ORIGINAL RESEARCH

Comparative assessment of digestive enzyme activities of five fish species from the southwestern Atlantic Ocean

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ABSTRACT. Due to the scarcity of data cited in the literature, digestive enzyme profiles of fish species from the southwestern Atlantic Ocean are of particular interest. In the present study, the viscera yield and digestive enzymes (acid and alkaline proteinase, lipase and amylase) of the stripped weakfish *Cynoscion guatucupa*, Brazilian codling *Urophycis brasiliensis*, Patagonian flounder *Paralichthys patagonicus*, southern eagle ray *Myliobatis goodei* and smallnose fanskate *Sympterygia bonapartii* were determined. All species exhibited high proteolytic activity (acid: 0.44-11.0 UE mg protein^{-1} and alkaline: 0.11-2.32 UE mg protein^{-1}) as well as moderate lipase (0.07-0.76 UE mg protein^{-1}) and amylase activity (0.03-0.24 UE mg protein^{-1}). Teleost fish exhibited higher enzyme activities than cartilaginous fish, with *U. brasiliensis* exhibiting the highest activities (proteinases, amylases, and lipases). High-activity enzymes from cold-temperate-adapted organisms, mainly from *U. brasiliensis* and *C. guatucupa*, may be the source of marine biotechnological bioactive compounds that are beneficial for biotechnological processes.

Key words: Digestive enzymes, marine fish, Argentine Sea.

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Evaluación comparativa de las actividades de las enzimas digestivas de cinco especies de peces del Océano Atlántico Sudoccidental

RESUMEN. Debido a la escasez de datos citados en la literatura, los perfiles de enzimas digestivas de especies de peces del Océano Atlántico Sudoccidental son de particular interés. En el presente estudio, se determinó el rendimiento por víscera y las enzimas digestivas (peptidasa ácida y alcalina, lipasa y amilasa) de la pescadilla de red *Cynoscion guatucupa*, la brótola *Urophycis brasiliensis*, el lenguado patagónico *Paralichthys patagonicus*, el chucho *Myliobatis goodei* y la raya mermorada *Sympterygia bonapartii*. Todas las especies exhibieron alta actividad proteolítica (ácida: 0,44-11,0 UE mg proteína^{-1} y alcalina: 0,11-2,32 UE mg proteína^{-1}), así como moderada actividad de lipasa (0,07-0,76 UE mg proteína^{-1}) y amilasa (0,03-0,24 UE mg proteína^{-1}). Los peces teleósteos exhibieron actividades enzimáticas más altas que los peces cartilaginosos, siendo *U. brasiliensis* el que presentó las actividades más altas (proteinasas, amilasas y lipasas). Las enzimas de alta actividad de organismos adaptados a climas fríos, principalmente de *U. brasiliensis* y *C. guatucupa*, podrían ser la fuente de compuestos bioactivos biotecnológicos marinos beneficiosos para los procesos biotecnológicos.

Palabras clave: Enzimas digestivas, peces marinos, Mar Argentino.

INTRODUCTION

Argentina's maritime territory extends over 4,000 km of coastline on the southwestern Atlantic. Demersal fish assemblages in the northern Argentine continental shelf (34° S-41° S), which corresponds to Buenos Aires Province coast, are extremely diverse (FAO 2011). They are distributed along the continental shelf, which exhibits latitudinal fluctuations in salinity and temperature (Piola et al. 2000). Their habitats vary from demersal to benthic, with depths ranging from 60 to 150 m. Eating habits also vary as they consume different proportions of fish, crustaceans and mollusks (Acuña Plavan and Verocai 2001; Molina and Cazorla 2015).

In the present study, we investigated three bony fish belonging to the Class Actinopterygii and two cartilaginous fish belonging to the Class Elasmobranchii. The selected Actinopterygians belong to three taxonomic orders (Perciformes, Gadiformes and Pleuronectiformes) while the two selected Elasmobranchians belong to two distinct taxonomic orders (Rajiformes and Myliobatiformes). The chosen species are representive of different taxa and are part of the fish diversity found in demersal communities from the southwestern Atlantic coasts. Furthermore, the studied species are subjected to industrial exploitation by marine fisheries. They are all captured and landed in great quantities and they all have commercial value (SSPyA 2024). These species are often caught unintentionally as bycatch and discarded before landing (Bovcon et al. 2013). Additionally, inland fish processing generates a considerable amount of waste. Instead of being discarded, digestive organs could be utilized for enzyme extraction, providing a wide array of enzymes with unique properties. This would reduce pollution and the environmental impact of fishing while also providing sources of marine bioactive compounds for biotechnological applications.

The digestion processes in fish is similar to those of other vertebrates. Proteinases, amylases, and lipases catalyze the hydrolysis of macronutrients, breaking down the ingested material into smaller size molecules (Karasov and Douglas 2013). Acid proteinases are found in the gastric chamber, whereas pancreatic proteinases, which act at higher pH levels, are present in the intestinal lumen. These enzymes play a key role in fish metabolism since aminoacid intake and assimilation are crucial for fish growth and overall metabolism. Pancreatic lipases, which depend on bile salts for the digestion of insoluble substrates, are the main lipid digestive enzyme in several fish species (Farrel 2011). Carbohydrate digestion proceeds at a very low rate in carnivorous fish, as they are not nutritionally important for their requirements and metabolism (Jiao et al. 2023; Yu et al. 2024). Omnivorous and herbivorous fish can vary their intestinal enzymatic activity in order to generate higher activities when more carbohydrates are ingested (Farrel 2011). Moreover, the five species studied in this work inhabit cold temperate waters below 20 °C (Piola et al. 2000). Enzymes from cold temperate-adapted ectothermic organisms could have great potential for use in the industry due to their high activity at low temperatures.

The nature of digestive enzymes varies depending on the habitat and climate in which fish live. Temperature adaptation of these enzymes involves differences in structure, substrate affinity, and activation energy, as well as changes in secretion rates and the production of isozymes-enzymes that catalyze the same reaction but with optimal efficiencies at different temperatures (Volkoff and Rønnestad 2020). Effects of water temperature on fish digestive enzyme activities appear to be species-specific, as the optimal temperature for enzyme activity generally falls within the thermal range of the species' natural habitat. Reports on enzymes from cold-adapted organisms have shown that adaptive strategies for functioning at low temperatures vary among enzymes. Analyses of structural features important for stability (e.g.

intra-molecular hydrogen bonds and ion pairs; proline, methionine, glycine, or arginine content; surface hydrophilicity; helix stability; and core packing) indicate that each cold-adapted enzyme or enzyme system employs a distinct combination of structural adjustments to increase molecular flexibility, thereby enhancing catalytic efficiency (Arne et al. 2000).

The aim of this study was to determine proteinase (acid and alkaline), lipase and amylase activities, as well as viscera yield in the striped weakfish *Cynoscion guatucupa* (Cg) (Cuvier, 1830), Brazilian codling *Urophycis brasiliensis* (Ub) (Kaup, 1858), Patagonian flounder *Paralichthys patagonicus* (Pp) (Jordan, 1889), smallnose fanskate *Sympterigia bonapartii* (Sb) (Müller and Henle, 1841), and southern eagle ray *Myliobatis goodei* (Mg) (Springer, 1939).

MATERIALS AND METHODS

Biological samples

Fishes were obtained during the Maritime Littoral Campaign (March-2018, Mar del Plata, Argentina), carried out by the Instituto de Investigaciones Marinas y Costeras (IIMyC) (Project PUE-CONICET) (Table 1). The fish were dissected ventrally. Viscera were removed and their contents emptied. Stomachs, intestines and pyloric caeca were used for subsequent tests. Each sample (stomach or intestine with pyloric caeca) was individually homogenized in chilled distilled water (1:3 w v^{-1}). The soluble protein fraction was obtained by centrifugation (Presvac EPF-12R, Argentina) for 30 min at 4 °C and 10,000 g. Supernatants were separated as extracts for all enzymatic assays. Enzymatic extracts from the intestine were frozen at -20 °C. Extracts from stomachs were maintained overnight at 4 °C to activate the pepsinogen and then stored at -20 °C until use (Friedman et al. 2020).

Biochemical analysis

Soluble protein in crude extracts of stomach and intestine (mg protein ml^{-1} extract) was measured according to Bradford (1976), using bovine serum albumin (Sigma A9647) as standard. Acid proteinase activity was determined in stomach simples whereas alkaline proteinase, lipase and amylase activities were evaluated in intestine samples. Acid proteinase activity was determined at pH 2.0 in a substrate solution containing 0.5% (w v^{-1}) bovine haemoglobin (Sigma H2625) in 200 mM Glycine-HCl Buffer according to Anson (1938). Al-

Table 1. Scientific name, abbreviation, taxonomic classification, number of samples used and distribution areas of studied fishes from the southwestern Atlantic Ocean.

Species	Abbreviation	Class, order, family	Samples	Distribution area
Cynoscion guatucupa	Cg	Actinopterygii, Perciformes, Sciaenidae	9	22° S-43° S (Caille et al. 1997)
Urophycis brasiliensis	Ub	Actinopterygii, Gadiformes, Phycidae	6	23° S-40° S (Cousseau 1993)
Paralichthys patagonicus	Pp	Actinopterygii, Pleuronectiformes, Paralichthyidae	4	23° S-43° S (Díaz de Astarloa 1994)
Sympterigia bonapartii	Sb	Elasmobranchii, Rajiformes, Arhynchobatidae	10	23° S-54° S (Menni and Stehmann 2000)

kaline proteinase activity was determined using 0.5% (w v^{-1}) azocasein (Sigma A2765) in 50 mM Tris-HCl Buffer, pH 7.5, according to García-Carreño (1992). Absorbance was recorded at 280 nm and 366 nm, respectively.

Lipase enzymatic activity was measured at pH 8.0 using the method described by Versaw et al. (1989) and its modification by Nolasco-Soria et al. (2018), using 20mM β-naphthyl caprylate as substrate. Activity was determined by measuring the release of β-naphthyl in a microplate spectrophotometer at 540 nm. Amylase activity was measured following Bernfeld (1955), as the rate of production of glucose from starch (1% w v^{-1}) as substrate at pH 7.5. The glucose produced during the incubation was measured by the DNS (Dinitrosalicylic Acid) method (Vega-Villasante et al. 1993).

All assays were performed in triplicate at room temperature. Specific enzymatic activities were expressed as units of activity per mg protein (UE mg $protein^{-1}$). Total enzymatic activities were expressed as units of activity per ml enzymatic extract (UE ml enzymatic $extract^{-1}$). The viscera yield (Y) was calculated as units of activity per g of tissue (UE gr $tissue^{-1}$). One unit of activity corresponds to the change in absorbance per minute (UE = ΔAbs min^{-1}).

Statistical analysis

Results were presented as means plus minus (±) standard error of the mean (SEM). Enzymatic activities among species were compared through Generalized Linear Mixed Models. Models were constructed using the function lme from the nmle package (Pinheiro et al. 2017) and they were validated using ANOVA. Differences between species for a specific activity assay were contrasted using Tukey. All statistical analyses were conducted in R 2.13.0.

RESULTS

The highest acid and alkaline proteinase specific activity was observed in *Urophycis brasiliensis* (11.0 ± 1.75 and 2.32 ± 0.41 UE mg $protein^{-1}$, respectively), which were statistically different from those of the other species (Table 2). Similarly, the highest specific lipase and amylase activities were observed for Ub (0.76 ± 0.11 and 0.24 ± 0.02 UE mg $protein^{-1}$, respectively). Lipase activity showed a trend of being more than 50% higher in *U. brasiliensis* compared to the other species (Table 3).

Table 2. Total and specific activity of acid and of alkaline proteinase of *Urophycis brasiliensis* (Ub), *Cynoscion guatucupa* (Cg), *Paralichthys patagonicus* (Pp), *Sympterigia bonapartii* (Sb) and *Myliobatis goodei* (Mg).

	Acid proteinase		Alkaline proteinase	
Species	TA	SA	TA	SA
Cg	2.34 ± 0.31^{a}	3.21 ± 0.38^{a}	0.7 ± 0.10^{a}	0.98 ± 0.10^{a}
Ub	2.69 ± 0.36^{ab}	11.0 ± 1.75^{b}	0.87 ± 0.11^{b}	2.32 ± 0.41^{b}
Pp	1.93 ± 0.11^{a}	2.51 ± 0.17^{ac}	0.64 ± 0.17^{ab}	0.94 ± 0.26^{a}
Sb	1.03 ± 0.14^{c}	0.44 ± 0.07^{c}	0.35 ± 0.06^{ab}	0.14 ± 0.22^{c}
Mg	1.67 ± 0.17^{bc}	0.76 ± 0.20^{c}	0.71 ± 0.18^{ab}	0.11 ± 0.03^{c}

Total proteinase activity (TA) and specific proteinase activity (SA) was expressed as UE mL^{-1} and UE mg $protein^{-1}$, respectively. Data are indicated as means (±) standard error of the mean (SEM). Different letters in the same column, indicate significant differences among species.

Viscera yield values varied significantly among species, but at the same other enzymatic results. *Urophycis brasiliensis* showed the highest yield values for all tested enzymes (Table 4). Regarding enzyme activity values and yield per viscera, *C. guatucupa* ranked second in importance, whereas *P. patagonicus*, although showing a tendency to occupy third place, this was not supported by statistical results, which could reflect the limited number of samples.

DISCUSSION

The five fish species analyzed here showed considerable activity differences between them. Enzyme activity values were in general substantially lower for cartilaginous than in teleostean fish. The different enzymatic activities can be related to the phylogenetic groups to which the fish belong (Chan et al. 2004; Chaudhuri et al. 2012). Variations in

Table 3. Total and specific lipase and amylase activity of *Urophycis brasiliensis* (Ub), *Cynoscion guatucupa* (Cg), *Paralichthys patagonicus* (Pp), *Sympterigia bonapartii* (Sb) and *Myliobatis goodei* (Mg).

	Lipase		Amylase	
Species	TA	SA	TA	SA
Cg	0.18 ± 0.02^{a}	0.38 ± 0.07^{ab}	0.08 ± 0.01^{a}	0.12 ± 0.01^{a}
Ub	0.29 ± 0.02^{ab}	0.76 ± 0.11^{b}	0.24 ± 0.01^{b}	0.24 ± 0.02^{b}
Pp	0.17 ± 0.01^{a}	0.21 ± 0.03^{a}	0.05 ± 0.01^{a}	0.05 ± 0.01^{ac}
Sb	0.12 ± 0.02^{a}	0.07 ± 0.01^{a}	0.09 ± 0.02^{a}	0.03 ± 0.01^{c}
Mg	1.24 ± 0.31^{b}	0.12 ± 0.03^{a}	0.03 ± 0.01^{a}	0.12 ± 0.03^{a}

Total enzyme activity (TA) and specific enzyme activity (SA) was expressed as UE mL^{-1} and UE mg $protein^{-1}$, respectively. Data are indicated as means (±) standard error of the mean (SEM). Different letters in the same column, indicate significant differences among species.

Table 4. Viscera yield of acid and alkaline proteinase, lipase and amylase of *Urophycis brasiliensis* (Ub), *Cynoscion guatucupa* (Cg), *Paralichthys patagonicus* (Pp), *Sympterigia bonapartii* (Sb) and *Myliobatis goodei* (Mg).

	Proteinase			
Species	Acid	Alkaline	Lipase	Amylase
Cg	2.08 ± 0.45^{a}	0.67 ± 0.08^{a}	0.23 ± 0.04^{a}	0.08 ± 0.01^{a}
Ub	8.27 ± 1.37^{b}	1.80 ± 0.30^{b}	0.80 ± 0.17^{b}	0.21 ± 0.02^{b}
Pp	1.17 ± 0.09^{a}	0.47 ± 0.13^{a}	0.11 ± 0.01^{a}	0.03 ± 0.00^{ac}
Sb	0.22 ± 0.04^{a}	0.06 ± 0.01^{a}	0.03 ± 0.02^{a}	0.02 ± 0.00^{c}
Mg	0.37 ± 0.08^{a}	0.05 ± 0.02^{a}	0.06 ± 0.01^{a}	0.06 ± 0.01^{ac}

Viscera yield (Y) was expressed as U gr^{-1} tissue. Data are indicated as means (±) standard error of the mean (SEM). Different letters in the same column, indicate significant differences among species.

values obtained could also be related to the species' feeding habits. Their digestive organs, particularly the intestine, reflect fish feeding habits. Fish digestive enzyme activities are closely related to the diet and the fish ability to digest and absorb different nutrients (Liu et al. 2021). Previous studies found that herbivorous fish such as Roho labeo (*Labeo rohita*) and Japanese eel (*Anguilla japonica*) have stronger amylase activity than carnivorous fish such as great white catfish (*Wallago attu*) (Agrawal et al. 1975) and rainbow trout (*Oncorhynchus mykiss*) (Hidalgo et al. 1999). In the present work, all fish species showed elevated proteinase activities. These elevated values could be associated with their carnivorous diet (Jiao et al. 2023; Yu et al. 2024).

Urophycis brasiliensis had the highest values for both enzyme activities (total and specific activity) and viscera yield, with *C. guatucupa* coming in second. Regarding feeding habits, *U. brasiliensis* preys on crustacean, fish, mollusks, and annelids, displaying opportunistic behavior (Acuña Plavan et al. 2007). *Cynoscion guatucupa* feeds mainly on crustacean, but as ontogeny progresses, individuals begin to consume fish (Sardiña and Lopez Cazorla 2005). On the other hand, *P. patagonicus* also preys mostly on pelagic fish (Troccoli et al. 2021). The skate *S. bonapartii* preys on various fish species (Castello et al. 1997), while *M. goodei* shows a uniform diet, feeding mainly on bivalves (Molina and Cazorla 2015). Differences in predatory behaviors and dietary items could explain distinct enzymatic activities. Generalist and opportunistic feeding behavior, along with a wide range of habitats, could be associated with high enzyme activity.

Friedman and Fernández-Gimenez (2024) recently conducted a systematic review of the state of knowledge regarding marine fish digestive enzymes at a global level. In this sense, the authors reported limited studies addressing species distributed exclusively in the southwest Atlantic Ocean, such as whitemouth croaker (*Micropogonias furnieri*), Parona leatherjacket (*Parona signata*) and Brazilian flounder (*Paralichthys orbignyanus*). In the case of the Brazilian flounder, the present study determined the enzymatic activity of intestinal proteinases using azocasein as a substrate under alkaline conditions. This study made no distinction between alkaline peptidases classes, nor did it evaluate their optimal biochemical conditions. However, Candiotto et al. (2018) reported the presence of trypsin, chymotrypsin, and aminopeptidases in the intestine using specific substrates and inhibitors, and observed optimal pH values of 9.5, 9.0, and 8.0, respectively, with an optimal temperature of 50 °C. Additionally, Friedman et al. (2022, 2023, 2024) stated that the most studied digestive enzymes in fishes are alkaline proteinases, with a general lack of information on other hydrolytic enzymes, making this an area of research vacancy. The same authors studied alkaline proteinases of several species from the Argentine Sea, including Argentine hake (*Merluccius hubbsi*), Brazilian flathead (*Percophis brasiliensis*), *U. brasiliensis*, and *C. guatucupa*. In this regard, Friedman et al. (2022) reported that stomach proteinases of *U. brasiliensis* and *C. guatucupa* exhibited the highest stability at pH range of 2.0-4.0. Alkaline proteinases of both species were highly stable between pH 7.0 and 11.5. Optimal temperatures were 30 °C and 50 °C, with stability at 10 °C and 30 °C for 150 min. The optimal temperature of intestinal enzymes was 50 °C, with high stability at 10 °C and 30 °C for 150 min. Furthermore, Friedman et al. (2023) conducted a kinetic characterization of crude extracts of alkaline proteinases from the intestine and pyloric caeca of *U. brasiliensis* and *C. guatucupa*, demostrating that maximum A440 values were dependent on both the species analyzed and the initial concentration of azocasein. Finally, Friedman et al. (2024) observed that the aspartic proteinase activity of these species was stable in the presence of Mn^{2+}, K^{+}, and Ca^{2+}, and that the surfactant Tween 20 increased enzyme activity in all cases, whereas Tween 80 had a positive effect only on proteinases from *U. brasiliensis*. Enzymes from both species were stable in the presence of hydrogen peroxide, and notably, the aspartic proteinases of *C. guatucupa* were compatible with the tested commercial detergents. While studies by Friedman et al. (2022, 2023, 2024) focused on advancing technological knowledge of proteinas-

es, the present work emphasizes a comparative analysis of different enzymes, acid and alkaline proteinases, amylases, and lipases, from five fish species of the southwest Atlantic Ocean.

Proteinase, amylase and lipase activities of a few other sciaenids (*C. othonopterus*, *C. parvipinnis* and *C. xanthulus*) have been reported (González-Félix et al. 2020). Regarding flatfishes, various enzyme measurements have been carried out in the genus *Paralychtys* spp., mainly *P. olivaceus*, in which different types of proteinases, carbohydrates degrading enzymes and lipases have been detected (Candiotto et al. 2018; Tian et al. 2002). Enzymes have also been detected in *P. orbignyanus* (del Valle et al. 2016) and *P. californicus* (Zacarias-Soto et al. 2006). Studies on members of the family *Phycidae*, particularly *Urophycis* sp., as well as on two chondrichthyan species, *Myliobatis* spp. and *Sympterygia* spp., are scarce. In this context, Lamas and Massa (2023) extracted and purified enzymes from the gastrointestinal tract of *M. goodei* using low-cost processes. Peptidases and lipases were characterized, demonstrating that purified enzymes were stable in the presence of commercial detergents, and that contributed positively to the removal of protein materials.

Enzymes are used in a wide range of bioprocesses (Fernández-Gimenez 2019) as those occurring in detergent, leather, food, agrochemical, pharmaceutical, chemical and waste treatment industries (Bougatef 2013; Homaei et al. 2016). Enzymatic extracts from marine organisms have been previously assessed for different biotechnological applications, demonstrating promising results as on active agent in antifouling paints (Lenchours Pezzano et al. 2024), in the production of hydrolysates with high nutritional content (Pereira et al. 2022), and as additives in aquaculture (Rodriguez et al. 2019, 2024; del Valle et al. 2022) and cheese making (Pereira and Fernández-Gimenez 2017). Thus, marine fish from cold temperate water, primarily from *U. brasiliensis*, followed by *C. guatucupa*, could be a source of compounds with unknown properties and novel biotechnological applications. Additional assays are required for the study of enzymes measured in these preliminary determinations. Stability in pH and temperature assays carried out with these enzymes would be of special interest for further studies, especially in the case of proteinases with high activities. Furthermore, determination of enzyme activity with different concentrations of industrial compounds, such as salts or detergents, would provide new useful information about their potential applications.

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Author contributions

María Cecilia Bonadero: formal analysis; data curation; writing-original draft. María Victoria Laitano: formal analysis; data curation; writing-review and editing. Juana Cristina del Valle: formal analysis; data curation; writing-review and editing. Yamila Eliana Rodriguez: formal analysis;data curation; writing-review and editing. Nair de los Ángeles Pereira: formal analysis; data curation; writing-review and editing. Analía V. Fernández Gimenez: formal analysis; data curation; writing-review and editing; supervision; project administration; funding acquisition.

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