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The effect of feeding on the secretion of pepsin, trypsin and chymotrypsin in the Atlantic salmon, *Salmo salar* L

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Abstract

Pepsin stored in the stomach mucosa of the Atlantic salmon (*Salmo salar* L.) increases within two days of the onset of starvation. Trypsin and chymotrypsin in the pyloric caeca/pancreas behave similarly, indicating that when no food is present in the gut, digestive enzymes accumulate in the secretory tissues. As a corollary, trypsin and chymotrypsin activities in the gut contents fell during starvation, indicating that secretion is greatly reduced when food is not present. At the onset of feeding, pepsin is rapidly synthesised in the mucosal tissues and then secreted. Twenty four hours after feeding, the pepsin levels of the mucosa are still low, suggesting that synthesis may be a rapid response to the presence of food in the stomach. Secretion of trypsin and chymotrypsin appears to take place as soon as digesta enters the intestine, between 4 and 14h after feeding, and resynthesis of enzyme precursors appears to be complete again within a further 11h. It is suggested that both synthesis and release may be under the control of cholecystokinin.

Introduction

In contrast to the situation in the higher vertebrates, very little information is available for fish on the role of food in stimulating the secretion of digestive enzymes. In the carp, total proteolytic enzyme activity in the pancreas decreased shortly after the commencement of feeding, but recovered 5h later (Onishi *et al.* 1973). Similarly, very little has been published on the activity of the glands which secrete digestive enzymes during periods when fish are not feeding. In the blackfish (*Gadopsis marmorcadus*) starvation resulted in an increase in peptic activity of the gastric mucosa probably due to storage of pepsinogen in the oxyntopeptic cells (Buddington and Doroshov 1986). The activity of both trypsin and chymotrypsin in the pancreas of catfish increased after 2 months starvation (Yoshinaka *et al.* 1981a). However, in the eel (*Anguilla japonica*) Yoshinaka *et al.* (1984) found a decrease in pancreatic trypsin and chymotrypsin activities after 1.5 months starvation. Secretion of digestive enzymes was found to decline in the carp (Onishi *et al.* 1973) and in the cod (Overnell 1973), whilst Yoshinaka *et al.* (1981b) showed that digestive enzyme activities in the intestinal contents, or digesta, also declined.

This study, a part of a detailed study on the digestive physiology of the Atlantic salmon (*Salmo salar* L.), was therefore undertaken to determine whether proteolytic enzyme activity declined during starvation and whether this was associated with a continued secretion of enzymes into the lumen of the gut, and to study the relationship between time

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of feeding, the time of digesta entering the intestine, and the time of release of these enzymes.

Material and methods

Effect of starvation on pepsin, trypsin and chymotrypsin storage and secretion

Enzyme storage was determined by assaying the mucosa of the stomach and the pancreas respectively, whilst secretion was determined by measuring the activity of the enzymes in the contents (digesta) of the gut lumen. Optimal conditions for enzyme assays were determined before undertaking this study, and are described in Einarsson (1994). The experimental fish were starved for 20 days, in water which ranged in temperature between 5 and 21°C. A control group was fed *ad libitum* for the same period.

The fish were in their second year of growth and contained both upper and lower modal individuals, with a forklength of 12.7–16.8 cm (22.8–45.0 g fresh weight). The mixed grouping may have contributed some variation to the data. Six starved and 6 fed fish were sampled after 0, 2, 4, 6, 8, 10, 13, 16 and 20 days, each time at 10.00h. The food was a commercial salmon food (freshwater smolt – 1,3 mm pellets, BP Nutrition – comprising 48% protein (95% of fishmeal origin), 22% fat and 12% carbohydrate), which preliminary experiments showed, contained no detectable pepsin, trypsin or chymotrypsin as contaminants.

The fish were killed by a blow to the head, and kept on ice. The abdomen was opened ventrally and the digestive tract removed. The stomach was removed, opened by a longitudinal incision and the contents (which comprised mucus in starved fish), gently sampled with a blunt scalpel. Stomach and digesta samples were then frozen at –20°C. The intestine, together with the fat surrounding the pyloric caeca was divided by a cut, posterior to the pyloric caeca. The luminal contents of the posterior section was mixed with the contents of the pyloric caeca. This was termed the intestinal digesta. The pyloric caecal section of the intestine and the intestinal digesta were then frozen at –20°C. The fish carcasses were then dried to constant weight at 80°C, and weighed to the nearest 0.1 mg. Preliminary experiments indicated that fish

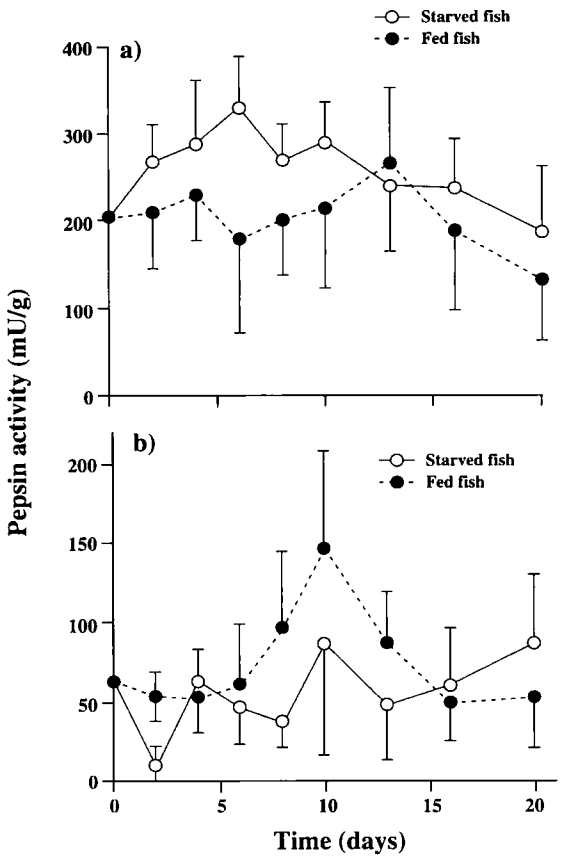

Fig. 1. Pepsin activity in the Atlantic salmon (*Salmo salar* L.) during a 20 day period of starvation (○) or feeding *ad libitum* (●) (*a*) in the stomach mucosa (*b*) in the stomach digesta/mucus. Pepsin activity is expressed in milliunits per g dry weight of fish (mU/g). Mean values ± SD (n = 6 in each case).

dry weight was preferrable as a normalising unit, since it varied less with the experimental treatments than the dry weights of stomach and intestine. Furthermore there was a linear correlation between weight of digesta and fish dry weight.

Samples were thawed and then kept on ice, and the tissue samples then weighed to the nearest 0.1 mg. To each stomach and stomach digesta sample was added 2.0 ml of Johnson and Lindsay buffer (Johnson and Lindsay 1930) pH = 4.25 and then homogenised after addition of CCl_4 to a final concentration of 5% (v/v). To each sample of pyloric caeca and intestinal digesta was added 5 ml of 0.9% w/v NaCl and 20 mM $CaCl_2$ after addition of CCl_4 to a final concentration of 20% (v/v) for the tissue samples and 10% for the digesta.

All samples were centrifuged at 9,000 × g for 4 min and enzyme activities determined in the super-

natant. The residual stomach digesta and intestinal digesta samples were then dried to constant weight at 80°C and weighed to the nearest 0.1 mg.

Pepsin activity in the samples from the stomachs was measured using azocasein as a substrate (Charney and Tomarelli 1947; Tomarelli *et al.* 1949; Brock *et al.* 1982). Two hundred µl of 5% (w/v) azocasein in distilled water was added to Johnson Linday buffer (Johnson and Lindsay 1939) pH = 4.25. Two hundred µl of supernatant was added and the buffer volume adjusted to make the total volume to 1.0 ml and a final azocasein concentration of 1.0%. The incubation time was 2h at 25°C. The reaction was stopped by the addition of 600 µl of 20% (w/v) trichloracetic acid (TCA). The blanks were incubated with the TCA and the pepsin inhibitor pepstatin A (Sigma: P4265) at a concentration of 0.17 mM. The blanks were prepared by pre-incubating the supernatants with the pepstatin A for 20 min at room temperature prior to the assay. After stopping the reaction, the incubation medium was left for 50 min at room temperature and then centrifuged for 6 min at 9,000 × g. One ml of NaOH was mixed with 1 ml of supernatant and readings taken on a spectrophotometer at 450 nm. Pepsin activity was expressed in milliunits (mU) per g dry weight of fish, where 1 mU is defined as 1 µg of azocasein hydrolysed in 1h.

Trypsin activity was measured with 1.1 mM D,L-B*p*NA (Erlanger *et al.* 1961) as substrate in 200 mM tris buffer pH 7.8, and chymotrypsin with 0.5 mM SAAPP*p*NA as a substrate in 100 mM tris buffer pH 9.0 (Del Mar *et al.* 1979). In each case, the assays were carried out at 25°C, and the released paranitroaniline (*p*NA) was measured at 504 nm. Prior to the chymotrypsin assay the samples were incubated with N*a*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), a specific inhibitor of trypsin, at a concentration of 30 mM, for 2 min at room temperature, to avoid interference by trypsin in the assay. The respective reaction rates were calculated in milliunits (mU), defined as 1 nmol of *p*NA released in 1 min. Trypsin and chymotrypsin activities were then expressed as mU per gram dry weight of fish.

Effect of feeding on pepsin synthesis and secretion

In this experiment, the time course of synthesis and secretion was determined when a single meal was given to fish which had been previously starved. The fish used were from the same population as in the previous experiment. They were starved for 20 days in water temperatures ranging from 5 to 21°C, and then divided into two groups which were kept at a mean temperature of 13°C. The experimental group was fed *ad libitum* for 1h and the control group remained unfed. Six fish from each group were sampled at 0, 1, 2, 4, 7, 13 and 25h after commencement of feeding of the fed group. The fish were processed and assay procedures were as above.

Effect of feeding on trypsin and chymotrypsin synthesis and secretion

The concept of this experiment was similar to that on pepsin synthesis and secretion, but there were changes in some details. The fish used were from a sibling population of lower modal fish in their 2nd year. The fresh weight ranged from 10.5–34.8 g. After 25 days of starvation, the fish were divided into 2 groups. The experimental group was fed *ad libitum* for 1.25h, whilst the control group remained unfed. Twenty fish from the experimental group and 10 fish from the control group were sampled at 0, 1, 4, 14, 25 and 54h after commencement of feeding in the experimental group. The fish were processed as before, except that during dissection the pancreas with its surrounding fat was scraped off the anterior intestine and the pyloric caeca, and frozen down for trypsin and chymotrypsin assays, which were carried out as before.

Results

Effect of starvation on pepsin, trypsin and chymotrypsin activity

The pepsin activity of the stomach mucosa was seen to rise in the starving fish within 2 days of starvation (Fig. 1(a)). The overall difference between the starving and the fed fish was significantly different (Two way ANOVA: $p < 0.0005$). The pepsin activity of the stomach mucosa was higher in the starving fish, than in the feeding fish (except at 13 days). Pepsin activity of the stomach digesta (Fig. 1(b)) was only significantly lower in

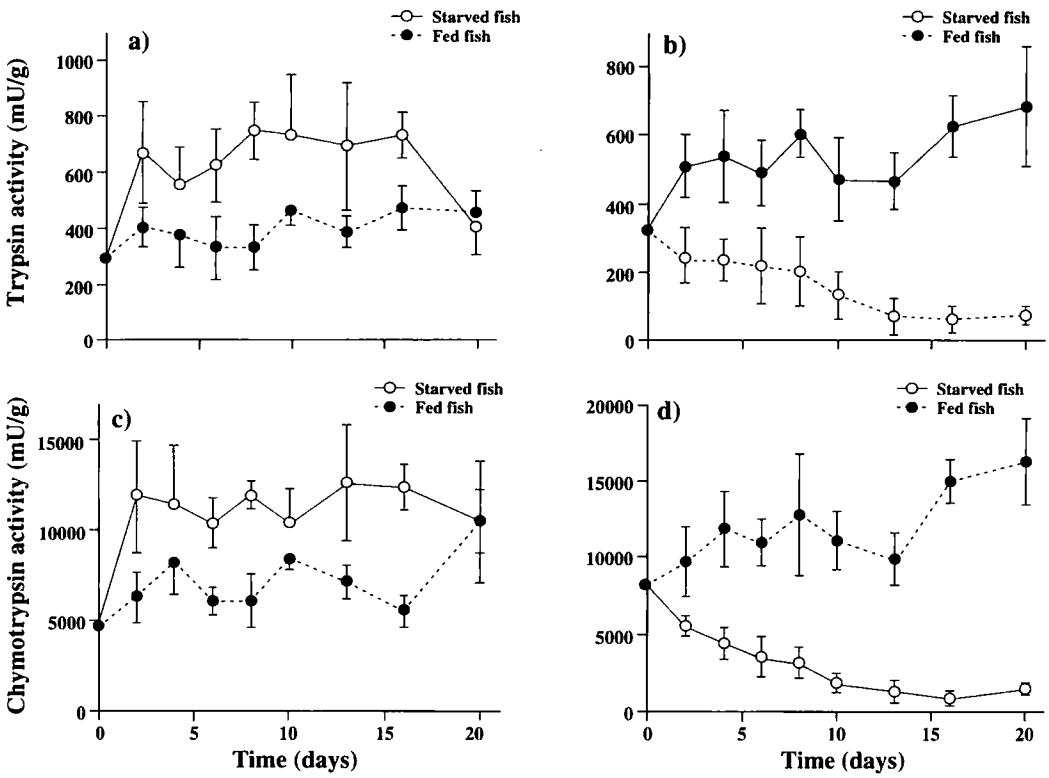

Fig. 2. Trypsin (*a* and *b*) and chymotrypsin (*c* and *d*) activity in Atlantic salmon (*Salmo salar* L.) during a 20 day period of starvation (○) or feeding *ad libitum* (●) (*a* and *c*) in the pyloric caeca/pancreas (*b* and *d*) in the intestinal digesta. Activity is expressed in milliunits per g dry weight of fish (mU/g). Mean values ± SD (n = 6 in each case).

the starving fish after 2 days ($p = 0.0002 < p = 0.005$; Bonferroni: 10 tests; one tailed test). No digesta, but some mucus, was observed in the stomach of the starving fish at and after 2 days, in contrast to the feeding fish which had digesta in their stomachs throughout the experiment.

The activity of both trypsin and chymotrypsin of the pancreatic tissue and pyloric caeca was seen to rise in the starving fish within 2 days of starvation (Fig. 2(a), 3(a)). The overall difference between the starving and the fed fish was significant (Two way ANOVA: $p < 0.0005$ in both cases). The activity in the pancreatic tissue and the pyloric caeca was always higher in the starving fish, except after 20 days. The trypsin and chymotrypsin activities of the intestinal digesta (Fig. 2(b), 3(b)) fell gradually in the starving fish, reaching a minimum after 16 days.

Effect of feeding on pepsin synthesis and secretion

Pepsin activity in the stomach mucosal tissues had risen after 1h of feeding and thereafter declined again (Fig. 4(a)). As expected, the pepsin activity in the control group of starved fish did not change. After the first hour the pepsin activity declined again and fell to a lower level than the control. There was significant overall difference in the pepsin activity between the experimental and the control groups throughout the experiment (Two way ANOVA: $p = 0.02$) but not between the two groups at the 1h sampling period ($p = 0.011 > p = 0.0036$; Bonferroni: 14 tests; one tailed test) or at any other time.

The pepsin activity in the stomach digesta (Fig. 5(a)) rose immediately after feeding and the overall level was significantly different from the control for the duration of the experiment (Two way ANOVA: $p < 0.0005$). The activity was signifi-

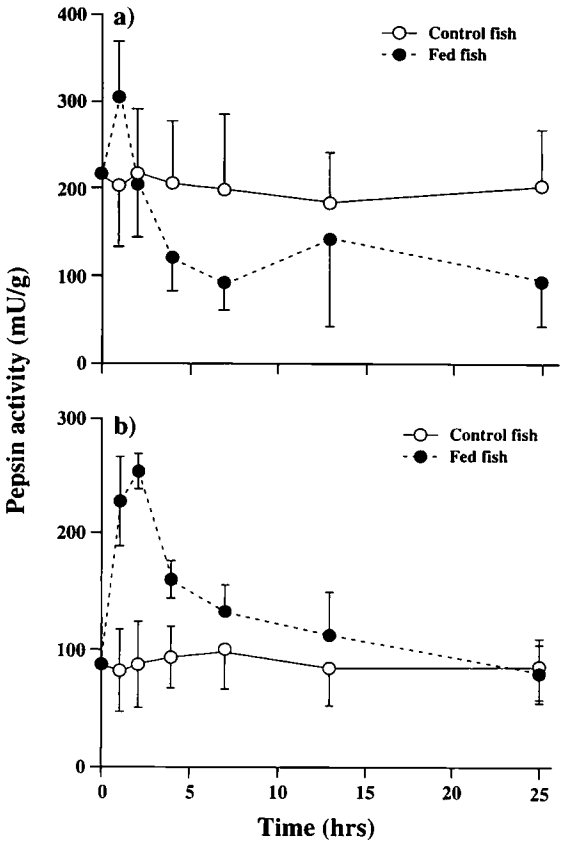

Fig. 3. Effect of a single meal on (*a*) the stored pepsin activity in the stomach mucosa and *b*) the secreted pepsin activity in the stomach digesta of Atlantic salmon (*Salmo salar* L.). The fish were fed for the first hour of the experiment, which followed 20 days starvation. Fed group (●) and control group, unfed (○). Pepsin activity was expressed as milliunits per g dry weight of fish (mU/g). Means ± SD (n = 6).

cantly higher in the fed than control groups at the 1, 2 and 4h sampling times ($p = 0.0005 < p = 0.0036$; Bonferroni: 14 tests; one tailed test), but not thereafter.

Effect of feeding on trypsin and chymotrypsin synthesis and secretion

Trypsin activity in the pancreatic tissue (Fig. 5(a)) showed no change until 13h after feeding, when it was significantly lower in the fed fish (Bonferroni: 29 tests; one tailed; $p = 0.0001 < p = 0.0017$). There was no significant difference at any other sampling time. By 25h post-feed the trypsin levels had returned to normal again. In the digesta, the trypsin activity increased to a maximum 13h after feeding (Fig. 5(b)), when it was significantly higher than in the starved fish (Bonferroni: 29 tests; one tailed; $p < 0.00005 < p = 0.0017$). The difference between the digesta from fed fish and the starved controls remained fairly constant for the remainder of the sampling time.

The activity of chymotrypsin (Fig. 6(a)) followed the pattern shown by trypsin. There was a significant lowering of activity in the pancreas of the fed fish 13h after feeding (Bonferroni: 29 tests; one tailed; $p = 0.0006 < p = 0.00172$) and this corresponded to an increase in activity in the digesta (Fig. 6(b)) (Bonferroni: 29 tests; one tailed; $p < 0.00005 < p = 0.00172$). The difference between the activities in the digesta from the fed fish and the controls remained fairly constant thereafter. Again by 25h after feeding, the levels of chymotrypsin in the pancreas had returned to the pre-feeding levels again.

Discussion

Effect of starvation on pepsin, trypsin and chymotrypsin storage and secretion

Starvation caused a slight rise in the activity of pepsin in the stomach mucosa, *i.e.*, the stored pepsin, suggesting that synthesis continues after secretion has been reduced following withdrawal of food. A similar result is evident in the experiments of Buddington and Doroshov (1986) in the blackfish (*Gadopsis marmorcadus*). In the present experiments, the activity of the pepsin in the stomach digesta fell after 2 days of starvation, suggesting a reduction of secretion, but thereafter there was no significant difference in the activity between the digesta of the fed and the control fish. This may represent the retention of a basal concentration of the pepsin in stomach mucus, possibly as a result of a markedly reduced frequency of stomach evacuation during starvation.

The activities of both trypsin and chymotrypsin in the pyloric caeca/pancreas increased during starvation, again indicating that as secretion is reduced when there is no food in the gut, these enzymes, or their precursors, accumulate in the pancreatic tissue. This agrees with observations on the rat (Girard-Globa *et al.* 1980) and on the catfish starved for 2 months (Yoshinaka *et al.* 1981a). Ba-

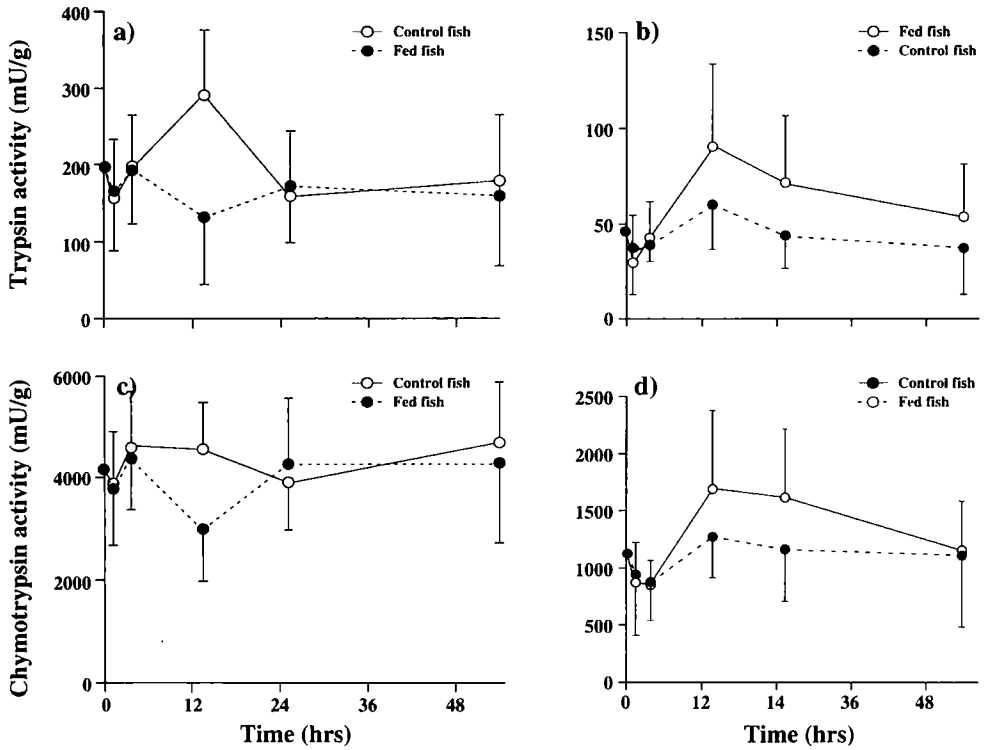

Fig. 4. Effect of a single meal on the stored trypsin (*a*) and chymotrypsin (*c*) activity in the pancreas, and the secreted trypsin (*b*) and chymotrypsin (*d*) activity in the intestinal digesta of Atlantic salmon (*Salmo salar* L.). The fish were fed the first 1.25h of the experiment, which followed 25 days starvation. Fed group (○) and control group, unfed (●). Activity was expressed as milliunits per g dry weight of fish (mU/g). Means ± SD (n = 20 for fed group, n = 10 for control, unfed group).

sal synthesis and secretion are known to occur in the pancreas of mammals (reviewed by Case 1978; Girard-Globa *et al.* 1980). Starvation also caused a gradual reduction of trypsin and chymotrypsin activities in the intestinal digesta indicating that secretion is greatly reduced upon withdrawal of food.

The effect of feeding on pepsin synthesis and secretion

The ingestion of food causes secretion of pepsin from the stomach mucosa within 1h and thereafter the levels were lower than in the starved fish. This suggests that pepsinogen is rapidly synthesised with the onset of feeding, and is then secreted, whereupon the pepsin activity of the digesta increases. The secretion may be triggered by the distension of the stomach, as demonstrated in the brown bullhead (Smith 1967). Recent studies suggest that serotonin, *i.e.*, 5-hydroxytryptamine (Holstein and Cederberg 1984) and/or some tachykinins (Holstein and Cederberg 1986) may be involved in the control of pepsin secretion in the Atlantic cod. Substance P (SP) is a member of the tachykinin family and acts in the rainbow trout by releasing serotonin (5-HT) from enteric neurones in the stomach (Holmgren *et al.* 1985).

The effect of feeding on trypsin and chymotrypsin synthesis and secretion

Secretion of trypsin and chymotrypsin from the pancreatic tissue appears to take place between 4 and 14h after feeding and this coincides with the entry of digesta into the intestine from the stomach (Einarsson, unpublished results) and with a reduction in the stored enzymes in the pancreas. Similar results were obtained by Pringle *et al.* (1992), who showed an approximately 50% reduction of trypsin activity in the pyloric caeca/pancreas when food entered the intestine. It is clear that these enzymes are stored in, and secreted from, the pancreas

(Einarsson and Davies 1996), but enter the gut through the pyloric caeca. Control of the secretion is probably by some cholecystokinin (CCK)-like peptide(s) which is/are very similar to the mammalian ones (Einarsson and Davies 1996). The role of CCK in the regulation of digestive enzyme release in mammals has been extensively documented (reviewed by Dockray 1989; Crawley and Corwin 1994), and porcine CCK has been found to stimulate secretion of trypsin and chymotrypsin from the pancreatic tissue of Atlantic salmon (Einarsson and Davies 1996).

The trypsin and chymotrypsin activity of the pancreas declined by 13h after feeding as a result of its secretion into the intestinal lumen. Thereafter the activity increased, as a result of resynthesis. This is similar to the observations of Onishi *et al.* (1973), who showed a decline, followed by an increase in proteolytic activity in the pancreas of carp. Similarly, in Atlantic salmon smolts which were fed one meal after a period of starvation, trypsin activity in the pyloric caeca/pancreas returned to normal 47h after commencement of feeding (Pringle *et al.* 1992). The pattern of rapid resynthesis of trypsin and chymotrypsin following secretion clearly contrasts with that of pepsin (see above), which appears to be synthesised in response to feeding.

In the present study, the resynthesis of trypsin and chymotrypsin in the pancreas may be under some form of homeostatic control, since activity levels in the fed fish matched those of the controls again after 25h. It has been suggested that CCK may act on the pancreas to increase the rate of synthesis following secretory release of enzymes (reviewed by Crawley and Corwin 1994). Such an increase, following stimulation by feeding has been demonstrated in frogs (Poort and Geuze 1969; Van Venrooij and Poort 1971) and snakes (Alcon and Bdolah 1975). In the rat, the rate of biosynthesis of pancreatic enzymes increased 8–10 fold, two hours after injection of a mixture of secretin and CCK (Reggio *et al.* 1971). It is possible therefore that CCK may regulate pancreatic enzyme levels in teleosts, by control of both synthesis and the secretion. Observations on cultured exocrine cells from the pancreas of pike appear to bear this out (Pilz and Plantikow 1992).

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