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Purification and characterization of pepsins A1 and A2 from the Antarctic rock cod *Trematomus bernacchii*

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The Antarctic notothenioid *Trematomus bernacchii* (rock cod) lives at a constant mean temperature of − 1.9 °C. Gastric digestion under these conditions relies on the proteolytic activity of aspartic proteases such as pepsin. To understand the molecular mechanisms of Antarctic fish pepsins, *T. bernacchii* pepsins A1 and A2 were cloned, overexpressed in *Escherichia coli*, purified and characterized with a number of biochemical and biophysical methods. The properties of these two Antarctic isoenzymes were compared to those of porcine pepsin and found to be unique in a number of ways. Fish pepsins were found to be more temperature sensitive, generally less active at lower pH and more sensitive to inhibition by pepstatin than their mesophilic counterparts. The specificity of Antarctic fish pepsins was similar but not identical to that of pig pepsin, probably owing to changes in the sequence of fish enzymes near the active site. Gene duplication of Antarctic rock cod pepsins is the likely mechanism for adaptation to the harsh temperature environment in which these enzymes must function.

One of the peculiarities characterizing Antarctic notothenioids is their ability to live at a temperature of − 1.9 °C with seasonal variations of about 0.2 °C [1]. To cope with such a harsh habitat, Antarctic fish have evolved a number of physiological adaptations, including the presence of antifreeze glycoproteins [2], the ability to keep tubulin polymerized at − 2 °C [3], and a substantial reduction of the hematocrit, a process which, in the Channichthyidae, reaches its extreme with the complete loss of both erythrocytes and hemoglobin [4]. In addition to this, cold-adapted organisms have developed a number of adjustments at the molecular level to maintain metabolic function at low temperatures. Among other features, they can produce enzymes characterized by a high turnover number or high catalytic efficiency.

In our studies on aspartic proteinases in notothenioids, a fish group endemic to Antarctica, we have focused our attention on pepsins because of their important nutritional role. Pepsins are a family of aspartic proteinases accomplishing important digestive functions in both invertebrates and vertebrates [5]. Like other aspartic proteinases, pepsin is produced as a zymogen. The primary structure of the zymogen includes a signal peptide (or presequence) and the so-called propart, whose autocatalytic cleavage leads to the formation of the active enzyme [6]. The catalytic mechanism depends on the presence of two aspartic acids positioned roughly in the center of a deep cleft forming the active site and covered by a hairpin loop (flap) protruding from the N-terminal lobe of the molecule. The active site cleft can accommodate about seven residues of a substrate. These residues are usually designated as P4-P3-P2-P1*P1′-P2′-P3′, with the scissile peptide bond between P1 and P1′ indicated by an asterisk and normally flanked by two hydrophobic residues. The corresponding subsites that constitute the topography of the active site cleft in each enzyme are

Abbreviations
ACN, acetonitrile; CK-MM, rabbit muscle creatine phosphokinase; IPTG, isopropyl thio-β-D-galactoside.

designated accordingly as S4-S3-S2-S1-S1′-S2′-S3′ [7]. The two major groups of immunogenetically and biochemically distinct pepsins are pepsin A and pepsin C, the latter also being known as gastricsin. These enzymes are active at extremely low pH values [8,9] in both the stomach and the duodenum, and are quickly denatured when the pH exceeds 5.5 [10], thus preventing continuous proteolytic action that may damage these organs. Pepsin C has a slightly higher optimal pH than pepsin A, but substrate specificity is nearly the same for the two enzymes [7]. Most of our knowledge of pepsin derives from studies on human and mammalian enzymes [11–14], and much less information is available on enzymes from other vertebrates, including fish [13,15,16].

We have previously [17] cloned and sequenced three forms of pepsin A (which were named A1, A2 and A3), and a single form of gastricsin (named pepsin C), from the gastric mucosa of the Antarctic notothenioid *Trematomus bernacchii* (rock cod). Note that the naming convention used here is tentative, because fish pepsin A is a close relative of mammalian pepsin A, pepsin F and chymosin, whereas fish gastricsin is close to a common ancestor of mammalian gastricsin and pepsin B. Phylogenetic analysis has shown that rock cod pepsin C and pepsin A form two distinct clades, and the three fish pepsin A isotypes result from two rounds of gene duplication leading to the most ancestral pepsin, pepsin A3, and to the most recent forms represented by pepsin A1 and pepsin A2 [17]. Molecular modeling studies have unraveled significant structural differences in these enzymes with respect to their mesophilic counterparts [17]. Rock cod pepsin A2 displayed local changes in the substrate-binding cleft, with a significant reduction of the hydropathic character and concomitant increase in the flexibility of this region with respect to the two isoforms A1 and A3, the gastricsin and the other fish pepsins [17]. In general, a reduced level of hydropathy and higher molecular flexibility characterize cold-adapted proteins [18]. Moreover, the central role of pepsin A in food digestion makes it an attractive candidate for genetic engineering and identification of amino acid residues modulating its activity or stability.

To better understand the molecular mechanisms responsible for adaptation of food digestion at temperatures below 0 °C, we produced the two *T. bernacchii* fish pepsin variants A1 and A2 (hereafter referred to as fish pepsins A1 and A2) by heterologous expression in *Escherichia coli*. The enzymes were purified, and their biochemical properties were studied in comparison to pepsin A from porcine stomach.

Results

Expression and purification of recombinant fish pepsinogens

Isopropyl thio-β-D-galactoside (IPTG) induction of *E. coli* BL21 (DE3) cells transformed with plasmid pET22b–PepA1 or plasmid pET22b–PepA2 resulted in the overexpression of recombinant fish pepsinogens A1 and A2 as inclusion bodies. The inclusion bodies were purified by several buffer washes and solubilized with 6 M urea, and the refolding was initiated by rapid dilution at alkaline pH as described in Experimental procedures. Refolded pepsinogens A1 and A2 were loaded onto a Sephacryl S-300 column, and each collected fraction was monitored for protease activity and absorbance at 280 nm (supplementary Fig. S1A). For both refolded fish pepsinogens, the peak of proteolytic activity eluted just before the end of the $A_{280\ nm}$ peak. Fractions 15–23 did not show proteolytic activity but contained most of the recombinant fish pepsinogen in association with several contaminants, as estimated by SDS/PAGE analysis (data not shown). Both recombinant fish pepsinogens eluted under the $A_{280\ nm}$ peak were assumed to be the incorrectly refolded zymogen forms [19]. The collected gel filtration fractions containing the active pepsinogen form (fractions 25–40) were pooled and further purified by anion exchange chromatography on a Resource Q column. Fish pepsinogens A1 and A2 were both eluted with 0.1 M NaCl (supplementary Fig. S1B). The enzyme-containing fractions were pooled and dialyzed against 20 mM Tris/HCl (pH 8.0).

The dialyzed solution containing pepsinogen A1 was found to be homogeneous by SDS/PAGE and MS (Fig. 1), and similar data were obtained for fish pepsinogen A2 (data not shown). To determine the purity and validate the correct mass of the purified fish pepsinogens, ESI MS was used. Because pepsins traditionally do not ionize well in the positive ion mode, the negative ion mode was used. Figure 1 shows representative ESI spectra for fish pepsinogen A1 (Fig. 1A), fish pepsin A1 (Fig. 1B) and, for comparison, pig pepsin (Fig. 1C). The mass spectra confirmed that the proteins were relatively pure, as the major peaks in the spectra were those of pepsinogen (Fig. 1A). Although a second band of higher molecular mass was seen in SDS/PAGE, MS did not indicate that this was a major contaminant.

Characterization of recombinant fish pepsins

Fish pepsins were prepared by activating purified recombinant pepsinogens by acidification with 2 M

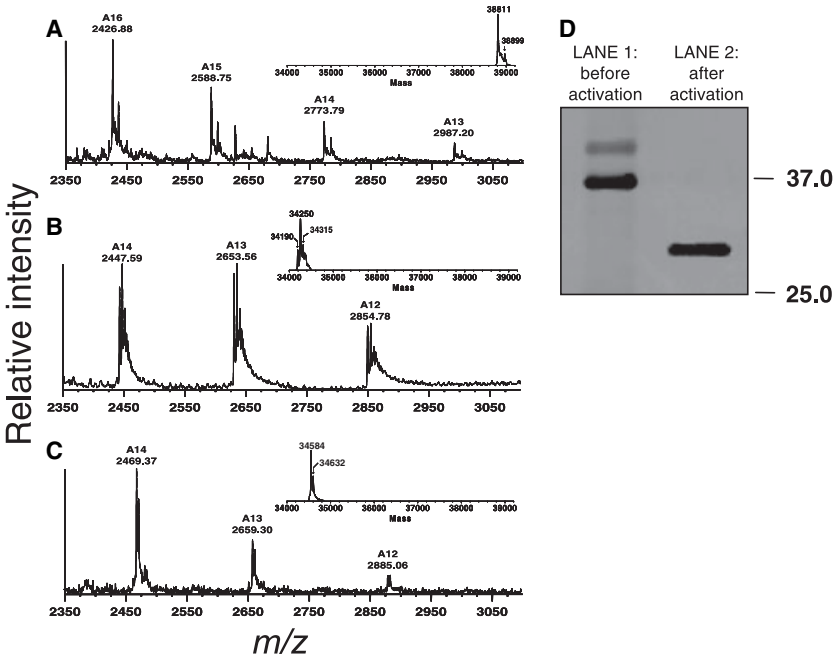

Fig. 1. Negative ion ESI mass spectra of (A) fish pepsinogen A1 and (B) activated fish pepsin A1. The measured molecular masses were 38 845 Da for the pepsinogen form (theoretical mass 38 850.6 Da) and 34 250 Da for the activated form (theoretical mass 34 241.2 Da). The conversion of pepsinogen to pepsin leads to a mass shift of ~4600 Da, due to the removal of N-terminal residues 1–38. (C) Negative ion ESI mass spectra of pig pepsin A from Sigma, as a control (theoretical mass 34 583.8 Da; measured mass 34 584 Da). (D) Commassie-stained SDS/PAGE gel of fish pepsin A1 before (lane 1) and after (lane 2) activation.

glycine-HCl (pH 2.0). The solution was neutralized by dialysis against sodium acetate buffer (pH 5.3). The recovered solution containing the active form of fish pepsin A1 showed a single band on SDS/PAGE (Fig. 1D, lane 2), and similar results were observed with activated fish pepsin A2 (data not shown). The conversion of pepsinogen to pepsin is expected to decrease the molecular mass by approximately 4600 Da, owing to the loss of the N-terminal enzyme propart (residues 1–38). The measured molecular masses of fish pepsinogen A1 and fish pepsin A1 were 38 845 Da and 34 250 Da, respectively (Fig. 1A,B). The observed experimental mass difference (4595 Da) is very close to the theoretical difference, indicating the removal of the N-terminal propart during the activation procedure. In addition, the N-terminal sequence determined by Edman degradation of the electroblotted recombinant fish pepsins was YQSGTESMTNDADLSYYGVI for both isoforms, confirming that the mature enzymes were generated by autoactivation of the refolded zymogen. Taken together, the SDS/PAGE, Edman degradation and ESI MS analyses all indicate that both fish pepsinogens were efficiently converted to the pepsin form at pH 2.0 by elimination of the N-terminal propart sequence.

The secondary structure of fish pepsins A1 and A2 was also investigated by far-UV CD spectroscopy (supplementary Fig. S2). The CD spectra for the two fish pepsin isoforms overlapped, indicating no substantial secondary structure differences. Secondary structure calculations from the far-UV CD spectral data showed that both Antarctic enzymes contained a high proportion of β-sheets (56.1% for A1 and 52.2% for A2), as reported for mammalian pepsins [20]. This result indicates that the secondary structure of the recombinant proteins was not altered during protein processing.

Alignment of Antarctic fish versus pig pepsins also revealed strong sequence identity (52.7%) between the three enzymes (Fig. 2). As compared to pig pepsin, both Antarctic fish enzymes lack the C-terminal sequence GMDVP (Fig. 2A,B). Interestingly, some of these residues belong to the subsites that constitute the topography of the active site cleft. Notably, the four amino acids E287, M289, V291 and T293 forming the loop EGMDVPT are residues of the S4-S1 subsites (E287 and M289) and S1′–S3′ subsites (V291 and T293) of pig pepsin that make contact with the residues of an ideal heptapeptide substrate [21].

pH and temperature studies

The enzymatic activity of fish pepsins A1 and A2 was investigated at different pH values and temperatures and compared with that of pig pepsin. A broad pH range was tested using hemoglobin as substrate (Fig. 3A). The mesophilic porcine enzyme exhibited 100% relative activity at pH 2.0, but a rapid reduction in activity at pH 2.5. In contrast, both Antarctic isoenzymes were less active at their optimum pH (2.5 for fish pepsin A1 and 2.0 for fish pepsin A2), and showed a slow decline of their relative activity at pH values

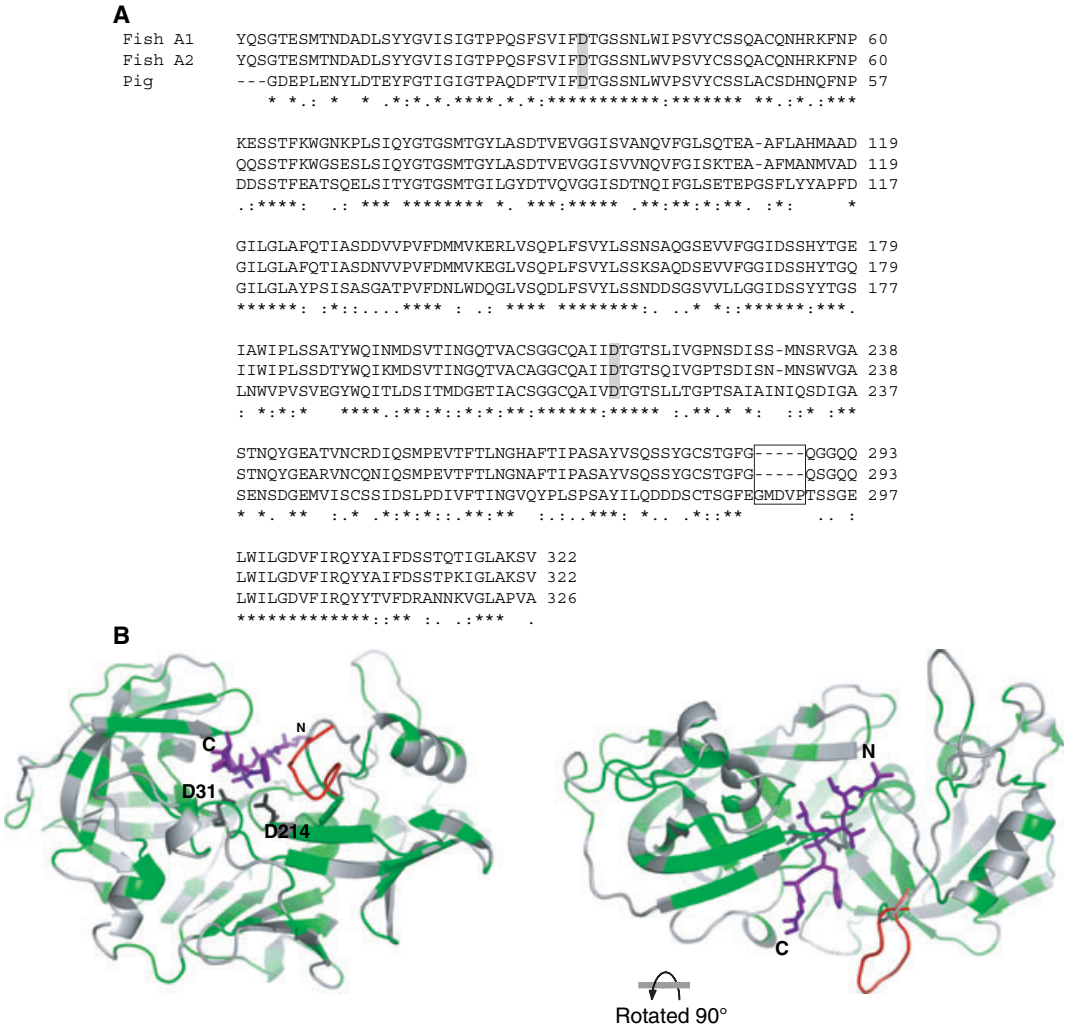

Fig. 2. (A) Alignment of amino acid sequences of the Antarctic recombinant pepsins with pig pepsin. Multialignment was achieved using the program CLUSTAL W version 1.83 [42]. The two aspartic acid residues present in the catalytic site are marked in gray. The missing residues in fish pepsin isoforms are boxed. (B) Model of fish pepsin A1 based on the crystal structure of pig pepsin (Protein Data Bank code 5PEP [21]), prepared as described previously [17]. The rendering on the right has been turned 90° towards the observer, as shown, to look down onto the top of the active site. The conserved residues between pig and fish pepsin, as determined by the alignment, are colored green. The extra residues in pig pepsin that are boxed in (A) are shown in the position that they occupy in the crystal structure of pig pepsin (5PEP) and are colored red. The remaining pig pepsin residues are virtually superimposable on the fish pepsin A1 model. The two conserved aspartic acids present in the catalytic site are drawn as sticks and labeled. The synthetic phosphonate inhibitor IVA-Val-Val-Leu^{P}-(○)Phe-Ala-Ala-OMe (IVA = isovaleryl; Leu^{P} = phosphinic acid analog of leucine; (○)Phe = L-3-phenyllactic acid) in the active site in this Protein Data Bank file is shown in stick form with the N-terminus and C-terminus labeled. Modeling of the inhibitor binding to the fish pepsin model was obtained by overlaying the crystal structure of the model with human pepsin 3A in complex with the synthetic phosphonate inhibitor (Protein Data Bank code 1QRP [23]).

above their optimum. The effect of the temperature on enzyme activity was also examined for fish and pig pepsins (Fig. 3B). The optimum temperatures (the temperatures at which there was the most activity) for Antarctic fish pepsins A1 and A2 were found to be 50 °C and 37 °C, respectively. At lower temperatures (4 °C, 10 °C and 25 °C), pig and fish pepsin A2 showed similar relative activities, whereas fish pepsin A1 was slightly less active. Pig pepsin exhibited 100% relative enzyme activity at 60 °C, and its activity dropped sharply to 20% at 70 °C. At the temperatures at which they were optimally active, the fish isoforms were less active than the pig enzyme, and they rapidly lost activity above 50 °C (Fig. 3B). This prompted an examination of the stability of all three enzymes at 50 °C and at pH 5.3 (at this pH, all three pepsins show

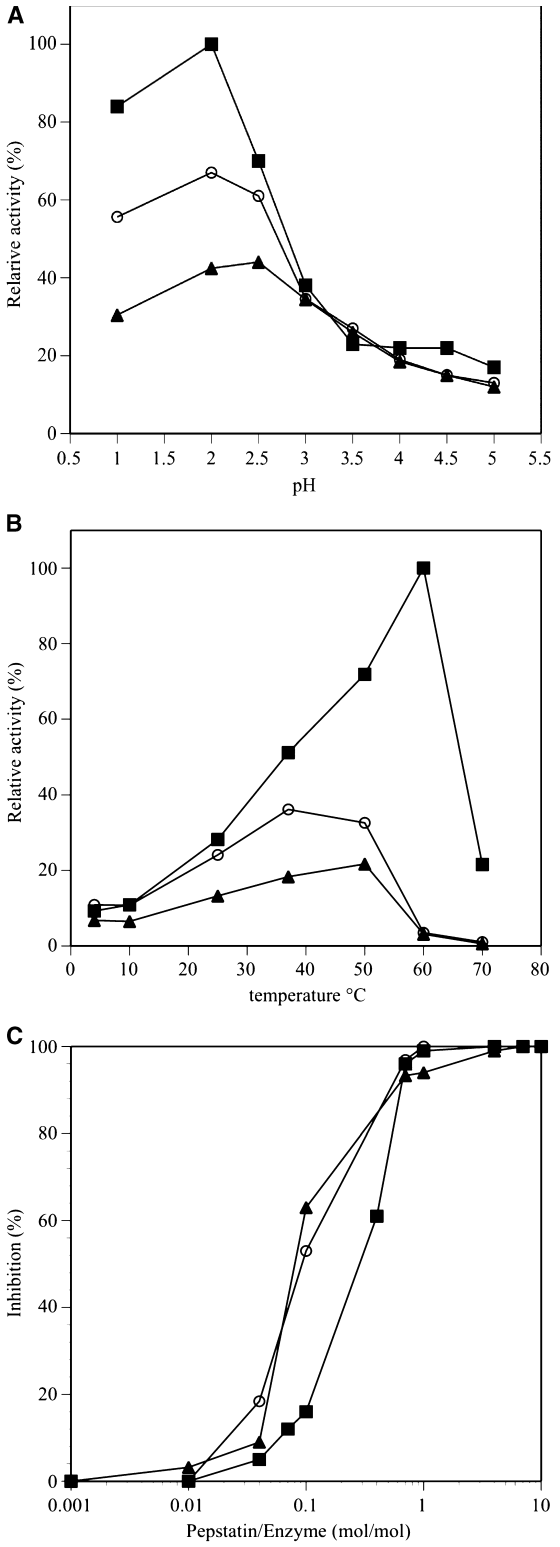

Fig. 3. (A) Effect of pH on the activity of commercial pig pepsin and recombinant fish pepsins A1 and A2. Active fish isoenzymes were obtained as described in Experimental procedures. The relative activity is expressed as a percentage of the highest activity over the pH range examined. Each data point represents the mean of three determinations. (B) Influence of temperature on enzymatic activity. Protease activity was determined using denatured hemoglobin as substrate. Pepsin obtained from pepsinogen activation was added to the reaction mixture containing sodium citrate buffer (pH 2.0). The assay was performed at various temperatures, as described in Experimental procedures. The results are representative of three independent sets of measurements. Relative activity is expressed as a percentage of the highest activity over the temperature range examined for the enzymes considered. (C) Effect of pepstatin A on fish and pig pepsins. Enzymes were assayed in the presence of increasing concentrations of pepstatin A using a 2.5% hemoglobin as substrate. Pepsin activity was determined at pH 2.0 and 37 °C. The fish and pig pepsin concentration in each assay was 0.021 μM. ■, activity of commercial pepsin; ▲, activity of fish pepsin A1; ○, activity of fish pepsin A2.

Table 1. Stability of pig and fish pepsins at 50 °C and pH 5.3. Aliquots were removed at appropriate times and the residual activity was measured at pH 2.0 and 37 °C using hemoglobin as substrate.

Enzyme	Half-life (min)
Pig pepsin	310 ± 1.55
Fish pepsin A1	270 ± 1.35
Fish pepsin A2	72 ± 0.46

low activity): aliquots were removed at appropriate times and the residual activity was measured at pH 2.0 using hemoglobin as substrate (Table 1). Under these conditions, a half-life of 310 min was observed for the mammalian enzyme. By comparison, the half-lives of Antarctic fish pepsins A1 and A2 under the same conditions were 270 and 72 min, respectively. As compared to pig pepsin and fish pepsin A1, fish pepsin A2 appeared to be more labile at the temperature at which it is maximally.

Effect of pepstatin A

Fish and pig pepsin activity was assayed in the presence of increasing concentrations of pepstatin A, using hemoglobin as substrate. Pepstatin A is a well-known acid protease inhibitor that binds in the active site cleft of the enzyme [12,22]. Figure 2B shows a ribbon representation of pig pepsin in complex with the phosphonate inhibitor IVA-Val-Val-Leu^{P}-(O)Phe-Ala-Ala-OMe [IVA = isovaleryl; Leu^{P} = phosphinic acid analog of leucine; (O)Phe = L-3-phenyllactic acid]. The occupancy as well as the hydrogen bond network between the enzyme and this inhibitor are essentially the same as those observed with pepstatin A [23]. Pepsin activity was determined at pH 2.0

and 37 °C for all three enzymes (Fig. 3C). Fish and porcine pepsins are inhibited completely by the equivalent molar amount of pepstatin, but fish isoforms showed different behavior in pepstatin A inhibition. Commercial pig pepsin required an inhibitor/enzyme ratio of 0.3 : 1 to produce 50% inhibition, whereas fish pepsins A1 and A2 required an inhibitor/enzyme ratio of 0.1 : 1 to reach about 50% and 60% inhibition, respectively.

Kinetic analysis

Kinetic parameters were determined for the purified Antarctic enzymes and pig pepsin using the synthetic chromogenic substrate Pro-Thr-Glu-Phe-(*p*-nitro-Phe)-Arg-Leu-OH (peptide 1) and are shown in Table 2. The K_m values were lower for fish enzymes, as might be expected for an enzyme from an Antarctic specie [24]. The measured k_{cat} values for fish pepsins A1 and A2 were 22 and 130 times smaller, respectively, than that of pig pepsin. Although the K_m values of fish pepsins were lower, the resultant values for the specificity constant (k_{cat}/K_m) of fish pepsins A1 and A2 were six and 12 times smaller, respectively, than that of pig pepsin. These data suggest that the differences in the 3D structure of fish and pig pepsins may well affect the kinetic behavior of the porcine and the fish enzymes. In porcine pepsin, there is a loop formed by the following residues: EGMDVPT (Fig. 2A,B). It contains four amino acids that are residues of the S4-S3′ subsites. This loop is missing in the Antarctic fish enzymes, as shown in the model of fish pepsin A1 in Fig. 2B. As a consequence, fish pepsins might accommodate the synthetic substrate in the catalytic pocket better than pig pepsin, due to a reduction in steric hindrance in fish pepsin. This hypothesis is corroborated by the fact that fish pepsins were more sensitive to pepstatin A than was pig pepsin (Fig. 3C). On the other hand, the specificity constants (k_{cat}/K_m) of fish pepsins were lower than that of the mammalian enzyme. This may be because the differences in subsite residues decrease the rate of hydrolysis. To explore the possibility that differences between fish and pig pepsins result from sequence changes that affect the substrate recognition region of the active site, we analyzed the digestion of two different substrates, as shown below.

Table 2. Kinetic parameters for the hydrolysis of a chromogenic substrate (peptide 1; see also Fig. 4A) by Antarctic and pig pepsins.

Enzyme	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($mM^{-1}·s^{-1}$)
Pig pepsin	0.26 ± 0.023	72.02 ± 3.67	277 ± 30.47
Fish pepsin A1	0.074 ± 0.006	3.32 ± 0.29	45 ± 6.75
Fish pepsin A2	0.025 ± 0.002	0.54 ± 0.043	22 ± 3.52

Specificity analysis

To determine whether changing the residues in proximity to the scissile peptide bond Phe–Phe would cause a difference in the activity of Antarctic pepsins, we used an HPLC assay involving two substrates that have different sequences on either side of the Phe–Phe scissile bond residues (Fig. 4A). In this assay, the *p*-nitro-Phe group acts as a reporter, and when the substrate is cleaved, the resulting smaller peptide containing the reporter has a different elution time in HPLC (Fig. 4B). Digestion of peptide 1 is shown in Fig. 4C and digestion of Phe-Gly-His-Phe-(*p*-nitro-Phe)-Ala-Phe-OMe (peptide 2) is shown in Fig. 4D. Both fish pepsin A1 and fish pepsin A2 were less efficient at cleaving peptide 1 than was pig pepsin, a result consistent with the previous data given in Fig. 3A,B and Table 2. However, both fish pepsins were unable to cleave peptide 2, and pig pepsin activity towards this substrate was reduced as compared to its digestion of peptide 1. These results are consistent with the hypothesis suggested above, whereby the missing loop in Antarctic pepsins (formed by residues EGMDVPT in pig pepsin) alters the substrate specificity of the fish pepsins by removing some of the residues near the scissile peptide bond.

The specificity of the Antarctic fish pepsins A1 and A2 for residues at the P1 and P1′ positions that flank the scissile peptide bond was determined by digesting a test protein with each enzyme (also with pig pepsin) and sequencing the resulting peptic peptide by MS. The digestion pattern of the test protein, rabbit muscle creatine phosphokinase (CK-MM), was different for digestion with pig versus fish pepsins (Fig. 5A). In general, the fish pepsins produced much larger pieces than the pig pepsin during the 3.5 min digestion time, again indicating the difference in the overall activity between the mesophilic and the Antarctic enzymes. The peptides in each part of the HPLC separation were visualized, compared (Fig. 5B), and then sequenced by tandem MS (MS/MS). An sample MS/MS spectrum is shown in Fig. 5C and is representative of the quality of the spectra obtained for the majority of the peptic peptides. High-quality MS/MS allowed the preparation of a digestion map of CK-MM, as shown in Fig. 6. The location of each peptic cleavage was tallied (supplementary Fig. S3 and supplementary Tables S1–S3), and the P1-P1′ amino acid preference for CK-MM was summarized (Fig. 7). The results of this analysis indicate that both pig pepsin and fish pepsins A1 and

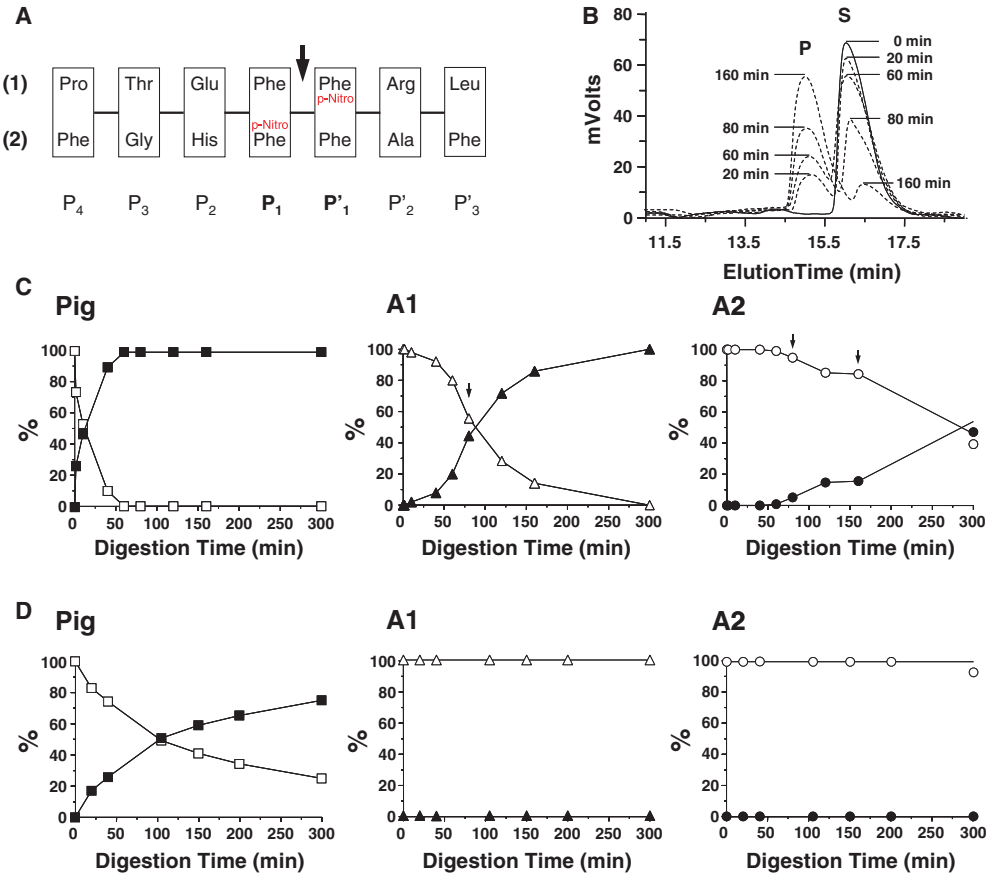

Fig. 4. Analysis of pepsin digestion of synthetic test peptides. (A) Amino acid sequence of the two synthetic peptides used in this study. The P4 to P3′ nomenclature refers to the position of each residue. The arrow indicates the position of the scissile peptide bond between P1 and P1′. (B) Example of the HPLC elution profile observed at 280 nm for pepsin A1 digestion of synthetic peptide 1; S, substrate; P, product. The digestion was performed in 100 mM sodium acetate buffer (pH 2.5) at 0 °C using a 1 : 10 protease/substrate ratio (w/w). At defined time points, 600 pmol was loaded onto a Jupiter 4u Proteo 90A column, and peptides were eluted using a 5–70% ACN gradient in 20 min. (C) Digestion of synthetic peptide 1 by pig pepsin, pepsin A1 and pepsin A2. Solid and empty symbols correspond to the substrate and the product, respectively. Arrows indicate time points at which additional amounts of fish pepsins were added (60 min, 4 μg of pepsin A1 and pepsin A2; 160 min, 4 μg of pepsin A2). (D) Digestion of synthetic peptide 2 by pig pepsin, pepsin A1 and pepsin A2. No digestion was observed with pepsin A1 and pepsin A2. Solid and empty symbols correspond to the substrate and the product, respectively.

A2 prefer hydrophobic residues in the P1-P1′ positions and that the overall specificity was similar among the three enzymes. However, important differences were apparent. Fish pepsin A1 could tolerate histidine or methionine in the P1 position, whereas fish pepsin A2 tolerated lysine and threonine in the P1 position. Pig pepsin was able to accommodate aspartic and glutamic acids in the P1 position, whereas neither of the fish pepsins produced any peptides that had these charged amino acids in the P1 position. As a result, the overall digestion profiles of the three pepsins on CK-MM were different. Figure 7B shows that pig pepsin produced many fragments in one portion of the substrate protein, whereas the fish pepsins preferred other regions.

Discussion

Antarctic fish are highly cold adapted and remarkably stenothermal [25], as a consequence of evolution of antifreeze glycoproteins and the higher catalytic efficiency of many enzymes at low temperatures [4]. For this reason, the study of enzymes in these poikilothermic species is of interest, especially in relation to the strategies adopted by these organisms to achieve a normal level of proteolysis at temperatures well below that of homeothermic species. Organisms can follow two strategies to achieve cold adaptation at the metabolic level: one way is to increase enzyme production in order to compensate for reduced kinetic efficiency; the other is via the

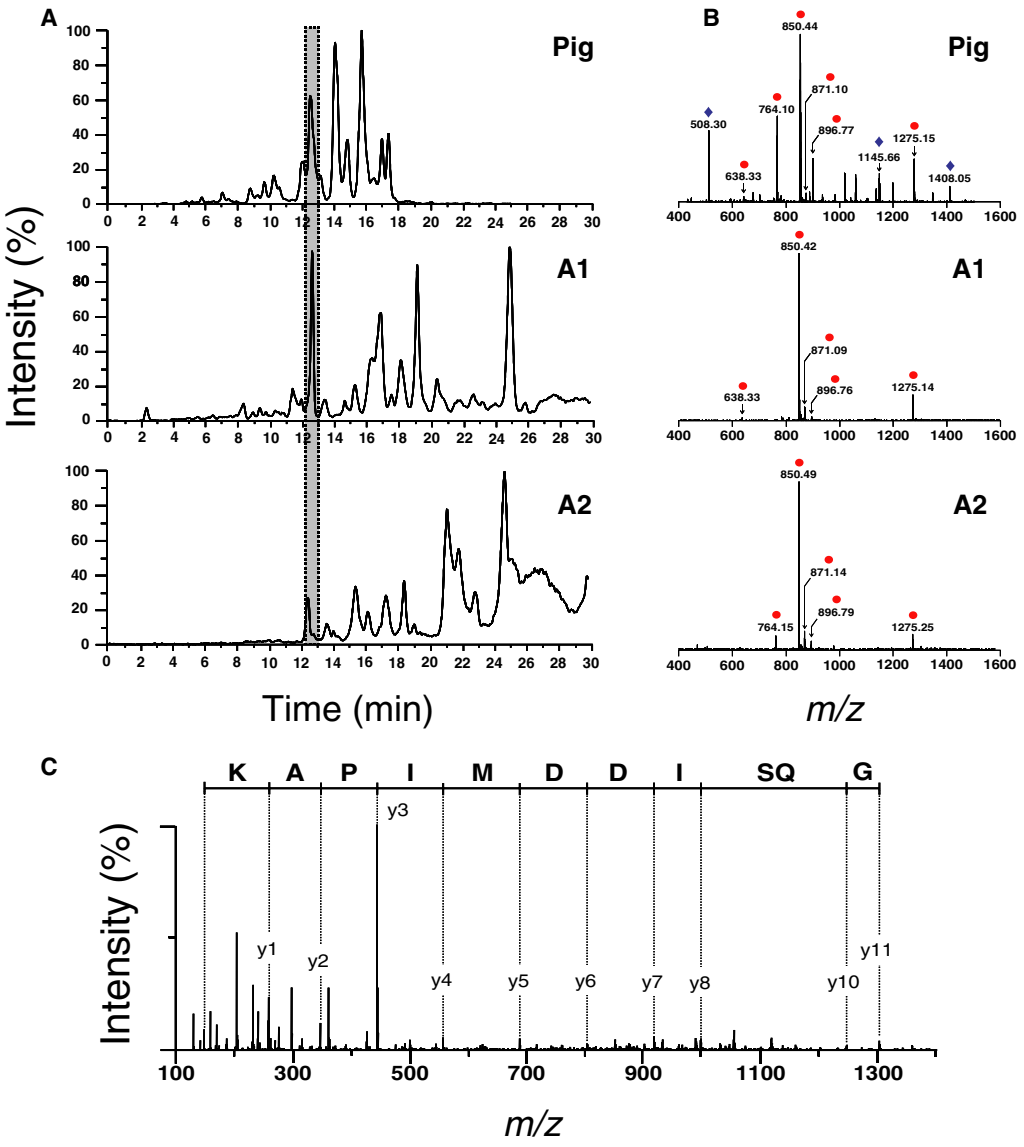

Fig. 5. (A) Comparison of the total ion chromatograms (TICs) obtained after digestion of CK-MM by pig pepsin, pepsin A1 and pepsin A2. The gray box corresponds to the region that was selected to compare the CK-MM peptide fragments generated after 3.5 min of digestion on ice (see below). (B) Representative mass spectra comparing the different peptide fragments eluted after 12 min of elution. Identical ions are indicated by a circle; nonidentical ions are indicated by a diamond. (C) MS/MS of the $[M + 3H]^{3+}$ ion (m/z 850.49) of the peptide Met360–Lys381, generated after pepsin A2 digestion.

expression of enzymes with relatively higher substrate turnover and the capacity to maintain ligand-binding properties at low temperature [26]. Multiple forms of type A pepsinogens are known to occur in various animals [27], and most of them are known to be products of different genes, as has been shown in humans [28] and cows [29]. The presence of multiple forms of pepsin A in some species may be correlated with the type of food or to the feeding habitat [30].

The present article describes the characteristics of the enzymatic properties of two recombinant pepsinogen isoforms, A1 and A2, from the Antarctic rock cod. The two Antarctic isoenzymes are, to our knowledge, the first Antarctic fish pepsinogens obtained using the strategy of expression in *E. coli* followed by refolding and purification. The effectiveness of this strategy was demonstrated by the fact that the purified fish pepsinogens were efficiently converted to the active form upon incubation in acidic conditions. The high

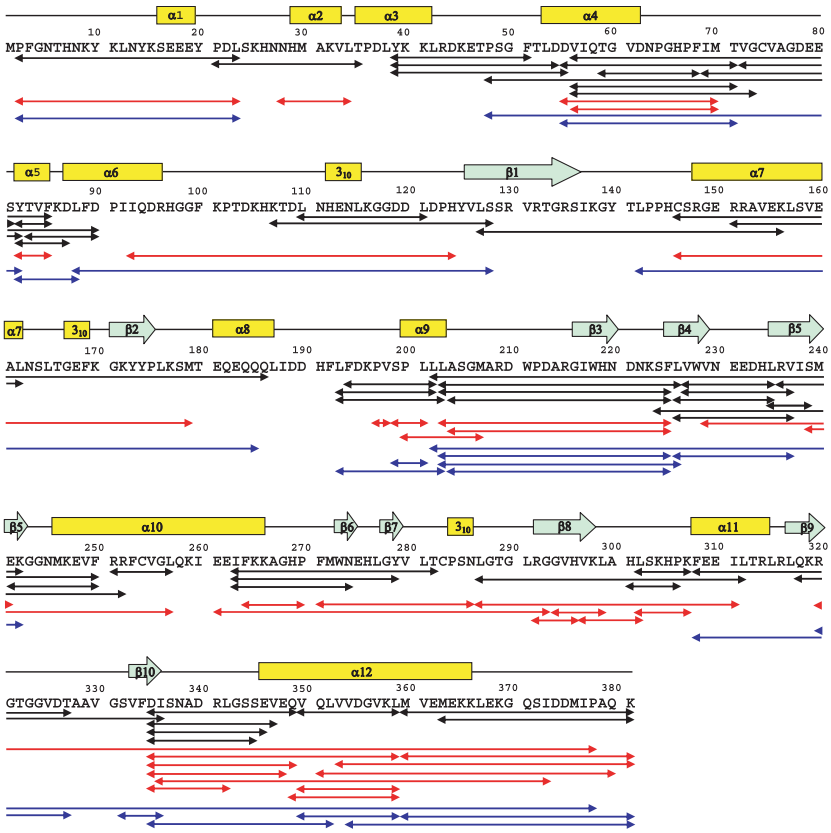

Fig. 6. CK-MM peptide map generated after 3.5 min of digestion on ice [protease/substrate ratio 1 : 1 (w/w)] by the pig pepsin (black arrows), pepsin A1 (red arrows) and pepsin A2 (blue arrows). Secondary structural elements of CK-MM determined by X-ray crystallography [43] are shown above the sequence.

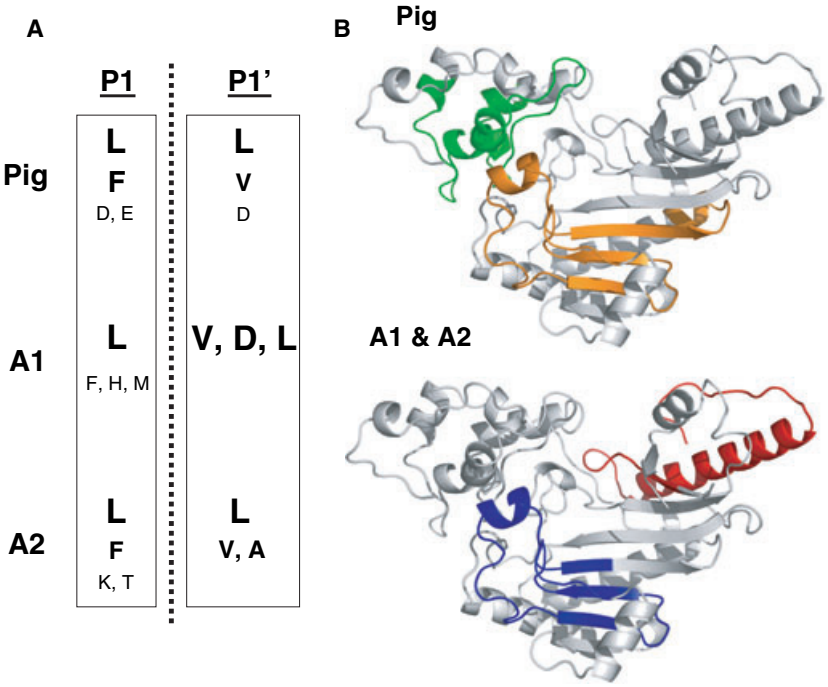

Fig. 7. (A) Cleavage preference of pig pepsin, pepsin A1 and pepsin A2 at the P1 and P1′ positions. The size of the letters indicates the probability of cleavage. (B) Three-dimensional structures of the monomeric form of CK-MM showing the regions that were extensively covered by the pig pepsin [Tyr39–Asp90 (green); Leu193–Phe250 (orange)], pepsin A1 [Asp335–Lys381 (red)] and pepsin A2 [Leu193–Val237 (blue)].

percentage of β-sheet structures, estimated by CD spectroscopy conducted on fish pepsins, was consistent with the literature data for properly folded forms of pepsin, including pig pepsins [20], suggesting that the Antarctic recombinant fish pepsins were correctly folded. The enzymatic assays further demonstrated

that the recombinant proteins were active and therefore properly folded.

All reported cold-adapted enzymes display modifications in their kinetic parameters that allow catalytic reactions to take place at low temperatures [31]. These changes may include a high specific activity at temperatures ranging from 0 °C to 30 °C [32]. Fish pepsins from Antarctic rock cod seem to constitute an exception, as their specific activities at low temperatures (4 °C, 10 °C and 25 °C) were similar to or lower than that of pig pepsin. On the other hand, as compared to pig pepsin and fish pepsin A1, fish pepsin A2 appeared to be more temperature sensitive, showing a half-life at 50 °C of 72 min (pig pepsin, 310 min; fish pepsin A1, 270 min). The full inactivation observed for pepsin A2 might be a consequence of the increased structural flexibility of the protein. Carginale *et al.* [17] reported that, apparently, fish pepsin A2 underwent local changes such as reduced hydropathy and increased flexibility at the level of the substrate cleft.

The most striking feature of the primary structure of Antarctic pepsin isoenzymes with respect to their mammalian counterparts is the absence of a loop near the active site. In the 3D structure of pig pepsin, the sequence EGMDVPT forms a loop near the active site, and this is missing in the fish isoforms (see red segment in the model of fish pepsin A1, Fig. 2). The loop is positioned close to the enzyme pocket and contains four amino acids (E, M, V and T) that are residues of the S4-S3′ subsites. Because these residues are missing in fish pepsins, they might accommodate substrates in the catalytic pocket better than pig pepsin. This hypothesis was corroborated by kinetic analysis carried out on fish and pig pepsins using peptide 1 (Fig. 4A). Fish pepsins showed lower K_m values than pig pepsin for this substrate. In addition, both fish isoenzymes required an inhibitor/enzyme ratio lower that that needed for pig pepsin to reach 50% inhibition. The specificity constants (k_{cat}/K_m) of fish pepsins were lower than that of the mammalian enzyme. This may be because the differences in subsite residues decrease the rate of hydrolysis.

In comparison experiments with two different substrate peptides (Fig. 4A), there was a significant difference between the activity of fish isoenzymes and that of pig pepsin. Fish pepsins were able to cleave peptide 1, with sequence PTEFF*RL, but were unable to cleave peptide 2, with the sequence FGHF*FAF (where the *p*-nitro group is indicated by the asterisk). Pig pepsin was active towards both substrates, with diminished activity towards peptide 2. These data suggest that the flanking residues on either side of the P1-P1′ position play a role in the specificity of the fish enzymes. To further investigate which residues fish pepsins preferred in the P1-P1′ positions, in comparison to pig pepsin, test digestion with CK-MM was performed. The results (Fig. 7A) suggest that the general specificities of all three enzymes at the P1-P1′ positions are highly similar, with some notable exceptions. Fish pepsin A1 could tolerate histidine or methionine in the P1 position, whereas fish pepsin A2 tolerated lysine and threonine in the P1 position. Pig pepsin was able to accommodate aspartic and glutamic acids in the P1 position, whereas neither of the fish pepsins produced any peptides that had these charged amino acids in the P1 position. In the P1′ position, pig pepsin and both fish pepsins were similar, except for the ability of fish pepsin A1 to accommodate aspartic acid. The size of the peptide population in this study was too small to allow any definitive conclusions about the contribution to specificity from amino acids in positions outside P1 and P1′ [33]. Taken together, our data indicate that the specificities of fish pepsins A1 and A2 are similar but not identical to that of pig pepsin, and we attribute the differences to the key changes in the active site of the fish enzymes.

In terms of the cold adaptation of pepsins from Antarctic fish, our study suggests that fish pepsin A2 meets the criterion for a cold-adapted enzyme showing limited stability at a moderate temperature (i.e. 50 °C). However, neither isoform meets the criterion that cold-adapted enzymes have a specific activity higher than that of their mesophilic counterparts over a temperature range from 0 °C to 30 °C. We now believe that Antarctic rock cod have adopted primarily gene amplification to accomplish efficient food digestion at low temperatures. Gene amplification increases enzyme production to compensate for the reduced kinetic efficiency. Antarctic rock cod have three forms of pepsin A [17] resulting from two gene duplication events: the first event consisted of the duplication of the pepsin A3 ancestor gene; the second duplication led to the appearance of pepsin A1 and A2 genes that contributed to a further increase in enzyme concentration. Through the production of isoenzymes that perform nearly the same type of digestion with similar specificity, Antarctic fish may accomplish a similar overall rate of digestion as their mesophilic counterparts.

Experimental procedures

Chemicals

Hemoglobin, pepstatin A, porcine pepsinogen and CK-MM were obtained from Sigma (St Louis, MO, USA). Sephacryl S-300 was obtained from Pharmacia Biotech

(Uppsala, Sweden). IPTG was obtained from Bio-Rad (Hercules, CA, USA). Immobilon-P membranes were obtained from Perkin-Elmer (Waltham, MA, USA). All other reagents were of analytical grade. The chromophoric peptides (peptides 1 and 2) were obtained from Bachem (Weil am Rhein, Germany).

Amplification, cloning and sequence analysis of the recombinant fish pepsinogen isoforms

The cDNAs encoding the propart and the mature regions of the two Antarctic fish isoforms (pepsin A1, GenBank accession number AJ550949; pepsin A2, GenBank accession number AJ550950), previously cloned in the pCR4–TOPO vector [17], were amplified by PCR using the forward primer 5′-GGAATTCCATATGTTCCACAAGATTCCCCT-3′ for fish pepsinogens A1 and A2, and the reverse primers 5′-ACCGGTCGACTTAGACAGACTTGGCCAA-3′ and 5′-ACCGGTCGACTTACACAGACTTGGCCAG-3′ for fish pepsinogens A1 and A2, respectively. The upstream primer contained an *Nde*I restriction site just before the start of the coding region. In the downstream primers, an adjacent *Sal*I restriction site followed the stop codon. Amplification was performed with 5 units of *Taq* DNA polymerase (Perkin-Elmer), 50 pmol of each of the above primers, and 0.2 mM dNTPs (final concentration). The mix was buffered with Perkin-Elmer PCR storage buffer (100 mM Tris/HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin). The reaction was carried out in a DNA Thermocycler Express (Hybaid, Ashford, UK), with an initial denaturation step at 95 °C for 3 min, 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final step at 72 °C for 15 min. The PCR fragments were gel-purified using a Qiagen gel extraction kit (Qiagen, Germantown, MA, USA) and subcloned in a pCR4–TOPO vector using a Topo TA Cloning kit (Invitrogen, Leek, the Netherlands). Pepsinogen cDNAs were sequenced with an automatic sequencer available at PRIMM (DNA Sequencing Service, Naples, Italy). The pCR4–TOPO vectors, containing fish pepsinogen A1 or fish pepsinogen A2, were digested with *Nde*I and *Sal*I, and the resulting fragments were ligated into the corresponding sites of expression vector pET22b(+) (Novagen, Madison, WI, USA). The resultant recombinant plasmids can produce the recombinant pepsinogen. The sequence of the fish pepsinogens cloned in pET22b(+) was again verified by sequencing in both directions.

Expression of recombinant pepsinogens

Competent BL21(DE3) *E. coli* cells were transformed with recombinant plasmid DNA containing either pepsinogen A1 or pepsinogen A2 cDNA to produce pET22b–PepA1 and pET22b–PepA2 cells. Transformed *E. coli* cells were grown overnight at 37 °C in LB broth supplemented with 100 $\mu g \cdot mL^{-1}$ ampicillin. The overnight culture was diluted 100-fold using 1 L of fresh LB broth containing ampicillin. The cells were then incubated at 37 °C until midexponential growth phase ($D_{600\ nm}$, 0.6). At this point, IPTG was added to a final concentration of 1.0 mM. After 10 h of induction at 37 °C, cells were collected by centrifugation at 7410 ***g*** for 20 min and washed twice in NaCl/P_i (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).

Isolation and solubilization of inclusion bodies

The bacterial pellet was frozen and thawed twice, resuspended in 50 mM Tris/HCl (pH 8.0) containing 150 mM NaCl, and subjected to sonication. Lysozyme (1 mg), RNase (1 mg), DNase (1 mg) and 30 mM $MgCl_2$ were added to the suspension and incubated for 30 min at 37 °C. The suspension was then diluted in 1 L of 50 mM Tris/HCl (pH 8.0) and 150 mM NaCl, and gently shaken for 2 h at 4 °C. After 30 min of centrifugation at 7000 ***g***, the pellet containing the inclusion bodies was resuspended in 1 L of 100 mM Tris/HCl (pH 8.0) and 50 mM β-mercaptoethanol. The suspension was gently shaken at 70 r.p.m. for 2 h at 4 °C, and then centrifuged at 7000 ***g*** for 30 min. The pellet was solubilized in 6 M urea, 100 mM Tris/HCl (pH 8.0), 1 mM glycine, 1 mM EDTA and 50 mM β-mercaptoethanol at 4 °C with gentle shaking for 12 h.

Refolding and purification

The solubilized material was centrifuged at 44 380 ***g*** for 2 h, using a 60Ti rotor. Renaturation of the supernatant containing pepsinogen A1 or pepsinogen A2 was achieved by a 200-fold dilution of the denaturant in 100 mM Tris/HCl buffer (pH 8.0), and the resultant solution was reconcentrated using a Prep/Scale-TFF cartridge, 10 kDa cut-off, tangential flow concentrator (Millipore Corporation, Billerica, MA, USA), and then further concentrated to about 50 mL in an Amicon ultrafiltration cell (Millipore). After removal of residual insoluble material by centrifugation at 20 000 ***g*** for 1 h, the clear supernatant was subjected to chromatographic purification using Sephacryl S-300 (1.5 × 45 cm) gel filtration. About 40 mg of total protein was loaded onto the Sephacryl S-300 column, which was equilibrated and eluted with 20 mM Tris/HCl (pH 8.0) at a flow rate of 0.8 $mL \cdot min^{-1}$. Fractions containing active pepsin were pooled and loaded onto a Resource Q column (30 × 6.4 mm; Pharmacia Biotech) equilibrated with 20 mM Tris/HCl (pH 8.0). Proteins were eluted with a linear gradient of 0–500 mM NaCl in 20 min at a flow rate of 1 $mL \cdot min^{-1}$.

Conversion of pepsinogen into the active form

Acidification was carried out by adding 5 mL of 2 M glycine-HCl (pH 2.0) to 40 mL of the pepsinogen preparation

and incubating the mixture at 25 °C for 2 h [34]. The solution was neutralized by dialyzing against 20 mM sodium acetate (pH 5.3) at 4 °C, using dialysis membranes with 12–14 kDa cut-off. The recovered solution containing the active form of pepsinogen was centrifuged at 20 000 ***g*** for 30 min at 4 °C, and used as purified Antarctic fish pepsin A1 or A2.

CD spectra of fish pepsins

CD spectroscopy was performed on homogeneous samples of recombinant fish pepsins A1 and A2 (0.1 $mg{\cdot}mL^{-1}$ in 2.5 mM sodium acetate buffer, pH 5.3), at 25 °C, using a J-710 spectropolarimeter (Jasco, Tokyo, Japan) calibrated with a standard solution of (+)-10-camphosulfonic acid. A cuvette with 0.1 cm path length was used to record spectra in the far-UV region (240–200 nm), and each spectrum was averaged six times. Photomultiplier high voltage did not exceed 600 V in the spectral region measured. The results are expressed in terms of residue molar ellipticity. The percentages of secondary structures were estimated according to the method of Yang *et al.* [35].

Negative ion ESI mass spectra of fish pepsinogens and pepsins

Negative ion mass spectra were acquired on a Waters LCT-Premier mass spectrometer equipped with a standard electrospray source, a capillary voltage of 2.8 kV, and a cone voltage of 50 V. The *m/z* scale was calibrated in negative mode with a 10 $ng{\cdot}\mu L^{-1}$ sodium formate solution in 90 : 10 isopropanol/H_2O. Pepsin samples were loaded onto a self-packed POROS 20R2 trap (POROS media; Applied Biosystems, Framingham, MA, USA) equilibrated with H_2O and 0.3% NH_4OH (pH 8.0), desalted for 10 min, and eluted in the mass spectrometer with a 5–50% isopropanol gradient in 20 min at 50 $\mu L{\cdot}min^{-1}$.

SDS/PAGE, western blotting and sequencing

In addition to MS, the purity of the pepsinogen and pepsin was examined by SDS/PAGE carried out according to Laemmli [36]. Samples were dissolved in buffer containing 5% β-mercaptoethanol. Gels were stained with Coomassie blue. Blotting from gels onto Immobilon-P membranes was performed as described by Matsudaira [37]. N-terminal sequencing was performed on the blotted protein by automated Edman degradation. The protein concentration was determined using two methods: (a) Bio-Rad protein assay, based on the Bradford method [38]; and (b) absorbance at 280 nm using absorption coefficients of 56 840 $M^{-1}{\cdot}cm^{-1}$ and 62 340 $M^{-1}{\cdot}cm^{-1}$ for pepsin A1 and pepsin A2, respectively. Extinction coefficients were determined using the PROTPARAM program on the ExPASy server [39].

Activity assay with hemoglobin

All enzyme assays were performed using a Cary 50 UV–visible spectrophotometer (Varian, Palo Alto, CA, USA). The proteolytic assay was performed using 2.5% denatured bovine hemoglobin as substrate, as described previously [40]. Pepsin activity was determined at acidic pH and 37 °C. After 60 min of incubation, the reaction was stopped by the addition of 5% trichloroacetic acid. The samples were centrifuged twice for 10 min at 16 000 ***g***, and the resulting supernatant containing the small peptides produced by proteolytic digestion was analyzed at 280 nm. One enzyme unit was defined as the amount capable of producing a $\Delta A_{280\ nm}$ $[A_{280\ nm\ (sample)} - A_{280\ nm\ (blank)}]$ of 0.1 in 60 min.

Determination of pH optimum

In order to determine the optimum pH values, 0.35 μg of fish pepsins (A1 or A2) were assayed in 100 mM HCl/KCl buffer at pH 1.0 and 150 mM sodium citrate buffer at pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0, using hemoglobin as substrate. Incubation was carried out at 37 °C for 60 min. Pig pepsin was also tested using the same experimental conditions.

Temperature studies

The optimum temperatures for pig and fish pepsin activities were determined by incubating pepsins in 0.15 M sodium citrate buffer (pH 2.0) at 4 °C, 10 °C, 25 °C, 37 °C, 50 °C, 60 °C and 70 °C with 2.5% hemoglobin. After 60 min, the reaction was terminated by the addition of 5% trichloroacetic acid. All the determinations were done in triplicate. The stability of pepsins at 50 °C in 20 mM sodium acetate (pH 5.3) was also investigated. Aliquots were removed at appropriate times, and the residual activity was measured at 37 °C and pH 2.0, using hemoglobin as substrate.

Inhibitory effect of pepstatin A

Fish and pig pepsins were assayed in the presence of increasing concentrations of pepstatin A in the range from 2.1×10^{-5} μM to 2.1×10^{-1} μM, using a 2.5% hemoglobin solution as substrate (the final hemoglobin concentration in the assay was 1%). Pepsin activity was determined at pH 3.0 and 37 °C. After 60 min of incubation, the reaction was stopped by the addition of 5% trichloroacetic acid. The samples were centrifuged twice for 10 min at 16 000 ***g***, and the resulting supernatant was analyzed at 280 nm. The

fish and pig pepsin concentrations used in each assay were 0.021 μM.

Cleavage of chromogenic substrates determined by following the rate of decrease in absorbance at 300 nm

The enzymatic cleavage of the Phe–(*p*-nitro-Phe) bond in the two chromogenic substrates peptide 1 and peptide 2 was followed spectrophotometrically at 300 nm, as described by Dunn *et al*. [41]. A stock solution (2.0 mM) of peptide substrate was prepared in 0.1 M acetic acid. The incubation mixture contained 0.2 mM chromogenic substrate and 0.35 μg of fish or pig pepsins in 0.15 M sodium citrate buffer (pH 2.0). The assay was performed by monitoring the decrease in absorbance at 300 nm ($\Delta A_{300\ nm}$·min^{-1}) at 25 °C for at least 10 min.

Determination of kinetic parameters

Peptide 1 was used as the substrate for kinetic analyses. The assay with the synthetic substrate was performed as described above. The kinetic constants K_m and V_{max} were determined from the plot of the reciprocal of the initial velocity versus the reciprocal of the concentrations of the synthetic substrate (up to 0.3 mM). k_{cat} values were obtained from the equation: $V_{max} = k_{cat}[E]$, where [E] is the enzyme concentration. The reported kinetic values correspond to the average of three independent determinations.

Analysis of chromogenic substrate cleavage by HPLC

The chromogenic substrates peptide 1 and peptide 2 were reconstituted in 30% acetic acid (pH 1.8) to a final concentration of 1 mg·mL^{-1}. Digestions were performed at 0 °C and 37 °C in 100 mM sodium acetate buffer (pH 2.5), with a 1 : 10 protease/substrate ratio (w/w). Fish pepsins and pig pepsin were diluted to a final concentration of 0.05 mg·mL^{-1}. At defined time points, 25 μL of the digestion mixture was removed and immediately loaded onto a Jupiter 4μ Proteo 90A column (50 × 1.00 mm; Phenomenex Corp., Torrance, CA, USA) equilibrated with 5% acetonitrile (ACN). Peptides were eluted in 20 min with a linear gradient of 5–70% ACN at 50 μL·min^{-1} on a Shimadzu (Columbia, MD, USA) HPLC column (LC-10ATvp). The elution profiles were recorded at 214 and 280 nm with a Shimadzu UV–visible detector (SPD-10Avp).

Protein digestions – identification of the generated peptide fragments

Creatine phosphokinase b from rabbit muscle was reconstituted in double distilled H_2O to a final concentration of 0.5 mg·mL^{-1}. Digestions were performed at 0 °C in 20 mM Tris/HCl buffer (pH 2.5), using a 1 : 1 protease/substrate ratio (w/w). After 3.5 min of digestion, samples were loaded onto a Magic C18 5μ 200Å column (1.0 × 50 mm; Michrom Bioresources, Auburn, CA, USA) equilibrated with 5% ACN and 0.05% trifluoroacetic acid. Peptide fragments were eluted into the mass spectrometer using a 5–50% ACN gradient in 30 min (for MS analysis) or in 50 min (for MS/MS analysis) at 50 μL·min^{-1} with a Shimadzu HPLC column (LC-10ADvp). Mass spectral analyses were performed on a Waters (Milford, MA, USA) Q-TOF Ultima mass spectrometer equipped with a standard electrospray source and a lockspray interface. The capillary and the cone voltages were set to 3.00 kV and 35 V, respectively. For each acquisition, a 250 fmol·μL^{-1} green fluorescent protein solution in 50 : 50 ACN/H_2O and 0.2% formic acid was continuously infused through the reference probe at 5 μL·min^{-1}. Data from the reference spray were used to calculate a mass correction factor in order to provide exact mass information. Peptide mass assignments were confirmed by exact mass measurement and/or MS/MS experiments.

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References

1 Brodeur JC, Calvo J, Clarke A & Johnston IA (2003) Myogenic cell cycle duration in Harpagifer species with sub-Antarctic and Antarctic distributions: evidence for cold compensation. *J Exp Biol* **206**, 1011–1016.

2 Chen L, DeVries AL & Cheng CH (1997) Evolution of antifreeze glycoprotein gene from a trypsinogen gene in Antarctic notothenioid fish. *Proc Natl Acad Sci USA* **94**, 3811–3816.

3 Detrich HW 3rd (1997) Microtubule assembly in cold-adapted organisms: functional properties and structural adaptations of tubulins from antarctic fishes. *Comp Biochem Physiol A Physiol* **118**, 501–513.

4 D'Avino R, Caruso C, Camardella L, Schninà ME, Rutigliano B, Romano M, Carratore V, Barra D & di Prisco G (1991) *An Overwiew of the Molecular Structure and Funtional Properties of the Hemoglobins of a Cold-Adapted Antarctic Teleost*. Springer-Verlag, Berlin.

5 Kageyama T (2002) Pepsinogens, progastricsins, and prochymosins: structure, function, evolution, and development. *Cell Mol Life Sci* **59**, 288–306.

6 Kageyama T, Ichinose M, Miki K, Athauda SB, Tanji M & Takahashi K (1989) Difference of activation processes and structure of activation peptides in human pepsinogens A and progastricsin. *J Biochem (Tokyo)* **105**, 15–22.

7 Fusek M & Vetvicka V (1995) *Aspartic Proteinases. Physiology and Pathology*. CRC Press, Boca Raton.

8 Dunn BM, Valler MJ, Rolph CE, Foundling SI, Jimenez M & Kay J (1987) The pH dependence of the hydrolysis of chromogenic substrates of the type, Lys-Pro-Xaa-Yaa-Phe-(NO_2)Phe-Arg-Leu, by selected aspartic proteinases: evidence for specific interactions in subsites S3 and S2. *Biochim Biophys Acta* **913**, 122–130.

9 Fruton JS (1970) The specificity and mechanism of pepsin action. *Adv Enzymol Relat Areas Mol Biol* **33**, 401–443.

10 Foltmann B (1981) Gastric proteinases: structure, function, evolution and mechanism of action. *Essays Biochem* **17**, 52–84.

11 Abad-Zapatero C, Rydel TJ & Erickson J (1990) Revised 2,3, a structure of porcine pepsin: evidence for a flexible subdomain. *Proteins* **8**, 62–81.

12 Fujinaga M, Chernaia MM, Tarasova NI, Mosimann SC & James MN (1995) Crystal structure of human pepsin and its complex with pepstatin. *Protein Sci* **4**, 960–972.

13 Gildberg A, Olsen RL & Bjarnason JB (1990) Catalytic properties and chemical composition of pepsins from Atlantic cod (Gadus morhua). *Comp Biochem Physiol B* **96**, 323–330.

14 Sanchez LM, Freije JP, Merino AM, Vizoso F, Foltmann B & Lopez-Otin C (1992) Isolation and characterization of a pepsin C zymogen produced by human breast tissues. *J Biol Chem* **267**, 24725–24731.

15 Gildberg A (1988) Aspartic proteinases in fishes and acquatic invertebrates. *Comp Biochem Physiol B* **91**, 425–435.

16 Tanji M, Kageyama T & Takahashi K (1988) Tuna pepsinogens and pepsins. Purification, characterization and amino-terminal sequences. *Eur J Biochem* **177**, 251–259.

17 Carginale V, Trinchella F, Capasso C, Scudiero R, Riggio M & Parisi E (2004) Adaptive evolution and functional divergence of pepsin gene family. *Gene* **333**, 81–90.

18 Marshall CJ (1997) Cold-adapted enzymes. *Trends Biotechnol* **15**, 359–364.

19 Lin XL, Wong RN & Tang J (1989) Synthesis, purification, and active site mutagenesis of recombinant porcine pepsinogen. *J Biol Chem* **264**, 4482–4489.

20 Cooper JB, Khan G, Taylor G, Tickle IJ & Blundell TL (1990) X-ray analyses of aspartic proteinases. II. Three-dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 A resolution. *J Mol Biol* **214**, 199–222.

21 Sielecki AR, Fedorov AA, Boodhoo A, Andreeva NS & James MN (1990) Molecular and crystal structures of monoclinic porcine pepsin refined at 1.8 A resolution. *J Mol Biol* **214**, 143–170.

22 Marciniszyn J Jr, Hartsuck JA & Tang J (1976) Mode of inhibition of acid proteases by pepstatin. *J Biol Chem* **251**, 7088–7094.

23 Fujinaga M, Cherney MM, Tarasova NI, Bartlett PA, Hanson JE & James MN (2000) Structural study of the complex between human pepsin and a phosphorus-containing peptidic-transition-state analog. *Acta Crystallogr D Biol Crystallogr* **56**, 272–279.

24 Genicot S, Rentier-Delrue F, Edwards D, VanBeeumen J & Gerday C (1996) Trypsin and trypsinogen from an Antarctic fish: molecular basis of cold adaptation. *Biochim Biophys Acta* **1298**, 45–57.

25 Cheng CH & Detrich WH (2007) Molecular ecophysiology of Antarctic notothenioid fishes. *Phil Trans R Soc Lond B Biol Sci* **B362**, 1–18.

26 D'Amico S, Claverie P, Collins T, Georlette D, Gratia E, Hoyoux A, Meuwis MA, Feller G & Gerday C (2002) Molecular basis of cold adaptation. *Phil Trans R Soc Lond B Biol Sci* **357**, 917–925.

27 Foltmann B (1981) Gastric proteinases – structure, function, evolution and mechanism of action. *Essays Biochem* **17**, 52–84.

28 Zelle B, Evers MP, Groot PC, Bebelman JP, Mager WH, Planta RJ, Pronk JC, Meuwissen SG, Hofker MH, Eriksson AW *et al.* (1988) Genomic structure and evolution of the human pepsinogen A multigene family. *Hum Genet* **78**, 79–82.

29 Lu Q, Wolfe KH & McConnell DJ (1988) Molecular cloning of multiple bovine aspartyl protease genes. *Gene* **71**, 135–146.

30 Kageyama T (2000) New world monkey pepsinogens A and C, and prochymosins. Purification, characterization of enzymatic properties, cDNA cloning, and molecular evolution. *J Biochem (Tokyo)* **127**, 761–770.

31 Feller G, Narinx E, Arpigny JL, Aiattaleb M, Daise E, Genicot S & Gerday C (1996) Enzymes from psycrophilic organisms. *FEMS Microbiol Rev* **18**, 189–202.

32 Feller G, Payan F, Theys F, Qian M, Haser R & Gerday C (1994) Stability and structural analysis of alpha-amylase from the antarctic psychrophile Alteromonas haloplanctis A23. *Eur J Biochem* **222**, 441–447.

33 Keil B (1992) *Specificity of Proteolysis*. Springer-Verlag, Berlin.

34 Shintani T, Nomura K & Ichishima E (1997) Engineering of porcine pepsin. Alteration of S1 substrate specificity of pepsin to those of fungal aspartic proteinases by site-directed mutagenesis. *J Biol Chem* **272**, 18855–18861.

35 Yang JT, Wu CS & Martinez HM (1986) Calculation of protein conformation from circular dichroism. *Methods Enzymol* **130**, 208–269.

36 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
37 Matsudaira P (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262**, 10035–10038.
38 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
39 Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD & Bairoch A (2003) ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res* **31**, 3784–3788.
40 Dame JB, Reddy GR, Yowell CA, Dunn BM, Kay J & Berry C (1994) Sequence, expression and modeled structure of an aspartic proteinase from the human malaria parasite Plasmodium falciparum. *Mol Biochem Parasitol* **64**, 177–190.
41 Dunn BM, Jimenez M, Parten BF, Valler MJ, Rolph CE & Kay J (1986) A systematic series of synthetic chromophoric substrates for aspartic proteinases. *Biochem J* **237**, 899–906.
42 Higgins DG & Sharp PM (1988) CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**, 237–244.
43 Rao JK, Bujacz G & Wlodawer A (1998) Crystal structure of rabbit muscle creatine kinase. *FEBS Lett* **439**, 133–137.

Supplementary material

The following supplementary material is available online:

Fig. S1. (A) Elution profile from a Sephacryl S-300 chromatography column of Antarctic recombinant pepsinogens.

Fig. S2. Far-UV CD spectra of fish pepsins A1 and A2.

Fig. S3. Frequency of cleavage at (A) the P1 site and (B) the P1′ site observed after rabbit muscle creatine phosphokinase digestion by pig pepsin, fish pepsin A1 and fish pepsin A2.

Table S1. Pig pepsin cleavage preference for rabbit muscle creatine phosphokinase.

Table S2. Fish pepsin A1 cleavage preference for rabbit muscle creatine phosphokinase.

Table S3. Fish pepsin A2 cleavage preference for rabbit muscle creatine phosphokinase.

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