Involvement of trypsin and chymotrypsin activities in Atlantic cod (Gadus morhua) embryogenesis

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Abstract

Proteases play a key role in yolk formation and degradation during embryogenesis of marine fish. This study presents the first clear data on the involvement of trypsin and chymotrypsin activities in the embryogenesis of Atlantic cod (Gadus morhua). Both enzyme activities were shown to be present in unfertilized eggs followed by a significant decline (P<0.01) in trypsin activity during the first 4 days post fertilization (dpf). Thereafter, the trypsin- and chymotrypsin activities increased to a maximum around day 9 pf. A decline in trypsin and chymotrypsin activities was observed from day 10 pf with minimal activity just prior to first feeding (day 15 pf). Western blot analysis, using polyclonal antibodies raised to Atlantic cod trypsins I and Y, mostly coincided with the trypsin activity profile. The novel trypsin Y was previously shown to have both trypsin- and chymotrypsin-like activities. Thus, some of the chymotrypsin activity observed in the samples may be originated from trypsin Y. The low trypsin and chymotrypsin activities just prior to first feeding (13–15 dpf) may indicate insufficient digestive function as trypsin has been shown to be a suitable short-term indicator reflecting the nutritional quality of marine fish larvae.

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1. Introduction

The survival rate of farmed Atlantic cod (Gadus morhua) during the first 14 weeks post hatch is generally very low, or between 10% and 40% (Steinarsson, 2004). Numerous genetic- and environmental factors affect the health and survival of marine fish larvae but starvation certainly has a major influence on their viability (Brown et al., 2003; Falk-Petersen, 2005). The nutritional requirement of fish larvae is closely linked to the digestion of yolk proteins and the digestive physiology during the embryonic development. Early larvae of Atlantic cod lack a functional stomach at first feeding (Hall et al., 2004). Therefore, digestion of proteins at this developmental stage relies primarily on the presence of pancreatic proteases such as trypsin and chymotrypsin, both of which are serine proteases. Trypsin is the only pancreatic protease that can activate its own precursor form as well as those of other pancreatic proteases (Corring, 1980). Thus, trypsin has a key position in controlling the activity of the pancreatic proteases.

A successful embryonic development of Atlantic cod depends on an internal balance of amino acids (Fyhn and Serigstad, 1987). As the building blocks for protein synthesis, amino acids are essential for cell renewal and organ development. Free amino acids are also important for osmotic control and the buoyancy of embryos (Fyhn...
et al., 1999; Finn et al., 2002). Last but not least, free amino acids are a major source of energy for the Atlantic cod during its early developmental phase (Fyhn and Serigstad, 1987; Finn et al., 1995). Consequently, an effective protein digestion is essential for the maintenance of embryonic health and survival.

Research on winter flounder (Pleuronectes americana) (Murray et al., 2004) and Atlantic salmon (Salmo salar) (Rungruansak-Torrissen et al., 1998) has shown that several varieties of trypsins are produced during the developmental period and at different environmental temperatures. The Atlantic cod is also known to produce different trypsin isozymes (Gudmundsdóttir et al., 1993). Furthermore, it produces a novel broad specificity trypsin, termed trypsin Y (Spilliaert and Gudmundsdóttir, 1999) showing both trypsin and chymotrypsin activities (Pálsdóttir and Gudmundsdóttir, 2004). Cod trypsins I and Y, monitored in this study, are analogous to the flounder trypsins III and I, respectively (Murray et al., 2004). Amino acid sequence identities between cod trypsins I and Y are only in the order of 45% (Spilliaert and Gudmundsdóttir, 1999). These trypsins have been classified into two different groups, termed Group I (trypsin I) and Group III (trypsin Y) (Spilliaert and Gudmundsdóttir, 1999; Roach, 2002). The Atlantic cod trypsin Y is the only biochemically characterized Group III trypsin (Pálsdóttir and Gudmundsdóttir, 2004; Gudmundsdóttir and Pálsdóttir, 2005). Seven other Group III trypsins from marine fish have been characterized from their cDNA sequences (Gudmundsdóttir and Pálsdóttir, 2005). The Group III trypsins have been suggested to play an important role in marine fish development (Roach, 2002).

A small number of studies have been published on trypsins in Atlantic cod larvae (Hjelmeland et al., 1984; Ueberschär, 1993; Perez-Casanova et al., 2006). Hiramatsu et al. (2002) showed that serine proteases and a cathepsin D-like protease play key roles in yolk formation and degradation in masu salmon (Oncorhynchus masou). Prior to the data presented in this paper, the involvement of serine proteases in Atlantic cod embryogenesis was unknown.

Cod embryos reared at 7 °C go through well defined developmental stages (Hall et al., 2004). These involve cell cleavage (0–1 days post fertilization, dpf), blastula (1–2 dpf), gastrulation (2–5 dpf), segmentation (organogenesis) (5–11 dpf) followed by hatching and early larval stages (Hall et al., 2004). Atlantic cod larvae hatch from small eggs (1.3–1.5 mm) (Martinsdóttir and Steinarsson, 1998) that contain a limited amount of yolk with a relatively short yolk-sac period (Fridgeirsson, 1978; Hall et al., 2004). The yolk-sac of Atlantic cod is absorbed in 3–6 days after hatching, depending on water temperature (Fridgeirsson, 1978; Hall et al., 2004). Competence at procuring and assimilating food prior to depletion of the endogenous energy source of the yolk-sac is crucial for the survival of marine fish larvae (Kim et al., 2001; Kjørsvik et al., 2004). One of the main challenges facing the hatchery production of Atlantic cod is to find ways to increase survival of early stage larvae. This is in line with the long-term aim of the study presented here.

2. Materials and methods

2.1. Sampling of eggs and embryos

Atlantic cod eggs were obtained from wild broodstock held in the fish farming facilities of the Department of Aquaculture, Hólar University College, Saudárkrókur, Iceland. In short, four female cod were stripped; egg clutches from each female were fertilized with milt from one male cod diluted in seawater (1:1 v/v). Immediately after fertilization, embryos were treated with a 0.03% glutaraldehyde solution for 10 min and stored in four incubators (transparent cylindrical tanks, 2 L, 30 cm × 10 cm) supplied with UV-sterilized seawater (at 34‰ salinity) at a flow rate of approximately 50 ml min⁻¹. The embryos were reared in continuous light (150 lux on the water surface) and the seawater temperature was maintained at 7.0±1 °C. The water in the incubators was continuously aerated. The assumption was made that eggs that sank to the bottom of the incubator during the first 5 days, were unfertilized.

Samples were first collected from the unfertilized eggs followed by daily sampling of embryos from each incubator until first feeding (15 dpf). The samples were collected in 1.5 ml plastic tubes, quickly frozen in liquid nitrogen and then stored at −70 °C until analysis. Dead embryos were removed daily from the incubators. One day after fertilization, 10 embryos from each incubator were collected for diameter measurements. Egg diameter (mm) was measured using an ocular micrometer in a Leica MZ12 dissecting microscope. Developmental observations of the four egg batches used in the experiments are shown in Table 1.

2.2. Preparation of cell lysates

The method used for the preparation of lysates from Atlantic cod eggs and embryos was based on a method by Magnadóttir et al. (2004) with minor modifications. Each sample was homogenized on ice in four volumes of ice-cold lysis buffer (50 mM Tris–HCl, pH 7.6, 0.3 M
NaCl, 0.5% Triton X-100 (v/v)) using a Teflon pestle. The homogenates were then centrifuged at 7000×g for 10 min at 4 °C. The supernatant, containing the soluble protein fraction was collected in 500 μl Eppendorf tubes and quick frozen in liquid nitrogen followed by storage at −70 °C. The protein content of each protein extract was estimated using a dye binding kit (BioRad, Hercules, CA, USA) based on the Bradford method (Bradford, 1976). Bovine serum albumin from Sigma (St. Louis, MO, USA) was used for trypsin activity measurement and N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma, St. Louis, MO, USA) was used for measuring the chymotrypsin activity. The activation assay for trypsin contained 20 μl of protein extract, 970 μl of 20 mM Tris buffer, pH 8.0, 1 mM CaCl2, and 10 μl of 25 mM synthetic substrate. The activation assay for chymotrypsin contained 20 μl of protein extract, 960 μl of 20 mM Tris buffer, pH 8.0, 1 mM CaCl2, and 20 μl of 25 mM synthetic substrate. The synthetic substrate was incubated in the buffer for at least 3 min at 23 °C prior to each measurement. The reaction was initiated with the protein extract and the enzymatic activity was monitored by measuring the absorbance at 410 nm at 23 °C for 10 s. The absorbance of the lysis buffer without the protein extract was measured under the same conditions and subtracted from the absorbance of the samples. The assays were repeated four times and the enzymatic activity was calculated by the following equation:

\[
\frac{[(\text{Abs}_{410}/\text{min}) \times \text{volume of reaction mixture (ml)} \times 10^6 \ \text{μmol/mol}]}{8800 \ \text{M}^{-1} \ \text{cm}^{-1} \times 1 \ \text{cm} \times \text{volume of enzyme extract (ml)}} \times \text{mg protein in the reaction mixture.}
\]

The enzymatic activity was expressed as specific activity (mU/mg protein). The molar extinction coefficient (ε) of para-nitroanilide is 8800 M⁻¹ cm⁻¹.

### 2.4. Western blot and SDS-PAGE analysis

Separation of proteins in the extracts of whole cod eggs and embryos was performed by SDS-PAGE analysis (Laemmli, 1970) in a SE 250 Series Mighty Small II Electrophoresis Unit (Hoefer, San Francisco, CA, USA) using the manufacturer’s instructions. The amount of acrylamide in the separation gel was 12% and 5% in the stacking gel. The protein content of the sample placed in each well of the SDS-PAGE gels was adjusted to 30 μg 20 μl⁻¹ (1.5 g l⁻¹) for SDS-PAGE protein analysis and 50 μg 20 μl⁻¹ (2.5 g l⁻¹) for preparation of Western blot analysis. Prior to sample loading on the gels, 20 μl of sample was diluted in 10 μl of 0.125 M Tris, pH 6.8, denaturing sample buffer containing 0.5% 2-mercaptoethanol and 2% SDS and boiled for 5 min. Thirty microliters of the sample extract were loaded in each well and electrophoresis was performed at a constant current (35 mA) at room temperature in an electrophoresis buffer consisting of 0.025 M Tris, 0.192 M Glycine and 0.1% SDS. The gels were stained with Coomassie Blue dye from Fermentas (St. Leon-Rot, Germany) following separation of the proteins by SDS-PAGE. Low molecular weight standards in the size range of 14.4–116 kDa from Fermentas (St. Leon-Rot, Germany) and the native trypsin I isolated and purified from Atlantic cod (Ásgeirsson et al., 1989) were used as standards in the SDS-PAGE. For Western blot analysis, proteins separated by SDS-PAGE analysis were transferred to a nitrocellulose membrane (NC, Hybond-ECL, Amersham Life Science, UK) using the trans-blot® SD semi-dry transfer cell from BioRad (Hercules, CA, USA) according to the manufacturer’s instructions. The transfer time allowed was 1 h at room temperature. After transfer onto the nitrocellulose membrane, residual sites were blocked with 5% semi-skimmed milk powder in 0.14 M Tris-buffered saline, pH 7.6, containing 0.1%...
Tween 20, at 4 °C overnight. The primary and conjugated secondary antibodies were diluted in the blocking solution and incubated with the membrane for 1 h at room temperature with extensive washing between each step. Detection was performed with polyclonal anti-trypsin I provided by Yves St. Pierre, (University of Quebec, Canada). Polyclonal antibodies raised to the novel recombinant trypsin Y were produced at the Institute for Experimental Pathology, University of Iceland, at Keldur under the supervision of Bjarnheidur Gudmundsdóttir. The blots were developed using the ECL system from Amersham Life Science (UK). Prestained molecular weight markers from Fermentas (St. Leon-Rot, Germany) and the native cod trypsin I (Ásgeirsson et al., 1989) were used as molecular weight standards.

2.5. Statistical analysis

The specific activity of trypsin and chymotrypsin in eggs and embryo extracts were expressed as the mean±S.E. (n=4). The biochemical data were tested for homogeneity of variances and normality of the data. The specific activity of the enzymes through the embryonic stage was investigated using a one-way analysis of variance (ANOVA) procedure. Multiple comparisons of specific activity over time were investigated using a Tukey Test comparison test. All statistics were conducted using SigmaStat 3.1 for Windows (SigmaStat® 3.1, USA). A significance level of \( P<0.05 \) was used.

3. Results

3.1. Developmental observations during embryogenesis

Developmental observations of the four cod egg batches used in the biochemical experiments are shown in Table 1. The average egg diameter was 1.40±0.018 mm. The average fertilization rate for the four batches was 72.30±8.22%, based on the assumption that sinking eggs were unfertilized. Hatch began 1 day later in batches 3 and 4, probably due to slightly lower rearing temperatures.

3.2. Trypsin and chymotrypsin activities in extracts of cod eggs and embryos

Interesting variations were observed in the trypsin activity during the embryonic period of development, especially from day 5 pf until hatch (12–13 dpf). Relatively high trypsin activity (85.09±3.32 mU/mg protein) was observed in unfertilized eggs (Fig. 1, point 0). A significant decline \( (P<0.01) \) in trypsin activity was seen on day 1 pf and it remained low until day 5 pf. However, no significant difference was observed in the trypsin activity of samples taken during the period between days 1 and 5 pf. The trypsin activity seen in the samples taken on day 1 pf \( (P<0.01) \), 2 pf \( (P<0.05) \), 3 pf \( (P<0.01) \) and 4 pf \( (P<0.05) \) was significantly lower than that of unfertilized eggs (Fig. 1). From day 5 pf, the trypsin activity increased gradually (62%) during the segmentation period reaching a maximum (106.23±3.77 mU/mg protein) at day 9 pf (Fig. 1, stage IV). During the segmentation period, tissues

![Fig. 1. Specific activity of trypsin (mU/mg protein) in extracts of Atlantic cod eggs and embryos measured towards the synthetic substrate N-CBZ-Gly-Pro-Arg-\( p \)-nitroanilide. The values are presented as means±standard error (S.E.). The Roman numbers indicate the different developmental stages of cod according to Hall et al. (2004). I=Cleavage Period, II=Blastula Period, III=Gastrula Period, IV=Segmentation Period (organogenesis), V=Hatching and Early Larval Period.](image-url)
and organs begin to differentiate within the embryo (organogenesis) (Hall et al., 2004). Despite a small decrease in trypsin activity (around 30%) between days 9 and 13 pf, the activity remained relatively high (around 70 mU/mg protein) during that period. From day 13 pf, a decrease was observed in the trypsin activity, with a minimum at day 15 pf of 14.74±5.20 mU/mg protein. The trypsin activity, measured on day 15 pf was significantly lower ($P<0.01$ and $P<0.05$) than at any other point during the experiment.

In general, the chymotrypsin activity reflected the trypsin activity profile with a considerably lower activity during the segmentation period (Figs. 1 and 2, stage IV), at hatch (12–13 dpf) and during the early larval period (Figs. 1 and 2, stage V). Chymotrypsin activity was present in unfertilized eggs (Fig. 2, point 0) with an increase during the segmentation period to a maximum of 74.30±9.97 mU/mg protein (from days 6 to 10 pf) and a substantial decrease around hatch and the early larval period (Fig. 2, stage V). The chymotrypsin activity in the embryo samples taken at day 14 pf was significantly lower than that found in the samples taken on days 2 pf ($P<0.01$), 4 pf ($P<0.05$), 8 pf ($P<0.01$) and 10 pf ($P<0.01$).

### 3.3. Western blot and SDS-PAGE analysis of cod egg and embryo extracts

Western blots of the cod egg and embryo extracts strongly indicated the presence of trypsin I (Fig. 3) and trypsin Y (Fig. 4) in unfertilized eggs as well as during the early embryonic stages. Bands of approximately 25 kDa, implicating the presence of trypsin I (Fig. 3) and trypsin Y (Fig. 4) were detected in the samples until days 9 and 11 pf, respectively. The presence of the trypsin bands mostly reflected the trypsin activity profile (Figs. 1 and 2). A smaller unknown band of about 15 kDa (Figs. 3 and 4), cross-reacting with the trypsin I and Y antibodies, was also present on the Western blots.

![Fig. 2. The specific activity of chymotrypsin (mU/mg protein) in extracts of Atlantic cod eggs and embryos measured towards the synthetic substrate N-Suc-Ala-Ala-Pro-Phe-$\mu$-nitroanilide. The values are presented as means±standard error (S.E.). The Roman numbers indicate the different developmental stages of cod according to Hall et al. (2004). I=Cleavage Period, II=Blastula Period, III=Gastrula Period, IV=Segmentation Period (organogenesis), V=Hatching and Early Larval Period.](image1)

![Fig. 3. Western blot analysis of extracts from Atlantic cod eggs and embryos using polyclonal antiserum raised towards the native cod trypsin I as a primary antibody. The first lane shows a sample taken from unfertilized eggs, lanes 2 to 6 show egg samples collected at 3, 5, 8, 9 and 11 dpf. Lanes 7 and 8 show yolk-sac larvae samples collected at 13 and 15 dpf. Lane 9 shows the native cod trypsin I standard. Protein molecular weight markers in the size range of 14.4–116 kDa were used for molecular mass determination.](image2)
This band may represent either trypsin breakdown products as the two trypsins are very sensitive to auto-
lysis during handling (Jónsdóttir et al., 2004) or one of the major yolk components, vitellogenin B (Magnadóttir et al., 2004). Due to their low amounts in the egg and embryo extracts, trypsin band corresponding to the cod trypsin I standard could not be identified on Coomassie Blue stained SDS-PAGE gels (results not shown).

4. Discussion

This study presents the first published results on the involvement of trypsin and chymotrypsin activities in the embryogenesis of Atlantic cod. The role of trypsin in the cleavage of yolk proteins during the final maturation of the oocyte is indicated by the high trypsin activity (85 mU/mg protein) observed in unfertilized cod eggs. This is further supported by Western blot analysis showing strong bands corresponding to trypsins I and Y in unfertilized eggs. Upon fertilization, the trypsin activity decreased to around 55 mU/mg protein and remained at that level during the blastula and gastrula periods. Western blot analysis indicates the presence of trypsins I and Y during these early developmental stages. The decreased trypsin activity at days 1–5 pf may be due to a gradual depletion of ovum-originated mRNAs encoding trypsinogen, as a variety of maternal mRNAs are stored in oocytes and transferred to fertilized eggs (Gilbert, 1997). After that stage, the embryo may be able to express its own mRNAs encoding these enzymes to ensure yolk utilization. The trypsin activity observed in the cod embryos during the early developmental phases suggests its possible involvement in the activation of other proteases. This is in agreement with Carnevali et al. (2001) who suggested that a serine protease might be involved in the initial activation of cathepsins D and L in sea bass (Dicentrarchus labrax), since their activities increased during the early stages.

A rapid increase in trypsin activity was observed in the cod embryos during the organogenesis reaching a maximum of 106 mU/mg protein at day 9 pf. These results may indicate preparation for hatching as the energy demand is raised at that time (Fyhn and Serigstad, 1987; Finn et al., 1995). A strong protein band, corresponding to trypsins I and Y, was observed in Western blot analysis at day 8 pf and a much weaker band was seen on day 9 pf where the highest trypsin activity was observed in the embryos. Despite a small decrease in trypsin activity (around 30%) between days 9 and 13 pf, the activity remained relatively high (around 70 mU/mg protein) during that period. However, this activity profile was not reflected on Western blots where weak protein bands, corresponding to trypsin, were observed at day 9. However, no such bands were detected at days 11 and 13 pf. This discrepancy between the trypsin activity profile and Western blot analysis may be explained by an increased activity of trypsin-like hatching enzymes (Li et al., 2006) from days 9 to 13 that are not detected by the cod trypsin antibodies.

The trypsin activity profile in the Atlantic cod embryos is in line with results presented by Hartling and Kunkel (1999). They found that proteolytic processing of yolk proteins in winter flounder was slow during the early embryonic development, but entered a more rapid phase between days 8 and 12 pf. Furthermore, Hiramatsu et al. (2002) demonstrated the involvement of serine proteases in the embryogenesis of salmonoids (masu salmon) and suggested that serine proteases play key roles in yolk formation and degradation.

A rapid decline in trypsin activity to approximately 14 mU/mg protein was observed in the cod embryos post hatch (13–15 dpf). These results were supported by Western blot analysis showing no bands of around 25 kDa with the trypsin I and Y antibodies. The Western blot results appear to be in contrast to Perez-Casanova et al. (2006) who demonstrated that trypsin-like enzyme activity and trypsinogen mRNA transcripts were present in Atlantic cod larvae as early as hatch. The trypsin activity profiles in Perez-Casanova et al. (2006) and this study cannot be compared in detail as different substrates were used to measure the trypsin activities in the two studies. It is also difficult to compare data generated by the RT-PCR technique and Western blot analysis since the molecular sensitivity of the former technique is

![Western blot analysis of extracts from Atlantic cod eggs and embryos using polyclonal antiserum raised towards the recombinant cod trypsin Y as a primary antibody.](image-url)
In general, the chymotrypsin activity reflected the trypsin activity profile with a slightly lower activity during embryogenesis. Chymotrypsin activity was present in unfertilized eggs. However, in contrast to the trypsin activity, no decline was observed in the chymotrypsin activity upon fertilization. Western blot analysis using antibodies to the novel trypsin Y strongly suggests its presence in unfertilized cod eggs as well as in embryos supporting its hypothetical role in marine fish development (Spilliaert and Gudmundsdóttir, 1999; Roach, 2002). The recombinant form of trypsin Y was previously shown to have trypsin and chymotrypsin activities (Pálsdóttir and Gudmundsdóttir, 2004). Therefore, some of the chymotrypsin activity observed in the Atlantic cod eggs and embryos may be due to the chymotrypsin activity of trypsin Y.

In conclusion, the results presented in this study show that trypsin and chymotrypsin activities are involved in the embryogenesis of Atlantic cod. Surprisingly, these enzymes seem to be produced in relatively low amounts just prior to first feeding, the critical time period at which the cod larvae must initiate feeding or face starvation. It may be possible to increase larval survival with external factors that could stimulate trypsin and chymotrypsin activities at first feeding.

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