Purification and Characterization of Trypsin from the Pyloric Caeca of Rainbow Trout (*Oncorhynchus mykiss*)

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Trypsin was purified from the pyloric caeca of rainbow trout (*Oncorhynchus mykiss*) by $(NH_4)_2SO_4$ fractionation, hydrophobic interaction chromatography, and affinity chromatography. The isolated enzyme had a single band on SDS-PAGE with an estimated molecular mass of 25.7 kDa. The trypsin was stable at pH 5-11 for 30 min at 30 °C, and its maximal activity against α-benzoyl-L-arginine *p*-nitroanilide (L-BAPNA) was between pH 9 and 10. The enzyme was stable up to about 40 and 50 °C for 30 min, at pH 5.4 and 8.0, respectively, but the thermal stability was highly calcium dependent (10-15 mM). Maximal activity of the enzyme against L-BAPNA was around 60 °C. The catalytic efficiency (k_{cat}/K_M) of the trout trypsin was about 12-15-fold higher than that of bovine trypsin, when measured at 10-20 °C, with L-BAPNA as a substrate, and its caseinolytic activity, at these temperatures, was about 1.6-2 times higher than that of the bovine enzyme, reflecting adaption of the activities of the fish trypsin to low temperatures.

INTRODUCTION

The growth of the fishing industry will in the near future increasingly depend on better utilization of the available raw material. Fish viscera is offal produced in large quantities by the fishing industry and represents a waste disposal and a potential pollution problem (Simpson and Haard, 1987). This material, however, is a rich potential source of various enzymes that may have some unique properties of interest for both basic research and industrial applications.

Fishes are poikilothermic, so their survival in cold waters required adaption of their enzyme activities to the low temperatures of their habitats. Enzymes from cold-adapted fish species thus often have higher enzymatic activities at low temperatures than their counterparts from warm-blooded animals (Simpson and Haard, 1987; Simpson et al., 1989; Asgeirsson et al., 1989; Raa, 1990). High activity of fish enzymes at low temperatures may be interesting for several industrial applications of enzymes, such as in certain food-processing operations that require low processing temperatures. Furthermore, relatively lower thermal stability, often observed with fish enzymes, may also be beneficial in such applications as the enzymes can be inactivated more readily, with less heat treatment, when desired in a given process (Simpson and Haard, 1987).

The study reported on here had the objective to investigate the properties of enzymes found in the viscera of rainbow trout (*Oncorhynchus mykiss*), which is by far the most important farmed fish in Denmark. The present paper describes the purification and some characteristics of the serine protease trypsin from the pyloric caeca of the trout and compares some of its properties to that of bovine trypsin and other fish trypsins that have been characterized to date.

MATERIALS AND METHODS

Materials. Rainbow trout viscera were obtained from a local Danish fish farm, frozen, and kept at -30 °C until use. N^{α}-Benzoyl-L-arginine *p*-nitroanilide (L-BAPNA), benzamidine, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), *p*-nitrophenyl *p*-guanidinobenzoate, soybean trypsin inhibitor (SBTI), aprotinin, Bowman-Birk inhibitor, bovine casein, and bovine trypsin (type III) were all purchased from Sigma Chemical Co. (St. Louis, MO). Phenyl-Sepharose CL-4B, benzamidine-Sepharose 6B, sodium dodecyl sulfate (SDS), *N,N,N',N'*-tetramethylethylenediamine (TEMED), ammonium persulfate, acrylamide, *N,N'*-methylenebis(acrylamide), Coomassie brilliant blue R-250, electrophoresis low molecular weight calibration kit, Ampholine PAG plates (pH 3.5-9.5), and p*I* markers (p*I* 2.4-5.65 and 4.7-10.6) were obtained from Pharmacia LKB (Uppsala, Sweden). All other chemicals were of analytical grade.

Purification Procedure. Pyloric caeca were dissected from the viscera, homogenized, and extracted with 5 volumes of 25 mM Tris-HCl buffer, pH 8.1, containing 0.3 M NaCl and stirred for 3 h. The extract was centrifuged at 19600*g* for 30 min. The supernatant was adjusted to pH 7 and $CaCl_2$ added to a final concentration of 20 mM. The mixture was stirred for 18 h and then allowed to stand for 2 h, and finally centrifuged at 19600*g* for 30 min (Asgeirsson et al., 1989). The supernatant was fractionated by $(NH_4)_2SO_4$, and the precipitate that formed at 30-70% saturation of $(NH_4)_2SO_4$ was collected by centrifugation at 19600*g* for 30 min. The precipitate was redissolved in 130 mL of 25 mM Tris-HCl, pH 7.7, made 1 M in $(NH_4)_2SO_4$, and applied to a phenyl-Sepharose CL-4B column (2.5 cm × 12 cm) and eluted with the same buffer (600 mL) until the nonbinding proteins had passed through the column. The column was then eluted with about 430 mL of the original buffer without $(NH_4)_2SO_4$ that released the trypsin activity from the column. The fractions containing trypsin activity were pooled and lyophilized. The redissolved sample (45 mL) was applied to a column of benzamidine-Sepharose 6B (0.9 cm × 10 cm) equilibrated with 25 mM Tris-HCl, pH 7.7, containing 10 mM $CaCl_2$ and eluted with the same buffer containing 0.5 M NaCl, until no change in absorbance at 280 nm was observed in the effluent (96 mL). Trypsin activity was eluted from the column with the original buffer containing 125 mM benzamidine. The pooled fractions containing trypsin were dialyzed overnight against about 300 volumes of the same buffer, without benzamidine, and then frozen in liquid nitrogen and stored at -80 °C until use. All steps in the purification were carried out at 5 °C, and the reported pHs are that of the buffers at that temperature.

Activity Measurements. Trypsin amidase activity was assayed by using L-BAPNA as a substrate (Erlanger et al., 1961). The activity was typically assayed at 25 °C against 0.5 mM

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L-BAPNA dissolved in 50 mM Tris-HCl, pH 8.2, containing 20 mM $CaCl_2$, and the release of *p*-nitroaniline was followed by the increase in absorbance at 410 nm (ϵ_{410} = 8800 M^{-1} cm^{-1}) using a Shimadzu UV 160 A recording spectrophotometer (Shimadzu Corp., Kyoto).

Electrophoresis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out by using a discontinuous buffer system in 12.5% (w/v) vertical slab gels, according to the procedure of Laemmli (1970). Gels were stained with Coomassie brilliant blue R-250. Isoelectric focusing was carried out at 4 °C in horizontal precast thin-layer polyacrylamide gels [Ampholine PAG plate (Pharmacia LKB)] containing Ampholine for the pH range 3.5–9.5. Isoelectric focusing calibration kits covering the p*I* ranges 2.4–5.65 and 4.7–10.6 were used for isoelectric point estimations.

Effect of pH on Stability and Activity. pH stability was assessed by determining the activity of the enzyme (0.1 mg mL^{-1}) before and after incubation for 30 min at 30 °C in universal buffer (pH 3–11) (Dawson et al., 1969) or glycine/NaOH buffer (50 mM) (pH 8.4–10.3). The effect of pH on the activity of the enzyme was determined by measuring initial rates against L-BAPNA dissolved in the same buffers adjusted to different pHs in the range pH 4–10.3.

Effect of Temperature on Stability and Activity. To determine the thermal stability, the enzyme (0.1 mg mL^{-1}) was dissolved in either 0.1 M sodium acetate buffer at pH 5.4 or 0.1 M Tris-HCl, pH 8.0; both buffers contained 10 mM $CaCl_2$. Samples were heated in sealed tubes at each temperature (10–65 °C) for 30 min and then cooled on ice and assayed for residual activity against L-BAPNA at 25 °C.

The effect of $CaCl_2$ on thermal stability was determined by heating aliquots of the enzyme (0.1 mg mL^{-1}) dissolved in 0.1 M Tris-HCl, pH 8.0, containing 1 mM EDTA, with or without 15 mM $CaCl_2$, at 55 °C. After a specific heating time, the samples were cooled on ice and assayed for remaining activity in the standard assay. The influence of temperature on the activity of the trypsin was determined by equilibrating the standard assay mixture at selected temperatures between 10 and 77 °C, before the enzyme was added and initial rates were determined. The pH of the buffer was adjusted such that it was 8.2 at each temperature tested.

The kinetic parameters K_M and k_{cat} for the amidase activity of the trout and bovine trypsin at 10–20 °C were determined from Lineweaver–Burk plots with L-BAPNA (0.075–0.75 mM) as a substrate. Active concentrations of the enzymes were determined by titration with *p*-nitrophenyl *p'*-guanidinobenzoate, according to the method of Chase and Shaw (1967).

The caseinolytic activity of the enzymes at 10–20 °C was determined with a Radiometer pH stat (consisting of a PHM 82 pH meter, TTT 80 titrator, and ABU 80 autoburet) (Radiometer, Copenhagen). The pH of the casein solutions used (5 mL of 18 mg/mL dissolved in 5 mM $CaCl_2$) was adjusted to 8.0 with 0.1 N NaOH. After this solution had been equilibrated at the given temperature, 50-μL aliquots of trypsin (dissolved in 5 mM $CaCl_2$) were added, and the time course of hydrolysis was monitored by measuring the amount of 0.01 N NaOH required to maintain the pH at 8.0. Activity was determined from initial rates of hydrolysis, as micromoles of NaOH added per minute per milligram of trypsin present in the assay.

All protein estimations were done according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Purification. The results of the purification of trypsin from the pyloric caeca of rainbow trout are summarized in Table I. By use of this procedure the enzyme was purified 70-fold and with a recovery of about 49% of the amidase activity present in the crude extract. The affinity-purified enzyme migrated as a single protein band, with an estimated molecular mass of 25.7 kDa, when analyzed by SDS–PAGE (Figure 1). Molecular masses of 23.5–28 kDa have been reported for trypsins isolated from various fish species (Murakami and Noda, 1981; Hjelmeland and Raa, 1982; Uchida et al., 1984; Genicot et al., 1988; Martinez et al., 1988; Asgeirsson et al., 1989; Simpson et al., 1989). Isoelectric focusing in the pH range 3.5–9.5 resolved the enzyme from the affinity column into two major bands having apparent isoelectric points of 4.6 and 4.9–5.0, respectively. Occurrence of such multiple forms of trypsin has also been observed when the enzyme was isolated from sardine (Murakami and Noda, 1981), capelin (Hjelmeland and Raa, 1982), chum salmon (Uchida et al., 1984), eel (Yoshinaka et al., 1985), anchovy (Martinez et al., 1988), and Atlantic cod (Asgeirsson et al., 1989; Raae and Walther, 1989). Further purification of those two forms was not attempted, and the affinity-purified enzyme was used in all experiments described in this study.

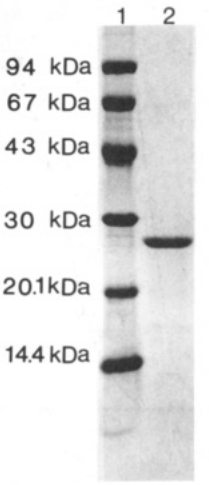

Figure 1. SDS–polyacrylamide gel electrophoresis of trypsin from rainbow trout. (Lane 1) Standard proteins; phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). (Lane 2) Trypsin from rainbow trout from benzamidine–Sepharose column.

Table I. Purification of Trypsin from the Pyloric Caeca of Rainbow Trout

purifn step	protein, mg	total units, μmol/min	sp act., units/mg	yield, %	purifn, *x*-fold
crude extract	4380	199	0.05	100	1.0
$(NH_4)_2SO_4$ (30–70%)	859	189	0.22	95.0	4.9
phenyl-Sepharose	341	170	0.5	85.4	11.1
benzamidine–Sepharose	30	97	3.2	48.7	70.4

Table II. Effect of Inhibitors on the Activity of Trypsin from Rainbow Trout

inhibitor	concn[a]	residual act., %
PMSF	1 mM	38.4
TLCK	1 mM	7.2
TPCK	1 mM	99.3
benzamidine	5 mM	7.6
SBTI	100 μg mL^{-1}	0.0
aprotinin	100 μg mL^{-1}	0.0
Bowman–Birk inhibitor	100 μg mL^{-1}	0.0

[a] Incubation was for 30 min at 25 °C.

Effect of Inhibitors. To confirm the identity of the purified enzyme, the effects of various protease inhibitors on its activity were determined (Table II). The enzyme was inhibited by PMSF, an inhibitor of the serine proteases, as well as the trypsin specific inhibitor TLCK, while TPCK, a site-specific inhibitor of chymotrypsin, had no significant effect on its activity. The trypsin inhibitors, benzamidine, SBTI, aprotinin, and Bowman–Birk inhibitor, all inhibited the enzyme, confirming its identity as a trypsin.

Effect of pH on Stability and Activity. The effects of pH on the activity and stability of the trout trypsin are

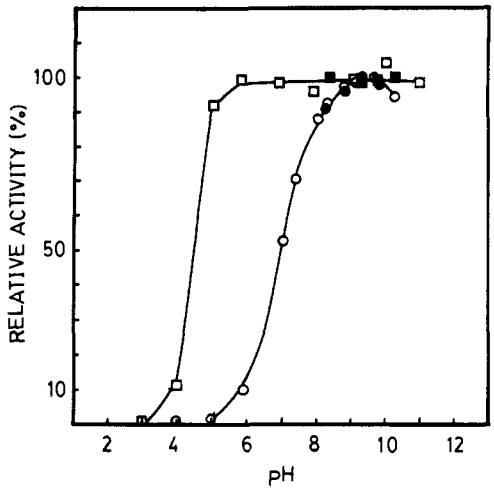

Figure 2. Effect of pH on the stability (□, ■) and activity against L-BAPNA at 25 °C (○, ●) of rainbow trout trypsin. Open symbols represent values obtained from measurements in universal buffer and solid symbols those in 50 mM glycine/NaOH buffer.

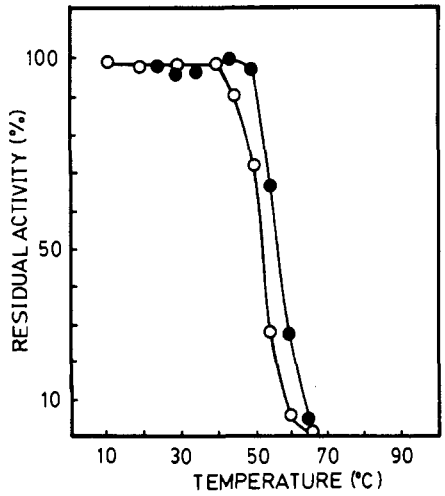

Figure 3. Thermal stability of rainbow trout trypsin at pH 5.4 and 8.0. Enzyme solutions (0.1 mg mL^{-1}) were heated for 30 min at the appropriate temperature before assays for remaining activity against L-BAPNA in the standard assay were performed. Buffers used were 0.1 M sodium acetate, pH 5.4 (○), or 0.1 M Tris-HCl, pH 8.0 (●), both containing 10 mM $CaCl_2$.

depicted in Figure 2. The enzyme was stable at 30 °C for 30 min in the pH range from approximately pH 5 to 11. At pH values below 5 the stability of the enzyme decreased sharply. Diminished stability at acidic pHs has been observed for trypsin from several fish species (Hjelmeland and Raa, 1982; Uchida et al., 1984; Yoshinaka et al., 1984, 1985; Genicot et al., 1988; Simpson et al., 1989) and is in marked contrast to the pH stability of bovine trypsin that is most stable at pH 3 (Lazdunski and Delaage, 1967; Keil, 1971). Maximum activity of the trout trypsin, measured as initial rates against L-BAPNA, was between pH 9 and 10 (Figure 2), which is somewhat higher than reported for most trypsins, which usually have pH optima centered around pH 8 (Simpson et al., 1989). More alkaline pH optima have, however, been reported for the enzymes from sardine (Murakami and Noda, 1981) and anchovy (Martinez et al., 1988).

Effect of Temperature on Stability and Activity. The thermal stability profile of the trout trypsin at pH 5.4 and 8.0 is shown in Figure 3. At pH 5.4 the enzyme was fully stable for 30 min at 40 °C, but at higher temperatures the stability decreased; the trypsin was fully inactivated after 30 min at 65 °C. At pH 8.0 the enzyme was

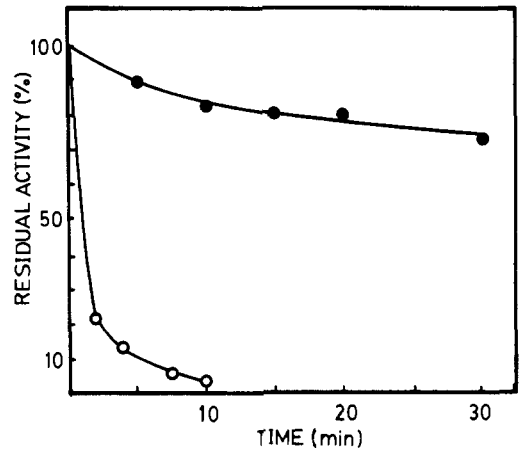

Figure 4. Effect of $CaCl_2$ on thermal stability of rainbow trout trypsin. The enzyme was dissolved in 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM EDTA with or without 15 mM $CaCl_2$. Heating was at 55 °C. Residual activity is expressed as relative to that of an unheated sample, in the presence of 15 mM $CaCl_2$ (●) or in the absence of $CaCl_2$ (○).

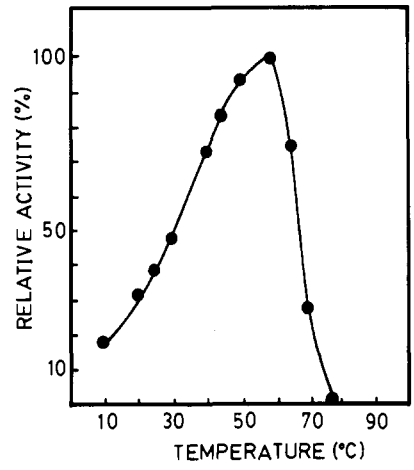

Figure 5. Effect of temperature on the amidase (L-BAPNA) activity of trypsin from rainbow trout. Activity was measured in the standard amidase assay, equilibrated at the appropriate temperature.

stable up to about 50 °C, above which the stability decreased sharply. The observed difference in thermal stability should reflect a difference in conformational stability of the enzyme at these pHs, as inactivation resulting from autolysis should be minimized at pH 5.4. Although thermal stabilities have not been determined for all of the fish trypsins described so far, it appears, as with the trout trypsin, that most of them are unstable to heating at temperature above 40–50 °C (Hjelmeland and Raa, 1982; Uchida et al., 1984; Genicot et al., 1988; Martinez et al., 1988).

The thermal stability of the trout trypsin was highly dependent on the presence of calcium (Figure 4). In the absence of added $CaCl_2$ the enzyme was almost fully inactivated after 10 min of heating at 55 °C, whereas in the presence of 15 mM $CaCl_2$ over 80% its original activity was retained under the same conditions. Binding of calcium to a single binding site in bovine trypsin significantly stabilized that enzyme against denaturation (Lazdunski and Delaage, 1967; Delaage et al., 1968; Bode and Schwager, 1975). Stabilization against thermal inactivation by calcium has also been reported for the trypsins from catfish (Yoshinaka et al., 1984) and eel (Yoshinaka et al., 1985).

The activity of the trout trypsin against L-BAPNA increased with temperature up to about 60 °C (Figure 5).

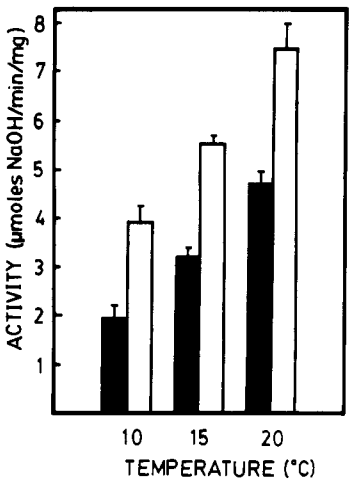

Figure 6. Comparison of caseinolytic activities of bovine (dark bars) and rainbow trout trypsin (open bars). Activity is expressed as micromoles of OH^- consumed per minute per milligram of enzyme at each temperature at pH 8.0, measured with a pH stat.

Table III. Comparison of Kinetic Parameters of Rainbow Trout and Bovine Trypsin for Activity against L-BAPNA at 10–20 °C and pH 8.2

enzyme	temp, °C	K_M, mM	k_{cat}, s^{-1}	k_{cat}/K_M, mM^{-1} s^{-1}
bovine	10	0.342	0.70	2.0
	15	0.469	1.26	2.7
	20	0.455	1.63	3.6
trout	10	0.061	1.70	27.9
	15	0.054	2.14	39.6
	20	0.077	3.28	42.6

Above this temperature the activity decreased sharply, presumably as a result of thermal inactivation. Similar results have been obtained for the bovine, trout, and the Antarctic fish *Paranotothenia magellanica* (Genicot et al., 1988) enzymes. Atlantic cod trypsin was in one study reported to have a temperature optima around 55 °C (Asgeirsson et al., 1989) but at 40 °C in other studies (Simpson et al., 1989). Temperature optima of 40–45 °C have been reported for the trypsin from capelin (Hjelmeland and Raa, 1982), sardine (Murakami and Noda, 1981), stomachless cunner (Simpson and Haard, 1987), and herring (Kalac, 1978), while that of Greenland cod was at 35 °C, when activity was measured against L-BAPNA (Simpson and Haard, 1984; Simpson et al., 1989).

The Michaelis–Menten kinetic parameters for the activity of trout and bovine trypsins against L-BAPNA at 10–20 °C are listed in Table III. The catalytic efficiency (k_{cat}/K_M) of the trout enzyme was about 12–15-fold higher than that of the bovine enzyme in that temperature range. While the turnover numbers (k_{cat}) for the trout enzyme were about twice as high as those of the bovine enzyme, more significant contribution to the higher catalytic efficiency of the trout trypsin comes from its lower apparent K_M values for this substrate. Similar results have been described for the amidase activity of trypsin from Atlantic cod against L-BAPNA (Asgeirsson et al., 1989). The catalytic efficiency of the cod enzyme was about 17-fold higher than that of bovine trypsin at 25 °C, where the major contribution came from about 8.5-fold lower K_M values (Asgeirsson et al., 1989). Such lowering of apparent K_M values as observed with those fish trypsins may thus be a part of the mechanism of adaption of poikilothermic organisms, to maintain high catalytic activity of their enzymes at low habitation temperatures.

The trout trypsin was also significantly more active than the bovine enzyme when their activity was measured against casein in the same temperature range (Figure 6). The difference in caseinolytic activity of the two trypsins corresponded approximately to the difference in the turnover numbers (k_{cat}) of the enzymes when assayed against L-BAPNA (Table III). The temperature response observed in the activity of the enzymes against the amide substrate therefore appears to be expressed when measured against the protein substrate. Under the substrate saturation conditions used in the casein assays, however, the higher proteolytic activity of the trout trypsin appears to result primarily from higher turnover numbers as compared to the bovine enzyme at those temperatures. Similar results have also been obtained for two chymotrypsin-like proteases isolated from rainbow trout (Kristjansson and Nielsen, 1991).

The relatively high proeteolytic activity of the trout enzyme at low temperatures suggests a potential use of the enzyme as a processing aid in various food processes where protein hydrolysis is required under low-temperature conditions. Some suggested applications in fish processing involve deskinning and descaling of fish, removal of membranes, and artificial or accelerated ripening of salt-cured herring products (Stefánsson and Steingrimstdóttir, 1989).

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LITERATURE CITED

Asgeirsson, B.; Fox, J. W.; Bjarnason, J. B. Purification and characterization of trypsin from the poikilotherm *Gadus Morhua*. *Eur. J. Biochem.* **1989**, *180*, 85–94.

Bode, W.; Schwager, P. The refined crystal structure of bovine β-trypsin at 1.8 Å resolution. II. Crystallographic refinement, calcium binding site, benzamidine binding site and active site at pH 7.0. *J. Mol. Biol.* **1975**, *98*, 693–717.

Chase, T.; Shaw, E. p-Nitrophenyl-p'-guanidinobenzoate HCl: A new active site titrant for trypsin. *Biochem. Biophys. Res. Commun.* **1967**, *29*, 508–514.

Dawson, R. M. C.; Elliott, D. C.; Elliott, W. H.; Jones, K. M. *Data for Biochemical Research*, 2nd ed.; Oxford University Press: London, 1969; p 485.

Delaage, M.; Abita, J. P.; Lazdunski, M. Physiochemical properties of bovine chymotrypsinogen B. A comparative study with trypsinogen and chymotrypsinogen A. *Eur. J. Biochem.* **1968**, *5*, 285–293.

Erlanger, B. F.; Kokowsky, N.; Cohen, W. The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* **1961**, *95*, 271–278.

Genicot, S.; Feller, G.; Gerday, C. Trypsin from antarctic fish (*Paranotothenia Magellanica* Forster) as compared with trout (*Salmo gairdnerii*) trypsin. *Comp. Biochem. Physiol.* **1988**, *90B*, 601–609.

Hjelmeland, K.; Raa, J. Characteristics of two trypsin type isozymes isolated from the arctic fish capelin (*Mallotus villosus*). *Comp. Biochem. Physiol.* **1982**, *71B*, 557–562.

Kalac, J. Studies on herring (*Clupea harengus* L.) and capelin (Mallotus *villosus* L.) pyloric caeca proteinases. II. Characterization of the anionic fraction of trypsins. *Biologia (Bratislava)* **1978**, *33*, 701–710.

Keil, B. Trypsin. *Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1971; pp 249–275.

Kristjansson, M. M.; Nielsen, H. H. Purification and characterization of two chymotrypsin-like proteases from the pyloric caeca of rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol.* **1991**, in press.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

Lazdunski, M.; Delaage, M. *Biochim. Biophys. Acta* **1967**, *140*, 417–434.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Martinez, A.; Olsen, R. L.; Serra, J. L. Purification and characterization of two trypsin-like enzymes from the digestive tract of anchovy *Engraulis encrasicholus*. *Comp. Biochem. Physiol.* **1988**, *91B*, 677–684.

Murakami, K.; Noda, M. Studies on proteinases from the digestive organs of sardine. I. Purification and characterization of three alkaline proteinases from the pyloric caeca. *Biochim. Biophys. Acta* **1981**, *658*, 17–26.

Raa, J. Biotechnology in aquaculture and the fish processing industry: a success story in Norway. In *Advances in Fisheries Technology and Biotechnology for Increased Profitability*; Voigt, M. N., Botta, J. R., Eds.; Technomic Publishing: Lancaster, PA, 1990; pp 509–524.

Raae, A. J.; Walther, B. T. Purification and characterization of chymotrypsin, trypsin and elastase like proteases from cod (*Gadus morhua L.*). *Comp. Biochem. Physiol.* **1989**, *93B*, 317–324.

Simpson, B. K.; Haard, N. F. Trypsin from Greenland cod (*Gadus ogac.*) Isolation and comparative properties. *Comp. Biochem. Physiol.* **1984**, *79B*, 613–622.

Simpson, B. K.; Haard, N. F. Cold-adapted enzymes from fish. In *Food Biotechnology*; Knorr, D., Ed.; Dekker: New York, 1987; pp 495–528.

Simpson, B. K.; Simpson, M. V.; Haard, N. F. On the mechanism of enzyme action: digestive proteases from selected marine organisms. *Biotechnol. Appl. Biochem.* **1989**, *11*, 226–234.

Stefánsson, G.; Steingrimstdóttir, U. Application of enzymes for fish processing in Iceland—Present and future aspects. In *Advances in Fisheries Technology and Biotechnology for Increased Profitability*; Voight, M. N., Botta, J. R., Eds.; Technomic Publishing: Lancaster, PA, 1990; pp 237–250.

Uchida, N.; Tsukayama, K.; Nishide, E. Properties of two main anionic trypsins from the pyloric caeca of chum salmon. *Bull. Jpn. Soc. Sci. Fish.* **1984**, *50*, 313–321.

Yoshinaka, R.; Sato, M.; Suzuki, T.; Ikeda, S. Enzymatic characterization of anionic trypsin of the catfish (*Parasilurus asotus*). *Comp. Biochem. Physiol.* **1984**, *77B*, 1–6.

Yoshinaka, R.; Sato, M.; Suzuki, T.; Ikeda, S. Purification and some properties of two anionic trypsins from the eel (*Anguilla japonica*). *Comp. Biochem. Physiol.* **1985**, *80B*, 5–9.

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