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Quantification of trypsin with a radioimmunoassay in herring larvae (*Clupea harengus*) compared with a highly sensitive fluorescence technique to determine tryptic enzyme activity

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Abstract. Enzymatic activity and quantity of the protease trypsin were measured in individual herring larvae (*Clupea harengus* L.). The enzymatic activity assay was done using a fluorescence technique, and a radioimmunoassay was used for quantification of trypsin. The results are compared and the differences between the techniques discussed. Both methods gave similar results, as high or low values in trypsin quantity were reflected in high or low values of tryptic activity. Quantity and activity were linearly and positively correlated, but small differences between methods were found at the lowest detection limits. Both techniques reflect high variability between individual larvae.

Introduction

During the larval period marine fishes are vulnerable to starvation or restricted food availability (O'Connell and Raymond 1970, Houde 1977, 1978, Werner and Blaxter 1981). Therefore, reliable methods for estimating the nutritional condition of larvae would be important tools for fishery biologists doing fish stock assessment or farming marine fish species. The methods still most commonly used are based on morphological (Ehrlich et al. 1976) and/or histological (O'Connell 1976, 1981, Theilacker 1986) characteristics. Recently, however, larval trypsin content and/or activity of trypsin have been highlighted as useful indicators of nutritional condition (Hjelmeland et al. 1984, Pedersen et al. 1987, Hjelmeland et al. 1988, Pedersen and Hjelmeland 1988, Ueberschär 1988, Pedersen et al. 1990, Ueberschär and Clemmesen 1990).

Due to the lack of a functional stomach in most marine fish larvae, the entire extracellular degradation of ingested prey takes place in the intestine with enzymes derived from the pancreas. Among these, the protease trypsin is a key enzyme (Corring 1980), and previous studies (Pedersen et al. 1987, Pedersen and Hjelmeland 1988) have shown that the content of trypsin and its precursor (trypsinogen) reflect the nutritional status of fish larvae.

Trypsin quantity in fish larvae has been assayed by means of a radioimmunoassay (RIA) (Hjelmeland and Jørgensen 1985), with specific antibodies against trypsin purified from pancreatic tissue of adult herring. The trypsin-RIA is sensitive enough to be used on individual larvae. In contrast, most conventional methods for measurements of tryptic activity demand pooled samples of larvae. However, a significant improvement was introduced by Ueberschär (1988), who applied a fluorescence technique for measurement of tryptic activity in larval fish. By using a fluorogenic substrate, tryptic enzyme activity in individual larvae could be measured and related to nutritional condition (Ueberschär and Clemmesen 1990).

Before choosing one or both of these methods, it is important to be aware of some basic differences between measurement of tryptic enzyme activity and of trypsin content. Whereas the RIA measures the total content of larval trypsin and trypsinogen molecules whether they are enzymatically active or not, the enzyme activity assay only detects the active form of trypsin including exogenous tryptic-like enzyme activity derived from ingested prey.

In addition, there are certain factors which may influence activity assays when crude larval homogenates are used. Trypsin inhibitors present in larval tissues (Hjelmeland 1983) will be free to react with trypsin in the homogenate, giving rise to a lower estimate of tryptic activity in the *in vitro* assay compared to the actual tryptic activity in the larva before homogenization. Intracellular enzymes and lysosomal proteases (Barret 1979) may cause an overestimation of extracellular trypsin in an enzyme activity assay. These two factors, trypsin inhibitors and intracellular proteases, will probably affect estimates of tryptic activity at low contents of trypsin in a larva.

In the present study, quantity (RIA) and activity (fluorescence technique) of trypsin were measured on identi-

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cal larvae in order to evaluate the relationship between the methods.

Material and methods

Larvae

Eggs from one female Clyde herring (*Clupea harengus*) were artificially fertilized with sperm from several males and incubated in running seawater at 7 °C until hatching was induced on Day 20 after fertilization as previously described (Pedersen et al. 1987). Newly hatched larvae were transferred to aquaria with a water temperature of 8 to 10 °C, a salinity of 30.2‰ and a 15 h light:9 h dark cycle. Larvae were fed nauplii and copepodites of *Acartia tonsa* from Day 4 after hatching; prey concentration was not controlled. Copepods were fed *Rhodomonas* sp., filtered (45 µm) and cooled before being offered to the larvae.

On Day 50 after hatching, larvae were transferred from a stock tank to a cylindrical aquarium of 50 liters void of food. The larvae were then starved for 68 h in order to reduce the intestinal trypsin content to prefeeding level (Pedersen and Hjelmeland 1988). On Day 53 after hatching, the larvae were sampled. First, eight starving larvae were sampled, each being pipetted onto a cellophane-covered glass slide from which seawater was removed with a pointed capillary tube. The larvae were immediately frozen at −26 °C. The remaining larvae were offered *Acartia tonsa* copepodites, and 25 larvae with copepods in the intestine were sampled as described above within 1 h from adding food to the tank, i.e., before any defaecation could occur (Pedersen 1984). Finally, nine larvae which had eaten were measured and then transferred to separate Eppendorf tubes without cellophane and frozen.

The eight starving larvae and the 25 fed larvae from the first sample were measured (standard length, 3 × magnification) and the number of ingested copepods was noted. Next, the pancreas was separated from the intestine as previously described (Pedersen et al. 1987) and individual intestines or pancreata each adherring to a small strip of cellophane were put into individual Eppendorf tubes and frozen. Samples were kept at −26 °C until the following day, then stored at −80 °C.

Individual samples of pancreata and intestines were homogenized in 800 µl buffer, pH 7.3, containing 130 m*M* NaCl, 8 m*M* Na_2HPO_4 and 20 m*M* KH_2PO_4.

Measurement of tryptic activity

The tryptic activity measurements were performed according to the fluorescence technique described by Ueberschär (1988) with some modifications. 500 µl of the substrate Nα-benzoyl-L-arginin-methyl-coumarinylamide, 0.20 m*M*, were added to 100 µl of the homogenate in a temperated cuvette (30 °C) and mixed well. The substrate was dissolved in a calcium-free TRIS-HCl buffer, 0.1 *M*, pH 8.00.

The relative fluorescence enhancement (excitation 380 nm, emission 440 nm) was recorded every 2 min over a maximum period of 10 min. The increase in fluorescence is directly proportional to the tryptic enzyme activity (Ueberschär 1988) and is given as a change in emission (%) per larva and standardized to 1 min.

Quantification of trypsin and trypsinogen

The content of trypsin and trypsinogen in individual herring larvae was analysed by a radioimmunoassay in which the anti-herring-trypsin antibody reacted with both the pancreatic form (trypsinogen) and the intestinal, active form (trypsin) of the enzyme (Hjelmeland and Jørgensen 1985). The linear range of the RIA standard curve was 0.3 to 24 ng of trypsin. The standard curve was produced with purified herring trypsin (0.13 mg enzyme protein ml^{-1}), which was also used to convert activity measurements into quantitative values.

Results

The number and standard length of the herring larvae from each group is shown in Table 1. A two-sample *t*-test of mean standard length of the sectioned feeders vs the sectioned starved larvae as well as vs the "whole larvae" was computed. No significant differences between means were found.

The mean values of trypsin quantity and tryptic enzyme activity for the groups of larvae are summarized in Table 2. In sectioned larvae (feeders and non-feeders) quantity of pancreatic trypsinogen tended to be higher than quantity of intestinal trypsin, which was the opposite of that which was found for tryptic activity. The correlation between trypsin quantity and enzyme activity was tested with a linear regression. Table 2 shows the estimated coefficients of the linear regressions of tryptic activity as a function of trypsin quantity for the different groups with their correlation coefficients. In all cases, a significant correlation was observed between enzyme quantity and enzyme activity, and the correlation coefficients indicate a strong positive linear correlation for all compared samples with the exception of the pancreas/feeder samples, which showed a rather weak correlation. Fig. 1 shows the relationship between trypsin content and tryptic activity in herring larvae. The data points represent individual values for the intestinal samples of fed and starved larvae and demonstrate a significant, positive linear correlation between enzyme quantity and enzyme activity. For the regression analysis the values of the starved larvae were not considered.

Conversion factor

In order to produce a conversion factor to the quantity measurement, the tryptic enzyme activity measurement was applied on purified herring trypsin. The activity measured was linear and positively correlated with the amount of herring trypsin (Fig. 2) and gave the following equation:

$$y = -0.130 + 0.00287\,x$$

Table 1. *Clupea harengus*. Number of larvae with their standard length for each of the three groups

	Sectioned larvae		Whole larvae
	Feeder	Non-feeder	Feeder
No. of observations	25	8	9
Length (average, mm)	21.02	19.96	17.91
SD (mm)	2.29	2.39	2.63
Min. (mm)	16.0	18.1	16.0
Max. (mm)	25.0	24.7	23.3

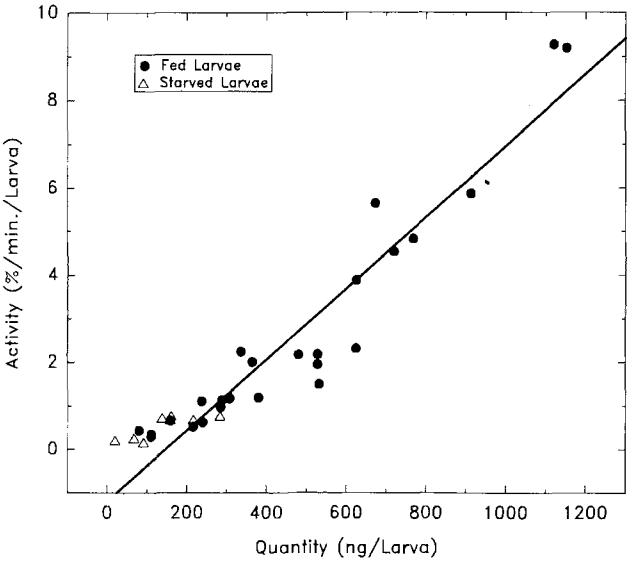

Fig. 1. *Clupea harengus.* Relationship between trypsin quantity and tryptic activity in the intestines of 53-d-old fed herring larvae. The curve was fitted by linear regression. Correlation coefficient and equation are shown in Table 2

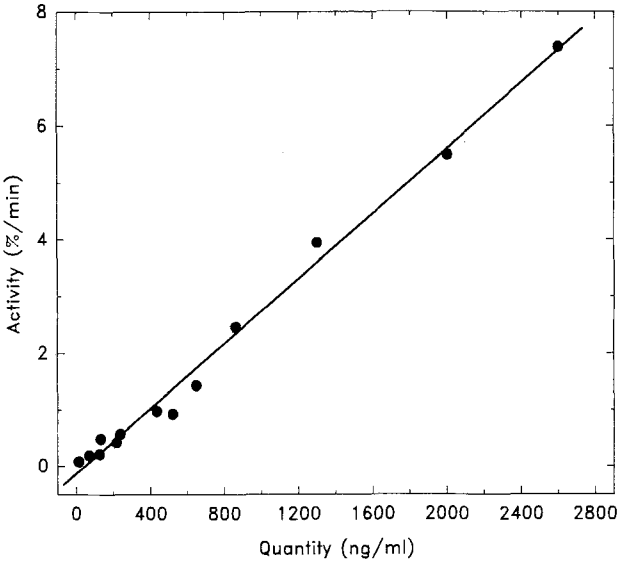

Fig. 2. *Clupea harengus.* Relationship between trypsin quantity and tryptic activity of purified herring trypsin. The curve was fitted by linear regression; the regression equation was used to convert activity values into trypsin quantity

Table 2. *Clupea harengus.* Summary statistic of the results of a comparison of trypsin(ogen) quantity and activity in the 53-d-old fed and starved herring larvae. Quantity is given as ng larva^{-1}; activity is given as % min^{-1} larva^{-1}

	Intestine, feeder		Pancreas, feeder		Intestine, non-feeder		Pancreas, non-feeder		Whole larvae	
	Quantity	Activity	Quantity	Activity	Quantity	Activity	Quantity	Activity	Quantity	Activity
No. of observations	25	25	25	25	8	8	8	8	9	9
Average	462.3	2.64	907.8	0.47	141.6	0.53	2144.6	0.53	675.9	1.49
SD	308.8	2.57	590.4	0.23	83.91	0.27	1250.1	0.27	717.7	2.04
Median	379.2	1.95	808	0.44	148	0.69	1960	0.47	331.2	0.53
Minimum	80	0.3	264	0.15	19.2	0.17	732.8	0.23	57.6	0.23
Maximum	1152	9.27	2400	0.91	283.2	0.78	4000	0.98	2000	6.35
Regression	$y=-0.98+0.0078x$		$y=0.23+0.00025x$		$y=0.16+0.0026x$		$y=0.087+0.00021x$		$y=-0.29+0.0026x$	
Correlation coefficient	0.942		0.665		0.816		0.966		0.928	
Significance ($p \leq 0.05$)	+		+		+		+		+	

Table 3. *Clupea harengus.* By using the conversion factor, enzyme activity values were converted into quantity values and compared with the values from the RIA measurements. The table shows the mean values with standard deviations for the sectioned and whole larvae, respectively

	Quantity (RIA) (ng larva^{-1})	Quantity (via activity) (ng larva^{-1})
Intestine, feeder	470.1 ± 308.8	764.5 ± 745.2
Pancreas, feeder	907.8 ± 590.4	161.1 ± 94.0
Intestine, starved	141.6 ± 83.9	177.5 ± 105.2
Pancreas, starved	2144.6 ± 1250.1	177.5 ± 105.2
Whole larvae, feeder	675.9 ± 717.7	444.7 ± 597.7

where x = quantity (ng ml^{-1}) and y = activity (% min^{-1}) with a correlation coefficient of $r=0.994$ ($n=13$). Linearity was found in the range of 12.8 to 2600 ng enzyme protein ml^{-1}.

By using the regression equation, activity measurements were converted into quantity of trypsin per larva and compared with the quantity values measured with the RIA (Table 3). The values are on the same order of magnitude in the case of intestinal samples of fed and starved larvae and for the group of the whole larvae, but larger differences were found in the pancreata samples, especially in starving larvae.

Discussion

A significant positive correlation between larval trypsin quantity (RIA) and larval tryptic activity (fluorescence technique) was found in all investigated samples. High or low quantities of trypsin correspond to high or low tryptic activities. However, quantity and activity of the enzyme are not closely linked in all samples of separated pancreatic and intestinal sections. In larval pancreatic tissue the vast majority of trypsin is in the form of enzymatically inactive trypsinogen, while most of the trypsin in the intestinal section will be enzymatically active. Hence, since the RIA measures both trypsinogen and

trypsin equally well and the fluorescence technique measures only the active form, trypsin content and tryptic activity will differ particularly in samples of pancreata and in homogenates of whole larvae. Thus, the calculated values of quantity via activity measurement compared with the quantity values measured by the RIA (Table 3) showed large differences in the case of pancreatic samples, particularly in those of starved larvae. In the intestinal sections of the feeders, the tendency towards higher values when using activity measurements may be attributed to tryptic-like enzymes from the ingested food. In the whole larvae the tendency towards higher RIA values can be explained by the pancreatic trypsinogen which is included in these samples and is measured in addition to intestinal trypsin by the RIA. Hence, the conversion factor between activity and quantity found by using purified herring trypsin cannot readily be adapted when measurements are done on homogenates of pancreatic samples or whole larvae. Theoretically, no activity should be measured in the pancreas homogenates, but a contamination of the pancreas with trypsin from the intestine during the preparation of the larvae may have occurred.

As methods for measuring larval fish condition, both the RIA and the fluorescence technique are sensitive enough to be used on individual larvae. The main difference between the two techniques is specificity. The RIA has a high specificity for trypsin and trypsinogen synthesized by the larva and does not measure other trypsin-like enzymes whether or not these are derived from ingested prey or are produced endogenously. Further, the RIA quantifies inhibitor-bound trypsin in a larval homogenate. However, the RIA does not discriminate between active and inactive forms of the enzyme. The enzyme measurement has a high specificity for trypsin-like enzymes which split a peptide bond at the carboxylic side of an arginine residue. Although most of the trypsin-like enzymes will come from the larval pancreas, the interference of larval cathepsin B and H from lysosomes (Barret 1979) and trypsin-like prey enzymes (Munilla-Moran et al. 1990) might be significant, but effects of enzyme inhibitors on the activity measurements do not seem to be significant in larval herring. Inhibitory effects would be expected to result in much lower calculated quantities (via activity) of trypsin compared to the RIA in the intestinal samples or in the homogenates of the whole larvae (Table 3).

The difference in sensitivity between the two methods is probably the reason why the regressions of activity as a function of enzyme quantity do not go through the origin. The RIA measures trypsin contents as low as 0.3 ng, while the linearity of the enzymatic measurement was tested from 1.28 ng onwards.

The differences in specificity and sensitivity indicate that the correlation between both methods is questionable when dealing with samples containing very low amounts of trypsin. However, from a methodological point of view it is of interest that a significant correlation exists within the range of values normally found in marine fish larvae.

The present study was designed to compare two highly sensitive techniques for the determination of the digestive protease trypsin in fish larvae. The high variability between individuals demonstrates the need for individual analysis. The results show that both assays are correlated and equally well suited to measure larval digestive capacity.

Since the amount of intestinal trypsin drastically decreases in larvae which have been starved for a few days, the easy-to-handle tryptic enzyme activity measurement should be applied in field studies monitoring the nutritional condition of marine fish larvae. In basal research on digestive capacity of fish larvae, particularly in sectioned samples, use of both methods is recommended, since the results will complement each other.

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