Digestive Enzymes in Marine Species. I. Proteinase Activities in Gut from Redfish (Sebastes mentella), Seabream (Sparus aurata) and Turbot (Scophthalmus maximus)

R. Munilla-Moran and F. Saborido-Rey
INSTITUTO DE INVESTIGACIONES MARINAS, C.S.I.C., E-36208 VIGO; SPAIN
Correspondence to: R. Munilla-Moran, lnstituto de Investigaciones Marinas, C.S.I.C., Eduardo Cabello, 6. E-36208 Vigo (Pontevedra). Spain. Tel. + 34-86-23-19-30; Fax + 34-86-29-27-62.

Abbreviations--PMSF, phenylmethanesulfonyl fluoride; TLCK, tosyl-lysine chloromethyl ketone; TPCK, tosylamido-2-phenylethyl chloromethyl ketone; DIFP, diisopropyl fluorophosphate; SBTI, soybean trypsin inhibitor; PCMB, p-hydroxymercuribenzoate; NBS, N-bromosuccinimide; TCA, tricholoroacetic acid.

ABSTRACT. The proteolytic activities of the digestive tract of three carnivorous fish species (Sebastes mentella, Scophthalmus maximus, Sparus aurata) have been studied. The activity found in the stomach showed a pH optimum of 2.0 for all species, whereas this was in the alkaline range (9.5-10.0) in the intestinal extracts. Similar optimal temperature was measured for all species, although higher residual activities were detected at low temperatures in redfish and turbot. Lower E_a values were detected in stomach than in intestine. The effect of HCl concentration on protein digestion differed between stomach and intestine being inhibitory in the former. The main proteolytic enzyme for the acid activity of the stomach of the three species seemed to be a pepsin. However, the enzymatic composition of the intestine was more species specific.

KEY WORDS. Protein digestion, proteases, carnivorous fish, stomach, intestine, Sebastes mentella, Scophthalmus maximus, Sparus aurata

INTRODUCTION

During the last decades much information on how fish use the food available in the environment, to be converted in matter and energy, has been gained. The basic sequence

of this process consists of ingestion of prey, digestion and assimilation of the products of digestion.

After the emergence of fish farming, many researchers started to study the way fish deal with ingested food. In this sense, much work has been carried out on gut evacuation rates and how it is influenced by different factors such as temperature, feeding frequency, food size and so on (1-4).

On the other hand, digestive processes in fish are believed to be very similar to those of mammals and other higher vertebrates. In fact, most of the peptides and hormones controlling digestive tract functions in higher vertebrates have also been found in fishes (4,5). Likewise, hydrolytic enzymes responsible for digestion of nutrients in mammals have also been detected in fish guts and in general have similar characteristics (6-9).

In fish, the levels of digestive enzymes may be influenced by the age of the fish (10), type of feeding (6,11,12), season and/or temperature of acclimatization (13) and so on. However, comparison of published data is often hampered by the use of heterogeneous techniques in these works. Comparative studies among several species are therefore highly advisable.

In this series, the ability to digest proteins, lipids, sugars and nucleic acids in three fish species has been investigated. These species are carnivorous active feeders, with a well-defined stomach and pyloric ceca, although their size and number varies with the species.

We deal with enzymatic degradation of dietary proteins; undoubtedly the best known in fish (4,8). The effect of the main environmental variables on protein enzymatic digestion has been studied and (by using specific inhibitors) identification of the principal proteinases of these species has been attempted. The activities of stomach and intestine were separately studied.

MATERIALS AND METHODS

Experimental Fish

Red fish (Sebastes mentella) were obtained during the EEC stratified bottom trawl survey on Flemish Cap Bank (NAFO Div. 3M) east of Newfoundland. Fish were sampled in summer from the same area, the East of Flemish Cap (Strata 14 and 15) at depths ranging between 420 and 480 m. Only fishes caught in summer were used in this study to avoid changes in the digestive system due to changes of season (14). The specimens, identified as S. mentella with the passage of the extrinsic gassbladder musculature between different ventral ribs (15,16), were frozen onboard at -30°C for further studies in the laboratory.

Turbot (Scophthalmus maximus) and seabream (Sparus aurata) adults were purchased from a commercial fish farm northwest of Spain (Cultivos de Peces, S.A., O Grove, Pontevedra). These had been kept in running sea water (≈15-18°C) and fed ad libitum on semi-moist diets. The specimens used for this study were obtained in October.

Fish were harvested by the farm staff, killed by a blow on the head and kept in the freezer until dissection of the gastrointestinal tract (usually done within 3 hr and never at temperatures above 0°C).

Number, size, weight of fish and food composition are given in Table 1. The size of the tanks where turbot and seabream were ongrown are also provided.

Preparation of Homogenates

All procedures were conducted in the cold (0-4°C), unless otherwise stated. Fishes were individually measured and weighed, and the whole gut was dissected. Guts were longitudinally cut open and all the gut contents removed by scrapping with a plastic slide. Then, after discarding the pyloric caeca, the stomachs and the intestines were sliced into small pieces, washed in distilled water, centrifuged at 2,000 rpm for 5 rain and frozen at -18°C until use. Stomachs of the same species were put together before freezing and the same was done for the intestines.

Tissues were thawed by stirring them with Tris-HCl 0.02 M buffer, pH 8.2 (1:4, wet weight:volume) for half an hour, at room temperature. The suspensions were

homogenized using an Ultraturrax at 25,000 rpm, for 2 x 1-rain pulses and the extracts were cleared by centrifuging at 14,000 rpm for 60 min and dialyzed overnight against ca. 150 volumes of buffer. Then, after being re-centrifuged at 14,000 rpm, the supernatants were used immediately or stored deep frozen (-77°C) in small aliquots (0.2 ml) until needed. These fractions were used for caseinolytic activity and protein content measurements.

Enzyme Activity

Total protease activity was measured using casein as substrate as described in Munilla-Moran and Stark (17). A typical assay was performed as follows: 0.25 ml of appropriate buffer, 0.25 ml of casein (10 mg/ml) and 0.05 ml of enzymatic extract were mixed and incubated for 1 hr under the specific conditions of the experiment (pH, temperature and so on). The reaction was stopped by adding 0.6 ml of chilled trichloroacetic acid (TCA) (8% in distilled water) to precipitate the non-digested protein. After 1 hr in the refrigerator, the suspensions were centrifuged at 15,000 rpm for 20 min at 7°C. The clear supernatant was measured in the spectrophotometer at 280 nm against blanks in which casein solution and enzymatic extract was substituted by distilled water. Controls were made in the same way, but the enzymatic extracts were added at the end of the incubation period and just before the TCA. The absorbance was converted in μg of tyrosine by using standard curves in which the enzymatic extract was substituted by different concentrations of tyrosine. Enzymatic extracts were diluted if required. One unit of activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per hour. Results are the mean of three independent experiments.

The effect of pH on the hydrolysis of casein was tested with the following 0.1 M buffers: glycine-HCl (pH 1.0-2.5), phosphate-citrate (pH 2.5-8.0), Tris-HCl (pH 8.0-10.5) and glycine-NaOH (pH 10.5-13.0).

The effect of temperature was measured at the optimal pH found for the respective segment.

The study of HCl concentration on the total protease activity was carried out by using the buffers with adequate concentrations of the salt.

Inhibitors were left to react with the enzymes for 30 min, at room temperature, before adding the exymic extract to the reaction mixture. When studying the effect of the inhibitors the optical readings were corrected according the absorbance at 280 nm due to the inhibitor.

Soluble protein contents of the homogenates were determined by the method of Miller (18), using bovine serum albumin as standard.

All chemicals used in this study were reagent grade and obtained from Sigma (St Louis, MO, U.S.A.) unless otherwise stated.

Statistics

Bartlett's test showed homogeneity of variances in samples. Data were submitted to Student's t-test or analysis of variance (ANOVA) for comparisons among samples. Tuckey's test in one-way ANOVA was also applied for samples comparisons. To test the effect of HCl concentration on relative activities, the Pearson test was used. The significant level was established at 95%. SYSTAT package (19) was used for statistical analysis of data.

RESULTS

In this work, three different marine fish species were used: redfish (S. mentella), seabream (S. aurata) and turbot (S. maximus). The stomach of redfish was sac-like and thin-walled. On the other hand, the stomach of turbot seemed to have strong muscular sheets surrounding a tube-like chamber with lower capacity (20). The seabream stomach has been studied elsewhere (21). The relative length of the intestines (referred to total body length) ranged between 0.5 and 1.0 for the three species. This pattern has been described as typical for carnivorous species (22,23).

Effect of pH

The effect of pH on the proteolytic activities was studied in the range of 1-13. The obtained results are shown in Fig. 1, A and B. In stomach, very acidic optimal pH (2.0) was observed for the three species (Fig. 1A), although a shoulder around pH 4 was detected in redfish and turbot. In intestines (Fig. 1B), the maximal activities were measured in the alkaline range (9.5-10.0). Nevertheless, acid activity (pH 2.0) was detected in intestinal homogenates of seabream (74.97 ± 2.88) and turbot (26.37 ± 1.15).

Effect of Temperature

The effect of temperature was studied in the range of 5-60°C. For the stomach homogenates (Fig. 2A), the optimal temperatures appeared to be between 35 and 40°C. In the case of redfish and turbot, a high proportion (45.77 ± 4.03%) of the maximal activity was retained at such a low temperature as 5°C. Meanwhile, only 13.31 ± 1.84% was retained at the same temperature in seabream. The results of the intestinal extracts are shown in Fig. 2B. As a rule, the optimal temperatures were higher (in ca. 10°C) to those found for their respective stomachs. Not much residual activity was measured at low temperatures (5.71 ± 0.63% for redfish, 8.91 ± 4.14% for turbot and 0.76 ± 1.32% for seabream). The activation energies (E_a), calculated with the help of Arrhenius plots, of these activities are presented in Table 2. No significant differences between the E_a values calculated for the intestinal activities ($P > 0.05$). A break point was found for the proteolytic activity of the stomach of seabream. Lower E_a values were found in stomachs than in intestines ($P < 0.01$).

In turbot stomach and intestine the caseinolytic activity was measured throughout a wide pH range (1-13) at three temperatures (20, 30 and 50°C). By increasing the assay temperature, a shift of the pH optimum to pH 3 was determined in stomach (Fig. 3A). Similarly, in intestine, the optimum pH decreased when temperature increased (Fig. 3B). This effect was clearer in intestine than in stomach.

Effect of Salt Concentration

All species used in this work are marine organisms. Salt will jointly be ingested with solid food. Therefore, cavital enzymes responsible for food digestion must act under a high saline environment. To study the effect of salt concentration on the proteolytic

activity of these species, the hydrolysis of casein was measured in the presence of several levels of sodium chloride. The results are shown in Fig. 4, A and B. HCl inhibited, proportionally to its concentration (Pearson test, $P < 0.05$), the activity in stomach of redfish and turbot. Also, the intestinal activity of redfish was proportionally reduced by salt concentration ($P < 0.01$). On the contrary, the intestinal activity of turbot was activated by HCl ($P < 0.05$). No relationship was found between HCl concentration and activation or inactivation in seabream.

As for temperature, a more detailed investigation was carried out in turbot (Fig. 5, A and B). However, no displacement of the pH optimum was detected in intestine when 0.05 and/or 0.5 M HCl was included in the assay mixture. In stomach, the maximum pH remained at 2.0. However, the relative activities measured at pH 4.0 and 5.0 at 0.5 M HCl were higher ($P < 0.0001$) than those measured at 0.05 M or in the absence of the salt.

Inhibitors

The ability to hydrolyze casein was studied in the presence of several compounds and the obtained activities compared with those measured in their absence. In stomach homogenates, the activity was measured at pH 2; meanwhile, in intestine, it was carried out at pH 9. The results are shown in Table 3. The intention of this experiment was to determine the differences, if any, in the proteolytic enzyme-class composition of these species. The reagents used here were inhibitors relationship of the four main classes of proteinases (24): serine (phenylmethanesulfonyl fluoride [PMSF], diisopropyl fluorophosphates [DIFP], tosyl-lysine chloromethyl ketone [TLCK], tosylamido-2-phenylethyl chloromethyl ketone [TPCK], soybean trypsin inhibitor [SBTI] and Aprotinin A), cysteine (iodoacetate and iodoacetamide), aspartic (Pepstatin) and metallo-proteases (EDTA). p-Hydroxymercuribenzoate (PCMB) and N-bromosuccinimide (NBS) were used as general SH-enzyme inhibitors. Because most proteinase inhibitors have been proved to be rather enzyme specific (24,25), it was arbitrarily decided that only high activity reductions (> 50%) will be considered as inhibitions.

In stomachs, proteolytic activity was fully inhibited by pepstatin in the three species. Also, chelating agents (EDTA) strongly reduced the activity in redfish and seabream but not in turbot. In seabream, similar inhibition by PCMB and NBS was found. Variable inhibition was observed with PMSF being the activity of redfish the most inhibited ($P < 0.05$). Surprisingly, TPCK inhibited the hydrolysis of casein in all species.

In intestines, SH-enzyme inhibitors had equal effects than that showed in the stomach. As expected, no inhibition was measured with pepstatin. In general, serine proteinase inhibitors (PMSF, DIFP, TLCK and TPCK) affected the proteolytic activity, although clear differences among species were noticed. Turbot activity appeared to be the less sensitive to artificial serine proteinase inhibitors and even retained a level of activity above 50% of the control. EDTA inhibited the proteinolytic ability in seabream only.

DISCUSSION

The aim of this work was to study the ability to digest dietary protein of three fish marine species possessing a well-developed stomach. These species showed enzyme activities able to hydrolyze the protein used as substrate in the assay. It is worth mentioning that one of the species used in this work (S. mentella) was caught from the wild; meanwhile, the other two were of aquacultural origin. It might well be that some comparisons of the results could be hampered by this origin difference.

Once the food has been ingested by the fish, it is kept in the gastric lumen where pepsin-like enzymes and, in some instances, HCl is secreted by the chief cells. These cells have been found in all gastric fish species so far investigated (4). Therefore, an acidic environment for gastric digestion must be expected. On the contrary, carbonate salts are secreted into the frontal part of the intestine to neutralize the acidity of the bolus. In fact, intestinal digestion is carried out under neutral-alkaline conditions.

A general common pattern has been found when the effect of pH on the caseinolytic activity of the stomach and intestine of these species has been studied. In the stomach, a maximum around pH 2.0 has been measured, although a shoulder (pH 4.0) was also found higher in redfish and turbot ($P < 0.001$) than in seabream (Fig. 1). It might well be that these two species start to digest the protein before full acidification of the gastric

lumen has been achieved. Two peaks of activity have been detected in gastric juice of several species, although it has been suggested that it might be an artefact due to isolation and assay procedures (26). In intestines, the optimal activity was detected when the pH was high (10.0) as for many other species. The acid activity detected in this segment varied among species ($P < 0.001$) and might be of gastric origin. This hypothesis might be supported by the fact that the highest level was found in seabream, which has the smallest pyloric caeca; meanwhile, redfish (with the largest ones) showed very low acid activity. It is likely that these appendices retain the bolus to allow the pancreatic secretions to inactivate the acid proteases.

Gut lumen temperature in fish is closely linked to that of the environment. Water temperature may have a manifold effect on fish digestion. Gut motility and hence gastric emptying rate is strongly dependent on temperature (1,27,28). Also, fishes acclimated to different temperatures showed distinct proteolytic levels (12,14). And the enzymes involved in protein digestion are influenced by temperature both on the enzyme activity or modifying the affinity of the enzyme by its substrate (29).

When studying the effect of temperature on the hydrolysis of casein, a general pattern was found. The optimal temperatures were lower in stomachs than in intestines ($P < 0.05$). Similar results have been found in two salmonids (30) and in Dover sole (31). The omnivore Clarias gariepinus showed similar maximal temperatures for pepsin, trypsin and chymotrypsin, although the authors did not mention the source of these activities (32).

According to Ugolev et al. (33), the lower the energy of activation value, the higher the efficiency of the enzyme. The E_a values calculated for acid proteolytic activities were lower than those of alkaline activities (Table 2) ($P < 0.05$). The alkaline proteinase activities had E_a values in the range reported for other authors using proteins as substrates (6,26,34,35). The E_a values calculated for acid activities were also in the range reported for other species (8,36). Redfish and turbot did not show a break point in the Arrhenius plots, indicating a high "adaptation potential to a wide temperature range" (37). On the contrary, seabream showed a break point at $20.3 \pm 0.1°C$. In the temperature range below this point, the E_a was about six times higher ($P < 0.001$) than above the break point. Following Ugolev's hypothesis (33), this species seems to have a

more efficient gastric digestion of protein in a warm environment. This can also be observed in Fig. 2. Turbot and redfish retained almost half of the activity at low temperatures (5°C). On the contrary, seabream had less than 3.5 times this activity ($P < 0.001$) at the same temperature. This suggests that the former species are better adapted to digest dietary proteins under colder environments than seabream.

In turbot, both in stomach and intestine, the optimum pH for casein digestion was shifted towards the neutral range. It must be borne in mind that the assay used in this work does not differentiate among all proteolytic enzymes, and it is likely that this effect is due to differences in optimal temperatures of these individual enzymes.

Bearing in mind that these species live in marine environments, luminal digestion of food, especially in stomachs, is likely to proceed under high salt concentrations (ca. 0.51 M). The results obtained in the present study showed a common pattern: the ability to degrade the dietary protein is more inhibited in stomach than intestine ($P < 0.05$). In fact, in turbot it is activated by salt levels.

There are just a few studies dealing with this subject, and their results differ. Sanchez-Chiang et al. (38) and Squires et al. (39) found an activator effect of salt on gastric proteases from salmon and cod. Fang and Chiou (40) tested NaCl concentrations up to 3.42 M with no effect on pepsin, trypsin and chymotrypsin activities from tilapia, although isoenzymic pattern varied depending on acclimatization to different salinities. Finally, the activity of acid proteases from sardine was reduced by 3.42 M NaCl (41). According to the results obtained in this work, it well may be assumed that enzymic gastric digestion of protein is depressed to some extent by the sea water surrounding the solid meal. However, esophagus has been proposed to play an active osmoregulatory role by decreasing the sea water strength (4).

The assay used in this work does not allow differentiation between specific proteases. However, the use of proteinase inhibitors may be a reliable tool for identifying different classes of proteases (25,42,43). The overall protease activity, irrespective of their origin, optimum pH or location, seemed to have SH groups essential for their action since PCMB and NBS strongly inhibited the activity. J6nfis et al. (6) found no effect of

PCMB on the proteolytic activities of fishes having different food regimes, but the authors did not mention the substrate used.

As expected, the acid activity of the stomachs was fully inhibited by pepstatin, a specific inhibitor for aspartic proteinases (pepsin, cathepsin D). Pepstatin also inhibited two pepsins isolated from Oncorhynchus keta (38).

The effect of the chelating agent EDTA was species specific. This compound inhibited the activity in stomach and intestine of seabream. On the contrary, it had little effect on turbot activities. In redfish, EDTA only inhibited the acid activity of the stomach. No reference has been found regarding the need of divalent cations for activity or stability of pepsin. However, in muscle of two fish species (44), cathepsin D was inhibited by pepstatin and some metal ions. These authors also reported an EDTA concentration-dependent inhibition of cathepsin D in milkfish (44). Therefore, it might be that gastric (acid) digestion is carried out, at least, by pepsin and cathepsin D in redfish and seabream; meanwhile, only pepsin is acting in turbot's stomach. Among alkaline proteases carboxipeptidases (A and B), elastase and aminopeptidases, some metal ion requirements have been reported (9,45,46).

High molecular weight (proteinaceous) serine protease inhibitors (Aprotinin and SBTI) inhibited the intestinal activity more strongly than low molecular weight (synthetic) inhibitors, probably due to their limited solubility and stability in aqueous solutions (47). Nevertheless, as stated before, the fact that the activities were measured with an unspecific substrate and that no purified enzymes have been used in this study likely hampered clearer results. Other authors have used similar conditions in their studies (7,48,49).

We thank J. L. Rodriguez, biologist of Cultivos de Peces, S.A. (0 Grove, Pontevedra, Spain), for turbot and seabream supplies and C. Barreiro and E. Calvar for technical assistance. This work was supported by a grant from the Xunta de Galicia No. XUGA402OIB90.

References

1. Flowerdew, M. W.; Grove, D. J. Some observations of the effects of body weight, temperature, meal size and quality on gastric emptying time in turbot, Scophthalmus maximus (L.) using radiography. J. Fish Biol. 14: 229-238; 1979.
2. Holmgren, S.; Grove, D. J.; Fletcher, D. J. Digestion and the control of gastrointestinal motility. In: Rankin, J. C.; Pitcher, T. J.; Duggan, R. T., eds. Control processes in fish physiology. London: Croom Helm; 1983: pp. 23-40.
3. Grove, D. J. Gastro-intestinal physiology: rates of food processing in fish. In: Nilsson, S.; Holmgren, S., eds. Fish physiology: recent advances. London: Croom Helm; 1986: p. 140-152.
4. Smith, L. S. Digestive functions in teleost fishes. In: Halver, J. E., ed. Fish nutrition, 2nd ed. London: Academic Press; 1989: p. 331-421.
5. Holmgren, S.; J6nsson, A. C; Holstein, B. Gastro intestinal peptides in fish. In: Nilsson, S.; Holmgren, S., eds. Fish physiology: recent advances. London: Croom Helm; 1986: p. 119-139.
6. Jonas, E.; Ragyanszki, M.; Olah, J.; Boross, L. Proteolytic digestive enzymes of carnivorous (Silurus glanis L.), herbivorous (Hypohthalmichthys molitrix Val.) and omnivorous (Cyprinus carpio L.) fishes. Aquaculture, 30: 145-154; 1983.
7. Krogdhal, A.; Holm, H. Pancreatic proteinases from man, trout, rat, pig, chicken, mink and fox. Enzyme activities and inhibition by soybean and lima bean proteinase inhibitors. Comp. Biochem. Physiol. 74B: 403-409; 1983.
8. Vonk, H. J.; Western, J. R. H. Comparative biochemistry and physiology of enzymatic digestion. London: Academic Press, 1984.
9. Yoshinaka, R.; Sato, M.; Tanaka, H.; Ikeda, S. Distribution of pancreatic elastase and metalloproteinase in several species of fish. Comp. Biochem. Physiol. 80B: 227-233; 1985
10. Il'ina, I. D.; Turetskiy, V. I. Development of the digestive function in fishes. J. Ichthyol., 28: 74-82; 1988.
11. Hofer, R.; Schiemer, F. Proteolytic activity in the digestive tract of several species of fish with different feeding habits. Oecologia, 48: 342-345; 1981.
12. Hofer, R. Protein digestion and proteolytic activity in the digestive tract of an omnivorous cyprinid. Comp. Biochem. Physiol., 72A: 55-63; 1982.
13. Kuz'mina, V. V. Evolutionary features of the digestive-transport function in fish. J. Evol. Biochem. Physiol., 27: 127-134; 1991.

14. Hofer, R. The adaptation of digestive enzymes to temperature, season and diet in roach, Rutilus rutilus and rudd Scardinius erythrophthalmus; Proteases. J. Fish Biol., 15: 373-379; 1979.
15. Ni, I.-H. Separation of sharp beaked redfish Sebastes fasciatus and S. mentella from Northeastern Grand Bank by morphology of extrinsic gassbladder musculature. J. North. Atl. Sci., 2: 7-12; 1981.
16. Power, D. J.; Ni, I.-H. Morphology of the extrinsic gassbladder musculature in the Golden Redfish, Sebastes marinus. J. North Atl. Sci., 3: 165-168; 1982.
17. Munilla-Moran, R.; Stark, J. R. Protein digestion in early turbot larvae, Scophthalmus maximus (L.). Aquaculture, 81: 315-327; 1989.
18. Miller, G. L. Protein determination for large numbers of samples. Anal. Chem. 31: 964; 1959.
19. Wilkinson, L. Systat: The system for statistics. Evanston, IL: Systat Inc.; 1985.
20. Munilla-Moran, R.; Stark, J. R. Metabolism in marine flatfish. VI. Effect of nutritional state on digestion in turbot, Scophthalmus maximus (L.). Comp. Biochem. Physiol., 95B: 625-634; 1990.
21. Cataldi, E.; Cataudella, S.; Monaco, G.; Rossi, A.; Tancioni, L. A study of the histology and morphology of the digestive tract of the sea-bream, Sparus aurata. J. Fish Biol., 30: 135-145; 1987.
22. De Groot, S. J. On the interrelationships between morphology of the alimentary tract, food and feeding behaviour in flatfishes (Pisces: Pleuronectiformes). Nether. J. Sea Res., 5: 121-196; 1971.
23. Khanna, S. S. Alimentary canal in some teleostean fishes. J. Zool. Soc. India, 13: 206-219; 1961.
24. Benyon, R. J.; Bond, J. S. Proteolytic enzymes. A practical approach. (Benyon, R. J.; Bond, J. S., editors). IRL Press, Oxford, England, 259 pages, 1989.
25. Laskowski, M.; Kato, I. Protein inhibitors of proteinases. Annu. Rev. Biochem. 49: 593-626; 1980.
26. Arunchalam, K.; Haard, N. F. Isolation and characterization of pepsin from Polar cod (Boregadus saida). Comp. Biochem. Physiol. 80B: 467-473; 1985.
27. Windel, J. T. Digestion and the daily ration of fishes. In: Gerking, S. D., ed. Ecology of freshwater fish production. Oxford: Blackwell; 1978: p. 159-183.

28. Fauconneau, B.; Choubert, G.; Blanc, D.; Breque, J.; Luquet, P. Influence of environmental temperature on flow rate of foodstuffs through the gastrointestinal tract of rainbow trout. Aquaculture, 34: 27-39; 1983.
29. Dabrowski, K. The role of proteolytic enzymes in fish digestion. In: Styczynska, E.; Backiel, T.; Jaspers, E.; Persoone, G.; eds. Cultivation of fish fry and its live food. Eur. Maricult. Soc., Spec. Publ. No 4. Belgium: Bredene; 1979: p. 102-126.
30. Torrissen, K. Characterization of proteases in the digestive tract of Atlantic salmon (Salmo salar) in comparison with rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol., 77B: 669-674; 1984.
31. Clark, J.; MacDonald, N.; Stark, J. R. Metabolism in marine flatfish. II. Protein digestion in Dover sole (Solea solea L.). Comp. Biochem. Physiol., 81B: 217-222; 1985.
32. Uys, W.; Hecht, T. Assays on the digestive enzymes of sharptooth catfish, Clarias gariepinus (Pisces: Claridae). Aquaculture, 63: 301-313; 1987.
33. Ugolev, A. M.; Egorova, V. V.; Kuz'mina, V. V.; Grudskov, A. A. Comparative-molecular characterization of membrane digestion in fish and mammals. Comp. Biochem. Physiol., 76B: 627-635; 1983.
34. Simpson, B. K.; Haard, N. F. Characterization of the trypsin fraction from cunner (Tautogolabrus adspersus). Comp. Biochem. Physiol., 80B: 475-480; 1985.
35. Ugolev, A. M.; Kuz'mina, V. V. Membrane hydrolases of fish enterocytes. Temperature adaptations. Comp. Biochem. Physiol., 106B: 433-452; 1993.
36. Twining, S, S.; Alexander, P. A.; Huibregtse, K.; Click, D. M. A pepsinogen from rainbow trout. Comp. Biochem. Physiol. 75B: 109-112; 1983.
37. Gelman, A.; Cogan, U.; Mokady, S. The thermal properties of fish enzymes as a possible indicator of the temperature adaptation potential of the fish. Comp. Biochem. Physiol., 101B: 205-208; 1992.
38. Sanchez-Chiang, L.; Cisternas, E.; Ponce, O. Partial purification of pepsins from adult and juvenile salmon fish Oncorhynchus keta. Effect of NaCl on proteolytic activities. Comp. Biochem. Physiol., 87B: 793-797; 1987.
39. Squires, E. J.; Haard, N. F.; Feltham, L. A. W. Gastric proteases of Greenland cod Gadus ogac. Structural properties. Biochem. Cell. Biol., 64: 215-222; 1986.
40. Fang, L. S.; Chiou, S. F. Effect of salinity on the activities of digestive proteases from the Tilapia fish, Oreochromis niloticus, in different culture environments. Comp. Biochem. Physiol., 93A: 439-443; 1989.

41. Noda, M.; Murakami, K. Studies of proteinases from the digestive organs of sardine. Purification and characterization of two acid proteinases from the stomach. Biochem. Biophys. Acta, 658: 27-32; 1981.
42. Braun, R.; Amesen, J. A.; Rinne, A.; Hjelmeland, K. Immunohistological localization of trypsin in mucus secreting cell layers of Atlantic salmon, Salmo salar. L. J. Fish Dis., 13: 233-238; 1990.
43. Salvesen, G.; Nagase, H. inhibition of proteolytic enzymes. In: Beynon, R. J.; Bond, J. S., eds. Proteolytic enzymes: a practical approach. Oxford: IRL Press; 1987: p. 83-104.
44. Jiang, S. T.; Her, Y. H.; Lee, J. J.; Wang, J. H. Comparison of the cathepsin D from mackerel (Scomber australasicus) and milkfish (Chanos chanos) muscle. Biosci. Biotech. Biochem., 57: 571-577; 1993.
45. Clark, J.; MacDonald, N.; Stark, J. R. Leucin aminopeptidase in the digestive tract of Dover sole (Solea solea L.). Aquaculture 61: 231-239; 1987.
46. Neurath, H. The diversity of proteolytic enzymes. In: Beynon, R. J.; Bond, J. S.; eds. Proteolytic enzymes: a practical approach. Oxford: IRL Press; 1989: p. 1-13.
47. North, M. J. Prevention of unwanted proteolysis. In: Beynon, R. J.; Bond, J. S.; eds. Proteolytic enzymes: a practical approach. Oxford: IRL Press; 1989: p, 105-124.
48. Jany, K. D. Studies on the digestive enzymes of the stomachless bonefish Carassius auratus gibelio (Bloch): Endopeptidases. Comp. Biochem. Physiol., 53B: 31-38; 1976.
49. Martinez, A.; Serra, J. L. Proteolytic activities in the digestive tract of anchovy (Engraulis encrasicholus). Comp. Biochem. Physiol., 93B: 61-66; 1989.

TABLE 1. Number of fish, morphometric measurements and rearing conditions of the species studied

Species	Number of fish	Size range (cm)	Weight range (g)	Tank (m^3)	Diet	Organic matter (%)	P: L: CH*
Redfish	18	22.0-40.0	320-810	Wild	Natural	-	-
Seabream	5	18.0-22.5	76-196	2.7	Semi-moist	83.95 ± 1.14	2:2:0.1
Turbot	6	26.5-32.0	401-585	20.0	Semi-moist	83.95 ± 1.14	2:2:0.1

*P: L: CH, proportion of protein: lipids : carbohydrates (of organic matter) of the semi-moist diets.

TABLE 2. Energy activation, E_a (kcal/mol/K) values of the caseinolytic activities of the different gut segments

	Stomach	Intestine
Redfish	4.73 ± 0.44^a	12.16 ± 0.48^a
Seabream	3.04 ± 0.17^b	12.97 ± 0.37^a
	18.68 ± 0.90^c	
Turbot	5.50 ± 0.33^a	12.13 ± 1.03^a

Within the column, the same superscript means no significant differences (P = 0.05).

TABLE 3. Effect of several inhibitors on the caseinolytic activity in stomach (measured at pH 2) and intestine (at pH 9) of redfish, seabream and turbot

	Stomach			Intestine		
Inhibitor	Redfish	Seabream	Turbot	Redfish	Seabream	Turbot
None	100	100	100	100	100	100
PMSF	38.0 ± 0.5^{a}	$66.8 + 2.5^{c}$	51.6 ± 1.0^{b}	53.7 ± 1.1^{b}	56.3 ± 4.8^{b}	88.5 ± 1.4^{d}
TLCK	67.1 ± 4.1^{a}	82.8 ± 1.8^{b}	81.2 ± 6.0^{b}	56.9 ± 1.4^{a}	$58.9 \pm 0.6^{a-c}$	69.2 ± 5.7^{c}
TPCK	16.9 ± 1.4^{a}	38.0 ± 3.7^{b}	30.9 ± 0.9^{c}	45.1 ± 0.8^{a}	57.9 ± 2.4^{e}	75.0 ± 2.8^{f}
DIFP	88.8 ± 1.4^{a}	94.7 ± 2.0^{a}	91.4 ± 0.8^{a}	1.9 ± 0.8^{b}	16.8 ± 0.8^{c}	58.6 ± 3.1^{d}
SBTI	57.7 ± 3.9^{a}	75.5 ± 4.4^{b}	89.2 ± 1.1^{c}	26.6 ± 4.4^{d}	4.6 ± 2.2^{e}	$15.0 \pm 6.1^{e,f}$
Aprotinin A	75.4 ± 1.9^{a}	96.9 ± 1.2^{b}	93.8 ± 1.1^{b}	48.7 ± 4.6^{c}	32.2 ± 0.8^{d}	28.6 ± 1.3^{d}
Iodoacetate	62.8 ± 0.9^{a}	65.7 ± 1.2^{a}	62.2 ± 1.2^{a}	63.3 ± 4.5^{a}	67.2 ± 3.7^{a}	64.9 ± 1.2^{a}
Iodoacetamide	67.1 ± 5.1^{a}	66.0 ± 1.8^{a}	59.7 ± 1.3^{a}	62.8 ± 5.9^{a}	65.5 ± 2.2^{a}	68.2 ± 3.1^{a}
Pepstatin	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.2 ± 0.2^{a}	87.2 ± 7.1^{b}	91.1 ± 11.3^{b}	95.5 ± 2.8^{b}
PCMB	35.9 ± 2.1^{a}	35.5 ± 0.3^{a}	33.1 ± 0.9^{a}	38.1 ± 2.4^{a}	34.9 ± 1.7^{a}	37.3 ± 0.7^{a}
NBS	51.1 ± 3.1^{a}	27.9 ± 8.2^{b}	44.1 ± 1.6^{a}	48.6 ± 4.1^{a}	26.3 ± 4.2^{b}	46.7 ± 1.8^{a}
EDTA	5.2 ± 1.1^{a}	15.1 ± 2.6^{c}	80.6 ± 1.2^{b}	80.5 ± 6.0^{b}	3.3 ± 3.1^{a}	75.3 ± 1.7^{b}

All inhibitors used were at a final concentration of 500 μM, but pepstatin that was at 5 μM. The given values are the percentage of inhibition compared with the control (assayed without inhibitor). The same superscript within the same row means no significant differences (one-way ANOVA, $P > 0.05$).

FIG. 1. The effect of pH on caseinolytic activity in stomach (A) and intestine (B) of redfish (O), seabream (Δ) and turbot (□).

FIG. 2. The effect of temperature on caseinolytic activity in stomach at pH 2.0 (A) and intestine at pH 9.0 (B) of redfish (O), seabream (Δ) and turbot (□).

FIG. 3. The effect of temperature on the pH optimum in stomach at pH 2.0 (A) and intestine at pH 9.0 (B) of turbot. Activities assayed at 20°C (O), 30°C (□) and 50°C (Δ).

FIG. 4. The effect of NaCl concentration on caseinolytic activity in stomach at pH 2.0 (A) and intestine at pH 9.0 (B) of redfish (O), seabream (Δ) and turbot (□).

FIG. 5. The effect of NaCl concentration on the pH optimum in (A) stomach at pH 2.0 and (B) intestine at pH 9.0 of turbot. Activities assayed at NaCl concentrations of 0 M (O), 0.05 M (□) and 0.5 M (Δ).