaved starch in their diets with reference to parameters such as hepatosomatic index, gut-somatic index and gut fluid volume (Table 7.4 and 7.5) were also identical. There was no change in gut fluid pH in either of the dietary treatments.

The body weights attained by the two treatment groups of carp did not differ significantly at the end of each week of the three week experiment (Table 7.3), however it was seen that the final weight attained by the fish fed Autoclaved starch diet was slightly lower (51.87g) than the weight of fish fed Raw starch Diet (54.85g). The Raw starch Diet had an apparent protein digestibility of 82% for carp, while the fish fed Autoclaved starch Diet had a protein digestibility of only 75%. The fishmeal protein was incorporated at equal levels in both the diets (Table 7.1) but it was not

subjected to any sort of heat treatment. Only the starch in the Autoclaved starch Diet had been heat treated prior to the diet preparation (see Section 7.2.2). This heat treatment may have altered the nature of some of the starch to a form unsuitable for efficient digestion. The carbohydrate digestibility was not altered significantly (97% and 95%) but the inclusion of heat treated starch reduced the digestibility of the proteins in the diet. Heating carbohydrates at sterilising temperatures such as the temperature (121°C) adopted in the present experiment, can lead to the formation of products such as laevulinic acid, reductones and 5-hydroxymethyl furfural (Bender, 1978). It is not known how these compounds affect digestibility in fishes, therefore their effect on reducing protein digestibility in this experiment can only be a speculation. Nevertheless the present digestibility values of 82% and 75% obtained with Raw and Autoclaved starch diets are close to the apparent protein digestibility for herring fishmeal protein in common carp reported in the literature, which is around 80% (Atack et al., 1979; Ogino and Chen, 1973).

The manifestation of substrate-induced enzyme synthesis and corresponding increase in enzyme activity has been discussed in earlier chapters. Such a response in alpha-amylase activity to the increased dietary carbohydrate was shown in earlier experiments and was again apparent in this trial. On feeding both dietary

treatments, there was a sharp increase of around 100% in the alpha-amylase activity in the gut contents (Fig.7.3). Similarly in the hepatopancreas and gut tissue the increase was in the order of 100 - 150% and 160 - 220%, respectively (Fig.7.4, 7.5). In bile such an increase was not evident, presumably due to the draining of the secreted enzymes into the intestinal lumen.

The variations recorded in amylase activity levels between the two treatments were not statistically significant. However, the alpha-amylase activities in gut contents of fish fed raw starch were consistently lower than those in fish fed autoclaved starch, particularly at the end of the first week. This had also been the case in carp fed diets containing wheat in Chapter 6 where fish fed Raw wheat Diet exhibited slightly lower activities than those fed Autoclaved wheat Diet. Although the magnitude of alpha-amylase activity in the present experiment was lower than that recorded in the trial using wheat, the trend in activities suggests that there could have been some reduction in amylolytic activity due to the adsorption of enzyme by raw starch. Such a reduction was demonstrated by Spannhof and Plantikow (1983) in the gut contents of trout fed native starch. Similarly, in the present experiment there was a 12% reduction in amylase activity in fish fed raw starch apparently due to this adsorption. However, in the earlier experiment carried out using wheat (Chapter 6) the

reduction in activity produced by Raw wheat Diet over the corresponding activity in fish fed the Autoclaved wheat Diet was in the order of 29%. This greater difference was probably due to adsorptive inhibition by raw starch in wheat and also due to the inhibitory activity of the wheat amylase inhibitor proteins.

The activity of alpha-amylase in the hepatopancreas and intestinal tissues of carp in the present experiment had increased significantly by the end of the first week. Unlike in the trial incorporating wheat in the diet (Chapter 6) where the raw wheat treatment groups had higher activity levels than the autoclaved wheat treatment, there were no difference between the magnitude of amylase activities in the fish fed raw and autoclaved starch. This further demonstrated that the sharp increase during the first week in carp fed raw wheat in the previous experiment was not only due to the increased carbohydrate content of the diet, but also because of the compensatory response of the fish to the presence of the inhibitor proteins of wheat in the gut lumen.

The crude protein content of the test diets was only 31 - 32% (Table 7.2) which was markedly lower then the holding ration containing about 50% protein (Appendix I). Despite this reduction in dietary protein content, there was a two fold increase in the proteolytic activities in the gut contents by the end of the first week and activity levels remained high for the duration

of the experiment (Fig. 7.6). In earlier experiments where carp were fed diets containing wheat (Chapter 6, Fig. 6.7) a similar increase in protease was recorded although it was of lower magnitude.

In the present experiment the concurrent increase in protease and amylase activities appears to support the hypothesis of 'parallel secretion'. This term was first suggested by Babkin (1906, cited by Rothman, 1977) and implies that various enzymes are secreted in parallel or at parallel rates. This occurs for only a short time and the continued feeding of the same diet will eventually lead to an increased synthesis of only the appropriate enzyme (Fabry, 1969). Also the increase in fish size could have been a contributing factor since protease secretion is known to increase with size in certain fish species such as Solea solea (Clark et al., 1985) and Acipenser fulvescens (Buddington, 1985), However, in carp (Morishita et al., 1964) and Rutilus rutilus (Hofer and Uddin, 1985) it is reported that trypsin-like activity is negatively correlated with fish weight.

Generally digestive enzyme adaptation to the diet occurs within 2 to 3 days and this adaptation is stabilised after 5 to 7 days in mammals (Ben Abdeljlil and Desnuelle, 1964; Corring and Saucier, 1972; Corring, 1975 cited by Corring, 1980). Adaptations of carbohydrase activity to diets in carp occur in less than a week (Kawai and Ikeda, 1972). The results of the present experiment

however show that in carp the process of enzyme adaptation can continue for longer periods. The main factor determining this period of adaptation appears to be the nature of the substrate and therefore a fixed adaptation period for all animals fed with various diets cannot be generalised.

It was concluded from this experiment that both raw and autoclaved wheat starch elicit similar changes in the activity levels of alpha-amylase and proteases in mirror carp.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS.

Purified wheat amylase inhibitor proteins significantly reduced the activity of mirror carp and Nile tilapia alpha-amylases when tested under *in vitro* conditions (Chapter 3). Furthermore, tilapia amylase was found to be more susceptible than carp alpha-amylase to this inhibitor (Table 3.10). The exact biochemical reason for this difference is not clear but it is a common feature that amylases of different origins are inhibited to varying degrees by wheat amylase inhibitor proteins (Silano *et al.*, 1975).

In the *in vitro* experiments, the magnitude of inhibition was also found to depend on whether the amylase was extracted from gut fluid, gut tissue or hepatopancreatic tissue of the fish. Hence, carp and tilapia gut fluid amylases were inhibited to a lesser extent than alpha-amylase from either intestine or hepatopancreas. Alpha-amylase activity in the samples from intestinal tissues were inhibited most in both carp and tilapia (Table 3.10). The alpha-amylases which are active in carp and tilapia gut lumen are a mixture of enzymes derived from the hepatopancreas and intestinal mucosal cells. Nevertheless the inhibitor had the least effect on alpha-amylase in gut fluid, probably because of

the counteracting effects of active proteases, starch from the diet itself and maltose from the hydrolysis of starch which are all known to reduce the activity of the wheat amylase inhibitor (O'Connor and McGeeney, 1981).

When carp and tilapia were fed diets containing starch mixed with proteins extracted from wheat grains (protein from 400g wheat per kg diet), the growth of carp was significantly reduced while tilapia did not show any such reduction in growth rate (Fig. 4.2 and Fig. 5.1). Furthermore when carp were fed a diet containing raw wheat, they exhibited a reduced growth rate during the first week of the trial but thereafter growth was at a rate similar to that of fish fed a diet containing autoclaved wheat (Fig. 6.1). Since it was later demonstrated that carp fed raw and autoclaved starch grow at equal rates (Table 7.3, Chapter 7), the difference observed in growth rates of carp fed raw and autoclaved wheat appears to be due to the effect of amylase inhibitors in the wheat rather than to the raw or gelatinized nature of the starch.

It is thought that tilapia is able to completely counteract the effect of the wheat inhibitors in the diet because of the presence of the enzyme pepsin in its stomach which is absent in the stomachless carp. Thus, despite tilapia alpha-amylase having been more susceptible to the action of wheat amylase inhibitors under <u>in vitro</u> conditions, when fed in the diet it

appears that the inhibitors were digested in the stomach by the protease pepsin. If this were the case then feeding wheat inhibitors to tilapia in a form resistant to gastric digestion should result in an effect similar to that seen in carp. Marci et al. (1977) fed gastric resistant microgranules containing wheat amylase inhibitor to chickens and found significant reduction in growth rates of the birds. The results of the experiments described in this thesis are also in general agreement to those of Lang et al., (1974) where the effects on rats were investigated.

Heat treatment by autoclaving at 121°C for 20 minutes denatured the inhibitors in wheat proteins and feeding such a preparation to carp did not retard growth (Fig.4.2). Alteration of the structure of proteins by mild heat does not affect the nutritive value of the protein, however, its specific biological properties are lost (Bender, 1978). Autoclaving ground wheat at sterilization temperature conditions for 20 minutes resulted in a small increase in carbohydrate digestibility in addition to inactivating the inhibitor proteins (Chapter 6). However, in this trial the protein quality appears to have been damaged by the heat process leading to lower protein digestibility. Heat treatment in excess of that required to destroy the antinutritional factors causes reduction in nutritive value. Aminoacids such as lysine, cystine, arginine, methionine, histidine etc.

become 'unavailable' and cannot be liberated during digestion as a result of various reactions that can take place when proteins are subjected to heat (Bender, 1978).

It is therefore considered essential to determine the optimum conditions required for destroying the inhibitors in wheat with minimum loss of nutritive value of the protein. Considerable amounts of work have been carried out to determine the optimum conditions for the treatment of soybean to inactivate the protease inhibitors it contains (Longnecker et al., 1964; Kakade and Evans, 1965;). Mustakas et al. (1970) examined the effects of a number of variables such as temperature, retention time and moisture conditions during extrusion cooking of soybean, on its nutritive value. They concluded that to obtain a satisfactory product where 89% of the trypsin inhibitor had been inactivated, the treatment conditions should be within the range of 121 to 138 °C for 1 - 2 minutes with 20 to 25% moisture. Similar high-temperature, short-time treatment may be suitable for wheat. Available lysine serves as a useful indicator of quality in overheated samples and the presence of inhibitor is an index of inadequate heat treatment for such toxin-containing feed ingredients (Bender, 1978).

Mirror carp fed diets containing amylase inhibitor exhibited elevated levels of glucose in blood plasma during the early part of the experiments (Fig. 4.3

and 6.2). There has been no specific work carried out to establish whether the nature of a diet could act as a 'stressor'. However, unbalanced diets and diets containing anti-nutritional factors may be expected to be more difficult to digest and utilise (Jauncey, pers. comm.). Recently Barton et al. (1988) have reported that post-stress hyperglycaemia was greatest in salmon fed high-lipid diets and therefore the type of diet fed to fish should be considered when interpreting the magnitude of hyperglycaemic stress response. Hence it is possible that the presence of inhibitor proteins in the diets of carp could have increased the stress response resulting from the effects of confinement, anaesthesia, handling etc.

In all the three trials with mirror carp, there were no clear trends or differences between treatments in the bile volumes recorded. Bile volume was recorded 4 or 5 hours after the last meal and in general ranged from 25 to 58 ul/100g fish. Compared to carp, tilapia had higher bile volumes of between 61 and 559 ul/100g fish, but again the volumes of individual fish were variable and did not exhibit any trend or differences between the treatments. Bile volume varies depending both on the diet fed to the fish and also the time after the meal that the volumes are recorded. Actively feeding fish have lower volumes compared to starving fish (Love, 1980). In addition, immediately after the meal, the volumes would be

lower than just before the feeding time. The mechanism of gall bladder contraction in carp and tilapia and the regulation of secretion of bile in these fish species have not been studied. In coho salmon (Vigna and Gorbman, 1977) and rainbow trout (Aldman and Holmgren, 1987) the hormone cholecystokinin (CCK) secreted by the endocrine cells in the intestinal mucosa, on the entrance of food, induces the gall bladder to contract and to empty the bile into the duodenum. It is possible that a similar mechanism operates in carp and tilapia.

The pH of the intestinal fluid of carp varied within a neutral or near neutral pH range of between 6.4 and 7.5 (Table 6.4). The contents were apparently well buffered by bile and none of the diets tested had any significant effect on gut fluid pH. Even though extremes of pH do not inactivate the inhibitor (Buonocore et al., 1977) a pH range of 5.8 to 7.0 is considered by O'Donnell and McGeeney (1976) to be ideal for inhibition of human pancreatic amylase by wheat amylase inhibitors. Thus the pH conditions in the intestine of carp was ideal for the action of the inhibitor fed to the fish in the diet.

Alpha-amylase activity increased when carp were fed all test diets. In the gut contents of carp fed diets containing active and inactive inhibitor the alpha-amylase activity at the end of first week was twice the initial level (Fig. 4.5). Similarly, in carp fed diets containing

raw wheat and autoclaved wheat, the activity increased to three times the initial level (Fig. 6.3). This process of substrate-induced enzyme synthesis is in accord with the findings of many earlier workers who have reported increased secretions of starch hydrolysing enzymes on feeding fish with diets containing carbohydrates (Fish, 1960; Nagase, 1964; Hofer, 1979a, 1979b; Hofer and Schiemer, 1981; Reimer, 1982).

The hepatopancreas in carp fed diets containing active wheat amylase inhibitor had twice the amylase activity recorded in carp fed diet with denatured inhibitor protein, and this high level of activity was observed only during the first two weeks of the experiment (Fig. 4.6). Hyperactivated hepatopancreas was also seen in carp fed a diet containing raw wheat (68% over the fish fed Autoclaved wheat Diet) at the end of the first week (Fig. 6.5). In the intestinal tissues increased amylase activities were also evident. The foregut, midgut and hindgut regions of carp fed diet containing active inhibitor showed 190%, 150% and 125% higher activity levels at the end of the first week than the activities in the corresponding regions of carp fed diet with denatured inhibitor.

It was apparent that carp fed diets containing active inhibitor increased their amylase secretions in order to overcome the effect of the antagonistic proteins in the diet by constantly replenishing and compensating

for the loss of amylolytic activity. On the other hand, fish fed denatured inhibitors could easily digest the carbohydrates in the diet with lower secretion levels of enzymes. Sturmbauer and Hofer (1986) also found that carp fed solely on native wheat for one week produced 3 to 4 times the amount of amylase enzyme secreted by carp fed extruded wheat. Earlier researchers working with chickens have also reported hyperactivity of the pancreas and marked increases in the production of alpha-amylase when this inhibitor was incorporated in the diet (Marci et al., 1977). Antitrypsin inhibitor present in soybean also elicits a similar response, but in this case the increased secretion is of the proteolytic enzymes (Chernick et al., 1948; Booth et al., 1960; Applegarth et al., 1964; Nitsan and Alumont, 1964; Lepkovsky et al., 1965; Konijn and Guggenheim, 1967; Schingoethe et al., 1970).

The hormone that stimulates pancreatic enzyme secretion physiologically is pancreozymin (cholecystokinin, CCK or CCK-PZ) (Davenport, 1982). When the alpha-amylase in the intestinal lumen is inactivated by the wheat inhibitor, the presence of undigested carbohydrates would induce secretion of more pancreozymin which would in turn stimulate further pancreatic amylase production until the food is digested efficiently. When such an excess of "protein" is secreted into the intestine, according to Rothman et al.'s proposal of

"enteropancreatic circulation" (Liebow and Rothman, 1975), a large percentage can be conserved by reabsorbtion and at least some of the amylases can be resecreted under normal circustances.

In carp fed diet containing active inhibitor the combined amylase activity level in gut fluids and solids remained stable at around 3000 uM/min up to the end of the second week of the trial. However, by the end of the the third week the level of activity had dropped to 2600 uM/min (Fig. 4.5). By contrast, such a drop in amylase activity in gut contents was not evident in carp fed diet containing denatured inhibitor. The hepatopancreatic tissues of carp fed diet with active inhibitor appeared to be producing lower amounts of enzymes by the end of the third week of this experiment. Compared to carp fed Inactive Diet, the amylase activities recorded in hepatopancreas from fish fed Active Diet was 17% lower (Fig. 4.6). Reduced alpha-amylase activity levels were also recorded in the bile of the former group of fish.

When this drop in amylase activities of gut contents is considered in conjunction with the concurrent lowering of activities in the hepatopancreas and bile in fish fed Active Diet, it indicates that the hepatopancreas has reduced its secretions, or in other words it had apparently become incapable of compensating for the continuous inhibition of amylase by the presence of large

amounts of inhibitor in every meal of the fish. By the third week the fish had almost stopped growing, their plasma protein content had increased and there was a sharp fall in hepatosomatic index (Figs. 4.2, 4.3, 4.4). All this indicates the mobilisation of body proteins. Hofer and Sturmbauer (1985) considered that the loss of digestive enzymes due to inhibition may negatively influence the protein balance of the fish.

Normal intestinal epithelium absorbs many intact protein molecules. It is known that in mammals (Beynan and Kay, 1976; Diamond, 1978) and possibly also in fish (Hofer and Schiemer, 1981), proteases are reabsorbed in the posterior regions of the alimentary canal. Liener (1979) suggested that the increase in faecal nitrogen (protein) observed in animals fed protease inhibitor containing diets, may be due to endogenous and dietary proteins, implying that bound proteases are reabsorbed to a lesser extent by the intestinal epithelium. Lyman and Lepkovsky (1957), again referring to trypsin inhibitor, considered that growth depression may be a consequence of an endogenous loss of essential aminoacids derived from the hyperactive pancreas that is responding in a compensatory fashion in order to overcome the depletion of enzymes caused by the inhibitor in the diet. Similar arguments can be applied to amylases and amylase inhibitors. Liebow and Rothman (1975) showed that amylases are reabsorbed with their activity intact.

Therefore the inactivated amylases are either digested by the proteases in the gut or excreted in the faeces since they can only be reabsorbed to a lesser extent compared to intact enzymes. In carp fed diet incorporating extracted wheat amylase inhibitor proteins (Chapter 4), the inhibitor contained in the diet appears to have reacted with, and bound to, appreciable quantities of amylase enzyme. The inhibitor binds with amylase in a 1:1 molar ratio (Buonocore et al., 1976). Therefore one molecule of inhibitor would bind with one molecule of amylase and if all such bound enzyme is excreted, the fish would not be able to cope with the large endogenous protein loss and would therefore lose body weight. Pancreatic enzymes are rich in sulphur-containing amino acids (Neurath, 1961), hence the body tissues could be drained of these essential amino acids in order to meet the increasing need for the synthesis of pancreatic enzymes. This hypothesis could explain the results of the experiment described in Chapter 4 but needs confirmation by measurement of endogenous protein losses possibly by the use of labelled proteins.

Histological examination of samples of hepatopancreas from carp fed diet containing amylase inhibitor did not reveal any tissue damage (Section 4.3.10, Plates 5 and 6). Hyperactivated pancreas resulting from feeding wheat amylase inhibitors or soybean protease inhibitors may lead to enlargement and

degenerative changes in the organ (Applegarth et al., 1964; Marci et al., 1977). However, some authors have not observed any hypertrophic changes in the pancreas due to feeding soybean protease inhibitor (Rackis et al., 1963; Saxena et al., 1963; Pubols et al., 1964). One of the reasons for the failure to establish such changes in the present study could be the relatively short duration of the trial, although in terms of enzyme secretion there was a reduction by the end of the third week. It is therefore possible that in longer experiments organ damage could become manifested.

In conclusion, wheat amylase inhibitor proteins are capable of significantly reducing carp and tilapia alpha-amylase activities under in vitro conditions. Under in vivo conditions tilapia is better able to tolerate and counteract this inhibitor but carp can be severely affected. In carp the amylase inhibitors of wheat not only reduce the digestibility of the dietary carbohydrate but may also 'lock up' significant portions of enzyme proteins of the fish thus rendering them unavailable for reabsorbtion and increasing faecal nitrogen. Thus instead of the carbohydrate component of the diet sparing protein it may cause increased losses of protein in the presence of this inhibitor. Nevertheless mirror carp can withstand and adapt to the small amounts of inhibitor present in wheat grains when incorporated in the diet below the threshold level at which they exert a

deleterious effect. In view of the results detailed in this thesis the future line of work would be to determine the exact threshold level of this inhibitor for carp and other fish with no peptic activity, in order to realise the full nutritive value of wheat in fish diet. Since any inhibition of the normal digestive processes could play a significant role in determining the overall nutritive value of the feed ingredient, selection of compatible ingredients for preparation of the fish diet becomes essential. For instance, a combination of raw soybean meal (containing protease inhibitor) with raw wheat (containing amylase inhibitor) could prove disastrous even for tilapia. This thesis provides some insight into the mechanisms of enzyme regulation and adaptation in fish which would be useful for developing the 'ideal diet' for cultivable fishes.

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APPENDIX I

Proximate composition (moisture free) of trout diets used as holding rations for fish stocks.

PARAMETERS	Lot 1	Lot 2	Lot 3 *
Moisture %	7.73	9.34	5.80
Crude protein %	52.47	53.50	51.25
Crude lipid %	12.62	10.66	20.68
Crude fibre %	2.14	3.06	1.11
Hydrolysable carbohydrates %	5.80	4.92	9.29
Total ash %	11.92	12.99	12.35
Acid insoluble ash %	0.24	--	--

* Lot 1 and 2 used for carp.

  Lot 3 for tilapia.

APPENDIX II

Effect of incubation temperature on the activity of Carp hepatopancreatic alpha-amylase.

Sample vol ul	Amylase activity (mU/min) at 25°C	37°C	Percent* Increase
50	131.73	227.68	72.84
100	252.47	436.16	72.76
150	369.71	658.02	77.98
200	480.41	832.14	73.21
250	592.48	979.64	65.35

$$* \% \text{ Increase} = \frac{(\text{Activity at } 37^\circ C - \text{Activity at } 25^\circ C)}{\text{Activity at } 25^\circ C} \times 100$$