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Isolation and characterisation of two chymotrypsins from *Allocyttus niger* (black oreo dory) viscera

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Abstract Two serine proteases from the viscera of deep-sea fish, black oreo dory (*Allocyttus niger*), were purified by hydrophobic, affinity, and cation exchange chromatography. They were designated as chymotrypsins on the basis of substrate specificity and susceptibility to inhibitors. The pH optima of chymotrypsin I and II were 8.6 and 10, respectively. Chymotrypsin II retained a remarkable 80% activity at pH 12.5. Thermal stability of both enzymes was enhanced in the presence of calcium ions. Both chymotrypsins were inhibited by high concentrations of substrate Suc-AAPF-NA.

Keywords chymotrypsin; deep sea fish; thermal stability; black oreo dory; *Allocyttus niger*; proteases; enzymes

INTRODUCTION

In general terms, the specific activity of an enzyme type is independent of the temperature at which it has evolved. In other words, at the respective growth optima of their source organisms, enzymes from mesophilic and thermophilic organisms have similar specific activities (Daniel et al. 1996). It follows that in terms of specific activity, the best source of enzymes which are to be utilised at low temperature are organisms that grow at low temperatures: enzymes from higher temperature organisms will have a lower specific activity because they will be operating below their "design" temperature, whereas those from even lower temperature organisms (if such were available) would be more active but very unstable (Daniel et al. 1996). Some food industry applications do require enzymes for low temperature use, or enzymes that can be readily denatured to eliminate residual activity after use (Haard 1992). Deep-sea fish represent a potential source of such enzymes, and the present paper describes the extraction, purification, and partial characterisation of two proteases from the viscera of black oreo dory (*Allocyttus niger*) caught in New Zealand waters. Whole viscera were used because these are raw by-product of the processing line. This species was chosen for two reasons—it is commercially caught by New Zealand vessels and it lives at depths below 600 m where the temperature of water is constant (2–4°C) throughout the entire live cycle. Because of the very stable temperatures there would be no evolutionary pressure to compromise enzyme efficiency at low temperatures with the need for stability at higher temperatures. Proteases from taxonomic order Zeiformes have not been previously described.

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MATERIALS AND METHODS

Reagents

N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), N-Succinyl-Ala-Ala-Pro-L-Phe-p-nitroanilide (Suc-AAPF-NA), N-Benzoyl-tyrosine Ethyl Ester (BTEE), N_{α}-Benzoyl-arginine Ethyl Ester (BAEE), N_{α}-Tosyl-l-arginine Methyl Ester (TAME), Phenylmethylsulfonyl fluoride (PMSF), N-Tosyl-L-phenylalanine Chloromethyl Ketone (TPCK), N_{α}-p-Tosyl-L-lysine Chloromethyl Ketone (TLCK), Sigma 7–9® (Tris[hydroxymethyl]aminomethane) were purchased from Sigma Chemical Company; $CaCl_2$, NaCl, NaOH, ethanediol, Trichloroacetic acid, HCl,

ethylenediaminetetra-acetic acid (EDTA), citrate, glycine were purchased from BDH; phenyl-Sepharose, electophoresis calibration kit for molecular weight determination of low molecular weight proteins, isoelectric focusing calibration kit for isoelectric point determination of proteins in the range pH 3–10, isoelectric focusing PhastGel® for isoelectric point determination of proteins in the range pH 3–9, 10–15% gradient PhastGel® for molecular weight determination, PhastGel® SDS buffer strips for electrophoresis, Mono S® FPLC® chromatographic column, were purchased from Pharmacia; Dimethyl sulphoxide was purchased from Serva, methanol was purchased from Ajax Chemicals. Apart from NaCl, all the reagents were of analytical grade.

Fish specimen collection

Specimens of *Allocyttus niger* (black oreo dory) were caught between 27 May and 2 June 1993, on the Chatham Rise fishing ground by a Sealord Products fishing trawler. They were brought to the Nelson fish processing plant on ice. On 4 June, 16.2 kg of viscera were collected from this plant. The viscera were chopped with a knife and mixed with 2.5 kg of NaCl to a final concentration of c. 2.5*M*. Then 50 g of $CaCl_2$ was added to give a final concentration of c. 20 m*M*, 144 g of HEPES buffer was added to give a final concentration of c. 30 m*M*, and the pH of the mixture was adjusted to 7.5 with concentrated NaOH. The viscera were then frozen at –35°C. About 7 h elapsed between taking fish from iced containers and freezing the viscera. During this time the material was kept at ambient temperature (c. 20°C). After 4 days at –35°C, frozen viscera were cut into three blocks and transferred to a –70°C freezer.

Homogenisation and extraction

The extraction method was developed from that of Ásgeirsson et al. (1989). Frozen oreo dory viscera (6 kg) were passed through a meat mincer fitted with a 3 mm strainer. The resulting paste was mixed with 5 volumes of 30 m*M* HEPES/NaOH pH 8. The suspension was stirred for 3 h at 4°C, then filtered through a double cheesecloth to remove large tissue fragments.

$CaCl_2$ was added to a final concentration of 20 m*M*. After stirring the suspension overnight NaCl was added to a final concentration of 3*M*. The mixture was centrifuged at 13 000*g* for 20 min at ambient temperature. The extract was recentrifuged after 15 h at 4°C at 18 000*g* for 30 min. Fat separated to the top of the supernatant and was strained out. The turbid supernatant was filtered through hollow fibre filtration system (Amicon DC 10LA) with a 0.8 μm filter cartridge (Amicon H5MP01–43) resulting in 30 litres of clear solution; the extract was maintained at pH 8 throughout this process. The temperature of the extract was maintained at 4°C by Julabo FT 401 cooling probe.

Hydrophobic interaction chromatography

Clarified extract (29 litres) was applied to a 28.5 × 7 cm phenyl-Sepharose column. Solvent front velocity was 63 cm × h^{-1}. The column was washed with the extracting buffer. The bound protease activity was eluted with 1300 ml of 70% ethanediol in 30 m*M* HEPES/NaOH 10 m*M* $CaCl_2$, pH 8. Fractions containing protease activity were dialysed against 38 litres of 30 m*M* HEPES/NaOH 10 m*M* $CaCl_2$ 3*M* NaCl, pH 8 using a spiral wound ultra filtration membrane system (Amicon DC101A) retaining proteins with molecular weight above 10 000 Da. The temperature of the extract was maintained at 4°C by Julabo FT 401 cooling probe.

The preparation was applied again to the same column washed with 30 m*M* HEPES/NaOH 10 m*M* $CaCl_2$ pH 8 with 3*M* NaCl. The initial elution was with sample buffer without NaCl, and then with 70% ethanediol in sample buffer without NaCl. Fractions were assayed for activity against N-Succinyl-Ala-Ala-Pro-L-Phe-p-nitroanilide (Suc-AAPF-NA). The 70% ethanediol elution fraction was chosen for further chymotrypsin purification.

Affinity chromatography on CBZ-D-Phenylalanine-TETA-//-Sepharose 4B

Material (250 ml) eluted with 70% ethanediol from the second phenyl-Sepharose column was diafiltered to a final concentration of 15% ethanediol and applied in 300 ml to a 4 × 1.6 cm CBZ-Phenylalanine-TETA-//-Sepharose 4B column. The column was washed with 10 m*M* HEPES/NaOH 10 m*M* $CaCl_2$, pH 8 buffer, then with the same buffer containing 3*M* NaCl to elute non-specifically bound proteins, and again with the same buffer without NaCl. Chymotrypsin activity was then eluted using a 150 ml linear gradient of 35–80% ethanediol.

Cation exchange chromatography on Mono S column

Fractions containing chymotrypsin activity eluted from CBZ-D-Phenylalanine-TETA-//-Sepharose 4B were combined, diafiltered to remove ethanediol

and applied to a 10 × 0.5 cm Mono S cation exchange column at 0°C. The sample buffer was 10 m*M* HEPES/NaOH 10 m*M* $CaCl_2$, pH 7.2, and the flow rate was 3 ml min^{-1}. Chymotrypsin activity was eluted with a 90 ml gradient of 0–0.36*M* NaCl in sample buffer. Three ml fractions were collected in tubes containing 1 ml of 100 m*M* HEPES with 10 m*M* $CaCl_2$, pH 8, to bring the samples to the assay pH. Fractions were immediately frozen in liquid nitrogen and kept at –70°C until further use.

Enzyme activity assays

The chymotrypsin amidase activity was assayed using N-Succinyl-Ala-Ala-Pro-L-Phe-p-nitroanilide (Suc-AAPF-NA) as substrate. The substrate specificity of purified enzyme was investigated following the same procedure but using different nitroanilide-linked peptides and amino acids. Stock solutions of substrates were prepared by dissolving nitroanilide-linked peptides in dimethyl sulphoxide to a concentration of 100 m*M*. These stock solutions were diluted in a 10% (v/v) solution of dimethyl sulphoxide in 30 m*M* HEPES/NaOH 5 m*M* $CaCl_2$, pH 8. Final concentration of Ca^{2+} in assay mixtures was 4.5 m*M*. To 700 µl of substrate solution an appropriate volume of enzyme sample was added and the increase in light absorption followed at 410 nm. Unless otherwise indicated the concentration of the substrate in the assay was 0.81 m*M* and the temperature of assay was 25°C. One unit of activity was defined as 1 µmole of p-nitroanilide released per min, using an extinction coefficient of at 410 nm of 8480 $M^{-1}cm^{-1}$ (Kristjànsson & Nielsen 1991).

For esterase activity measurements, the substrates were dissolved in 30 m*M* HEPES/NaOH, pH 8, containing 5 m*M* $CaCl_2$ and 1% methanol.

The reaction was spectrophotometrically monitored at 256 nm for N-Benzoyl-l-tyrosine Ethyl Ester (BTEE) and N_α-Benzoyl-l-arginine Ethyl Ester (BAEE) and at 247 nm for N_α-Tosyl-l-arginine Methyl Ester (TAME). The activity unit was 1 µmole of ester hydrolysed per min.

Determination of protein concentration

Protein concentration was determined by the method of Bradford (1976).

Electrophoresis

Molecular weights were estimated by SDS/PAGE on 10–15% gradient PhastGel® using Pharmacia Phast System, by comparison with standard marker proteins (Low Molecular Weight Marker Kit), molecular weights of the markers were: 94, 67, 43, 30, 20.1, and 14.4 kDa. Buffer system in the gel is of 0.112*M* acetate (leading ion) and 0.112*M* Tris pH 6.4. Buffer system in the strips is of 0.20*M* tricine (trailing ion), 0.20*M* Tris, and 0.55% SDS pH 7.5. The buffer strips are made of 2% agarose. Isoelectric focusing was carried out using the Pharmacia Phast System and PhastGel® IEF 3–9. This gel is a homogenous polyacrymide gel (5% T, 3% C) containing Pharmolyte® carrier ampholytes. Pharmolyte® generates stable pH gradient during run and prefocusing step. The gels were run according to Phast System Technique File No. 100. Sample proteins are separated under 2000 V potential. Isoelectric focusing calibration kit for isoelectric point determination of proteins in the range pH 3–10 was used to generate standard curve from which isoelectric point of chymotrypsin was read. Proteins were fixed into the gels in 10% trichloroacetic acid immediately after the run and gels were silver stained according to the instructions of Pharmacia's Phast system.

RESULTS

Purification of two chymotrypsins from *Allocyttus niger*

Chymotrypsin activity was eluted from the Mono S column in two peaks designated as chymotrypsin I and chymotrypsin II with the maximum activity eluted at about 100 and 200 m*M* respectively (Fig. 1).

The overall recovery of chymotrypsin activity from clarified extracts was 49% (Table 1). Assays of the extract before clarification were relatively inaccurate because of the large amount of particulate material present, so the loss of activity during clarification of the extract is unknown. Even after the clarification, the accuracy of the activity figures for the early purification stages is uncertain because of the likelihood of substrate inhibition (e.g., Freeman et al. 1993; Toogood et al. 1995). For example, in some purification steps more than 100% of chymotrypsin activity appears to have been recovered. Specific activities and purification factors of purified chymotrypsins were 38.7 and 55.8 Units mg^{-1}; and 176 and 253 for chymotrypsin I and II, respectively.

There was still some degree of cross contamination between the two chymotrypsins after the Mono S step. SDS-Page gel revealed two bands of unequal density in fractions collected from each peak. An SDS/PAGE gel showed c. 20% cross

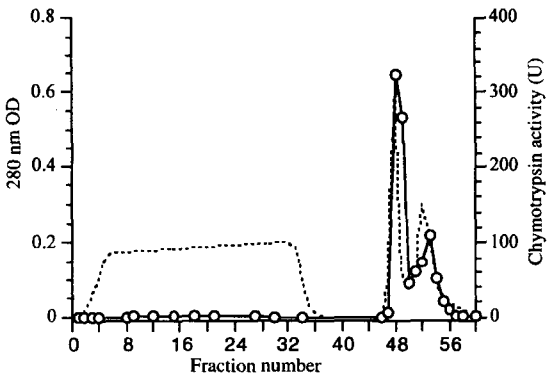

Fig. 1 Elution profile of the final purification step—cation exchange chromatography on the Mono S gel. - - - - = 280 nm OD; —O— = chymotrypsin activity. Fractions: 1–33, loading in 10 m*M* HEPES, 10 m*M* $CaCl_2$ pH 7.2; 34–42, wash with the same buffer; 43–60, linear gradient of 0–360 m*M* NaCl. 1U = 1 µmole of Suc-AAPF-NA hydrolysed per min. 5 µl of eluted fractions were added to 700 µl of Suc-AAPF-NA solution. Duration of activity assay was 1 min. Assay mixture contained 5 m*M* Ca^{2+}. Graph points are the mean of triplicate assays.

contamination of each chymotrypsin by the other. On isoelectric focusing gel (IEF) chymotrypsin I stained as a single band with silver staining, the chymotrypsin II concentration was too low to detect on IEF gel.

Molecular weight determination and assessment of purity

SDS-PAGE showed bands corresponding to molecular weights of 27000 Da for chymotrypsin I and 30 000 Da for chymotrypsin II.

Isoelectric point determination

Silver staining of IEF gel revealed a single band in the chymotrypsin I fraction track corresponding to a pI value of 7.4.

Effect of inhibitors

The overall effect of inhibitors on the activity of the proteases from *Allocyttus niger* (Table 2) enables designation of the proteases as chymotrypsins. The inhibitor PMSF almost completely inhibited both enzymes, which places them in the serine protease class. The enzymes were also strongly inhibited by the inhibitor TPCK and chymostatin, confirming their identity as true chymotrypsins. Activity of chymotrypsin I from *Allocyttus niger* was increased almost 2-fold after incubation with the trypsin-specific inhibitor TLCK, whereas that of chymotrypsin II was unaffected.

Substrate specificity

Of six chromophore-linked peptide substrates (N-Suc-L-Phe-NA, N_α -Benzoyl-L-Arg-NA, Suc-AAPF-NA, L-Ala-NA, N-Suc-L-Phe-NA, N-Glutaryl-Phe-NA), only Suc-AAPF-NA was hydrolysed at a significant rate. This peptide was specifically designed as a chymotrypsin substrate. Esterase activity was assayed against three esters (TAME, BAEE, BTEE). The first two were designed as trypsin substrates and were not hydrolysed by either of the purified enzymes, but BTEE, the chymotrypsin substrate, was hydrolysed. This confirms that the purified enzymes are true chymotrypsins.

pH optima

Observed pH optima against Suc-AAPF-NA at 35°C for chymotrypsin I and chymotrypsin II were

Table 1 Chymotrypsin I and II purification data. (All figures were multiplied to represent total recovery from 6 kg of wet weight of viscera, if all clarified extract had been taken through all stages of purification.)

Stage	Chymotrypsin relative activity (%)	Chymotrypsin total activity (U)	Total protein (mg)	Specific activity ($U \times mg^{-1}$)	Purification factor
Clarified extract	100	6645	30365	0.22	1
First phenyl-Sepharose	105	6977	2477	2.88	13
Second phenyl-Sepharose	74	4917	820	5.33	24
Affinity column	38	2525	422	5.98	27
Chymotrypsin I recovered from Mono S	28	1860	48	38.75	176
Chymotrypsin II recovered from Mono S	21	1395	25	55.80	253

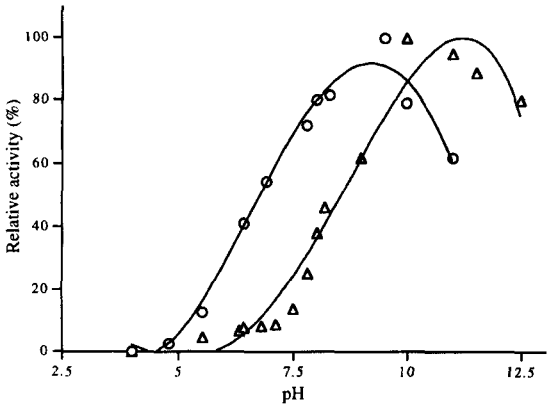

Fig. 2 Effect of pH on amidase activity of chymotrypsin I (○) and II (Δ). Following buffer systems were used: Citrate/NaOH-pH4–8; HEPES/NaOH pH6.5–8.5; Sigma7–9® (Tris[hydroxymethyl]aminomethane)/HCl pH7–9; glycine/NaOH pH8–13. In all buffers, concentration of Ca^{2+} was 5 m*M*. Duration of activity assay was 15 s. Activity was assayed at 35°C. Graph points are the mean of triplicate assays.

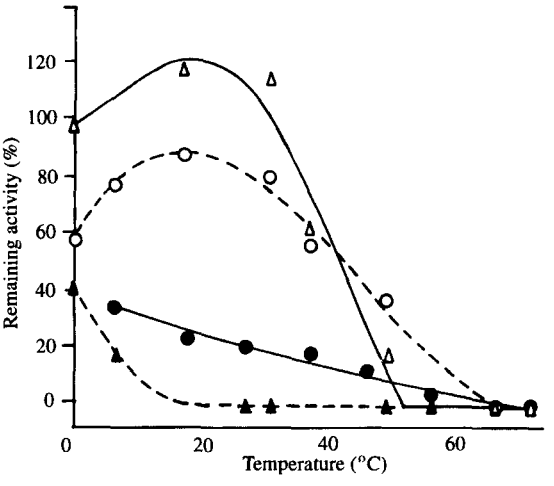

Fig. 3 Thermal stability of: chymotrypsin I in presence of 10 m*M* Ca^{2+} (○); chymotrypsin I in absence of Ca^{2+} (●); chymotrypsin II in presence of 10 m*M* Ca^{2+} (△); and chymotrypsin II in absence of Ca^{2+} (▲). Enzymes were incubated for 30 min in presence of 10 m*M* Ca^{2+} or in buffer devoid of Ca^{2+} containing 10 m*M* EDTA at temperatures between 0 and 65°C, then assayed for residual activity at 25°C. Duration of activity assay was 1 min. Assay mixture contained 5 m*M* Ca^{2+}. Graph points are the mean of triplicate assays.

c. 9 and 10, respectively (Fig. 2). Chymotrypsin I appeared to be relatively more active at lower pH values. Chymotrypsin II retains a remarkable 80% activity at pH 12.5. Unfortunately, assaying activity above pH 12.5 was not possible owing to instability of the substrate.

Effect of temperature on stability

Chymotrypsin I is more stable than chymotrypsin II. The rapid loss of activity above 50°C was likely to be the result of both denaturation and autolysis rather than solely denaturation.

After removing calcium from enzyme solutions, both enzymes were significantly less stable, but chymotrypsin I was far more stable than chymotrypsin II (Fig. 3). Interestingly both enzymes increased their activity after 30 min incubation in the presence of 10 m*M* Ca^{2+} at temperatures between 0 and c. 25°C.

Kinetics of the hydrolysis of Suc-AAPF-NA

The Michaelis-Menten relationship showed substrate inhibition above a Suc-AAPF-NA concentration of c. 0.3 m*M* for chymotrypsin I and

Table 2 Effect of inhibitors on purified chymotrypsins. (Pre-incubation was for 30 min at 20°C. Substrate was Suc-AAPF-NA.)

Inhibitor	Incubation concentration of inhibitor	Assay concentration	Remaining activity of chymotrypsin I(%)	Remaining activity of chymotrypsin II(%)
TPCK	1 m*M*	0	3	6
TLCK	1 m*M*	0	193	109
PMSF	15 m*M*	0	2	1
EGTA	15 m*M*	15 m*M*	46	17
EDTA	100 m*M*	100 m*M*	34	51
Benzamidine	5 m*M*	5 m*M*	69	69
Chymostatin	20 μg × ml^{-1}	20 μg × ml^{-1}	0	0

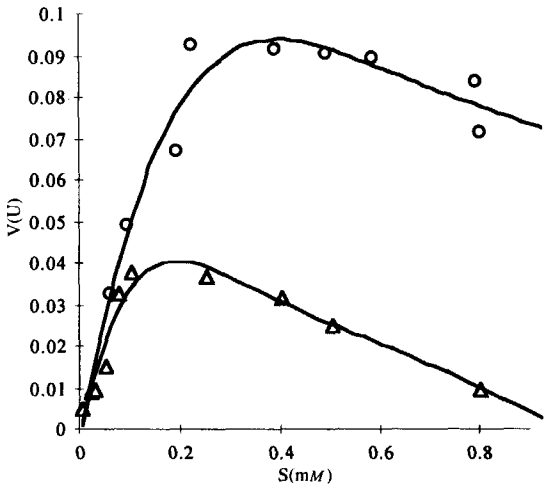

Fig. 4 Michaelis-Menten plot of chymotrypsin I (○) and chymotrypsin II (△) with Suc-AAPF-NA as a substrate at 25°C. Duration of activity assay was 1 min. Assay mixture contained 5 m*M* Ca^{2+}. Graph points are the mean of triplicate assays.

0.1 m*M* for chymotrypsin II (Fig. 4), making accurate determination of K_m difficult. Calculated approximate K_m values for chymotrypsin I fell between 100 and 150 μ*M*, and for chymotrypsin II between 20 and 50 μ*M*.

DISCUSSION

Recovery of more than 100% of activity in purification steps is not uncommon for proteases (e.g., Freeman et al. 1993; Toogood et al. 1995). Other proteins or peptides present in the extract may be inhibiting the chymotrypsin. Any actual loss of activity in each step is thus difficult to estimate. Data such as yield and total activity in the earlier stages of purification must therefore be regarded with caution.

Values of specific activity against Suc-AAPF-NA (38.7 and 55.8 Units mg^{-1}) are very similar to those reported by Kristjànsson & Nielsen (1991) for rainbow trout chymotrypsins (30.3 and 45.8 Units mg^{-1}). Purification factors were higher than those reported by Kristjànsson & Nielsen (1991) (47.3 and 71.6), but as they purified enzyme from pyloric caeca, their initial extract was probably much richer in chymotrypsin.

The established molecular weights of black oreo dory chymotrypsins are similar to those of other fish chymotrypsins: rainbow trout (*Oncorhynchus mykiss*) 28200 and 28800 Da (Kristjànsson & Nielsen 1991); Atlantic cod (*Gadus morhua*) 27 000 Da, 26 000 Da ± 1500 (Àsgeirsson & Bjarnason 1991) (Raae & Walther 1983); carp (*Cyprinus carpio*) 25 000 Da (Cohen et al. 1980); and sardine (*Sardinops melanosticta*) 28 700 Da (Murakami & Noda 1980).

The pI of black oreo dory chymotrypsin is rather higher than that found for rainbow trout (4.9–5.0) (Kristjànsson & Nielsen 1991), Atlantic cod (5.6 and 6.4, 5.8 and 6.2) (Raae & Walther 1989) (Àsgeirsson & Bjarnason 1991), and sardine (5.3) (Murakami & Noda 1980).

Chymotrypsins purified from rainbow trout showed a similar pattern of inhibition sensitivities but were only 62 and 51% inhibited by PMSF (Kristjànsson & Nielsen 1991). Inhibition by metal chelators suggests Ca^{2+}-dependent stability of black oreo dory chymotrypsin and a similar effect has been reported for other fish proteases (Raae & Walther 1989; Kristjànsson & Nielsen 1991).

Very similar values of pH optima were described for two rainbow trout chymotrypsins (Kristjànsson & Nielsen 1991).

Bovine chymotrypsin's catalysis requires a basic group that must be unprotonated and an acidic group that must be protonated for the reaction to occur. The imidazole side chain of the histidine 57 residue is the prime candidate to account for the pH dependence of chymotrypsin at lower pH values. It was established that both acylation and deacylation (but not the initial binding step) require that this histidine be in its unprotonated neutral state. The acidic group with an apparent pK of 8.5, which is responsible for the sharp decrease of chymotrypsin activity at a pH above 9, has been identified as the protonated α-amino group of isoleucine 16, which forms a salt bridge to aspartate 194 required for structural stability of the active site of the enzyme. The drop in activity above this pH is the result of an unfavourable effect on K_s (Hess 1971). However, oreo dory chymotrypsins described here have their pH optima above 8, and one is still 80% active at pH 12.5. Higher activity at alkaline pH values has also been reported for chymotrypsins from carp (Cohen et al. 1980) and trout (Kristjànsson & Nielsen 1991). The above results would imply that different amino acid residues are involved in stabilising the active site conformation of fish and bovine chymotrypsins (Kristjànsson & Nielsen 1991).

Substrate inhibition of chymotrypsin by Suc-AAPF-NA has not been previously reported

although substrate inhibition is a common feature of proteases when hydrolysing large substrates such as azocasein (e.g., Cowan & Daniel 1982; Saravani et al. 1989; Freeman et al. 1993). Substrate inhibition of trypsin by small ester substrates was reported by Yoshinaka et al. (1984, 1985). Estimated K_m values are of the same order as those of rainbow trout chymotrypsins, 23 and 35 μ*M*, and bovine chymotrypsin 53 μ*M* (Kristjànsson & Nielsen 1991). Published differences in K_m values of fish and bovine chymotrypsins against Suc-AAPF-NA are significant, but not nearly as large as for fish and bovine trypsins against BAPNA substrate, which are 2445 μ*M* for bovine trypsin, 201 μ*M* for rainbow trout trypsins, and 118 μ*M* for *Paranotothenia magellanica* trypsins (Genicot et al. 1987). Reported "physiological efficiency" of proteolytic enzymes from fish expressed as k_{cat}/K_m are usually much higher than those of corresponding enzymes from homoeothermic organisms (Genicot et al. 1987). The major contribution to enhanced physiological efficiencies of atlantic cod and rainbow trout trypsins come from lower K_m values (Àsgeirsson et al. 1989) (Kristjànsson 1991). However, under physiological conditions in the gut digestive enzymes are likely to operate under conditions of substrate saturation so the values of K_m have no influence on the overall rate of reaction. The concept of physiological efficiency as understood above seem thus to be dubious in the instance of these enzymes. In the instance of chymotrypsins from rainbow trout increased "efficiency" resulted from both lower K_m values and higher catalytic activity (k_{cat}) (Kristjànsson & Nielsen 1991). The high k_{cat}/K_m values of chymotrypsin from atlantic cod is mainly a result of higher catalytic activity (k_{cat}) (Àsgeirsson & Bjarnason 1991).

The higher thermal stability of chymotrypsin I may be the result of stronger binding of Ca^{2+} (Kristjànsson & Nielsen 1991) or to higher intrinsic stability of this isoenzyme. Stability of rainbow trout chymotrypsin is also Ca^{2+} dependent (Kristjànsson & Nielsen 1991). In vivo Ca^{2+} concentration in marine fish intestine is probably between 10 m*M* (concentration of Ca^{2+} in sea water swallowed by fish during feeding) (Barnes 1954) and 3 m*M* (typical concentration in intercellular fluids of marine fish) (Prosser 1973).

The Ca^{2+} dependent increase in activity after 20 min of incubation at 25°C is probably an effect either of activation of some chymotrypsinogen remaining in the sample, or of dissociation of an inactive or partially inactive enzyme-protein complex. A similar temperature-dependent "activation" was observed by Cowan & Daniel (1982) for the thermophilic protease caldolysin. They concluded that this activation is too slow to be caused entirely by kinetic dissociation and that it may involve some proteolytic cleavage. The phenomenon of heat-induced activation has also been detected in enzymes other than proteases (Hickey & Daniel 1979). The stability profiles of chymotrypsins from rainbow trout show a similar tendency but they displayed maximal activity after 30 min incubation at temperatures of c. 30 and 40°C (Kristjànsson & Nielsen 1991). These differences may be caused by black oreo dory being adapted to live in an environment of virtually constant water temperature of 2°C, whereas trout during their life cycle may experience temperatures ranging from nearly 0 to 20°C. All reported fish serine proteases have a lower temperature stability than corresponding bovine proteases.

The thermal stability data suggests that a mixture of the two proteases will not be readily inactivated at low temperatures; indeed even in the absence of Ca^{2+} chymotrypsin I retains 10% of its activity after 30 min at 45°C. Temperatures above 60°C are likely to be necessary for removal of >90% of activity in less than 30 min. However, chymotrypsin II has widely differing stabilities depending on whether Ca^{2+} is present, opening the possibility of use in presence of Ca^{2+}, followed by the action of a Ca^{2+}-chelator to allow rapid denaturation even at 20°C. Separation of the two enzymes is therefore likely to be necessary for uses where a readily denatured protease is required.

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