Food Chemistry 116 (2009) 40–46

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Proteolytic activities of ventral muscle and intestinal content of North Sea herring (*Clupea harengus*) with full and emptied stomachs

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ARTICLE INFO

Article history:
Received 9 October 2008
Received in revised form 18 December 2008
Accepted 2 February 2009

Keywords:
Herring (*Clupea harengus*)
Belly bursting
Gelatin zymography
Electrophoresis
Protease
Emptied stomach

ABSTRACT

The aim of the work was to examine the effect of allowing herring (*Clupea harengus*) to empty the stomachs prior to being taken on board the fishing vessels, on the proteolytic activities in extracts of ventral muscle and of intestinal content. The proteolytic activities were examined by gelatin zymography and by incubating the extracts with isolated myosin heavy chain (MHC) protein. Extracts from herring with full stomachs showed strong gelatinolytic and MHC degrading activities particularly in extracts from intestinal contents. Extracts from fish that had been allowed to empty their digestive system for 19 h had reduced activities, which was most noticeable in the ventral muscle extracts. The activities from the ventral muscle did not originate endogenously *post-mortem*, as shown by the fact that ice storage for 24 h of isolated ventral muscle did not display the activities, while ventral muscle extract from ice-stored whole fish for 24 h did.

1. Introduction

Herring (*Clupea harengus*) is a significant pelagic species in North-Atlantic fisheries, and one important challenge to fishermen is to ensure the good quality of their catch. However, during heavy feeding, rapid degradation of the ventral wall may take place in this and other pelagic species. This quality deterioration is known as belly bursting, it lowers the price of the catch, and in extreme cases may render the fish unsuitable for human consumption. Several authors have investigated the belly bursting phenomenon in different species of pelagic fish over the years. As early as in 1926; Almy (1926) concluded that trypsin leaking from the *pyloric caeca* was causing belly bursting in herring (*C. harengus*). Baalsrud (1951) hypothesized that belly bursting in herring could be attributable to four causes: (1) bacterial activity, (2) autolysis by enzymes in the muscle of the fish, (3) diffusion of digestive enzymes, and (4) enzymes from the prey. Herring feed on several species of zooplankton, including *Calanus* spp., amphipods and euphausiids (Dalpadado, Ellertsen, Melle, & Dommasnes, 2000; Last, 1989; Prokopchuk & Sentyabov, 2006; Segers, Dickey-Collas, & Rijnsdorp, 2007). Baalsrud (1951) suggested that the species of prey present in the fish are the indirect cause of belly bursting due to increased secretion of digestive enzymes when the prey contains elements that are hard to digest (e.g., shells), and therefore concluded that the digestibility of the prey and not the digestive enzymes *per se*, was the cause of the belly bursting process. He further claimed that the secreted proteases will degrade the intestinal wall of the fish *post-mortem* leading to a build-up of an enzyme-rich fluid in the peritoneal cavity which will degrade the ventral wall. Gildberg (1982) found that belly bursting in capelin (*Mallotus villosus*) could probably be due to leakage of both gastric acid, with subsequent weakening of the ventral tissue, and pepsins. In an earlier work, Gildberg (1978) had already discussed additive effects of heavy feeding in summer capelin such as a high glycogen level leading to low *post-mortem* muscle pH weakening the connective tissue and a simultaneous high production of digestive enzymes. Lavéty and Love (1972) have shown that fish that have been feeding heavily seem to have a weaker muscle connective tissue than starved fish. Gilberg (1978) also showed that proteases can leak from seemingly intact digestive organs, and that proteases active at alkaline pH leaked faster than those active in acid pH, which could be due to differences in molecular structure of the proteases or the fact that the wall of the stomach might be less susceptible to leaking than the alimentary canal. Martinez and Gildberg (1988) found that leakage of alkaline/neutral proteases from the *pyloric caeca* and intestine were the probable cause of belly bursting in anchovy (*Engraulis encrasicholus*).

Although there is a general agreement that one of the main causes of the degradation are the digestive enzymes from the fish

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doi:10.1016/j.foodchem.2009.02.001

itself, leaking to surrounding tissues, Botta, Kennedy, Kiceniuk, and Legrow (1992) claimed that belly bursting in capelin is more related to physical handling practices (frozen storage time, thawing time and length of dockside storage) than factors like fish size, amount of red feed (zooplankton) in the digestive system or roe content. The fact that belly bursting in fish may not depend on the amount of prey ingested was discussed by Marvik (1974) who had observed that batches of fish seemingly deprived of prey were affected by belly bursting. However, this author does not mention the method used to estimate the amount of ingested prey.

A practice sometimes used by fishermen to avoid belly bursting in some pelagic species during the feeding season is to keep the fish alive in the purse seine for a certain period of time after capture before it is taken on board. If this is done optimally, the fish will still be schooling and the water remains oxygenated, but the crowding of the fish in the seine will prevent further supply of prey and therefore leads to evacuation of the prey already present in the digestive system of the fish (Broms, 2007). Broms (2007) has recently shown a 50% evacuation of the stomach contents in Norwegian spring spawning herring in about 11–20 h and total evacuation in 27–57 h. The gastric evacuation rate in pelagic species depends on physical and biological factors such as variations in temperature, the level of stress of the fish and the type of prey ingested (Broms, 2007; Marvik, 1974; Temming, Bøhle, Skagen, & Knudsen, 2002).

The present work is part of our studies characterising the causative enzyme(s) of belly bursting in herring. 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 most probably in the upper part of the intestine (Felberg & Martinez, 2006; Veliyulin, Felberg, Digre, & Martinez, 2007). In this work we describe, for the first time, the effect of allowing herring to empty their stomachs on the gelatinolytic and myofibrillolytic activities of extracts from intestinal content and ventral muscle.

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 (two locations) and July 2007 (one location). The herring were frozen immediately after catch and stored at −20 °C until the analyses were performed unless otherwise stated.

The fish were captured at two different locations in 2006; N57°58′ E02°21′ and N59°47′ E02°04′. The former had their stomachs full, and the latter were allowed to empty their stomach contents for 19 h. Average lengths and weights for the full and emptied fish were 21.7 ± 1.5 cm and 128.6 ± 19.4 g (full fish, $n = 15$) and 23.2 ± 1.5 cm and 144.8 ± 24.8 g (emptied fish, $n = 15$), respectively.

In order to examine the development of *post-mortem* protease activities in the isolated ventral muscle of herring, fish captured in 2007 (N59°56′ E02°44′), with partially full stomachs, were treated as follows: the ventral muscle of 20 fish was dissected immediately after capture and cut in two pieces, one piece was immediately frozen and the other was wrapped in aluminium foil, stored in ice for 24 h and then frozen. Twenty additional fish were stored round in ice for 24 h prior to dissection of the ventral muscle. All samples were frozen stored and transported at −20 °C.

2.2. Enzyme extraction

All the extracts used in this study were performed on tissues dissected in ice from partially thawed fish. Two procedures were used, based on the method described by Lødemel and Olsen (2003). The whole procedure was performed in ice and ventral muscle and intestinal content of both full and emptied fish were used. In the first procedure, 0.1–0.5 g of tissue were accurately weighted into a 2 mL Eppendorf tube and homogenized with 0.5–1 mL of 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) and the supernatants were collected, pooled (yielding a total volume of 1.5 mL) and stored at −80 °C until further use. Protein concentrations were determined by the Bradford method (Bradford, 1976).

A second simplified procedure was used to obtain the extracts shown in Figs. 3 and 4. As before, the whole procedure was performed in ice and ventral muscle and intestinal content of both full and emptied fish were sampled. Samples (0.5–0.6 g) were accurately weighted in 2 mL Eppendorf tubes in ice. Two volumes of cold extraction buffer (50 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$ and 0.25% Triton X-100, in a ratio sample:buffer 1:2 w/v) were added and the samples were homogenized with pellet mixer for approximately 20 s each. The samples were kept in ice for 15 min and then centrifuged at 6000*g* for 5 min at 4 °C. The supernatant was decanted and stored at −20 °C until further use. Protein concentrations were determined by the Bradford method (Bradford, 1976).

2.3. Gelatin zymography

Substrate gel electrophoresis was performed according to the method described by Lødemel and Olsen (2003). Samples were diluted in a 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 cm × 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 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, the 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 standards were used as internal standards to monitor run-to-run differences between the zymograms, and therefore the two strongest bands, a (in the upper part of the gel) and b (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.

2.4. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

The extracts were analysed by SDS–PAGE according to Laemmli (1970) in 8 cm × 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;

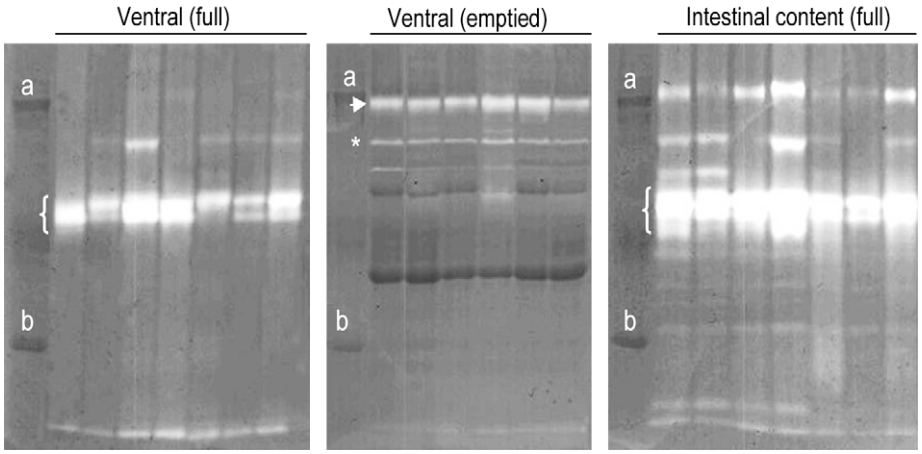

Fig. 1. Gelatin zymograms of ventral muscle extracts from full (left panel, dilution 1:200) and emptied herring (middle panel, dilution 1:2) and from intestinal content extracts (right panel, dilution 1:6000 and 1:8000). Each lane represents one individual herring. "a" and "b" denote the two most visible bands of the low molecular weight standard. Asterisks and arrowheads indicate bands referred to in the text.

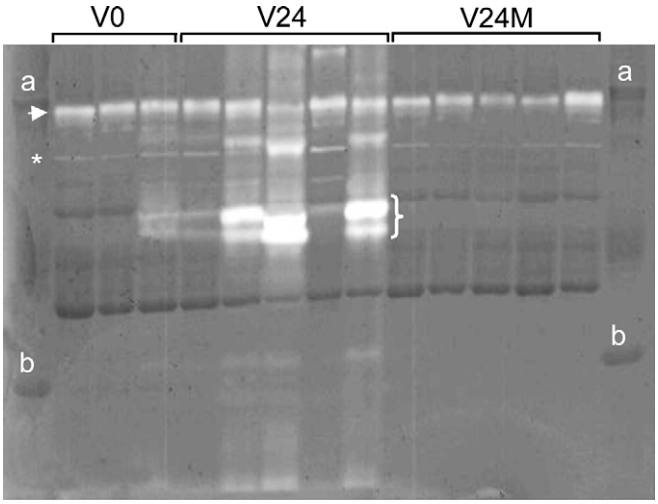

Fig. 2. Gelatin zymogram of ventral muscle samples from fish caught in 2007. Each lane represents one individual fish. V0: ventral muscle dissected immediately after catch, V24: ventral muscle dissected from fish stored in ice for 24 h after catch, V24M: ventral muscle dissected immediately after catch and stored in ice for 24 h. Ten micrograms of protein of each sample was loaded. "a" and "b" denote the two most visible bands of the low molecular weight standard. Asterisks and arrowheads indicate bands referred to in the text.

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), dried between two sheets of cellophane, scanned and examined by visual inspection. The Low Molecular Weight Calibration Kit for SDS Electrophoresis (GE Healthcare) was used as marker. It contains a mixture of markers of molecular mass of 97, 66, 45, 30, 20.1 and 14.4 kDa.

2.5. Isolation of myosin heavy chain (MHC)

Myosin heavy chain (MHC) from salmon was selected because we wished to detect its fragments after enzyme hydrolysis using a rabbit polyclonal anti-MHC preparation raised against a salmonid, Arctic charr (*Salvelinus alpinus*), MHC (Martinez & Pettersen, 1992). Myofibrillar proteins were prepared by homogenizing about 0.5 g of salmon (*Salmo salar*) muscle with a pellet mixer in 3 mL of 62.5 mM Tris–HCl, pH 6.8, 5% SDS and 100 mM DTT. The mixture was boiled for 2 min, mixed by vortexing, kept for 20 min at room temperature, mixed again and boiled. The extract was then centrifuged at 16,100*g* for 5 min to remove tissue debris, and the supernatant was collected in a new tube. The extract was diluted 1:10 in Laemmli sample buffer and applied to a preparative gel (8 cm × 10 cm × 1 mm thick slab gel), consisting of a 7.5% acrylamide and 0.2% piperazine diacrylamide separating gel and a 3% acrylamide and 0.08% piperazine diacrylamide stacking gel (Anderson et al., 1973; Hochstrasser, Harrington et al., 1988; Hochstrasser, Patchornik et al., 1988; Laemmli, 1970). The gels were run at 175 V for approximately 1 h, stained for 2 min in 2% Coommassie Brilliant Blue R250 (in 45% ethanol and 10% acetic acid) and then destained in several changes of distilled water until the myosin heavy chain (MHC) band was visible. The band was cut and frozen before being eluted from the gel-slices.

To elute the MHC, the excised pieces of gel containing the band were placed into a 1.5 mL Eppendorf tube and homogenized in 200 μL of Laemmli sample buffer without glycerol and without bromophenol blue. The tubes were submitted to three cycles of 10 min incubations at 60 °C followed by strong vortexing and resting for 30 min at room temperature. The tubes were left overnight at room temperature and centrifuged the following day at 14,000*g* for 10 min. The liquid supernatants containing the eluted MCH were collected and the protein content in the supernatants was estimated by their OD_{280} absorption (Etienne et al., 2000). The samples were diluted with Laemmli sample buffer and boiled for 5 min prior to analysis by SDS–PAGE (as described in Section 2.4) to evaluate their purity.

2.6. Evaluation of proteolytic activity using MHC as substrate

Intestinal and ventral muscle extracts from one full and one emptied herring were used. The extracts were diluted 1:4 in extraction buffer (50 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$ and 0.25% Triton X-100) and 10 μL of these dilutions were mixed with 20 μL of the MHC substrate and 70 μL of incubation buffer (50 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$). Twenty microlitres of substrate containing 1.2 mg/mL (MHC) was used for the examination of proteolytic activity and yielded 2.4 μg (MHC) of substrate per reaction

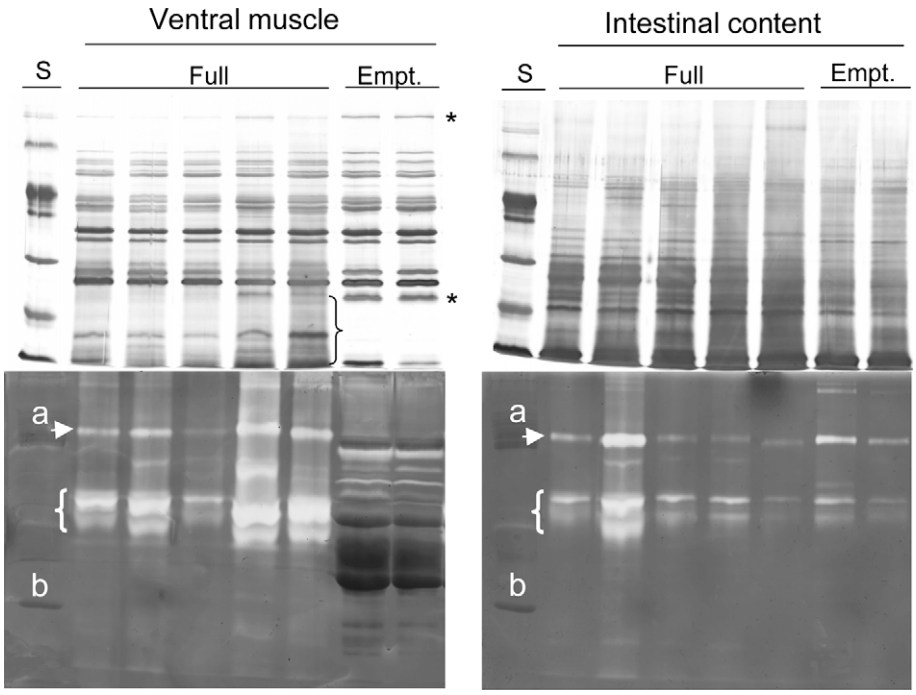

Fig. 3. SDS–PAGE (above) and gelatin zymograms (below) of ventral muscle extracts from five full and two emptied herring and from intestinal contents of full and emptied herring as indicated. Five micrograms of protein was loaded for SDS–PAGE analysis. For gelatin zymography, 5 μL of the following dilutions were loaded: ventral muscle of full herring: 1:500; ventral muscle of emptied herring: 1:1.5; intestinal content of full herring: 1:8000; intestinal content of emptied herring: 1:5000. Lane S: LMW standard. "a" and "b" denote the two most visible bands of the low molecular weight standard. Asterisks and arrowheads indicate bands referred to in the text.

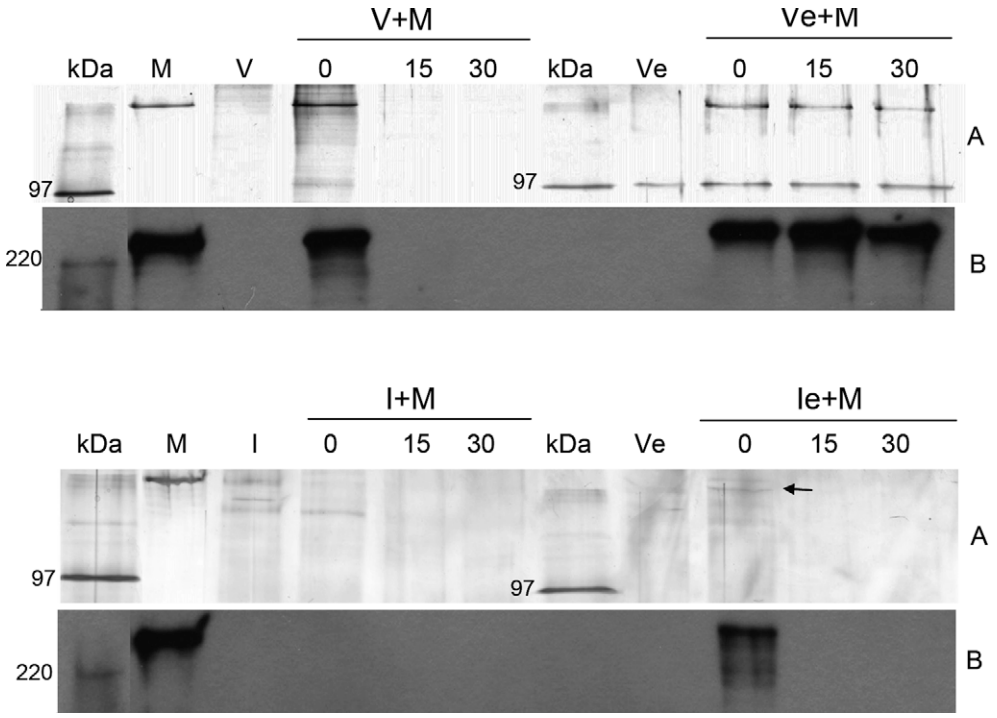

Fig. 4. SDS–PAGE analysis (A) and western blotting with anti-MHC antibodies (B) of extracts of ventral muscle and intestinal contents from full (V and I) and emptied (Ve and Ie) herring, respectively, immediately after the addition of MHC (M) as substrate and after 15 and 30 min of incubation, as indicated on top of the lanes. Each lane contained 0.6 μg MHC protein. kDa, LMW standard in the SDS–PAGE gels and MagicMark Western Blot standard in the blots. The arrow indicates presence of a weak MHC band. Controls are: lane M; sample with MHC only, lanes V and Ve; ventral muscle extract from fish with full and emptied stomach, respectively, lane I and Ie; intestinal content from fish with full and emptied stomach, respectively.

lane. Controls were prepared by mixing the substrate with 10 μL of extraction buffer instead of 10 μL of extract and by mixing 10 μL of extract with 20 μL of extraction buffer instead of substrate.

Some reactions were stopped immediately after mixing and were referred as zero time while others were incubated at 20 °C for additional 15 and 30 min. The reactions were stopped by mix-

ing 10 μL of the incubation mixtures with 30 μL Laemmli buffer and boiling immediately for 5 min. The effects of ventral and intestinal extracts of both full and emptied fish on MHC were examined by SDS–PAGE (as described in Section 2.4). By loading 10 μL per lane, each lane contained approximately 1.6 μg protein of ventral extracts and 0.69 μg protein of intestinal extracts.

2.7. Immunodetection of MHC incubated with herring extracts

The effect of the extracts on the MHC substrate was examined by SDS–PAGE and by MHC immunodetection. Fifteen microlitres of each extract-MHC incubation mixture were loaded in the sample wells of 8 cm × 10 cm, 0.5 mm thick slab gels (as described in Section 2.4). SDS–polyacrylamide gel electrophoresis was performed according to Laemmli (1970) at a constant voltage of 175 V for approximately 1 h. Gels were transferred to a pure nitrocellulose membrane (Trans-Blot® Transfer Medium, 0.45 μm) for 1 h at constant 150 mA using a Mini-Trans Blot Cell (Bio-Rad) following the instructions of the manufacturer. The transfer buffer was 380 mM glycine, 50 mM Tris, 20% methanol and 0.1% SDS (Towbin, Staehelin, & Gordon, 1979). Membranes were afterwards saturated for 1 h with blocking solution which contained 5% w/v milk (Blotting Grade Blocker Non-fat dry milk, Bio-Rad) in phosphate buffered saline (PBS). The membranes were then washed with PBS containing 0.1% w/v Tween 20, incubated with the primary antibody for 1 h, washed in PBS/Tween, incubated with the secondary antibody for 1 h, and washed again in PBS/Tween. The primary antibody was a polyclonal rabbit anti-serum anti-myosin heavy chain from Arctic charr (Martinez & Pettersen, 1992), and the secondary antibody was a peroxidase-conjugated goat anti-rabbit IgG (Sigma). Both antibodies were diluted in 5% milk in PBS. Finally, the membranes were incubated with substrate (SuperSignal West Pico Chemiluminescent Substrate kit, Pierce) and exposed to X-ray film (CL-XPosure™ Film (Pierce)).

3. Results and discussion

Most of the fish with gastric content had full, or partially filled, stomachs with dark brown, reddish or pink material and intact or partly intact peritoneum. Signs of leakage of digestive juices visible as yellow–brown fluid in the peritoneal cavity, and often found on the internal part of the ventral muscle, were observed. The intestine frequently adhered to the ventral muscle and ruptured easily. Also, the ventral muscle of these fish disintegrated rapidly during thawing. Most emptied fish displayed little or no stomach content. They had intact peritoneum and a better external appearance with more scales and shinier skin. There was little discolouration of the inside of the ventral muscle, and the intestine did not rupture easily. Although the stomachs were almost empty, the intestine usually had some content with a red–brown colour. The stomachs of all the fish were separated from the oesophagus, intestine, *pyloric caeca* and all surrounding fat and weighed. The stomachs of full fish had a weight of 3.9 ± 0.9 g (n = 15) (3.1% of the total fish weight) and the stomachs of emptied fish had a weight of 2.1 ± 0.5 g (n = 15) (1.3% of the total fish weight).

We have earlier discarded pepsin and leakage of gastric acid as probable causes of belly bursting in herring (Felberg & Martinez, 2006; Veliyulin et al., 2007). In addition, we have described enzymatic activities in the ventral muscle of herring during the heavy feeding season that degrade gelatin and whose intensity increase *post-mortem* with storage time in ice (Felberg, Batista, Nunes, & Martinez, 2008). Also in this work we chose to use gelatin zymography as our method of investigating presence of tissue-degrading proteases due to its convenience and ability to render fast and reproducible results for gelatin degrading enzymes (Snoek-van Beurden & Von den Hoff, 2005). Gelatin is partly denatured collagen, and therefore an enzyme's ability to degrade gelatin implicates a possible ability to degrade collagen, the most important structural protein in connective tissue.

The left and central panels of Fig. 1 show the differences in gelatinolytic activities of the ventral muscles of several full and emptied herring, respectively. The samples of the full fish had to be diluted 1:200 to avoid overloading the zymogram and this high dilution causes the protein content to remain under the level of detection of Coommassie Blue staining. The ventral muscle samples from full fish displayed only a few individual differences in the activities, a common feature being some stronger activities located above the middle part of the zymogram (two or more bands, labelled by brackets). These samples were all, as mentioned earlier, diluted 1:200 and it is therefore not unexpected that different fish have different amounts of activity due to for instance slight differences in amount or area of tissue extracted or individual expression of enzymes. The gelatinolytic activities in the intestinal contents of several full herring (Fig. 1, right panel) have similar electrophoretic mobilities, but these samples required a several thousand dilution (up to 1:8000) to display comparable intensities in the gels, which supports the hypothesis that the tissue-degrading enzymes found in ventral muscle of full fish possibly leak from the intestinal area.

The ventral muscle extracts of emptied herring did not contain strong gelatinolytic activities and were only diluted 1:2 (Fig. 1, middle panel). The gelatinolytic activity seen in these samples in the upper part of the gel close to the "a" band of the low molecular weight standard (labelled with an arrowhead in Fig. 1) is similar to an activity seen in blood samples from herring (Felberg & Martinez, 2006). An activity band with similar mobility is also seen in all muscle and intestinal samples in Figs. 2 and 3 (also labelled with an arrowhead). It is possible that this band originates from blood, since this fish were not bled and therefore differences in intensity might be due to different amounts of blood retained in the muscle samples. Another gelatinolytic band was found in the ventral muscle of emptied fish (labelled with an asterisk in Figs. 1 and 2), with the same electrophoretic mobility as one previously described by our group in dorsal muscle extracts from herring and that also seems to be present as a weak band in blood samples (Felberg & Martinez, 2006; Felberg et al., 2008).

The extracts used in this work were made from frozen round fish or frozen fish tissues and it is therefore important to consider the effect of freezing and thawing. Botta et al. (1992) claimed that the length of frozen storage and freezing/thawing conditions have a pronounced effect on belly bursting in capelin. Freezing of tissues has several adverse consequences including that water-soluble constituents become concentrated in the unfrozen phase, and that water converted to ice increases 9% in volume (Fennema, 1996). Thus, while lowering of the temperature will decrease the reaction rates, the increase in concentration of reactants and enzymes due to freezing may actually increase the reaction rates (Fennema, 1996). The formation of ice crystals and their expansion may cause freeze-damage to the ultra-structure of fish tissues, e.g. disruption of membranes (Ashton, 2002). It has been shown that leakage of some enzymes of the cell interstitial fluid from the muscle of rainbow trout (*Oncorhynchus mykiss*) is more severe in fish stored in ice with subsequent subjection to a freeze-thaw cycle than fish only stored in ice (Nilsson & Ekstrand, 1993), indicating damage to cellular membranes. It was impossible to examine gelatinolytic activities on freshly caught North Sea herring on board the fishing vessels and therefore the fish had to be frozen for transport and storage. During the cruise in 2007, the ventral muscle samples were dissected immediately or 24 h after catch. The tissues were frozen separately, and extracted in our laboratory no later than 2 months after the cruise. As shown in Fig. 2, the ventral muscle

from fish that were dissected directly after catch had little gelatinolytic activity, regardless of whether they were frozen immediately after dissection (V0), or after 24 h of ice storage (V24M). Muscles dissected after 24 h of ice storage in the round fish (V24), on the other hand, displayed activity bands similar to those of ventral muscles and intestinal content of full herring shown in Fig. 1 (labelled with brackets in the figure). No *post-mortem* increase in gelatinolytic activities in the ventral muscle was observed when it was stored dissected in ice for 24 h prior to enzyme extraction. Increased activities were only noticed when the fish was stored intact for 24 h before removing the ventral muscle, supporting the hypothesis that enzymatic degradation is due to action of enzymes leaking from surrounding organs.

The protein content in extracts from ventral muscle and intestinal contents of full and emptied herring and their corresponding gelatinolytic activities are shown in Fig. 3. The protein profiles, as determined by SDS–PAGE, of ventral muscle extracts from full fish were very similar to each other, but different from those of ventral muscle from emptied herrings. The disappearance of two bands (labelled with asterisks in Fig. 3) from the extracts of emptied herring and the darker background of the lanes from the extracts of full herring, indicate that some proteolytic degradation had taken place in the latter samples. The protein patterns of intestinal contents varied more amongst samples, as could be expected, since it will be dependant on the feed ingested in addition to the individual level of digestive enzymes of the herring, but full and emptied fish had similar protein patterns. The gelatinolytic activities observed in ventral muscle of full herring presented the same patterns (number of bands and their electrophoretic mobility) as extracts from intestinal contents from both full and emptied herring, the differences observed seemed to be more quantitative than qualitative, and this may be due to the difficulties in calculating the exact dilution that would give identical activity loadings. The ventral muscles of emptied fish also contained some gelatinolytic activities, but not only were they negligible regarding their intensity compared to the ones of full fish, they also had different electrophoretic mobilities indicating that they are different enzymes. In addition, no apparent differences in the number, relative intensities or electrophoretic mobilities of the activity bands were observed between the two extraction methods (standard procedure: Figs. 1 and 2, simplified procedure: Fig. 3) used in this work. In this figure, the extracts of only two emptied herring are shown since there was almost no variation in the activities and protein patterns amongst all emptied fish examined.

The ability of the extracts to hydrolyse isolated MHC is shown in Fig. 4. Only the extract from ventral muscle of emptied herring lacked the ability to digest the MHC band, while the proteolytic ability of the intestinal content extract of full herring was so high that the MHC band was completely digested upon mixing. The other two extracts showed intermediate activities and the MHC was completely digested in less than 15 min of incubation with both ventral muscle of full herring and intestinal content extract of emptied herring. The degradation of MHC determined by SDS–PAGE (Fig 4A) was confirmed by western blotting using the antibodies directed against MHC (Fig. 4B).

Comparison of the gelatinolytic activities and the SDS–PAGE protein patterns (Fig. 3) and MHC degrading ability (Fig. 4) of full and emptied fish showed that stronger gelatinolytic activities seemed to correspond to more degraded protein patterns in the SDS–PAGE gel and, not surprisingly, that the samples with higher gelatinolytic activities also were more efficient to digest the MHC (Fig. 4). All the results shown in Figs. 1–4 seem to indicate a positive effect of allowing the fish to empty their digestive system for some hours prior to being taken onboard the fishing vessels.

The kind of prey thought to promote belly bursting are "the black prey" ("kruttåte"/"svartåte" in Norwegian), red feed ("rødåte" in Norwegian) and some also report that "small shrimps" can be causative. The black prey is the most feared, and constitutes close to 10% of the food of herring in the Norwegian Sea (Melle, Ellertsen, & Skjoldal, 2004). They are shelled gastropods of the genus *Limacina* that possess an unpigmented shell, but dark body pigmentation which can stain the flesh of predatory fish (Melle et al., 2004). *Limacina retroversa* is reported to cause fast *post-mortem* deterioration of fish when present in their digestive system (Baalsrud, 1951). Two species of *Limacina* can be found in the Norwegian Sea: *L. helicina* which is considered a more Arctic species and *L. retroversa* which can be found in Atlantic and coastal waters of the Norwegian Sea (Melle et al., 2004). The red feed is identified as *Calanus* spp. and the "small shrimps" mentioned are probably krill. Crustaceans have hard chitinous exoskeletons which might be hard to digest, especially in large concentrations. The possible problem of digesting the shell of *Limacina* spp. has been discussed, which is thought to lead to strong secretion of digestive enzymes (Baalsrud, 1951). Since experience shows that the species of prey have an impact, we believe that the structural features of the prey, the fact that they are present in large amounts, and also possibly the presence of prey enzymes, may contribute to the occurrence of belly bursting. However, this remains to be investigated.

Another factor concerning the digestion of prey is the effect of the dilation of the stomachs in herring that have been feeding heavily. During our cruises we have observed that the *pyloric caeca* and the upper part of the intestine (close to the *pyloric caeca*) are easily ruptured in full herring *post-mortem*. This is mentioned in the work of Almy (1926), who also noted that both the *pyloric caeca* and the intestine are located in close proximity to the ventral wall, which implies that any leakage from these organs could easily affect the ventral muscle. The blind sac of the stomach can become quite dilated when herring are gorging themselves with prey and will take up a large amount of space in the peritoneal cavity. This can put pressure on the other organs, and the *pyloric caeca* and intestine would be located even closer to the ventral wall. Other organs will also swell due to food intake and it has been shown that the size of the viscera of herring that have not been feeding heavily (winter herring) are reduced to 1/5 of the viscera size of summer herring (Baalsrud, 1951). Emptied fish used in our work were observed to have a marked reduction in stomach dilation compared to full fish.

In summary, we have shown that the proteolytic activities capable of degrading gelatin and MHC in extracts of ventral muscle are reduced by allowing the fish to empty their stomach contents prior to being taken on board. This, together with the fact that isolated ventral muscle did not develop the characteristic gelatinolytic activities, suggest in agreement with Almy (1926) and Baalsrud (1951) that the activities originate in the digestive system of the fish from where they leak to weaken and disrupt the digestive structures and ventral wall. Some elements that might facilitate the leakage are the high level of digestive enzymes (possibly very high due to induction of secretion by elements that are hard to digest) during heavy feeding and the crowding of the peritoneal cavity by organs swollen with prey and digestive juices.

Acknowledgements

We are grateful for the help of NIFES in gathering samples of herring in 2006 and the crew of M/S Libas and Lars Olav Lie for having us on board, for all the help in sampling and transportation of fish and for invaluable discussions and comments. The financial support of the Norwegian Research Council to projects 165332/I10 and 179460/I10 and of FHF to the project "*Pelagisk kvalitet*" is gratefully acknowledged.

References

Almy, L. H. (1926). The role of the proteolytic enzymes in the decomposition of the herring. *Journal of the American Chemical Society, 48*, 2136–2146.

Anderson, C. W., Baum, P. R., & Gesteland, R. F. (1973). Processing of adenovirus 2-induced proteins. *Journal of Virology, 12*(2), 241–252.

Ansorge, W. (1983). Fast visualization of protein bands by impregnation in potassium permanganate and silver nitrate. In D. Stathakos (Ed.), *Electrophoresis '82* (pp. 235–242). Berlin, New York: Walter de Gruyter & Co.

Ashton, I. P. (2002). Understanding lipid oxidation in fish. In H. A. Bremner (Ed.), *Safety and quality issues in fish processing* (pp. 254–285). Cambridge: Woodhead Publishing Limited.

Botta, J. R., Kennedy, K. M., Kiceniuk, J. W., & Legrow, J. (1992). Importance of redfeed level, fish size and roe content to the quality of roe capelin. *International Journal of Food Science and Technology, 27*, 93–98.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry, 72*, 248–254.

Broms, C. T. Å. (2007). Distribution and life cycle of *Calanus* species in relation to the seasonal phytoplankton development and predator-prey interactions with Norwegian spring spawning herring in the physical setting of the Norwegian Sea ecosystem. *PhD Thesis*. Faculty of Mathematics and Natural Sciences, University of Oslo, Norway.

Baalsrud, K. (1951). Proteolytisk nedbrytning av sild. *Tidsskrift for kjemi, bergvesen og metallurgi, 5*, 71–73.

Dalpadado, P., Ellertsen, B., Melle, W., & Dommasnes, A. (2000). Food and feeding conditions of Norwegian spring–spawning herring (*Clupea harengus*) through its feeding migrations. *ICES Journal of Marine Science, 57*, 843–857.

Etienne, M., Jérôme, M., Fleurence, J., Rehbein, H., Kündiger, R., Mendes, R., et al. (2000). Identification of fish species after cooking by SDS–PAGE and urea IEF: A collaborative study. *Journal of Agricultural and Food Chemistry, 48*, 2653–2658.

Felberg, H. S., & Martinez, I. (2006). Protein degrading enzyme activities in herring (*Clupea harengus*) muscle and stomach. In J. B. Luten, C. Jacobsen, K. Bekaert, A. Sæbø, & J. Oehlenschlager (Eds.), *Seafood from fish to dish (quality, safety and processing of wild and farmed fish)* (pp. 275–282). The Netherlands: Wagening Academic Publishers.

Felberg, H. S., Batista, I., Nunes, M. L., & Martinez, I. (2008). Gelatin degrading enzyme activities in herring (*Clupea harengus*) and sardine (*Sardina pilchardus*) ventral and dorsal muscles. In *Proceedings of the 2006 nor-fishing technology conference* (pp. 94–100). Trondheim: ISSN 189-6737.

Fennema, O. R. (1996). Water and ice. In O. R. Fennema (Ed.), *Food chemistry* (pp. 17–94). New York: Marcel Dekker Inc..

Gildberg, A. (1978). Proteolytic activity and the frequency of burst bellies in capelin. *Journal of Food Technology, 13*, 409–416.

Gildberg, A. (1982). Autolysis of fish tissue – general aspect. *Dr. Scient. Thesis*. Institute of Fisheries, University of Tromsø, Norway.

Hochstrasser, D. F., Harrington, M. G., Hochstrasser, A. C., Miller, A. C., & Merril, C. (1988). Methods of increasing resolution of two-dimensional protein eletrophoresis. *Analytical Biochemistry, 173*, 424–435.

Hochstrasser, D. F., Patchornik, A., & Merril, C. R. (1988). Development of polyacrylamide gels that improve the separation of proteins and their detection by silver staining. *Analytical Biochemistry, 173*(2), 412–423.

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

Last, J. M. (1989). The food of herring, *Clupea harengus*, in the North Sea (1983–1986). *Journal of Fish Biology, 34*, 489–501.

Lavéty, J., & Love, R. M. (1972). The strengthening of cod connective tissue during starvation. *Comparative Biochemistry and Physiology Part A, 41*, 39–42.

Lødemel, J. B., & Olsen, R. L. (2003). Gelatinolytic activities in muscle of Atlantic cod (*Gadus morhua*), spotted wolffish (*Anarhichas minor*) and Atlantic salmon (*Salmo salar*). *Journal of the Science of Food and Agriculture, 83*, 1031–1036.

Lødemel, J. B., Mæhre, H. K., Winberg, J.-O., & Olsen, R. L. (2004). Tissue distribution, inhibition and activation of gelatinolytic activities in Atlantic cod (*Gadus morhua*). *Comparative Biochemistry and Physiology Part B, 137*, 363–371.

Martinez, A., & Gildberg, A. (1988). Autolytic degradation of belly tissue in anchovy (*Engraulis encrasicholus*). *International Journal of Food Science and Technology, 23*, 185–194.

Martinez, I., & Pettersen, G. W. (1992). Temperature-induced precocious transitions of myosin heavy chain isoforms in the white muscle of Arctic charr (*Salvelinus alpinus*). *Basic and Applied Myology, 2*, 89–95.

Marvik, S. (1974). Stengforsøk (internal report). *The Research Laboratory of the Norwegian Canning Industry* (8 pages). Stavanger, Norway.

Melle, W., Ellertsen, B., & Skjoldal, H. R. (2004). Zooplankton: The link to higher trophic levels. In H. R. Skjoldal (Ed.), *The Norwegian Sea ecosystem* (pp. 137–202). Trondheim: Tapir Academic Press.

Nilsson, K., & Ekstrand, B. (1993). The effect of storage on ice and various freezing treatments on enzyme leakage in muscle tissue of rainbow trout (*Oncorhynchus mykiss*). *Zeitschrift für Lebensmittel-Untersuchung und -Forschung, 197*, 3–7.

Prokopchuk, I., & Sentyabov, E. (2006). Diets of herring, mackerel, and blue whiting in the Norwegian Sea in relation to *Calanus finmarchicus* distribution and temperature conditions. *ICES Journal of Marine Science, 63*, 117–127.

Segers, F. H. I. D., Dickey-Collas, M., & Rijnsdorp, A. D. (2007). Prey selection by North Sea herring (*Clupea harengus*), with special reference to fish eggs. *ICES Journal of Marine Science, 64*(1), 60–68.

Snoek-van Beurden, P. A. M., & Von den Hoff, J. W. (2005). Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *BioTechniques, 38*, 73–83.

Temming, A., Bøhle, B., Skagen, D. W., & Knudsen, F. R. (2002). Gastric evacuation in mackerel: the effects of meal size, prey type and temperature. *Journal of Fish Biology, 61*, 50–70.

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America, 76*, 4350–4354.

Veliyulin, E., Felberg, H. S., Digre, H., & Martinez, I. (2007). Non destructive nuclear magnetic resonance image study of belly bursting. *Food Chemistry, 101*, 1562–1568.