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Partial characterisation of gelatinolytic activities in herring (*Clupea harengus*) and sardine (*Sardina pilchardus*) possibly involved in *post-mortem* autolysis of ventral muscle

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ABSTRACT

The present work aims at identifying enzymatic activities that may contribute to the *post-mortem* autolysis of the ventral muscle, known as belly bursting, in herring (*Clupea harengus*). Gelatinolytic proteases extracted from several herring tissues and also from a sardine (*Sardina pilchardus*) tissue were partially characterised using gelatin zymography, inhibitor analysis, immunodetection with anti-trypsin antibody and MALDI-TOF/TOF peptide sequencing. The results indicate the presence of gelatinolytic trypsin-like serine proteases and metalloproteases in several samples including the ventral muscle of herring. MS/MS analysis gave partial sequences of peptides from some of the proteases, and these were identified as elastase, trypsin and aspartyl aminopeptidase. It is likely that belly bursting in herring is caused by leakage of enzyme-rich fluids from the intestine and/or *pyloric caeca* which may also contain exogenous proteases from the digestive systems of the prey.

1. Introduction

Post-mortem autolysis of the ventral wall of pelagic fish, also known as belly bursting, usually takes place during the heavy feeding season. Analyses of the autolytic activities related to this phenomenon in different pelagic fish species over the years have lead to different conclusions. In some species trypsin-like activities seem to be the most relevant (Almy, 1926; Baalsrud, 1951; Gildberg, 1978; Martinez & Gildberg, 1988), whereas gastric acid leakage and pepsin activity appear to be the main cause of autolysis in others (Gildberg, 1982). Although some studies seem to agree that the cause of the degradation is of proteolytic origin, Botta, Kennedy, Kiceniuk & Legrow claimed that physical handling practices were the main factors determining the occurrence of belly bursting (Botta, Kennedy, Kiceniuk, & Legrow, 1992).

Digestive proteases in fish are found in the gastric mucosa, *pyloric caeca*, pancreatic tissues and intestinal mucosa (Fänge & Grove, 1979; Kapoor, Smit, & Verighina, 1975). The gastric mucosa and the diffuse pancreatic tissues secrete proteases into the lumen of the alimentary canal, whilst the intestinal mucosa produces proteases which are located on the brush border of the epithelium (Fänge & Grove, 1979). The aspartic protease pepsin, found in the stomachs of teleosts (Fänge & Grove, 1979; Kapoor et al., 1975), is highly active and stable at acid pH (Simpson, 2000). Conversely, the major serine proteases found in the pancreatic tissues, *pyloric caeca* and intestines; trypsin, chymotrypsin and elastase, are active and stable in neutral to slightly alkaline pH and unstable or inactive in acid pH (Fänge & Grove, 1979; Simpson, 2000). The digestive metalloproteases in fish include carboxypeptidase A and B, which are secreted from the pancreas (Fänge & Grove, 1979), as well as the aminopeptidases, which might be present in a variety of tissues and are also thought to be produced in the intestinal mucosa in teleosts (Fänge & Grove, 1979; Taylor, 1993). Leucine aminopeptidase has been found in the *pyloric caeca* of sardine (*Sardina pilchardus*) (Martinez & Serra, 1989). In addition to the digestive proteases produced by the fish, it has been indicated that a part of the total amount of proteases present in the digestive system of the fish might be derived from the food or prey (Dabrowski & Glogowski, 1977; Fänge & Grove, 1979; Kuz'mina & Golovanova, 2004).

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Other proteases that have been implicated in the autolysis of fish tissues and softening of fish muscles *post-mortem* are the intracellular calpains and the lysosomal cathepsins, especially cathepsins D and L (Huss, 1995). Cathepsin L and calpains belong to the cysteine protease group, whilst cathepsin D is an aspartic protease. The physiological role of some matrix metalloproteases is extracellular matrix degradation and remodelling, but lately they have also been implicated in fish muscle softening (Bracho & Haard, 1995; Kubota et al., 2001; Woessner, 1991). The matrix metalloproteases belong to the metalloprotease family.

The work presented here aims at identifying and characterising the enzymes involved in the *post-mortem* autolysis of the ventral wall of the pelagic fish herring (*Clupea harengus*). In addition, analyses of samples of the *pyloric caeca* of sardine (*S. pilchardus*) are included for comparative purposes. We have previously found that the enzymatic activities leading to the rupture of the ventral wall in herring do not originate in the stomach, but more likely in the upper part of the intestine (Felberg & Martinez, 2006; Veliyulin, Felberg, Digre, & Martinez, 2007). The activities are present in the ventral muscles of spring herring which are feeding, but not in autumn herring with empty stomachs, and the activities in the ventral muscle increase in intensity during ice storage (Felberg, Batista, Nunes, & Martinez, 2008). We have also shown that spring herring which have been allowed to empty their stomachs do not exhibit strong gelatinolytic activities in their ventral muscles and that the activities seen in fish with full stomachs do not seem to be endogenous to the ventral muscle and are able to digest myosin heavy chain (Felberg et al., 2009).

Continuing this line of work we present here, for the first time to our knowledge, the partial characterisation and peptide sequencing of some gelatinolytic activities found in the ventral muscle of spring herring and the activities found in stomach content and intestine of herring and in the *pyloric caeca* of both herring and sardine. For the characterisation of the activities we have used inhibitor studies, peptide mapping and peptide sequencing of the isolated activities by matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometry (MALDI-TOF MS/MS) on partially purified proteins subsequent to polyacrylamide gel electrophoresis.

2. Materials and methods

2.1. Fish samples

Herring (*C. harengus*) were sampled aboard the purse seiner Libas in the North Sea in June 2006. The herring were frozen immediately after catch and stored at −20 °C until the analyses were performed. The fish were captured at two different locations, N57°58′ E02°21′ and N59°47′ E02°04′. The former herring had full stomachs, whilst the latter were allowed to empty their stomach contents for 19 h (the emptied fish were used only in the SDS–PAGE and Western blot analyses). Average lengths and weights for the full and emptied fish were 21.7 + 1.5 cm and 128.6 + 19.4 g (n = 15) and 23.2 + 1.5 cm and 144.8 + 24.8 g (n = 15), respectively.

Sardine (*S. pilchardus*) were captured off the Algarve coast of Portugal by ring net in March 2006. The fish were immediately iced and stored for 24 h before freezing at −80 °C. Ten of these fish were used and they had an average weight of 46.9 ± 8.4 g and length of 14.9 ± 1.2 cm.

2.2. Enzyme extraction

All the extracts used in this study were made from tissues and stomach- and intestinal content samples dissected/sampled in ice from partially thawed fish. "Ventral muscle" was sampled by dissection of the belly flap, avoiding skin and peritoneum, and "dorsal muscle" was sampled by dissection of the herring light dorsal muscle. "Stomach content" was sampled by opening the stomach and carefully collecting the contents, without scraping the stomach lining, and "intestinal content" was sampled by applying gentle pressure to the dissected intestine and collecting the contents. "*Pyloric caeca*" was sampled by dissecting the *pyloric caeca* from the intestine and removing fat and nematodes, if present, before homogenisation.

The procedure for extracting herring samples was based on the method described by Lødemel and Olsen (2003). The entire procedure was performed in ice, and dorsal muscle, ventral muscle, stomach content, intestinal content and *pyloric caeca* were sampled. About 0.1 to 0.5 g of sample was accurately weighed into 2 ml Eppendorf tubes and homogenised in 0.5 to 1 ml cold extraction buffer containing 50 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$ and 0.05% Brij 35 (Lødemel, Mæhre, Winberg, & Olsen, 2004), using a pair of scissors or a pellet mixer (VWR, No. 431-01000). The extracts were incubated for 1 h (4 °C) and then centrifuged for 30 min (6000*g*, 4 °C). The supernatants were collected. The pellets were resuspended in 0.5 ml of extraction buffer, centrifuged for 20 min (16,100*g*, 4 °C). The supernatants of each extract were pooled (yielding a total volume of 1–1.5 ml) and stored at −80 °C until further use.

For sardine samples, 0.5 g of *pyloric caeca* were sampled as described for herring and homogenised in ice with 2 ml buffer (50 mM Tris–HCl pH 7.5, 10 mM $CaCl_2$ and 0.5 ml CCl_4). The homogenates were centrifuged at 26,000*g* (4 °C) and the supernatants were collected.

Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard.

Extracts from each sample were pooled from either 15 herring or 10 sardines, except in the SDS–PAGE and Western blot analyses, where the extracts of two individual sardines were used.

2.3. Affinity isolation

The gelatin- and benzamidine-binding proteins were separated using gelatin Sepharose 4B and benzamidine-Sepharose 6B (GE Healthcare), respectively. Samples of ventral muscle, stomach content and intestinal content from herring and of *pyloric caeca* from both herring and sardine were subjected to gelatin Sepharose, whilst only samples of ventral muscle and intestinal content from herring were subjected to benzamidine-Sepharose. The crude extracts (1 ml) were added to 0.5 ml of gelatin- or benzamidine-Sepharose that had been preequilibrated in washing buffer (50 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$, 0.05% Brij 35 and 0.02% NaN_3). The binding step was performed for 1 h (4 °C) with gentle agitation. The samples were subsequently centrifuged (100*g*, 1 min, 4 °C) and the supernatant was removed (unbound proteins). The Sepharose beads were washed with 1 ml washing buffer 15 times and bound proteins were subsequently eluted several times with 0.5–1 ml washing buffer containing 10% DMSO for gelatin binding proteins or 50% ethyleneglycol for benzamidine-binding proteins. The eluted fractions were concentrated using Microcon YM-10 centrifugal filter units (10 kDa, Millipore). Eluted fractions from several parallel experiments were pooled and concentrated for each tissue sample. The affinity chromatography was performed both in tubes and in microcolumns, but since the two methods gave similar results, the tube method was mainly used due to its simplicity.

2.4. Gelatin zymography

Substrate gel electrophoresis was performed according to the method described by Lødemel and Olsen (2003). Samples were di-

luted in loading buffer containing 62.5 mM Tris–HCl pH 6.8, 4% SDS, 25% glycerol and some bromophenol blue. Non-heated, non-reduced samples were loaded into wells of 8 × 10 cm, 0.5 mm thick home-made slab gels which consisted of a 9% acrylamide and 0.24% bisacrylamide separating gel and a 5% acrylamide and 0.13% bisacrylamide stacking gel. The separating gel contained 0.1% gelatin.

Electrophoresis took place for 30 min at 100 V followed by 75 min at 150 V in a MiniProtean III electrophoresis cell (Bio-Rad) placed in an ice bath. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 15 min prior to incubation overnight in 50 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$ and 0.02% NaN_3 at 38 °C. The gels were subsequently stained with 0.1% Coomassie Brilliant Blue R250 in 45% ethanol and 10% acetic acid. To maximise the contrast between clear bands and the background, gels were destained in 40% ethanol and 10% glycerol. The gelatinolytic activities were identified as clear zones against a blue background. The molecular weight markers used were the Low Molecular Weight Calibration Kit for SDS Electrophoresis (GE Healthcare), and they were treated as the samples: dissolved in the same sample buffer described above with no reductants or heat denaturation. These markers were used as internal standards to monitor run-to-run differences between the zymograms. The two strongest bands, m1 (in the upper part of the gel) and m2 (lower part), are labelled in the figures for gel-to-gel comparison purposes. Gels were dried between two sheets of cellophane, scanned and examined by visual inspection.

2.5. Inhibition

The inhibitors used in this work, all of them purchased from Sigma–Aldrich, were: soybean trypsin inhibitor (SBTI), leupeptin, *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), N_α-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), pepstatin A and 1,10-phenanthroline. The inhibitors were added to the Triton X-100 washing solution and to the incubation buffer used in gelatin zymography. The concentration of each inhibitor added to the incubation buffer is shown in Table 1, and half of these amounts were added to the corresponding Triton X washing solutions.

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

The extracts were analysed by SDS–PAGE according to Laemmli (1970) in 8 × 10 cm, 0.5 mm thick home-made slab gels, where the separating gel contained 15% acrylamide and 0.087% piperazine diacrylamide and the stacking gel 5% acrylamide and 0.13% piperazine diacrylamide (Anderson, Baum, & Gesteland, 1973; Hochstrasser, Harrington, Hochstrasser, Miller, & Merril, 1988; Hochstrasser, Patchornik, & Merril, 1988). The extracts were diluted in Laemmli sample buffer (4.8% SDS, 1 mM ethylenediaminetetraacetic acid (EDTA), 125 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol (DTT), 20% glycerol and some bromophenol blue). The samples were boiled for 5 min. Electrophoresis was carried out at 75 V for 10 min and 150 V until the front reach about 0.5 cm from the bottom of the gel. The gels were silver stained (Ansorge, 1983) and dried between two sheets of cellophane. The Low Molecular Weight Calibration Kit for SDS Electrophoresis (GE Healthcare) was used as marker. It contains phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Bovine trypsin was used as a control in the SDS–PAGE and the immunoblot, and it was purchased from Sigma–Aldrich (T7309).

2.7. Preparative lithium dodecyl sulphate polyacrylamide gel electrophoresis (LDS–PAGE) and in-gel digestion

To prepare samples for peptide analysis by MS, the extracts were subjected to LDS–PAGE. The samples were diluted in loading buffer (NuPAGE 4X LDS Sample Buffer, Invitrogen) and run in 1.5 mm 10% acrylamide NuPAGE gels (Invitrogen) for 55 min at 200 V using MOPS running buffer. The molecular mass marker used was the SeeBlue Plus2 Pre-Stained Standard (Invitrogen). The gels were silver stained with the ProteoSilver Plus Silver stain kit (Sigma–Aldrich) according to the manufacturer's protocol. Bands of interest were excised and further processed with trypsin digestion as described by Shevchenko, Wilm, Vorm, and Mann (1996). Briefly, gel bands were washed twice in 50 mM ammonium bicarbonate (NH_4HCO_3), 50% acetonitrile (CH_3CN), dehydrated in CH_3CN and completely dried under vacuum. The proteins in the dried gel bands were reduced by 10 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 for 45 min at 56 °C, followed by alkylation in 55 mM iodoacetamide, 50 mM NH_4HCO_3 for 45 min at room temperature. Washing and dehydration were repeated as above and 12.5 ng/μl trypsin (trypsin modified sequencing grade, Promega) in 50 mM NH_4HCO_3 was added. Trypsin digestion was performed overnight at 37 °C. Peptides were extracted twice with 50% CH_3CN, 5% formic acid (HCOOH) and once with CH_3CN. Peptides were dried under vacuum and further desalted before MS analysis.

2.8. Immunodetection of trypsin-like enzymes in extracts of herring and sardine

SDS–PAGE was performed according to Laemmli (1970) as described in Section 2.6 at a constant voltage of 175 V for approximately 1 h. Proteins were transferred to a nitrocellulose membrane (Trans-Blot® Transfer Medium, 0.45 μm) for 50 min at constant 150 mA using a Mini-Trans Blot Cell (Bio-Rad) and transfer buffer containing 380 mM glycine, 50 mM Tris and 20% methanol (Towbin, Staehelin, & Gordon, 1979). Membranes were blocked

Table 1
Effect of the inhibitors on the protease activities as detected by gelatin zymography.

Inhibitor	Inhibits	Concentration	Effect
1,10-Phenantroline	Metalloproteases, chelates divalent metal ions	1 mM	Complete inhibition of upper activity bands shown in Fig. 1b. No effect on middle bands
E-64	Cysteine proteases (calpain, several cathepsins)	10 μM	No visible inhibition
Pepstatin A	Acid proteases (pepsin, renin, cathepsin D)	1.5 μM	No visible inhibition
TPCK	Chymotrypsin (+bromelain, papain, ficin), not trypsin	5 μM	No visible inhibition
Leupeptin	Serine & cysteine proteases (calpain, cathepsins B, H, L & trypsin)	2.5 μM	Strong inhibition of middle bands shown in Fig. 1a and b. No effect on the upper bands shown in Fig. 1b
SBTI	Trypsin and to a lesser extent chymotrypsin. Proteases with trypsin-like mechanism	10 μg/ml	Results shown in Fig. 1c. Complete inhibition of middle bands shown in Fig. 1a and b. No effect on the upper bands shown in Fig. 1b
TLCK	Trypsin (+bromelain, papain, ficin), not chymotrypsin	100 μM	Strong inhibition of middle bands shown in Fig. 1a and b. No effect on the upper bands shown in Fig. 1b

for 1 h with in 5% w/v dry milk (Blotting Grade Blocker Non-fat dry milk, Bio-Rad) in phosphate buffered saline (PBS) containing 0.1% w/v Tween 20. Membranes were washed twice in PBS/Tween prior to incubation with the primary rabbit anti-bovine pancreas trypsin antibody (Abcam, cat. no. ab1879, 1:1000 in blocking buffer) for 1 h. After four washes in PBS/Tween, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (Sigma–Aldrich cat. no. A0545, 1:16,000 in blocking buffer) for 1 h, and washed again four times in PBS/Tween. Membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to X-ray film (CL-XPosure™ Film, Pierce).

2.9. Mass spectrometry analysis

Subsequent to in-gel digestion, dried peptides were resuspended in 10 μl 0.5% acetic acid, 0.02% heptafluorobutyric acid and desalted using home-made Stage-Tips made from C18 material (Rappsilber, Ishihama, & Mann, 2003). Briefly, resuspended peptides were loaded on the column and washed with 0.5% acetic acid, 0.02% heptafluorobutyric acid. Peptides were eluted with 70% CH_3CN, 0.1% trifluoroacetic acid (TFA) and mixed 1:1 with matrix suspension (7 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) in ethanol:acetonitrile (1:1)) before depositing on the MALDI target. Peptide mass fingerprints and MS/MS analysis were acquired using a ULTRAFLEX III MALDI-TOF/TOF (Bruker Daltonics) in reflectron mode and LIFT mode.

The peptide mass fingerprints were analysed using NCBI database search with MASCOT software (Matrix Science). The amino acid sequences in the LIFT spectra were deduced both manually and by using the manufacturer's software. The most probable sequences were chosen and compared to known sequences using the NCBI Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment of amino acid sequences from the samples to known sequences was performed using the Clustal X, which is the downloadable version of the Clustal W computer program (Thompson, Higgins, & Gibson, 1994).

3. Results

Fig. 1a shows the gelatinolytic activities in the different crude extracts used in this study. The main activities in all the samples of herring, except the dorsal muscle which contains almost no activity, were located in the middle of the zymograms (marked with a bracket in Fig. 1a). The main activity of sardine *pyloric caeca* extract (marked with an arrow) was located slightly above the main activity in the herring extracts. Notably, the extracts had to be diluted differently (from 1.5 to 6000 times) in order to achieve comparable bands of enzymatic activities. It is interesting to note that the activities of the ventral muscle, stomach content, intestinal content and *pyloric caeca* of herring have quite similar electrophoretic mobilites.

Fig. 1b shows the gelatin- and benzamidine-binding fractions of the samples shown in Fig. 1a (benzamidine-binding fractions only from ventral muscle and intestinal content of herring). In addition, the effect of the most potent inhibitor (SBTI) on these samples is shown in Fig. 1c. SBTI inhibited almost completely the "middle bands" in the zymogram (denoted MB in the figure), indicating trypsin-like activity. The "upper bands" (denoted UB in the figure) remained active in the herring samples. SBTI also inhibited almost completely the main activities (marked with arrows in the figure) of the sardine extracts.

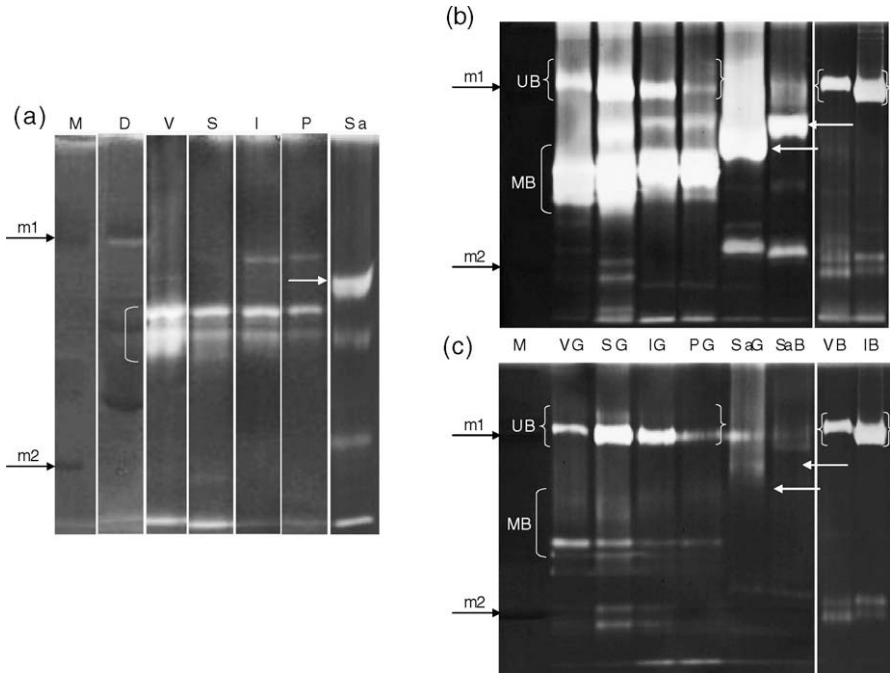

Fig. 1. Gelatin zymograms of herring and sardine extracts: (a) crude extracts; (b) gelatin- and benzamidine-binding fractions and (c) the same samples as (b) but treated with SBTI. The abbreviations used for the samples were as follows: for herring samples: D, dorsal muscle; V, ventral muscle; S, stomach content; I, intestinal content and P, *pyloric caeca,* and for sardine: Sa, *pyloric caeca.* In panels (b) and (c) the name of the sample is followed by a G or B denoting the gelatin- and benzamidine-binding fractions, respectively. The dilutions used were: panel (a) D, 1:1.5; V, 1:500; S, 1:500; I, 1:6000; P, 1:5000, and Sa, 1:3000 and in panels (b) and (c) VG, 1:2; VB, 1:4; SG, 1:3; IG, 1:21; IB, 1:31; PG, 1:21 and SaG and SaB 1:21. The main activity bands in the herring samples are marked with brackets, and the main activities in the sardine samples are marked with white arrows. The bands in the area marked with brackets in the upper part of the zymograms (denoted UB in the figure) are referred to as "upper bands" in the text. The bands enclosed by a bracket in the middle of the zymograms (denoted MB in the figure) are referred to as "middle bands". M, molecular mass markers. "m1" and "m2" denote the two most visible bands of the marker.

In addition to the effect of SBTI, other inhibitors were also tested using zymography analysis (results not shown) and a summary of the results can be found in Table 1. SBTI, leupeptin and TLCK inhibited the main activities in the crude extracts shown in Fig. 1a and the middle bands (MB, Fig. 1b) of gelatin binding proteases of the samples from herring and also the main activities (shown with arrows in Fig. 1b) of the gelatin binding proteases from sardine. SBTI gave a more complete inhibition of these bands than leupeptin and TLCK. The "upper bands" (denoted as UB) in Fig. 1b were completely inhibited by 1,10-phenanthroline. The lack of effect of the cysteine and aspartyl protease inhibitors, E-64 and pepstatin A, respectively, excluded the presence of cysteine- and aspartyl proteases with gelatinolytic activity. TPCK was did not inhibit any of the bands, and therefore excluded active chymotrypsin. Bovine trypsin was used as a control in many of the experiments, and it was shown to be completely inhibited by SBTI, TLCK and leupeptin (results not shown).

The crude extracts were also examined by SDS–PAGE and anti-trypsin immunoblot (Fig. 2). In the upper panel of Fig. 2, the SDS–PAGE gel is shown, whilst the immunoblot is shown in the lower panel. Bovine trypsin was used as a control. It is known that trypsin can autoproteolyse and that preparations of trypsin can include the enzyme in several different forms where the polypeptide backbone has been clipped, which still retain variable amounts of catalytic activity (Barrett, Rawlings, & Woessner, 2004). This might explain the presence of several bands in the bovine trypsin control sample. In the immunoblot in Fig. 2, the areas with the largest cross-reactivity with anti-trypsin antibody are denoted as U (upper area) and L (lower area). These areas probably consisted of several bands. The bovine trypsin control exhibited strong cross-reactivity in the U and L areas, and also in an area just below U. Almost all the samples showed strong cross-reactivity in the U area, except the samples of ventral muscle of herring with emptied stomachs (Ve) and stomach content of herring with full stomachs (Sf), which showed slight cross-reactivity, and the stomach content of herring with emptied stomachs (Se) which showed no cross-reactivity. The intestinal content of herring with full stomachs (If) also gave strong cross-reactivity in the L area, but this reactivity was weaker in the sample of intestinal content of herring with emptied stomachs (Ie). The same pattern was seen in the samples of *pyloric caeca* of herring with full and emptied stomachs and also in the individual samples of sardine *pyloric caeca*, although the sardine samples were taken from to individual fish treated in the same way. The Sa1 sample also contained the band below the U area seen in bovine trypsin. Several cross-reactive bands of weaker intensity were observed in the higher molecular mass region in the samples of ventral muscle from herring with emptied stomachs (Ve), stomach content from herring with full stomachs (Sf) and slightly in the stomach content of herring with emptied stomachs (Se). The anti-bovine trypsin antibody cross-reacted with the 45 kDa marker protein, which is ovalbumin from chicken egg white. The reason for this is unknown, but it might be attributed to the presence of a contaminant copurifying with ovalbumin and cross-reacting with the polyclonal anti-trypsin antibody. The presence of a weaker band immediately below the 45 kDa marker protein which is present in all preparations of this particular marker supports our hypothesis, and this band has been labelled with a white arrowhead in Fig. 2.

Fig. 3 shows the LDS–PAGE analysis of the gelatin- and benzamidine-Sepharose affinity purified extracts. Visible bands after silver staining were subjected to in-gel trypsin digestion and MALDI-MS and MS/MS analysis. Bands 1 to 6.1, 8.1, and 8.2 had apparent molecular mass of about 20–25 kDa. Band 7 was located slightly above bands 1–6.1 in the gel, closer to 28 kDa. Multiple bands were

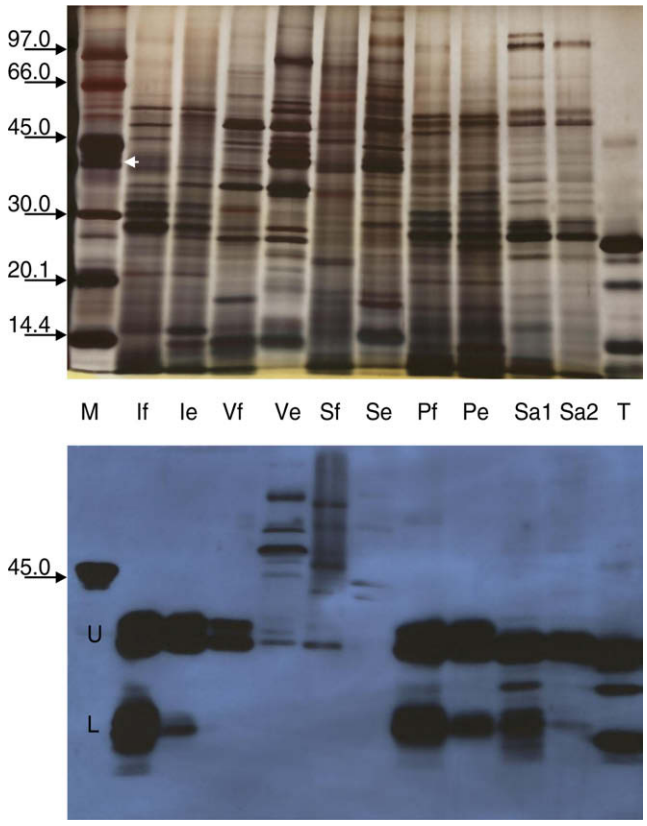

Fig. 2. SDS–PAGE (upper panel) and immunoblot (lower panel) of several extracts of herring and sardine. The abbreviations used for the samples were as follows: for herring samples: V, ventral muscle; S, stomach content; I, intestinal content and P, *pyloric caeca,* and for sardine: Sa, *pyloric caeca*. Sa 1 and 2, are extracts of *pyloric caeca* of two individual sardines. T, bovine trypsin. The "f" and "e" following the sample abbreviations indicate samples taken from herring with full or emptied stomachs, respectively. For SDS–PAGE analysis approximately 1 μg of protein of each sample was loaded. For the western blot analysis, 6–8 ug of protein of full herring, 7.5–11 μg protein of samples of emptied herring, 10 μg protein of *pyloric caeca* of sardine and 0.2 μg protein of bovine trypsin. The main areas of cross-reactivity are marked with the letters U (upper area) and L (lower area). The arrowhead indicates a possible contaminant of the marker mixture.

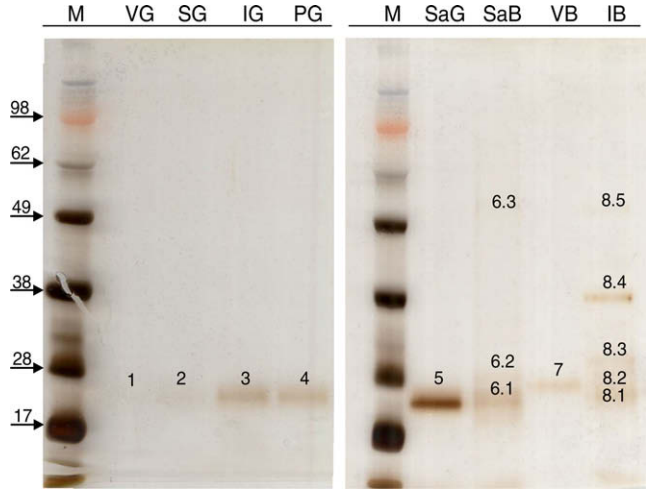

Fig. 3. SDS–PAGE of the gelatin- (G) and benzamidine- (B) binding fractions of the extracts of herring and sardine. The samples, applied from left to right were: M, molecular mass marker (values in kDa), herring samples: V, ventral muscle; S, stomach content; I, intestinal content; P, *pyloric caeca*. Sardine samples: Sa, sardine *pyloric caeca*. The numbers of the bands are placed slightly above or on top of the bands excised.

observed in the benzamidine-binding fractions of the sardine *pyloric caeca* and of the intestinal content of herring, including a common band around 50–55 kDa (bands 6.3 and 8.5) that was not observed in the other samples.

Six of the protein bands (3, 4, 5, 6.1, 6.2, and 7) isolated from the gel yielded unambiguous sequences when analysed in MS/MS mode. The MS/MS spectra of peptides from these samples are shown in Supplementary Fig. 1. All sequences were compared to similar sequences from *Homo sapiens*, *Bos taurus*, *Rattus norvegicus* and *Salmo salar* if available, and in addition, the first sequence matches from the BLAST homology searches were included. Bands 3 and 4 gave quite similar peptide sequences, and the BLAST search and subsequent sequence alignment revealed sequence homology to an internal peptide in elastase-1-precursor from several species (Fig. 4a). Bands 5 and 6.1 matched with trypsin (Fig. 4b), and band 6.2 matched with aspartyl aminopeptidase (Fig. 4c). No significant sequence homology was identified for the two sequences found in peptides from band 7. However, the sequences are quite unambiguous and are thus included for future reference. An overview over the sequences found, their first matches in homology searches and the BLAST scores and *E*-values are listed in Table 2.

4. Discussion

The major gelatinolytic activities found in all samples examined except the dorsal muscle of herring and the *pyloric caeca* extract of sardine have similar electrophoretic mobilities in the zymograms (Fig. 1a). The dorsal muscle has very low gelatinolytic activity, mainly represented by a band in the slow migrating region, as corroborated by our previous studies (Felberg & Martinez, 2006; Felberg et al., 2008). The similarities between the activities of the intestinal content, *pyloric caeca* and ventral muscle of herring seem to indicate a possible leakage of gelatinolytic enzymes from the digestive organs to the ventral cavity. Interestingly, the extract of the stomach content also displays the same activities.

Ingested prey is known to have a considerable buffering effect on the gastric pH, including alkalisation by ingestion of prey with calcareous shells (Kapoor et al., 1975). It is thus likely that the large amount of prey in the stomachs of heavily feeding herring might buffer and partially neutralise the gastric pH. This may contribute to preserve the activities of exogenous alkaline proteases until they enter the *pyloric caeca* and the intestine. This is also in agreement with previous work, indicating that the collection of active proteases in the digestive systems of fish feeding on natural food or live prey actually is a mixture of endogenous proteases and exogenous ones derived from the food (Dabrowski & Glogowski, 1977; Fänge & Grove, 1979; Kuz'mina & Golovanova, 2004). It has furthermore been shown that the major proteases active at alkaline pH in *Calanus finmarchicus*, a major prey for herring (Dalpadado, Ellertsen, Melle, & Dommasnes, 2000; Prokopchuk & Sentyabov, 2006; Segers, Dickey-Collas, & Rijnsdorp, 2007), are serine proteases and metalloproteases (Solgaard, Standal, & Draget, 2007).

Gelatin zymography under alkaline conditions is well suited to investigate proteases involved in *post-mortem* autolysis in herring tissue for several reasons: (1) zymography is fast and reproducible, and is also compatible with the use of inhibitors, (2) gelatin is a product of collagen degradation, and proteases able to digest gelatin might contribute to the digestion of collagen, (3) we have previously demonstrated that leakage of acid proteases from the stomach is an unlikely reason for *post-mortem* autolysis in herring since the stomach seems to be intact during degradation of the ventral wall (Veliyulin et al., 2007). However, as shown in Fig. 1a and b, the stomach content of herring contains alkaline proteases which are active even after storage in the herring stomach and freezing and thawing of the fish, and which are probably derived from the prey. Since these proteases apparently survive the conditions of the stomach, it is very likely that they remain active during and after transport to the *pyloric caeca* and the intestine and contribute to proteolytic activity of the enzyme-rich fluid which leaks to the ventral muscle. We thus hypothesise that the amount and the characteristics of the proteases of the given prey species present are important determinants of the *post-mortem* autolysis.

Due to the several 1000-fold dilutions of some of the extracts required to avoid overloading of the zymograms and to the presence of gelatin in these gels, it was impossible to submit the gelatinolytic activity bands from these gels directly to sequencing. Therefore, the activities were partially purified by their affinities to gelatin- and benzamidine-Sepharose. Benzamidine-Sepharose was chosen because inhibitor studies and cross-reactivity with the anti-trypsin antibody indicated the presence of serine proteases, possibly trypsin. Gelatin Sepharose was chosen to isolate the metalloproteases that the inhibitor studies indicated might be present in the extracts. It is interesting to note that the gelatin Sepharose affinity chromatography which is often used to isolate metalloproteases, isolated both metalloproteases and serine proteases, whilst the benzamidine affinity chromatography, used to isolate trypsin-like enzymes, isolated mostly a metalloprotease (Fig. 1b).

Marine trypsins are known to be inhibited by SBTI and TLCK, whilst marine pancreatic elastases are strongly inhibited by SBTI, partially by TPCK and not inhibited by TLCK (Simpson, 2000). Our studies showed that the main activities in the crude extracts and the "middle bands" in the gelatin binding fractions were strongly inhibited by leupeptin, SBTI and TLCK, but not by TPCK, essentially excluding chymotrypsin (Table 1, effect of SBTI shown in Fig. 1c). When interpreting the results of the inhibitor studies of marine proteases, one must take into account that homologous proteases may exhibit inter-species variations in response to specific inhibitors which, in addition, can be season-dependent within certain species (Simpson, 2000). Nevertheless, the results shown in Fig. 1c and Table 1 indicate that serine proteases, most probably trypsin-like, and metalloproteases ("upper bands") were present in the samples.

The immunoblot shown in Fig. 2 indicated the presence of several possible trypsin-like proteases. The antibody used was raised against bovine pancreatic trypsin since no antibody raised against fish trypsin was available to us. However, since trypsin, chymotrypsin and elastase are sequence-homologous proteases that have risen from a common ancestor with tryptic specificity (De Haën, Neurath, & Teller, 1975) cross-reactivities may occur. Polyclonal antibodies raised against trypsin from one species might very well also cross-react with trypsin-like enzymes from another species.

Peptide mass fingerprints of the samples were insufficient to identify the bands using NCBI database search with MASCOT software (Matrix Science) due to lack of sequence information for the species examined in this work. However, MS/MS analysis of the peptides yielded confident sequence information for several of the peptides by combining automated and manual inspection of the spectra. Moreover, we found that the peptide sequences in our experiments corresponded to partially conserved sequences of digestive proteases from different organisms. These results, together with the gelatinolytic activities, response to inhibitors and anti-trypsin immune reactivity of the proteases further, strengthen the identification of the peptides by *de novo* sequencing.

The peptide sequence from intestine and *pyloric caeca* of herring (Fig. 3, bands 3 and 4), gave a positive match in the BLAST database search with an elastase precursor from only two fish species; *Paralichthys olivaceus* and *Epinephelus coioides*. The alignment of this protease with elastases from other species, however, showed some similarities that strengthened this identification (Fig. 4a). The sequences of the samples from sardine *pyloric caeca* (Fig. 3, bands 5

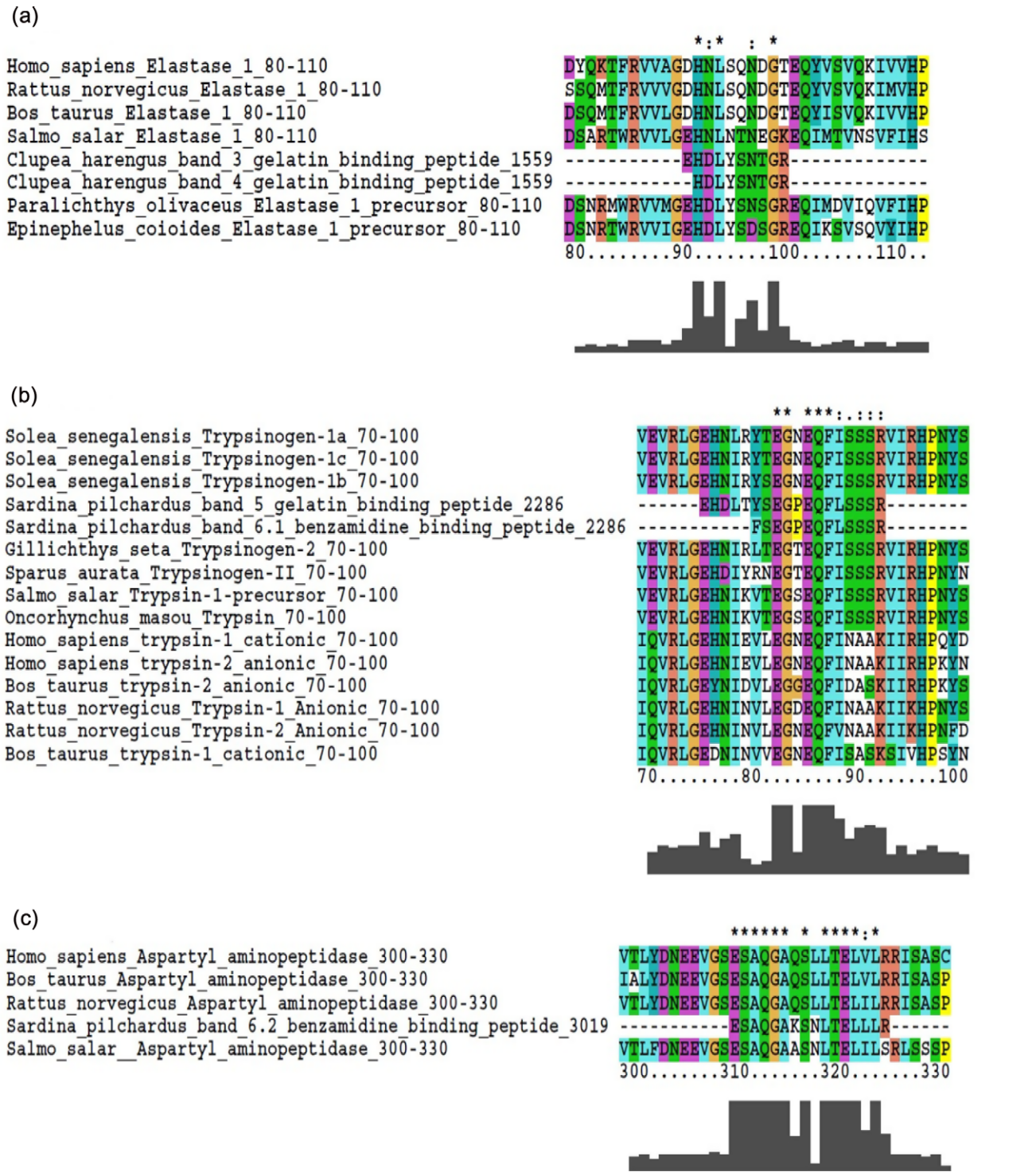

Fig. 4. Alignments of the sequences derived from the spectra in Supplementary Fig. 1. (a) Alignment of the sequences found in peptides of the gelatin binding fractions of intestinal content (band 3 in Fig. 3) and *pyloric caeca* (band 4 in Fig. 3) of herring. (b) Peptide sequence alignment of the gelatin- (band 5 in Fig. 3) and benzamidine-binding (band 6.1 in Fig. 3) fractions of sardine *pyloric caeca*. (c) Peptide sequence alignment of the benzamdine binding fraction (band 6.2 in Fig. 3) of sardine *pyloric caeca*. Individual residues are coloured according to Clustal X colour coding.

and 6.1), gave matches with trypsin sequences from many fish species (Fig. 4b) and band 6.2, also derived from sardine *pyloric caeca*, displayed sequence similarity with aspartyl aminopeptidases from several species, both from fish and mammals (Fig. 4c). By using this kind of technique to identify proteases from non-sequenced species, only sequences which are conserved between species will give matches. A possible reason why the benzamidine-binding fraction of ventral muscle did not give a match is that the sequences obtained correspond to non-conserved domains of the protease. Alternatively, the putative protease we have found has not yet been identified. Although all the samples were purified enough for successful MS/MS analysis, the low protein content in some of the bands, e.g. bands 1 and 2 in Fig. 3, made further analysis impossible.

Table 2
Sequences found in some of the samples compared with the first match found in NCBI BLAST.

Band	Sequence[a]	Peptide mass	Match species[b]	Match protease[b]	Match sequence[b]	BLAST score	Blast *E*-value
IG (band 3)	EHDLYSNTGR	1559	*Paralichthys olivaceus*	Elastase-1 precursor	EHDLYSNSGR	32.9	3.4
PG (band 4)	HDLYSNTGR	1559	*P. olivaceus*	Elastase-1 precursor	HDLYSNSGR	29.5	32
SaG (band 5)	EHDLTYSEGPEQFLSSSR	2286	*Solea senegalensis*	Trypsinogen-1a	EHNLRYTEGNEQFISSR	40.5	0.0017
SaB (band 6.1)	FSEGPEQFLSSR	2286	*S. senegalensis*	Trypsinogen-1b	YTEGNEQFISSR	31.2	11
SaB (band 6.2)	ESAQGAKSNLTELLLR	3019	*Salmo salar*	Aspartyl aminopeptidase	ESAQGAASNLTELILSR	38	0.098
VB (band 7)	ENTVGYSGSNSALNWR	1754	–	–	–	–	–
	WRSSGDTLFVR	2457	–	–	–	–	–

[a] The MS/MS technique is unable to differentiate between leucine (L) and isoleucine (I) and for simplicity the L/I amino acid residue is always denoted as L in this paper. BLAST searches with I instead of L in some sequences gave a higher score. In Fig. 4, the sequences of several species for each sequence found are included for comparison.
[b] The species, protease and sequence match mentioned in this table refer to the first significant hit in the BLAST database. Hypothetical or predicted proteins were omitted from the sequence homology search.

Unexpectedly, only elastase was identified by MS/MS in the samples of gelatin binding fractions of intestine and *pyloric caeca* from herring. A collagenolytic serine protease has been found in cod intestine which displayed a 55% N-terminal amino acid sequence homology to cod elastase (Kristjánsson, Gudmundsdóttir, Fox, & Bjarnason, 1995). In addition, according to Martinez and Serra (1989), elastase activity is quantitatively very important in intestine and *pyloric caeca* of another pelagic fish, the anchovy (*Engraulis encrasisholus*). However, one would also expect to find sequences corresponding to trypsin in these samples from herring, as were found in sardine *pyloric caeca*. The reason for not identifying trypsin remains elusive, but one possibility might be differences in how strongly the different proteases are bound to their substrates in the digestive tract. The herring were feeding heavily and had a great amount of prey in their digestive systems whilst the sardines were only moderately feeding, implying that the digestive enzymes in herring could be saturated with substrate, and thus less extractable (Gildberg & Raa, 1980). In addition, enzymes from the prey might constitute a substantial amount of the total proteases in the digestive system of the fish, which would proportionally reduce the contribution of the fish's enzymes to the total activities in the extracts.

Thus, the differences found between sardine and herring may be attributed to several factors: (1) to species-specific differences, (2) to quantitative and qualitative differences in their ingested prey and their corresponding proteases, (3) to slight differences in the composition of the extraction buffers. Whilst the fat in the sardine samples was eliminated by CCl_4, it was only solubilised by Brij in the samples of herring, and there was also a slight difference in the pH values of the buffers used (7.5 vs. 8). Therefore, the different extraction methods may have had a slight effect on the relative amount of proteins and enzymes extracted from the two species.

In conclusion, the gelatinolytic activities we have found in ventral muscle of herring seem to be trypsin-like serine proteases and a metalloprotease. The main activities (located in the middle of the zymograms) were inhibited by serine protease and trypsin inhibitors and reacted with commercial anti-trypsin antibody. Comparison of the gelatinolytic activities in the ventral muscle to the ones of the extracts of stomach content and the enzyme-rich extracts of intestinal content and *pyloric caeca* showed that they are quite similar, which supports the theory that leakage of proteases to the ventral muscle is a probable cause of ventral tissue degradation. Due to lack of reported sequences to databases, it was not possible at this time to identify sequences of the partially purified proteases found in ventral muscle. However, sequences from a gelatin binding protease from intestine and *pyloric caeca* of herring indicated the presence of an elastase, and sequences from proteases of the *pyloric caeca* of sardine indicated presence of trypsin and an aspartyl aminopeptidase. Since it is likely that the *post-mortem* autolysis in herring is caused by leakage of enzyme-rich fluids from the intestine and/or *pyloric caeca*, it is most probable that the cause would be due to the added activity of the herring's endogenous enzymes together with exogenous proteases from the digestive systems of the prey.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.07.012.

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