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KINETIC CHARACTERIZATION OF DIGESTIVE PROTEINASES EXTRACTED FROM THE PROCESSING WASTE OF SOUTH ATLANTIC FISH

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Abstract

In Argentina, the fishing industry is highly developed, producing large quantities of waste products from fish processing which contain digestive enzymes with potential biotechnological uses. Among these enzymes are proteinases that hydrolyze proteins and can convert them into functional ingredients. To use these value-added compounds in industrial processes, kinetic characterization of these proteinases under specific conditions, such as contact time, pH, and temperature, must first be carried out. The objective of the present work was to perform a kinetic characterization of crude extracts of alkaline proteinases from intestine-ceca of *Merluccius hubbsi, Percophis brasiliensis, Urophycis brasiliensis* and *Cynoscion guatucupa.* Results showed that the reached maximum values of A440 depended on both the species analyzed and the initial concentration of azocasein. The maximum concentration of hydrolysis products obtained at long times was proportional to the initial concentration of the target protein. *P. brasiliensis* was the most suitable tested species to obtain high hydrolysis rates at high azocasein concentrations, and exhibited the highest proteinase activities, so it can be considered a candidate species for future biotechnological applications.

Keywords: Fish, Waste, Digestive proteinases, Kinetic modeling

Statements and Declarations

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Introduction

Argentina has enormous marine wealth, being a reference country due to its fishing resources and the great development of its fishing industry (Sánchez-Carnero et al., 2022). Industrial fisheries export almost all their catches. In 2020, the fishing sector exported 464,730 tons to more than 100 countries, making shrimp, hake, and squid the main exported species (Rozycki et al., 2021). The Argentine hake (*Merluccius hubbsi*) is the main fishing resource, with nearly 284 mil tonnes captured in 2022. In the same year, the Brazilian flathead (*Percophis brasiliensis*) and the stripped weak-fish (*Cynoscion guatucupa*) captures were 6076 t and 5043 t, respectively (MAGyP, 2022). Besides, Brazilian codling (*Urophycis brasiliensis*), Brazilian flathead, and stripped weak-fish appear as by-catch in the hake fishery (Bovcon et al., 2013).

As a general rule, fish processing yields large quantities of protein-rich wastes with a high nutritional value (Aspevik et al., 2017). Although a fraction of these residues is used in the elaboration of fishmeal and fish oil, in some cases wastes are discarded directly into the environment without any processing (González et al., 2010; Pereira and Fernández-Jiménez, 2016). However, instead of being discarded and contributing to environmental pollution, these wastes could become a new source of profit for fishing and other related industries (Zhu et al., 2020). These residues could be incorporated as components in industrial processes to obtain value-added food products or as functional ingredients (Pal and Suresh, 2016; Haryati et al., 2021).

Several high-value enzymes are present in fish waste, being proteinases among the most used ones in industrial bioprocesses (Atta et al., 2017). Proteinase-based processes have been widely used in the food industry, such as dairy, brewing, beverages, and dietary supplements (Fernandes, 2010). Besides the food industry, proteinases are also used in other industries such as leather, pulp, detergent, textiles, and personal care (Tapal and Tiku, 2019). In particular, two types of proteinases are distinguished in the digestive tract of fish: exoproteases that hydrolyze an amino acid from the N-terminus (amino peptidases) or from the C-terminus (carboxypeptidases), and endoproteases or proteinases that break peptide bonds distributed along the chain (Klomklao, 2008). The inclusion of these proteinases in the development of new industrial, agricultural, food, and pharmaceutical products offers promising alternatives that lead to a circular economy (Osorio et al., 2021).

In the case of proteinases from fish waste, it is necessary to perform a kinetic characterization under specific conditions to evaluate the suitability of their use in industrial processes. In general, hydrolysis kinetics has been studied in combination with the production of protein hydrolysates. Several authors report that different factors, such as enzyme concentration, temperature, pH, ionic strength, time, and initial substrate concentration, are key parameters that significantly affect the degree of hydrolysis (DH) (Márquez and Vázquez, 1999; Qi and He, 2006; Chalamaiah et al., 2012; Hardt et al., 2013; Butré et al., 2014; Lassoued et al., 2015; Najafian and Babji, 2015). Considering that the main problems associated with the enzymatic hydrolysis of proteins are the production of a

bitter taste, coagulation, and the high cost of enzymes, the prediction and control of DH are crucial to optimize a given bioprocess (Tapal and Tiku, 2019).

The quantitative evaluation of proteinase activity is usually performed by spectrophotometric procedures (Landi et al. 2011). In the case of alkaline hydrolysis, azocasein is used as a substrate to detect proteinase activity because of its intense color and accessible availability in commercial format (Nolasco and Soria, 2021). The azocasein colorimetric method was developed many years ago by Charney and Tomarelli (1947) and then underwent modifications to optimize it. Azocasein is an azoprotein synthesized by the diazotization of the aromatic amino acids, mainly tyrosine, present in the casein (Akpinar and Penner, 2002). The proteolytic hydrolysis of azocasein releases dye-containing short peptides as the hydrolysis product. To quantitatively analyze these products, the remaining (e.g., unhydrolyzed) azocasein must be separated by denaturation with trichloroacetic acid (TCA), and then, precipitated by centrifugation the precipitated. Finally, soluble dye-containing short peptides can be evaluated at 440 nm (Coelmont et al. 2023).

In a systematic review presented by Friedman and Gimenez-Fernandez (2023) on the state of knowledge about biotechnological uses of digestive enzymes of marine fishery resources, those authors reported that enzyme kinetics assays were scarce among the articles where fish digestive enzymes were characterized. Although some works report kinetic parameters such as the Michaelis-Menten constant (K_m), and the maximum hydrolysis rate (V_m), the development of new models using azocasein as the substrate is not studied. In this sense, among the intestinal enzymes, the most studied were trypsins with the substrate BAPNA (Nα-benzoyl-dl-arginine-p-nitroanilide). Besides, it is important to note that most authors usually work with purified enzymes but not with crude extracts (Jellouli et al. 2009; Bougatef et al. 2010; Hayet et a. 2011; Ou et al. 2011; Arvizu-Flores et al. 2012; Carretas-Valdez et al. 2020). However, the main advantage of using crude extracts instead of isolated enzymes relies on the great cost reduction associated with the purification step.

The development of models to explain the hydrolysis of azocasein are not common in fish enzyme studies. Previously, Fernandez and Riera (2013), and Trusek-Holownia and Noworyta (2015) developed kinetic models to describe the composition of protein hydrolysates. These works can be taken as a starting point to describe the progress of azocasein proteolysis by fish enzymes.

In a previous work (Friedman et al., 2021), a partial characterization of crude extracts of alkaline proteinases obtained from intestine-ceca of *Merluccius hubbsi* (Argentine hake), *Percophis brasiliensis* (Brazilian flathead), *Urophycis brasiliensis* (Brazilian codling), and *Cynoscion guatucupa* (Stripped weakfish) was performed. In that work, it was determined that proteinases obtained from these species were highly stable within a pH range from 7 to 11.5, and temperatures between 10 and 50 ºC during 150 min. In all cases, results demonstrated that proteinases were inactive at 70 ºC after 150 min. To include them in different commercial formulations to obtain value-added products, it is necessary to deepen their characterization. For this reason, the objective of the present work was to complement this previous work of characterization with the study of the kinetics of alkaline

proteinases present in the viscera of the above-mentioned South Atlantic fish species. The results of this study can contribute to the design of bioengineering processes based on the recovery and selection of fish proteinases and to the proposal of new uses for fishery wastes from different commercial species.

MATERIALS AND METHODS

Fish samples and preparation of crude enzyme extracts

Specimens of *M. hubbsi*, *P. brasiliensis*, *U. brasiliensis*, and *C. guatucupa* were fished off the coast of Mar del Plata (38°04´S, 57°30´W) by a commercial fleet. Samples were taken at this site because Mar del Plata is the main port of Argentina, where 50% of the total catch is landed (Gianelli et al. 2023). Besides, both fish processing plants and laboratories where we worked are located near this port. Small intestine and pyloric ceca were immediately extracted and kept on ice until docking. Then, samples were transported to our laboratory within 30 min for further processing. Samples were rinsed with cold distilled water and then stored in polyethylene bags at -20 °C. Viscera of four adult fishes of each species were used in this study. The preparation of enzymatic crude extracts was performed according to Friedman et al. (2021). Firstly, undigested food in the digestive tract was removed. Then, intestine-ceca samples were minced and crushed in a glass-Teflon tissue homogenizer with distilled water (1:4 w/v). The resulting materials were centrifuged at 10,000g for 30 min at 4 °C (Giumelli Z127, Argentina). Finally, supernatants (e.g., crude extracts) were stored at -20 °C until their evaluation.

Kinetic evaluation of proteinase activity of the obtained crude extracts

The proteinase activity of the obtained crude extracts was evaluated according to Garcia-Carreño and Haard (1993). 50 µl of crude extract were mixed with 250 µl of universal buffer (57 mM boric acid, 36 mM citric acid, 38 mM monobasic sodium phosphate, 1 N sodium hydroxide, pH 8) in Eppendorf tubes. Then, the reaction started with the addition of 250 µl of azocasein (Sigma A2765) dissolved in the above-mentioned universal buffer at the concentration of 0.05 to 0.5% w/v. Tested initial azocasein (AZC) concentrations in the reaction mixture ranged from 0.23 to 2.27 g/L. The mixture was incubated at 25 °C and at predefined intervals (30 to 360 min) the reaction was stopped by adding 250 µl of 20% w/v trichloroacetic acid (TCA). Then, the tubes were centrifuged at 10,000g for 5 min (Giumelli Z127, Argentina) to remove the remaining AZC along with non-hydrolyzed peptides. Finally, TCA-soluble peptides were evaluated at 440 nm (A440) in a spectrophotometer (SPECTROstar Nano BMG LABTECH, Germany). Blank assays were prepared in the same manner except that the tested crude extract was added just before TCA precipitation. Therefore, these blanks accounted for the presence of small peptides and proteins in the tested crude extract and AZC. The negative control of hydrolysis consisted in replacing the crude extract with a similar amount of universal buffer. This assay accounted for the possibility of non-enzymatic

hydrolysis of AZC. However, results demonstrated that the autolysis of AZC was negligible under the tested conditions. To determine the kinetic evaluation of the proteinase activity of crude extracts, a model was developed based on the work of Guérard et al., (2001) and Trusek-Holownia & Noworyta (2015).

All experiments were performed in duplicate and data were reported as mean and standard deviation.

Dynamical simulations and fitting procedure

All dynamical simulations and fittings were performed using the software GEPASI 3.30 (Mendes, 1993). GEPASI integrates the systems of differential equations with the routine LSODA (Livermore Solver of Ordinary Differential Equations). LSODA algorithm switches the integration method between the Adams integration method with variable step size and variable order up to 12th order within nonstiff regions and the Gear (or BDF) method with variable step size and variable order up to 5th order for stiff regions. Concerning the fitting procedure, the Multistart Optimization algorithm with Levenberge Marquardt local optimization was selected. Multistart is a hybrid stochastic-deterministic optimization method. Rather than run a simple local optimization, Multistart runs several of them. The first start takes for an initial guess the parameter values entered by the user. Then, initial guesses for the subsequent starts are generated randomly within specific boundaries that the user can set. The local optimizer used is the Levenberge Marquardt method, which has proved the most efficient gradient optimizer available in GEPASI (Mendes and Kell, 1998). Details concerning GEPASI can be found in Supplementary Data, Item 1.

Results and Discussion

Hydrolysis of AZC by crude extracts

Figure 1 shows the hydrolysis of AZC by the tested crude extracts. As a general rule, the absorbance at 440 nm (A440) increased as a function of time, indicating the production of TCA-soluble peptides released by the hydrolysis of AZC. In all cases, reached maximum A440 values ($A440_{max}$) depended on both the tested species and the initial AZC concentration. The monotonic progress of the hydrolysis process shown in Figure 1 was also reported by other authors (Mahmoud et al., 1992; Camacho et al., 1998; Kristinsson et al., 2000; Pagán et al., 2013). Although the presence of an absorbance plateau ($A440_{max}$) could suggest a gradual inactivation of the proteinases present in the crude extracts, previous results demonstrate that the decrease of the proteinase activity of all tested intestine-ceca samples was negligible under the studied conditions (Friedman et al., 2021). Finally, Figure 2 shows that $A440_{max}$ increased as a function of the initial AZC concentration in the reaction mixture (S_{T0}), suggesting that the maximum concentration of

hydrolysis products (P) obtained at long times was proportional to the initial concentration of the target protein.

Modeling the enzymatic hydrolysis of azocasein

Based on the above-mentioned results, a kinetic model was developed to represent the hydrolysis process of AZC by the tested crude extracts. The model developed in the present work is based on the works by Guérard et al. (2001), and Trusek-Holownia and Noworyta (2015). According to those authors, among all peptide bonds of a given protein (AZC in this work), only a few of them are the actual substrate for proteinases. Thus, one of the main factors controlling the hydrolysis rate is the concentration of available hydrolyzable bonds. It is worth mentioning that these hydrolyzable bonds depend on the tested protein and the studied proteinase. In this sense, for a certain crude extract that contains proteinases, AZC would have hydrolyzable and non-hydrolyzable peptide bonds under the assay conditions. Consequently, AZC would be composed of two fractions, a hydrolyzable (S_H) and a non-hydrolyzable (S_{NH}) one:

$$S_T = [S_H] + [S_{NH}] \quad (1)$$

where S_T represents the total AZC concentration. While $[S_{NH}]$ is, by definition, a constant, the cleavage of the hydrolyzable bonds of AZC causes a decrease of $[S_H]$ (and thereof, S_T), producing a mixture of TCA-soluble peptides (P) which are responsible for the absorbance of the supernatant. Conversely, because S_{NH} is removed from the solution by TCA, S_{NH} cannot contribute to the absorbance.

The definition of the hydrolysis rate (R_H) is crucial during the development of a hydrolysis model. In principle, for given experimental conditions (e.g., pH, temperature, ionic strength), R_H is a function of both the enzyme and the hydrolyzable fraction of AZC (S_H) concentrations (Camacho et al., 1998; Pagán et al., 2013; Trusek-Holownia and Noworyta, 2015). As a general rule, R_H is assumed to follow either a Michaelis–Menten or first-order kinetics with respect to the target protein concentration (Mahmoud et al., 1992; Márquez and Vázquez, 1999; Trusek-Holownia and Noworyta, 2015). In some cases, proteolysis includes a demasking stage, e.g., a transition of masked bonds to a demasked state, where peptide bonds become accessible to the enzyme attack. In these cases, proteolysis seems to be a two-phase process with consequent demasking and hydrolysis stages (Vorob'ev, 2009). However, Figure 1 shows a monotonic increase of the absorbance as a function of time, suggesting that the hydrolysis process can be represented as a single step as follows:

$$E + S_H \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES_H \overset{k_3}{\rightarrow} E + P \quad (2)$$

This sequence of reactions represents the classical Michaelis-Menten mechanism for enzymatic catalysis. Using the steady-state approximation for ES_H, and taking into account the mass balance for the total enzyme concentration (E_T), the following expression for R_H can be obtained (Frey and Hegeman, 2007):

$$R_H = -\frac{d[S_H]}{dt} = \frac{d[P]}{dt} = V_m \frac{[S_H]}{K_S + [S_H]} \quad (3)$$

where $V_m = k_3 E_T$ is the AZC maximum hydrolysis rate at saturating substrate concentration, and $K_S = \frac{k_2 + k_3}{k_1}$ is the half-saturation constant. In the cases when the decay rate of the intermediate complex (via k_2 and/or k_3) is much higher than its formation rate, the concentration of ES_H is negligible, thus $[E] \cong E_T$. According to these considerations, the formation of the intermediate complex would be the rate-limiting step of the hydrolysis process

$$R_H = -\frac{d[S_H]}{dt} = \frac{d[P]}{dt} \approx -k_{app}[S_H] \quad (4)$$

where $k_{app} = k_1 E_T$ is the apparent first-order rate constant that depends on the initial enzyme concentration. Equation (4) demonstrates that the first-order approximation used by other authors (Mahmoud et al., 1992; Pagán et al., 2013; Trusek-Holownia and Noworyta, 2015) to represent the hydrolysis kinetics is a special case of the Michaelis-Menten mechanism.

Because in the azocasein method, the available data are the change of absorbance (A440) as a function of time (Fig. 1), it is necessary to connect the formation of hydrolysis products (P) with the change of A440. Assuming that at t = 0, P = 0 (e.g., hydrolysis products are absent at the beginning of the experiment), P and S_H can be associated as follows:

$$[P] = [S_H]_0 - [S_H] \quad (5)$$

where $[S_H]_0$ is the initial concentration of the hydrolyzable portion of AZC. Besides, considering that S_{NH} is a constant, and using eq. (1), it is straightforward to show that

$$[P] = S_{T0} - S_T \quad (6)$$

where S_{T0} is the initial AZC concentration in the reaction mixture. Taking into account the results shown in Figure 2, A440 and P can be related as follows:

$$A440 = \alpha + \beta P \quad (7)$$

where α is the y-intercept and β a slope that links the concentration of P with A440. Then, combining eqs. (6) and (7), the following expression can be obtained

$$A440 = \alpha + \beta S_{T0}(1 - f_S) \quad (8)$$

where $f_S = \frac{S_T}{S_{T0}}$ represents the remaining fraction of the native AZC.

With regard to β, early works demonstrated that a solution of AZC that is completely digested has the same color intensity as the native AZC (Charney and Tomarelli, 1947; Coêlho et al., 2016). According to these considerations, a value for β = 1.079 ± 0.017 a.u. L gAZC^{-1} was calculated as the slope of the straight line obtained from the plot of A440 as a function of the native AZC concentration (Fig. 2, blue triangles). It is important to note that as the hydrolysis proceeds, f_S decreases from 1 to a certain minimum value (f_{Smin}) that depends on the non-hydrolyzable fraction of AZC (S_{NH}) under the tested conditions. If the consumption of S_H is complete, the hydrolysis stops and A440 reaches a given maximum value ($A440_{max}$ in Figure 2). Under this condition, and considering eq. (1), the following can be obtained:

$$f_{Smin} = \frac{S_{NH}}{S_{T0}} \quad (9)$$

According to eq. (9), the minimum value of f_S under the tested conditions represents the non-hydrolyzable fraction of the native AZC. Combining eqs. (1) and (8), and considering that at t = 0, $f_S = 1$, the initial value for $[S_H]$ can be calculated as follows:

$$[S_H]_0 = S_{T0}(1 - f_{Smin}) \quad (10)$$

First-order (eq. 4) and Michaelis-Menten (eq. 3) models, along with eqs. (8) and (10), were implemented in GEPASI 3.30 (Mendes, 1993). Implementation details can be found in the Supplementary Data, Item 1. Then, these models were fitted to the experimental data to obtain the corresponding coefficients of the tested models. Fitting results are shown in Table 1.

Figures 3 to 6 demonstrate that, as a general rule, the Michaelis-Menten model (eq. 3) better describes the experimental data in comparison with the first-order model (eq. 4). This result was expectable since eq. (3) has one more adjustable coefficient than eq. (4). Although in the cases of *P. brasiliensis* and *U. brasiliensis,* the difference between those models was negligible, the better performance of the Michaelis-Menten model in comparison with the first-order model is evident in *M. hubbsi* and *C. guatucupa* (for more details, see Figs. A1 to A4 in the Supplementary Data). In

those cases, the mean error (ME) corresponding to the Michaelis-Menten model (eq. 3) was about half of the corresponding to the first-order model (eq. 4) (Table 1).

Table 1 also shows that in terms of V_m, intestine-ceca crude extracts from *P. brasiliensis* and *U. brasiliensis* exhibited the highest proteinase activities, followed by *M. hubbsi*, and finally, *C. guatucupa.* However, it is important to notice that according to eq. (3), the rate of hydrolysis (R_H) not only depends on V_m, but also on K_S and on the actual substrate concentration ($[S_H]$), which in turn also depends on f_{Smin} (eq. 10). Using the coefficients depicted in Table 1, calculations demonstrate that for initial native AZC concentrations lower than about 0.2 g/L, R_H corresponding to *M. hubbsi*, *P. brasiliensis*, and *U. brasiliensis* were similar. Under this condition, the hydrolysis rate corresponding to intestine-ceca crude extracts from *C. guatucupa* was about half of the above-mentioned species. Finally, calculations also demonstrate that for high AZC concentrations, *P. brasiliensis* was the most suitable tested species to obtain high hydrolysis rates (Supplementary Data, Fig. A5).

Apparent degree of hydrolysis (DH_{app})

The degree of hydrolysis (DH) is a key parameter in the study of proteinases and is defined as the fraction of the total number of peptide bonds of the target protein that are cleaved during the hydrolysis process. Direct techniques to evaluate DH include pH-stat, trinitrobenzene sulfonic acid, o-phthaldialdehyde, trichloroacetic acid soluble nitrogen, and formol titration methods. As a general rule, these methods are based on the direct quantification of N-terminal moieties corresponding to the new peptides released during the hydrolysis process (Rutherfurd, 2010). Conversely, the AZC method employed in the present work to evaluate the proteinase activity of the tested crude extracts relies on the change of absorbance of the reaction mixture as a function of time due to the presence of TCA-soluble hydrolysis products (Fig. 1). For this reason, we use the term apparent degree of hydrolysis (DH_{app}) to point out that this value was not obtained by any of the above-mentioned direct methods, but using an indirect evaluation of the hydrolysis process:

$$DH_{app}(\%) = 100\left(\frac{A440}{A440_{AZC}}\right) \quad (11)$$

where A440 is the absorbance of the reaction mixture after a certain hydrolysis time, and $A440_{AZC}$ represents the absorbance corresponding to the native AZC at the tested initial concentration. It is important to point out that eq. (11) is based on the assumption that a solution of AZC that is completely digested has the same color intensity as the native AZC (Charney and Tomarelli, 1947; Coêlho et al., 2016). For this reason, A440 values could range from 0 at the beginning of the experiment ($DH_{app} = 0\%$) to $A440_{AZC}$ in the cases when AZC is completely digested ($DH_{app} = 100\%$).

Figure 7 shows some typical examples of the apparent degree of hydrolysis (DH_{app}, eq. 11) as a function of time corresponding to intestine-ceca crude extracts obtained from the tested species. In all cases DH_{app} increased as a function of time, reaching a maximum value that depended on both, the tested species and the initial AZC concentration. While maximum DH_{app} values corresponding to *M. hubbsi*, and *P. brasiliensis* ranged between 40 and 60%, samples corresponding to *U. brasiliensis*, and *C. guatucupa* only achieved about 15 - 35%. Taking into account that among the tested species, intestine-ceca crude extracts obtained from *M. hubbsi*, and *P. brasiliensis* also had the highest hydrolysis rate (see Section "Modeling the enzymatic hydrolysis of azocasein", and Table 1), results obtained in the present work suggest that these species are the best source of peptidases.

Specific fish proteinase activity (SFPA)

From a technological point of view, it is crucial to determine the best species to obtain the maximum proteinase activity per mass of fish. This information may help designers to optimize the extraction process of fish proteinases by selecting the most suitable fish species. The key parameter is the maximum hydrolysis rate at saturating substrate concentration (V_m). For clarity, V_m in Table 2 was expressed in terms of milligrams of AZC hydrolyzed per minute per liter of the reaction mixture. According to the extraction technique (Section "Fish samples and preparation of crude enzyme extracts"), 4 mL of extraction buffer per gram of processed tissue was used. Then, the mixture was centrifuged to remove the cellular debris, and the supernatant (e.g., the crude extract, CE) was recovered. Considering that during the evaluation of the proteinase activity, 50 µL of this crude extract (V_{CE}) was employed and that the total volume of the reaction mixture (V_{RM}) was 550 µL (Section "Kinetic evaluation of proteinase activity of the obtained crude extracts"), the ratio between V_{RM} and V_{CE} was 11. Thus, the product between V_m and the above-mentioned ratio is the specific enzyme content of the crude extract (q_{CE}), being this coefficient a measure of the amount of enzyme per unit volume of CE. Besides, assuming that about 4 mL of crude extract (CE) per gram of tissue was recovered from the centrifugation step (Section "Fish samples and preparation of crude enzyme extracts"), the product between this value and q_{CE} represents the proteinase activity per unit mass of processed tissue (q_{Tissue}).

Table 2 shows that up to this point, similar conclusions regarding the best species in terms of activity can be obtained from the values of V_m or q_{Tissue}, e.g., that intestine-ceca crude extracts from *P. brasiliensis* and *U. brasiliensis* exhibited the highest proteinase activities. However, according to a previous work (Friedman et al., 2021), the tissue yield (TY, mass of tissue per unit mass of fish) depends on the tested species, being *U. brasiliensis* the most favorable species in terms of TY (Table 2). Thus, the specific fish proteinase activity (SFPA) was obtained as the product between TY and q_{Tissue}. Table 2 shows that *P. brasiliensis* and *U. brasiliensis* were similar in terms of SFPA, being SFPA corresponding to *M. hubbsi* about 25% of the above-mentioned species. Finally, Table 2 demonstrates that *C. guatucupa* cannot be recommended as a source of proteinases.

The present study provides new information concerning the proteinases obtained from the residues of the studied fish species. From this work, it is possible to determine which fish species are the most suitable as a source of proteinases. Additionally, the knowledge of the biochemical characteristics of these enzymes can be used to optimize efficiency and yield and, in the future, increase the potential production of the processes in which these enzymes are used. From the results of this study, a comparison between proteinases can be established taking into account the hydrolysis rate and the effect of the initial AZC concentration on the hydrolysis rate. The information obtained from the enzyme kinetic modeling could be used to obtain peptides with functional properties that can be incorporated as bioactive ingredients in commercial products. Although results obtained in the present study are promising, further research is in progress to evaluate the performance of the crude extracts under actual conditions, such as in laundry detergents and for enzymatic treatment of soybean meal as functional feed for aquaculture.

Conclusions

In the present work, a kinetic characterization of crude extracts of alkaline proteinases obtained from intestine-ceca of *Merluccius hubbsi* (Argentine hake), *Percophis brasiliensis* (Brazilian flathead), *Urophycis brasiliensis* (Brazilian codling), and *Cynoscion guatucupa* (stripped weakfish) was performed. This characterization included results about the hydrolysis of azocasein by crude extracts from these species, the suitability of first-order (eq. 4), and Michaelis Menten (eq. 3) models to represent the hydrolysis process, the calculation of the apparent degree of hydrolysis and the determination of the specific fish proteinase activity. We can conclude that the maximum concentration of hydrolysis products obtained at long times was proportional to the initial concentration of the target protein. The Michaelis-Menten model better describes the experimental data compared to the first-order model. Although crude intestine-ceca extracts obtained from *M. hubbsi*, and *P. brasiliensis* presented the highest rate of hydrolysis, *P. brasiliensis* and *U. brasiliensis* had the highest specific proteinase activity. Finally, while all four commercial fish species are a source of digestive enzymes, proteinases from *P. brasiliensis* species may be chosen as the best candidates for potential implementation in industrial processes.

Tables

Table 1. Coefficients of the tested models corresponding to intestine-ceca crude extracts from *Merluccius hubbsi*, *Percophis brasiliensis*, *Urophycis brasiliensis* and *Cynoscion guatucupa*.

		Species			
Model	**Coefficient**	***Merluccius hubbsi***	***Percophis brasiliensis***	***Urophycis brasiliensis***	***Cynoscion guatucupa***
First-order (eq. 4)	α (a.u.)	0.140 ± 0.010	0.176 ± 0.007	0.111 ± 0.008	0
	f_{Smin}	0.46 ± 0.08	0.57 ± 0.01	0.63 ± 0.01	0.45 ± 0.36
	k_{app} x10^3 (min^{-1})	3.26 ± 0.73	12.47 ± 0.71	7.39 ± 0.70	1.15 ± 0.89
	ME* (a.u.)	0.0634	0.0352	0.0385	0.0435
	r^2	0.9009	0.9762	0.9444	0.8134
Michaelis-Menten (eq. 3)	α (a.u.)	0.080 ± 0.007	0.160 ± 0.007	0.085 ± 0.007	0
	f_{Smin}	0.58 ± 0.01	0.57 ± 0.01	0.67 ± 0.01	0.65 ± 0.03
	V_m x10^3 (gAZC L^{-1} min^{-1})	3.43 ± 0.16	15.92 ± 3.46	5.34 ± 0.53	1.23 ± 0.05
	K_S (gAZC/L)	0.105 ± 0.028	0.722 ± 0.265	0.144 ± 0.050	0.141 ± 0.036
	ME* (a.u.)	0.0360	0.0309	0.0342	0.0142
	r^2	0.9628	0.9787	0.9600	0.9564

*Mean Error: $ME = \frac{1}{N}\sum|A440_{exp} - A440_{model}|$, where N is the total number of data

Table 2. Calculation of the specific fish proteinase activity (SFPA) corresponding to the tested South Atlantic fish species. For calculation details, see the text.

	Species			
Coefficient	***Merluccius hubbsi***	***Percophis brasiliensis***	***Urophycis brasiliensis***	***Cynoscion guatucupa***
V_m (mgAZC LRM^{-1} min^{-1})	3.43 ± 0.16	15.92 ± 3.46	5.34 ± 0.53	1.23 ± 0.05
V_{RM}/V_{CE} (LRM/LCE)	11			
q_{CE} (mgAZC LCE^{-1} min^{-1})	37.73 ± 1.76	175.12 ± 38.06	5[illegible]74 ± 5.83	13.53 ± 0.55
CE/Tissue Ratio (LCE/KgTissue)	4			
q_{Tissue} (mgAZC KgTissue^{-1} min^{-1})	150.92 ± 3.10	700.4[illegible] ± 15[illegible].24	234.96 ± 23.32	54.12 ± 2.20
TY x 10^3 (kgTissue/KgFish)*	7.0 ± 1.7	6.7 ± 0.8	18.0 ± 2.2	10.8 ± 5.4
SFPA (mgAZC KgFish^{-1} min^{-1})	1.1 ± [illegible]3	4.7 ± 1.2	4.2 ± 0.7	0.6 ± 0.3

* Adapted from Friedman et al. (2021)

Figures

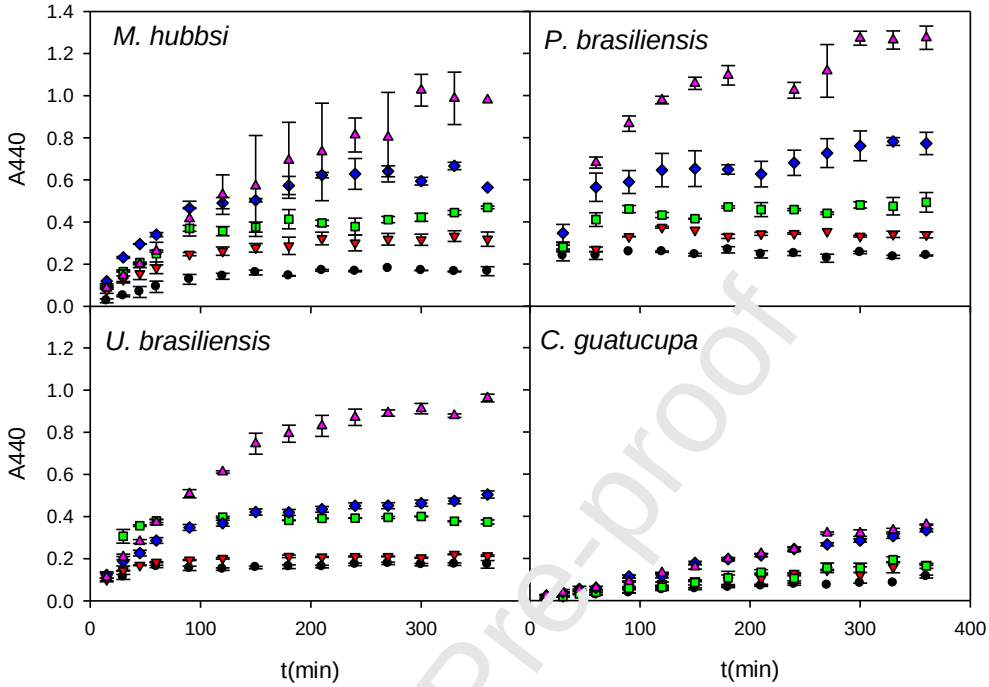

Fig. 1 Hydrolysis of AZC by intestine-ceca crude extracts of a) *M. hubbsi*, b) *P. brasiliensis*, c) *U. brasiliensis*, and d) *C. guatucupa.* In all cases, T = 25 ^{o}C, pH = 8, and the tested initial AZC concentration in the reaction mixture were (in g/L) 0.23 (black circles), 0.45 (red triangles), 0.68 (green squares), 1.14 (blue diamonds), and 2.27 (pink triangles). Bars indicate the standard deviation of duplicates.

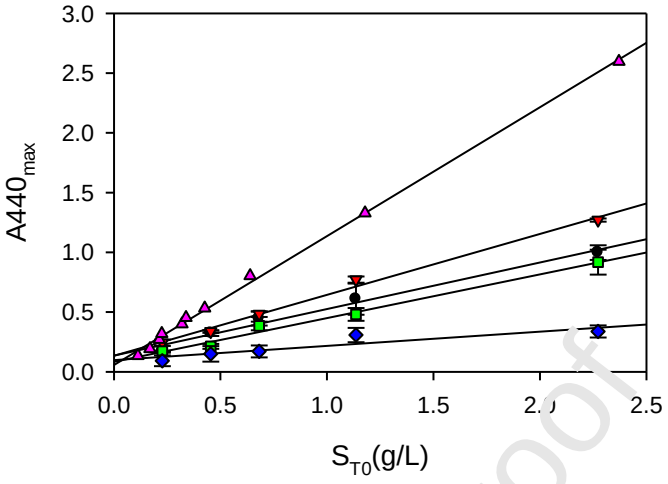

Fig. 2 Effect of the initial AZC concentration in the reaction mixture (S_{T0}) on the maximum absorbance ($A440_{max}$) by intestine-ceca crude extracts of *M. hubbsi* (black circles), *P. brasiliensis* (red triangles), *U. brasiliensis* (green squares), and *C. quatucupa* (blue diamonds). In all cases, T = 25 ^{o}C, pH = 8, and hydrolysis time 300 - 360 min. Bars indicate the standard deviation of duplicates. Pink triangles show the absorbance corresponding to the native (e.g., unhydrolyzed) AZC. Lines represent linear regression.

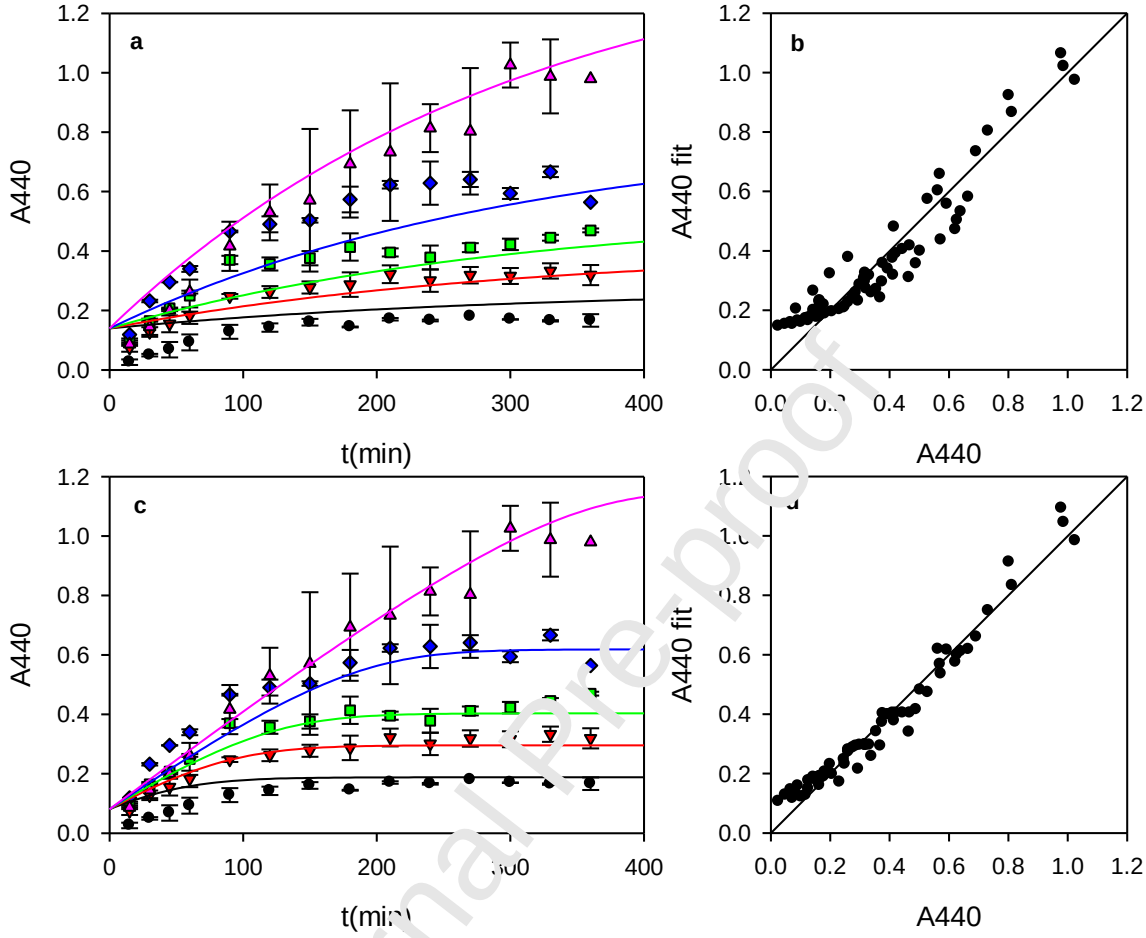

Fig. 3 Hydrolysis of AZC by intestine-ceca crude extract corresponding to *M. hubbsi.* In all cases, T = 25 ^{o}C, pH = 8. Tested initial AZC concentration in the reaction mixture were (in g/L) 0.23 (black circles), 0.45 (red triangles), 0.68 (green squares), 1.14 (blue diamonds), and 2.27 (pink triangles). Bars indicate the standard deviation of duplicates. Lines in a) represent the 1st order model (eq. 4). Lines in c) represent the Michaelis-Menten model (eq. 3). Lines in parity plots (b, d) represent the perfect correlation line.

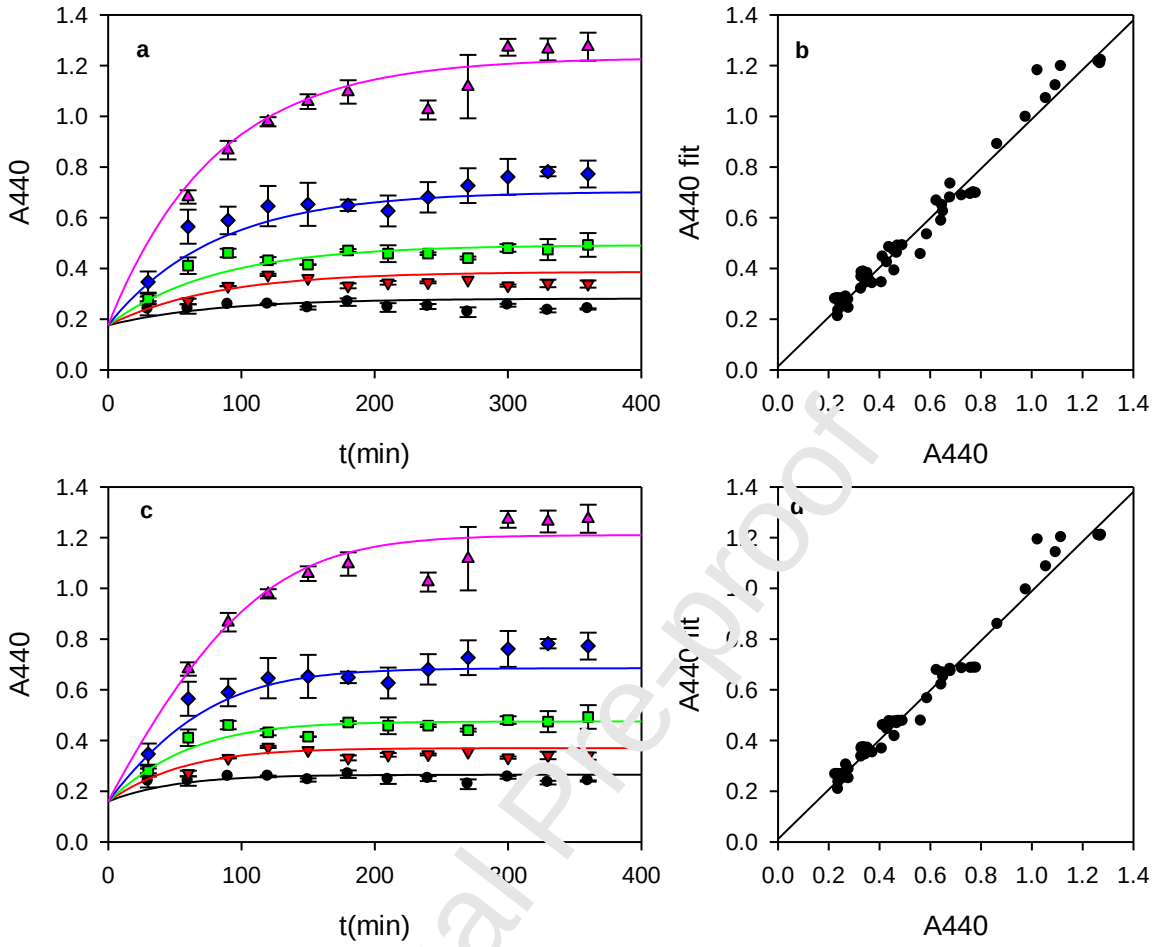

Fig. 4 Hydrolysis of AZC by intestine-ceca crude extract corresponding to *P. brasiliensis.* In all cases, T = 25 ^{o}C, pH = 8. Tested initial AZC concentration in the reaction mixture were (in g/L) 0.23 (black circles), 0.45 (red triangles), 0.68 (green squares), 1.14 (blue diamonds), and 2.27 (pink triangles). Bars indicate the standard deviation of duplicates. Lines in a) represent the 1st order model (eq. 4). Lines in c) represent the Michaelis-Menten model (eq. 3). Lines in parity plots (b, d) represent the perfect correlation line.

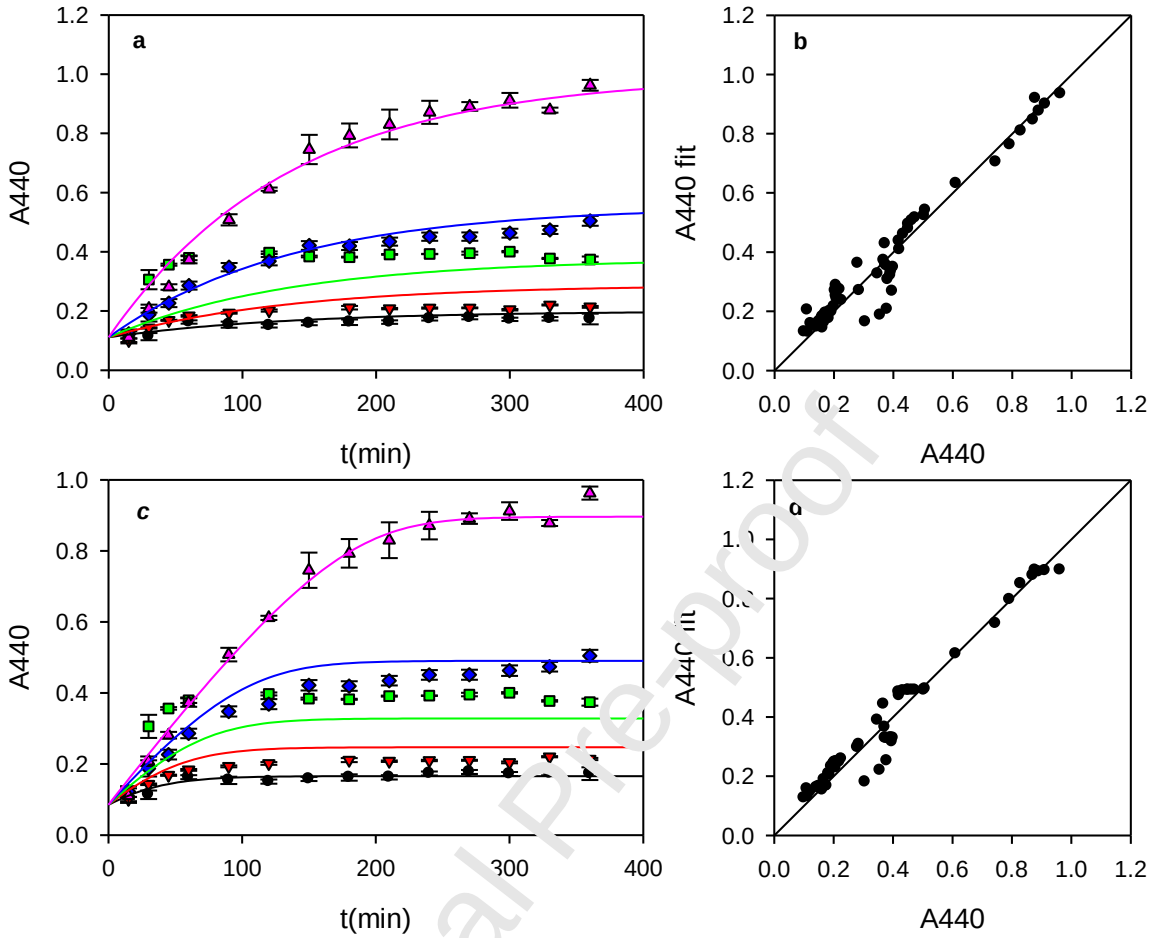

Fig. 5 Hydrolysis of AZC by intestine-ceca crude extract corresponding to *U. brasiliensis.* In all cases, T = 25 °C, pH = 8. Tested initial AZC concentration in the reaction mixture were (in g/L) 0.23 (black circles), 0.45 (red triangles), 0.68 (green squares), 1.14 (blue diamonds), and 2.27 (pink triangles). Bars indicate the standard deviation of duplicates. Lines in a) represent the 1st order model (eq. 4). Lines in c) represent the Michaelis-Menten model (eq. 3). Lines in parity plots (b, d) represent the perfect correlation line.

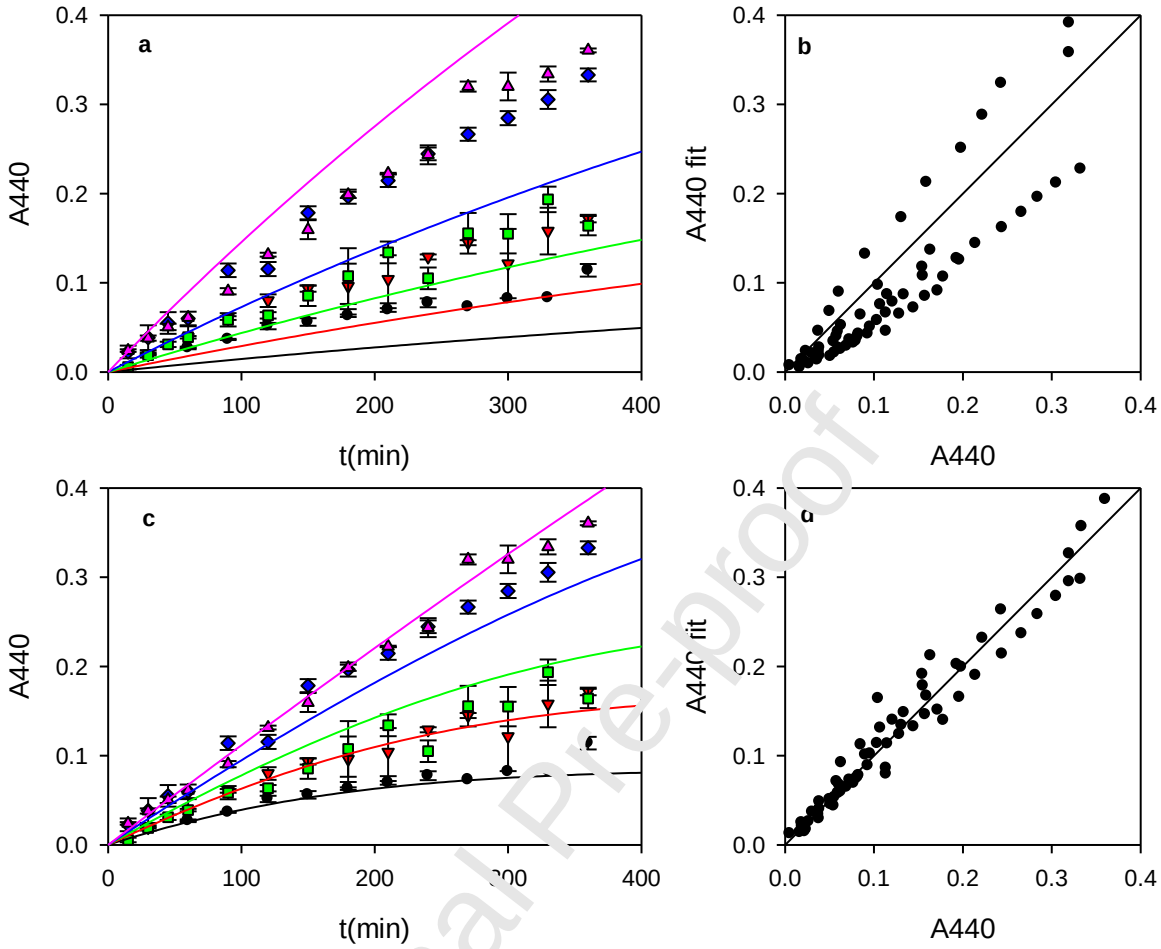

Fig. 6 Hydrolysis of AZC by intestine-ceca crude extract corresponding to *C. guatucupa.* In all cases, T = 25 ^{o}C, pH = 8. Tested initial AZC concentration in the reaction mixture were (in g/L) 0.23 (black circles), 0.45 (red triangles), 0.68 (green squares), 1.14 (blue diamonds), and 2.27 (pink triangles). Bars indicate the standard deviation of duplicates. Lines in a) represent the 1st order model (eq. 4). Lines in c) represent the Michaelis-Menten model (eq. 3). Lines in parity plots (b, d) represent the perfect correlation line.

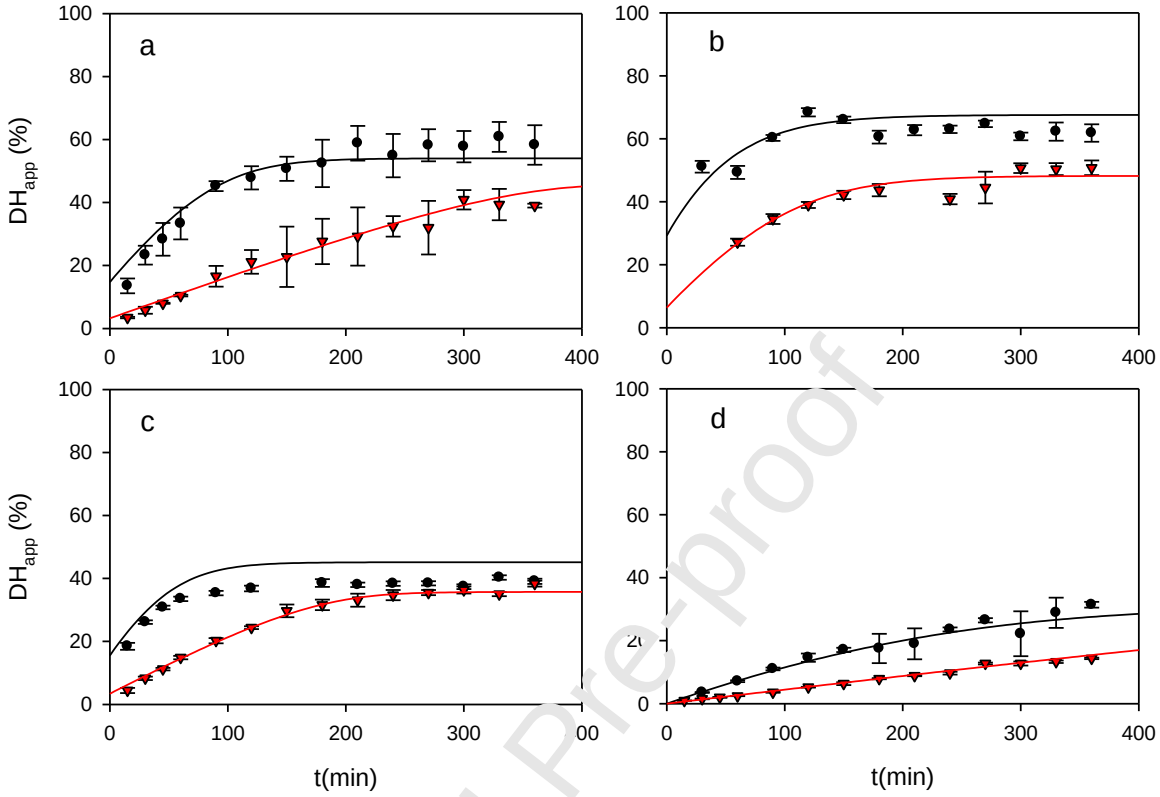

Fig. 7 Typical examples of the apparent degree of hydrolysis (DH_{app}, eq. 11) as a function of time corresponding to a) *M. hubbsi*, b) *P. brasiliensis*, c) *U. brasiliensis*, and d) *C. guatucupa.* In all cases, T = 25 °C, pH = 8. Initial AZC concentrations in the reaction mixture were (in g/L) 0.45 (black circles), and 2.27 (red triangles). Bars indicate the standard deviation of duplicates. Lines represent the Michaelis-Menten model (eq. 3) using the coefficients shown in Table 1.

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CRediT authorship contribution statement

Ivana Soledad Friedman: conceptualization, methodology, investigation, writing—original draft, review, and editing. **Analia Verónica Fernández Giménez:** conceptualization, supervision, funding acquisition, review, and editing. **Edgardo Martín Contreras:** conceptualization, methodology, modeling, software, supervision, writing—original draft, review, and editing.

Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Contreras, E.M. reports financial support was provided by CONICET. Friedman I.S. reports financial support was provided by CONICET. Fernandez-Gimenez A.V. reports financial support was provided by CONICET.

Fish waste of four species of South Atlantic Sea (intestine-ceca)

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Enzyme crude extract

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Alkaline proteinase activity

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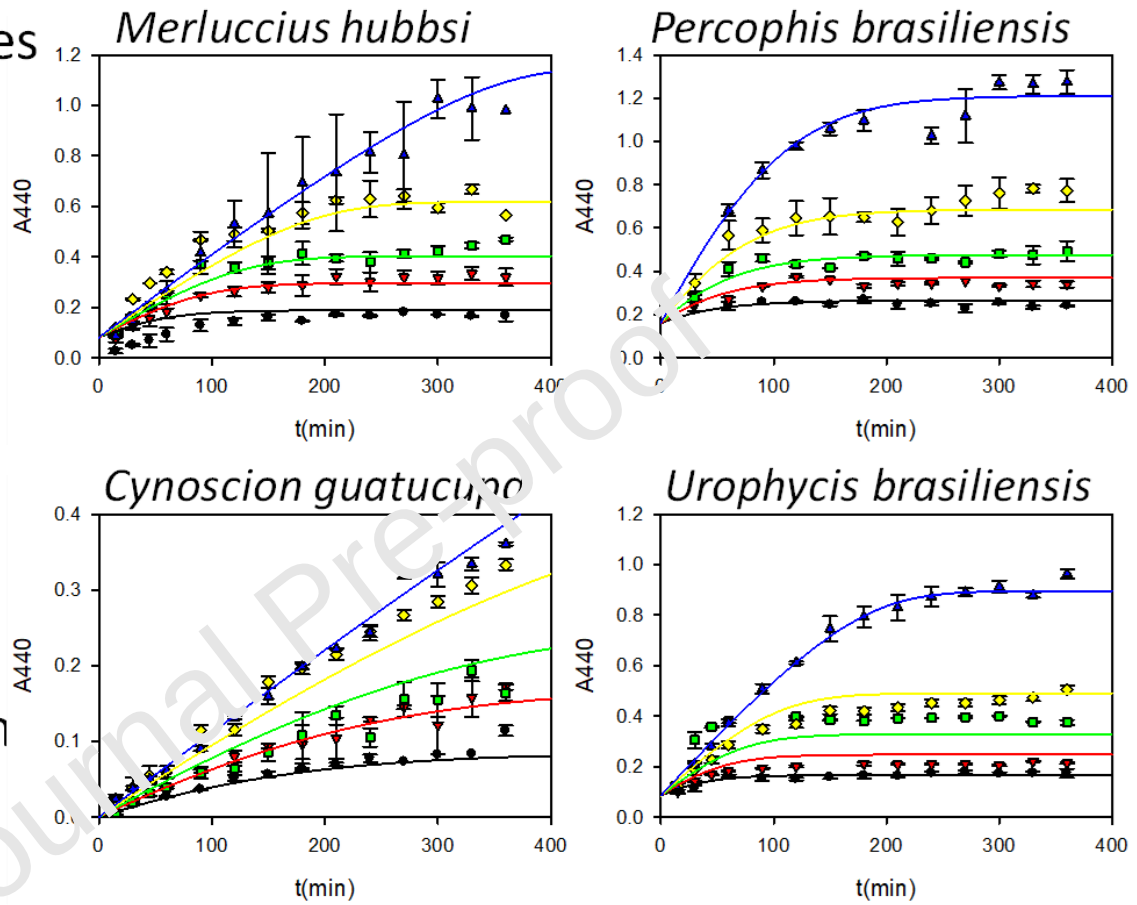
Kinetic characterization and modeling

↓

Specific Fish Proteinase activity



→ *P. brasiliensis*, *U. brasiliensis* > *M. hubbsi* > *C. guatucupa*

Highlights

* Proteinase activity of crude extracts from four fish species was evaluated

* Azocasein method was used to evaluate the proteinase activity

* A model was developed to obtain kinetic information regarding the hydrolysis process

* The model adequately represented the obtained results

* *Percophis brasiliensis* exhibited the highest specific fish proteinase activity