

Short Communication

Effects of pH and ionic strength on the protease activity of gastric extracts from the Coho salmon *Oncorhynchus kisutch*

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ABSTRACT. The activity of fish acid proteases is strongly dependent on the pH, and many basic and applied works had focused on researching the pH-profile of digestive proteases using different buffered media. Nevertheless, enzymatic activity is also known to be affected by the ionic strength (IS) of the medium. The present work is aimed at researching the combined effects of pH (2.5, 3.0, 3.5, 3.75 and 4.0) and IS (50, 100 and 200 mM) on the protease activity of gastric extracts of juvenile Coho salmon *Oncorhynchus kisutch*. It is concluded that the activity of Coho salmon gastric proteases is maximal at pH 2.5-3.0, drops towards pH 4.0 irrespective of ionic strength, and they showed a lower activity at IS 50 mM in comparison to IS 100 or 200 mM. The present results point out to the convenience of considering the effect of ionic strength when measuring the activity of fish gastric proteases.

Keywords: *Oncorhynchus kisutch*; Coho salmon, gastric proteases; enzymatic activity; pH, ionic strength

Many of the fish species with commercial interest possess a functional stomach from the end of the metamorphosis onwards. In vertebrates, the stomach controls the delivering of chyme into the duodenum and promotes the breakdown of polypeptides into oligopeptides (Grabner & Hofer, 1989). In larval digestion, the action of pepsin determines an important increase in the assimilation of proteins in *Hippoglossus hippoglossus* (Tonheim *et al.*, 2005) and a moderate improvement in the *in vitro* digestibility of fish muscle (Tonheim *et al.*, 2007) and is considered one of the cues to introduce inert diets in feeding protocols for fish larvae (Rønnestad *et al.*, 2013). The gastric digestion of proteins is based upon two secretory processes, the acidification of the gastric lumen and the secretion of zymogens, mainly acidic proteases, carried out by oxynticopeptic cells located in the glands of the stomach

of fishes (Bomgren *et al.*, 1998). The activity of gastric proteases depends on the degree of acidification attained in the lumen of the stomach, this is the reason why the activity of acidic proteases is measured in relation to gastric pH (Márquez *et al.*, 2012). For example, when dealing with feeding protocols (Chiu & Pan, 2002; Campoverde *et al.*, 2017; Bravo *et al.*, 2018), the inclusion of new protein sources (Sotoudeh *et al.*, 2016) and the utilization of feed additives (Castillo *et al.*, 2014) in larval or juvenile fish. In general, the proteolytic activity of gastric extracts from fish shows a maximum value in the pH range 2.0-3.0 and a subsequent steep drop before the pH reaches 4.0 (Munilla-Morán & Rey-Saborido, 1996a; Alarcón *et al.*, 1998; Chong *et al.*, 2002; Xiong *et al.*, 2011; Peña *et al.*, 2015). Thus, the range of pH between 2.0 and 4.0 is particularly interesting to understand the proteolytic capacity of the stomach in fish.

Nevertheless, in addition to the apparent effect of pH, the enzymatic activity is also affected by the ionic strength, a factor not so frequently investigated about digestive enzymes of fish. It is known that the ionic strength (IS) determines the folding of proteins in solution (Gabrielczyk *et al.*, 2017) and/or the protein/ligand binding affinity (Papaneophytou *et al.*, 2014), and there is no a priori reason to rule out these effects in the case of digestive enzymes of fish or any other animal taxon. Indeed, the addition of NaCl to the reaction buffer changes the amylolytic and proteolytic activities of gut extracts from marine fish, the size of the change being dependent on the kind of enzymatic activity and the fish species under research (Munilla-Morán & Rey-Saborido, 1996a,b). Data on the ionic environment in the gut of fish are scarce, although a value for gastric IS over 200 mM can be inferred from the detailed work by Bucking & Wood (2009) on the postprandial distribution of Na⁺, K⁺, Ca²⁺, Mg²⁺ and Cl⁻ along with the gut of juvenile rainbow trout. The present work is aimed at disclosing the combined effects of pH and ionic strength on the proteolytic activity of gastric extracts of the Coho salmon, *Oncorhynchus kisutch*, a cultivated species of commercial importance in Chilean aquaculture. According to SERNAPESCA (2019), the Chilean production of Coho salmon reached approx. 15 and 19% of the total biomass of salmonid species cultured in 2016 and 2017, respectively.

Juveniles of Coho salmon were stocked in 500 L tanks connected to a freshwater flow-through system at the facilities of Departamento de Ciencias Agropecuarias y Acuícolas of Universidad Católica de Temuco (Chile). Fish received a daily ration (1% body weight) of a 53/17 protein/lipid diet. The tank was regularly cleaned from feces and uneaten feed. Sampling protocols always included anesthesia of fish with isoeugenol (Aqui-s; Bayer Animal Health, Santiago, Chile) prior of being euthanized according to Chilean legal requirements on experimental animals (Ministerio de Salud de Chile, Ley 20380). Crude gastric extracts were obtained from the stomachs of seven fish weighing 92 ± 11 g after dissection and homogenization of the tissue in 0°C distilled water (1:3 w/v). Extracts were centrifuged at 15,800 g for 15 min at 4°C, and the supernatant stored in aliquots at -20°C until used in assays of acid protease activity by the method of Anson (1938). The substrate used was bovine hemoglobin 0.5% w/v in citric buffer 30 mM. The buffer was prepared at pH values 2.50, 3.00, 3.50, 3.75 and 4.00 and the ionic strength (IS) to 50, 100 and 200 mM (with NaCl), thus resulting in a 5×3 factorial experiment. The experimental range of pH was selected according to i) the recorded values of postprandial gut

pH in salmonids (Bucking & Wood, 2009; Bravo *et al.*, 2018) and, ii) the general pattern of activity of fish gastric proteases in relation to pH, with a maximum at 2.0-3.0 and a subsequent substantial drop before pH reaches 4.0. The range of ionic strength was selected by considering the postprandial ionic composition of the gastric chyme in salmonids (Bucking & Wood, 2009), implying an ionic strength over 200 mM, and also the reaction buffers used to test acid proteases *in vitro* which often, contrary to the fluid phase of the gastric chyme, do not contain additional ions affecting ionic strength. Bovine hemoglobin was chosen as the suitable protease substrate since it is frequently utilized in works dealing with fish gastric proteases. Extracts were diluted in distilled water 1:8 v/v before the assay, which was carried out for 15 min at 25°C. The reaction was stopped with 0.5 mL of 20% w/v trichloroacetic acid and centrifuged at 15,800 g for 15 min at 20°C. The supernatant was collected and the absorbance at 280 nm was measured (Perkin-Elmer Lambda 25 UV/Vis Spectrophotometer). Two experimental assays and one blank assay were performed for each combination of factor levels and each fish, resulting in more than 300 spectrophotometric measurements. The activity was estimated from the difference in absorbance between the experimental assays and the blank, and setting the extinction coefficient of L-tyrosine at 0.008 mL (μg cm)⁻¹. The protease activity was expressed as μg L-Tyr released per min and g of gastric tissue. Data on protease activity were analyzed using a two-factor (pH, IS) 5×3 repeated-measure ANOVA. Type I error was fixed at 0.05.

Both factors, pH and the ionic strength of the buffer, exerted a significant effect on protease activity (Tables 1 and 2). The activity showed a maximal value in the pH range 2.5-3.0 and dropped as pH increased from 3.0 to pH 4.0 for IS 50 mM and 100 mM, following a convex pattern for all the levels of ionic strength tested. In the case of IS 200 mM, protease activity showed a general decreasing pattern in the pH range 3.0-4.0, barring the pH sub-interval 3.50-3.75, for which the activity seemed to maintain its value close to 12,000 units. On the other hand, the activities obtained at 50 mM of IS were always lower than those for 100 and 200 mM, respectively. The interaction term was significant, most probably indicating that values for IS 200 mM tended to be below values for IS 100 mM just in the pH interval 2.5-3.5, but the reverse pattern was apparent in the pH interval 3.5-4.0. It is useful to check the ratio (activity at pH 3.0)/(activity at pH 4.0), to understand the relationship between the effects of pH and IS on the activity of gastric proteases, whose values were 2.34, 2.19 and 1.88 for IS values of 50, 100 and 200 mM, respectively. In addition, the complementary ratio (activi-

Table 1. Acid protease activities of gastric extracts from juvenile Coho salmon (*Oncorhynchus kisutch*) concerning pH and ionic strength (mM). Activities are expressed as mean values ($n = 7$) \pm SEM (standard error of the mean). The activity unit is defined as 1 μ g of tyrosine released per min and g of gastric tissue.

pH	Ionic strength (mM)		
	50	100	200
2.50	12,539 \pm 1,071	15,028 \pm 1,279	14,351 \pm 917
3.00	12,382 \pm 897	15,351 \pm 987	15,010 \pm 742
3.50	10,714 \pm 757	13,219 \pm 794	11,686 \pm 968
3.75	8,312 \pm 596	10,852 \pm 652	12,090 \pm 841
4.00	5,281 \pm 308	6,997 \pm 391	7,989 \pm 416

vity at ionic strength 200 mM)/(activity at ionic strength 50 mM) was 1.21 and 1.51 for pH values 3.0 and 4.0, respectively.

The pH-profile of gastric proteases of the Coho salmon resembles that reported for other fish species (Alarcón *et al.*, 1998; Castillo-Yáñez *et al.*, 2004; Xiong *et al.*, 2011). The same basic pattern was obtained for IS ranging 50-200 mM, although the activity at 50 mM stood below those at 100 and 200 mM. From the point of view of the gastric function, this result is in keeping with the known ionic conditions in the stomach lumen after the ingestion of commercial pellets by juvenile trout, where the IS due to the main ions is above 200 mM (Bucking & Wood, 2009). Nevertheless, the direction of the effect of IS is probably species-specific, since a decrease in the caseinolytic activity of gastric extracts from three species of marine fish was reported by Munilla-Morán & Saborido-Rey (1996a) when the concentration of NaCl in the reaction medium (100 mM HCl-glycine buffer, pH 2.0) raised from 0 to 200 mM. Alternatively, it can be thought that the pH-profile is dependent on the chosen protease substrate (which is usually bovine hemoglobin when following the Anson protocol (Anson, 1938)). The pH-profile of fish gastric proteases in the pH range 3.0-4.0 is of particular interest because it usually implies a change from optimal to suboptimal activity. The interactive effect between pH and IS (Table 2) is probably related to the following result: the protease activity at ionic strength 100 mM is above that one at ionic strength 200 mM provided the pH is \leq 3.50, but the opposite is true when pH is \geq 3.75.

This result is in keeping with the potential effects of ionic strength on protein structure and protein-ligand binding affinity (Papanephytou *et al.*, 2014). The activity ratios (pH 3.0)/(pH 4.0) and (ionic strength 200 mM)/(ionic strength 50 mM) are beneficial to illustrate the way both factors combine to determine the activity

Table 2. Repeated-measures ANOVA on total acid protease activity ($U\ g^{-1}$ stomach) from Coho salmon (*Oncorhynchus kisutch*) stomach homogenates by pH and ionic strength. Both factors are treated as repeated measures factors. IS: ionic strength in mM; df: degrees of freedom; MS: mean squares; F: F-value; P: P-value.

Source	df	MS	F	P	Homogeneous groups
IS	2	6.8 \times 10 ⁷	37.8	<0.001	50 vs 100, 200
pH	4	2.0 \times 10 ⁸	97.4	<0.001	2.5, 3.0 vs 3.5 vs 3.75 vs 4.0
IS \times pH	8	3.1 \times 10 ⁶	4.7	<0.001	-

of gastric proteases. The revision of both ratios indicates that the further one of the factors is from its optimal range, the stronger is the effect of a change towards sub-optimal values in the other one. In this way, IS can soften the relative drop of activity associated with an increase in gastric pH, as long as IS maintains around 200 mM. An interesting derived hypothesis is the potential effect of the mineral composition and solubility of the diet on the protease activity at the beginning of digestion, when the gastric pH is high, through the ionic environment of the gastric chyme. In species such as the rainbow trout *Oncorhynchus mykiss*, the period for which the gastric pH keeps at high values (4.0-5.0) extends for many hours after feed ingestion (Bucking & Wood, 2009).

On the other hand, considering that in other fish species the ratio of acid protease activity (pH 3.0)/(pH 4.0) can be higher than 5.0 (Alarcón *et al.*, 1998), it can be concluded that this ratio may differ among fish families. Another line to be explored in the future is the consequences on the solubility of dietary proteins in the stomach of fish. Previous *in vitro* results indicate that the solubility of glycinin, one of the more abundant proteins in soybean depends strongly on both pH and IS (Lakemond *et al.*, 2000), which is important for protein digestion because solubilized proteins can be more easily accessed by proteases.

In summary, the results are here presented to support the following facts in relation to the gastric proteases of the Coho salmon: i) an optimum pH in the range 2.5-3.0, ii) a significant effect of ionic strength (IS) on the activity of gastric proteases, with the lowest values when IS is below 100 mM, and iii) a mild interaction between the effects of pH and IS. Thus, we propose taking into account, not only the pH but also the ionic strength of the reaction medium when assaying the activity of fish gastric proteases.

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