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Alkaline trypsin from the viscera and heads of *Engraulis anchoita*: partial purification and characterization

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Abstract

Marine by-products contain valuable protein fractions. To ensure a profitable utilization of wastes from the fish industry, the knowledge about their quality and composition is necessary. Fish digestive proteolytic enzymes from cold-adapted ectothermic organisms have found applications in several industries because their temperature requirements and other characteristics differ from those of homologous proteases from warm-blooded animals. Herein, we describe detection, partial purification, and characterization of proteolytic enzymes from the viscera and heads of *Engraulis anchoita*. Enzymatic activities of the by-products were assayed using azocaseín as a substrate. To characterize the alkali protein fraction, the activity against inhibitors and their molecular weights were studied. The crude protein extract exhibited maximal activity at pH 8.0 and 60°C. Results relative to the substrate-specific Na-benzoyl-DL-arginine-p-nitroanilide and the 25 kDa molecular weight indicated that the recovered protease was trypsin. The activity showed an increment in presence of SDS and a slight decrease when it was incubated with EDTA. Increasing the concentration of NaCl up to 5% did not significantly decrease the protein's activity. The results obtained suggest that by-products of anchovy industry could be used in the detergents industry.

Key words: alkaline proteases, trypsin, *Engraulis anchoita*, viscera, heads

Introduction

Fishing of the anchoita (*Engraulis anchoita*) species is of prominence in the fishing industry in Argentina. This species is widely distributed in the Southwest Atlantic Ocean (Pastous Madureira et al., 2009). Argentina is a pioneer in the exploitation and manufacture of this species for human consumption (Czerner and Yeannes, 2010). In the salting and ripening of anchovy, the specimens are beheaded, partially gutted, and salted. This processing generates a considerable amount of wastes corresponding to more than 40% of the total weight of the processed material. The fishing industry wastes are regarded as a source of high biological value proteins, lipids rich in omega-3 polyunsaturated fatty acids, vitamins, and other bioactive compounds with functional properties (Gbogouri et al., 2004). The value of fish by-products, specifically as a source of enzymes, can be increased by using such materials for different purposes in areas like medicine, food, and pharmaceutical industries (Shahidi and Kamil, 2001; Burkert et al., 2004; Castro Ceseña et al., 2012; Lamas et al., 2015). Several studies have shown that these enzymes exhibit unique properties of activity and stability due to the adaptation of these marine organisms to extreme environmental conditions; wide ranges of temperature and pH; fluctuant availability of oxygen; the presence of surfactants, oxidizing agents, and heavy metals (Haard, 1992; Diaz-Lopez and Garcia-Carreño, 2000; Joo et al., 2001; Ketnawa et al., 2013).

Approximately 60% of the enzymes used in the industrial bioprocesses are proteases (Ferraro et al., 2010; Salazar-Leyva et al., 2014). Therefore, an intensive effort was done to develop isolation and purification methods

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for fish proteolytic enzymes. Typically, reports of purification describe the precipitation by way of salt and organic compounds, chromatography, or column isolation (Heu et al., 1991; Ishida et al., 1995; Michail et al., 2006; Siringan et al., 2006; Klomklao et al., 2007; Castro Ceseña et al., 2012; Blanco et al., 2014).

Pepsin and trypsin are the most important proteolytic enzymes obtained from aquatic invertebrates. This is because they are, among other things, widely used in different bioprocesses such as meat tenderization, wastewater treatment, or production of laundry detergents (Simpson, 2000; Castillo-Yañez et al., 2005; Shahidi, 2007; Klomklao and Songklanakarin, 2008; Ferraro et al., 2010; Rawdkuen et al., 2012; Ferraro et al., 2013; Blanco et al., 2014).

Trypsins of marine origin have been isolated and characterized from several fish tissues and species such as hepatopancreas of carp (*Cyprinus carpio*) (Cao et al., 2000), hepatopancreas of crawfish (*Procambarus clarkii*) (Jeong et al., 2000), viscera of true sardine (*Sardinops melanostictus*), and pyloric caeca of arabesque greenling (*Pleuroprammus azonus*) (Kishimura et al., 2006), yellowfin tuna spleen (*Thunnus albacores*) (Klomklao et al., 2006), pyloric caeca of sardine (*Sardinops sagax*) (Castillo-Yañez et al., 2005), and pancreas of small-spotted catshark (*Scyliorhinus canicula*) (Blanco et al., 2014).

Two trypsin-like enzymes were purified from the pyloric caeca and intestines from the members of the *Engraulidae* family (Martinez and Gildberg, 1988). Heu and coworkers (1995) reported an alkaline protease isolated from the viscera of *Engraulis japonica*, which was further identified as trypsin. Whereas the use of visceral by-products has been successfully investigated, the rest of the wastes such as heads and tails are not proportionally well studied (Regeinstein and Regeinstein, 1991; Shahidi and Kamil, 2001; Michail et al., 2006). Considering a large quantity of by-products generated during the beheading and gutting of anchovy, the aim of this work was to extract the proteolytic enzymes from the viscera and heads of *Engraulis anchoita*. In addition to that, the recovered proteolytic enzymes were partially purified and characterized.

Materials and methods

Biological samples

Samples of *E. anchoita* were collected during the research cruises performed by the National Institute for Fisheries Research and Development (INIDEP). Specimens were obtained off the coast of Buenos Aires province, of the area placed between 34°S and 41°S. Six adult individuals of commercial size (19-21 cm total length) were selected. The viscera and heads were separated and placed in vacuum-sealed polyethylene bags. Then, the samples were frozen at −80°C until they were subsequently used for enzyme extraction.

Preparation of crude enzyme extracts

For the extraction of enzymatic fractions, the viscera and heads were thawed and homogenized in chilled distilled water (1:4 w/v), using the Omni Mixer Homogenizer. The homogenization was repeated three times. The homogenates were centrifuged at 10 000 g for 30 min at 4°C. The lipid phases were discarded and the supernatants collected were referred to as "crude enzyme extract". The proteins of crude enzyme extract were quantified using the Lowry method (1958), with bovine albumin as the protein standard. The absorbances were measured at 500 nm using a Shimadzu UV spectrophotometer.

Partial purification of protein extracts

The partial purification of protein extracts was carried out following the procedure developed by Michail and coworkers (2006), with slight modifications. The crude enzyme extracts were first subjected to acetone precipitation. Acetone, which was precooled to 15°C, was slowly added to the crude enzyme extracts until the enzyme solutions and acetone ratio was 1:0.75. Then, the mixture was stirred for 10 min. The obtained precipitates were separated from the supernatants by centrifugation at 10 000 g for 10 min at 4°C. Precooled acetone was added to the supernatants till the ratios of 1:1 and 1:1.25 between enzyme solutions and acetone were obtained. The solutions were stirred for another 10 min before centrifugation at 10 000 g for 10 min at 4°C. The collected precipitates were dissolved in TRIS-HCl buffer, pH 8.

Enzyme activity assays

Total proteolytic activity was evaluated according to a method developed by Castro Ceseña and coworkers (2012), with some modifications: 500 µl of 0.5% w/v azocasein in 50 mM TRIS-HCl was used as a substrate. Aliquots (15 µl) of crude and purified protein extracts and the substrate solutions were mixed and incubated in Eppendorf tubes for 30 min at 60°C. The reactions were

stopped by adding 500 µl of 20% trichloroacetic acid (TCA), and then stored for 10 min at 0-4 °C. The tubes were centrifuged for 5 min at 10 000 g. The supernatants were separated and the progress of reactions was measured by monitoring the absorbance at 366 nm. A blank activity was run by adding the enzyme solution after TCA was added.

The substrate hydrolysis units per mg of protein were calculated using the following equation:

$$\text{U/(mg protein)} = (\Delta\text{Abs/min})\ (\text{final reaction volume})/(\text{mg protein}).$$

Effects of temperature, pH, and NaCl on proteinase activity

The effect of temperature on proteinase activity was determined by incubating enzymatic reactions at different temperatures ranging from 40 to 80 °C. The reactions were also evaluated at different pHs in the range 6.0-10.0. The effect of NaCl on crude proteolytic activity was studied. NaCl was added into the standard reaction assay to obtain the final concentrations of 2.5, 5, 10, 15, and 20% w/v. The residual activity was determined at 60 °C and pH 8.0 for 30 min using azocasein as a substrate.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracts

SDS-PAGE was carried out in gels with 30% of acrylamide/bisacrylamide (37.5 : 1) solution according to the Laemmli procedure (1970). Aliquots of the crude extracts and purified extracts were denatured with equal volumes of a denaturalization buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol) and boiled for 10 min. The samples (15 µl) were loaded on the gel made of 4% stacking and 12.5% separating gels and subjected to electrophoresis at a constant current of 100 V per gel for 2 h. After the electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and destained with 7% acetic acid. The molecular weight of the enzyme extracts was estimated using low molecular weight calibration kit as markers (24, 29, 36, 45, and 66 kDa).

Evaluation of trypsin-specific activity

Trypsin activity was evaluated according to a method described by Castillo-Yañez and coworkers (2005), using Na-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as a substrate, with slight modifications: 100 µl of protein extract was mixed with 10 µl of 0.5 M Tris–HCl (pH 8.0), 0.1 ml of 0.2 M $CaCl_2$; and 40 µl of 0.02 M BAPNA in dimethyl sulfoxide (DMSO). The final reaction volume was adjusted to 1 ml with distilled water.

Assays were performed at 30 °C. One unit of activity was defined as the production of 1 mol/min of free p-nitroanilide, which was measured by monitoring the change in absorbance at 410 nm for 10 min.

The units of substrate hydrolysis per mg of protein were calculated using the following equation:

$$\text{U/(mg protein)} = (\Delta\text{Abs/ min})\ (1000)\ (\text{final reaction volume})/(8800)\ (\text{mg protein}),$$

where 8800 is the molar extinction coefficient of p-nitroanilide in $M^{-1}\ cm^{-1}$.

Effects of EDTA and SDS on trypsin activity

The effect of metalloprotease inhibitor ethylenediaminetetraacetic acid (EDTA) on proteinase activity was determined by incubating the enzyme solution with an equal volume of inhibitor to obtain the final concentrations shown in Table 2. The mixtures were allowed to stand for 30 min at room temperature. The residual enzyme activity of the mixtures was measured at 30 °C and pH 8.0 using BAPNA as a substrate. Controls and blanks were assayed under the same conditions. The activity of the enzyme without inhibitors was considered as 100%.

The effect of sodium dodecyl sulfate (SDS) on enzyme activity was studied by incubating the enzyme solution with the same volume of SDS solution to obtain the final concentrations designated (0.5 mM SDS and 1 mM SDS). The mixtures were incubated for 60 min at 40 °C. The activity of the enzyme without a surfactant was considered as 100%. The residual enzyme activity was measured at 30 °C and pH 8.0 using BAPNA as a substrate.

Kinetic studies

The activity of the purified trypsin was evaluated at 30 °C with different final concentrations of BAPNA, ranging from 0 to 2000 µM. The determinations were performed in triplicate and kinetic parameters (K_m and V_{max}) were calculated using the Lineweaver-Burk plots (Lineweaver and Burk, 1934). The determinations were performed twice and the respective kinetic parameters, including K_m and V_{max}, were calculated using the Lineweaver-Burk plots (Lineweaver and Burk, 1934).

The values of the turnover number (k_{cat}) were calculated from the following equation:

$$V_{max} / [E] = k_{cat},$$

where E is the active enzyme molar concentration and V_{max} is the maximal velocity.

The active enzyme molar concentration was estimated, using its molecular weight as determined by SDS-PAGE (Heu et al., 1995; Copeland, 2000).

Statistical analysis

All experiments were performed in triplicate and the results are expressed as the mean value ± standard deviation.

The data were subjected to the analysis of variance (ANOVA), and the differences between the means were carried out using Duncan's multiple range tests, being statistically different at the significance level of 5%.

Results and discussion

Temperature and pH optimum

The pH and the temperature profile of the crude extracts are shown in Figure 1. The highest proteinase activity was obtained at 60 °C. The optimal temperature of the crude protein extract obtained from the viscera and heads of *E. anchoita* was similar to the crude proteinase and autolytic activity of the extract made from an entire *Indian anchovy* fish (Siringan et al., 2006; Siringan et al., 2007).

The proteinase activity was strongly affected by temperature. The thermal profile of the activity of the protein extract obtained from the viscera and heads showed that the proteolytic enzymes were highly stable at temperatures equal to or below 60 °C, but were inactivated at higher temperatures.

The crude protein extract was active at pH ranging from 6.0 to 10.0, with an optimum of around 8. As shown in Figure 2, at temperatures less than 60 °C, the relative activities at pH 9.0 and 10.0 were about 82% and 75%, respectively. Therefore, we concluded that the crude protein extract exhibited a maximum activity at alkaline pH. Our observation is supported by Simpson (2000), who reported that the most important enzymes in the viscera of fish are alkaline proteases. In the industrial processes, the use of alkaline proteases has increased remarkably, as they are stable and active under

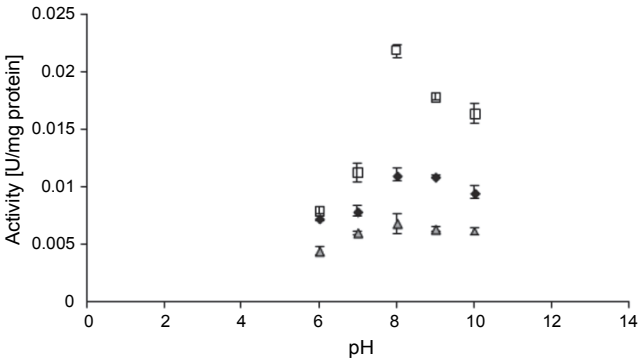

Fig. 1. pH and temperature profile of the crude protein extract from *Engraulis anchoita* viscera and heads. Temperature assayed: ◇ 40 °C, □ 60 °C, ▵ 80 °C. Results are mean values ± standard deviation (n = 3)

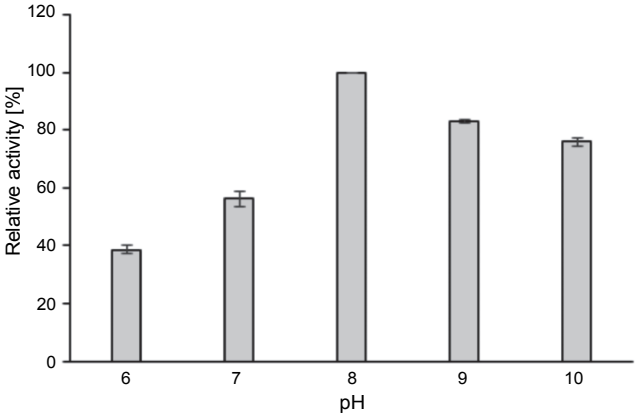

Fig. 2. Relative activity at different pH of the crude protein extract from *Engraulis anchoita* viscera and heads, with temperature fixed at 60 °C. Results are mean values (n = 3). Error bars represent the standard deviations of mean values

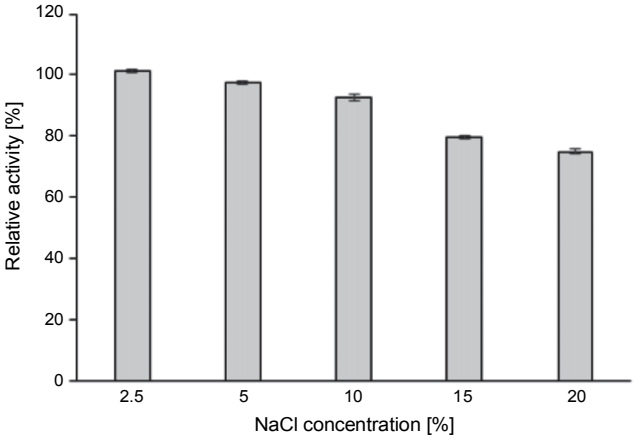

Fig. 3. Effect of NaCl concentration on proteolytic activity of the crude protein extract from *Engraulis anchoita* viscera and heads, with pH and temperature fixed at 8.0 and 60 °C respectively. Results are mean values (n = 3). Error bars represent the standard deviations of mean values

drastic conditions of temperature and pH and in the presence of surfactants or oxidizing agents (Joo et al., 2001). Our results suggest that the *E. anchoita* viscera and heads could be a potential source of alkaline proteases for certain industrial applications as one of the most important variables in the selection of proteases for detergent industry is their optimum pH (Banerje et al., 1999). The alkaline proteases are known to improve the effectiveness of laundry detergents due to their ability to aid in the removal of protein stains (Anwar and Saleemudin, 1998; Gupta et al., 2002).

Effect of NaCl on proteolytic activity of extracts

The effect of NaCl on crude proteolytic activity of extracts obtained from the viscera and heads of *E. anchoita* incubated at pH 8.0 and 60 °C temperature is shown in Figure 3. A low concentration (2.5% w/v) of salt added to a reaction slightly increased the enzymatic activity. Then, we observed that the enzyme activity decreased slightly with an increase in NaCl concentration. The proteolytic activity at 15% w/v NaCl was 80%. The decrease in the activity may be due to the denaturation effect the salt evokes on the enzymes. The results suggested that enzymes from *E. anchovy* by-products were active, even at a moderately high salt concentration.

Klomklao and coworkers (2008) reported that endogenous proteinases were still active in the presence of high concentrations of NaCl (25% w/v) and that the proteolytic activity of crude extracts decreased with high concentrations of salt at 30% w/v in true sardine (*Sardinops melanostictus*). In our study, we observed that the crude extract recovery process (at a high NaCl concentration), which is commonly used in *E. anchoita* processing, retains the proteolytic activity of enzymes.

Purification of the E. anchoita proteolytic extract

The proteolytic extracts of the viscera and heads of *E. anchoita* were recovered and purified using cold acetone. All the results are summarized in Table 1. Under optimum enzymatic conditions, the 1 : 1 (crude extract: cold acetone) purified extract achieved a proteolytic activity of 0.0206 U/mg of protein and the recovery of proteinase activity was 76.22%. The 1 : 1.25 (crude extract: cold acetone) purified extract showed a slightly higher proteolytic activity (0.0224 U/mg of protein); however, no statistically significant differences were detected between both the purified extracts.

The achieved high percentages of protein recovery indicated that cold acetone is an effective agent for the initial step of protease purification. These results are in accordance with previous studies of precipitation of proteolytic enzymes from trout (*Salmo gairdnerii*) heads using acetone (Michail et al., 2006) and of partial acetone purification of proteolytic enzymes from sardine by-products (Castro Ceseña et al., 2012).

The differences observed in proteolytic activities between the crude and purified extracts could be attributed to the presence of lipids in the crude extracts. Matsushita and coworkers (1970) reported that lipid oxidation inhibits the activity of protease. Likewise, Castro Ceseña and coworkers (2012) concluded that the lipid content was removed by acetone purification. Therefore, after filleting, lipids must be separated as soon as possible, because their presence accelerates protein oxidation, resulting in a significant detriment in protein quality (Kanner and Rosenthal, 1992).

SDS-PAGE

The molecular weights of the enzyme extracts were estimated using the SDS-PAGE (Fig. 4). The main band with a molecular weight of about 25 kDa appeared on the protein gel in all the extracts analyzed. Trypsin was reported within the range 23-30 kDa for some fish species (Simpson, 2000). Two trypsin-like enzymes, designated trypsin A and B, were purified from the pyloric caeca and intestines of anchovy *Engraulis encrasicholus* with molecular weights of 27 and 28 kDa, respectively (Martinez et al., 1988). Castillo-Yañez and coworkers (2005) identified a 25 kDa protein band representing trypsin from the pyloric caeca of Monterey sardine (*Sardinops sagax caerulea*). Kurtovic and coworkers (2006) identified a trypsin band of 28 kDa molecular weight from the salmon pyloric caeca (*Oncorhynchus tshawytscha*) using SDS-PAGE. Molecular weights of other fish trypsins present in the viscera, estimated using SDS-PAGE, were 24 kDa for skipjack tuna (*Katsuwonus pelamis*) (Klomklao et al., 2007) and 25 kDa for sardine (*Sardina pilchardus*) (Bougatef et al., 2007). The molecular weight of trypsin extracted from the intestines of *B. capriscus* was estimated to be 23.2 kDa (Jeloulli et al., 2009).

Trypsin-specific activity

All the analyzed extracts hydrolyzed BAPNA efficiently. The trypsin activity of the crude extract was

Table 1. A summary of the proteolytic and trypsin activity from *Engraulis anchoita* viscera and heads at 60 °C and pH 8.0

Fraction	Proteolytic activity [U/mg]	Activity [U]	Purified fold	Recovery [%]
Crude extract (CE)	0.0218 ± 0.0025^{b}	0.3189 ± 0.022^{b}	1	100
Acetone: CE 1 : 1	0.0206 ± 0.0030^{a}	0.2431 ± 0.015^{a}	0.945	76.22
Acetone: CE 1 : 1.25	0.0224 ± 0.0010^{b}	0.2468 ± 0.010^{a}	1.028	77.40
	Trypsin activity [U/mg]			
Crude extract (CE)	0.0955 ± 0.0012^{a}	1.397 ± 0.023^{a}	1	100
Acetone: CE 1 : 1	0.134 ± 0.0020^{b}	1.581 ± 0.014^{b}	1.40	113.17
Acetone: CE 1 : 1.25	0.175 ± 0.0030^{c}	1.929 ± 0.018^{c}	1.83	138.3

Results of activity are mean values ± standard deviation (n = 3); different letters in the same column indicate significant differences ($P < 0.05$) (a – means the lowest value, b – means the highest value)

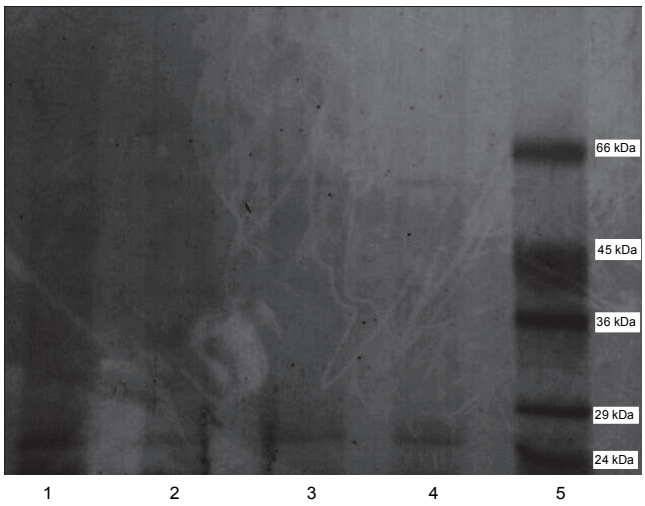

Fig. 4. SDS-page of the extracts from *Engraulis anchoita* by-products: Lane 1 – crude enzyme extract; Lane 2 – crude enzyme extract; Lane 3 – 1 : 1 crude extract: cold acetone purified extract; Lane 4 – 1 : 1.25 crude extract: cold acetone purified extract; Lane 5 – standard proteins marker of different molecular weights

0.0955 U/mg (Table 1). This result is in agreement with the trypsin activity (0.11 U/mg) from the spleen of yellowfin tuna *Thunnus albacores* (Klomklao et al., 2006). Blanco and coworkers (2014) reported a slightly higher activity (0.137 U/mg) in the homogenate of trypsin from *Scyliorhinus canicula* pancreas of a small-spotted catshark.

Acetone precipitation noticeably increased trypsin's activity in extracts purified from the viscera, to 0.113 U/mg and 0.168 U/mg in the 1 : 1 and 1 : 1.25 (acetone: crude extract) extract fractions, respectively. Jellouli and coworkers (2009) reported similar results for the specific trypsin activity using acetone precipitation in the intestines of gray triggerfish (*Balistes capriscus*).

The purification fold was 1.83 in the 1 : 1.25 acetone: crude extract fraction. A 3.27-fold increase in purified trypsin activity from the viscera of sardine by-products (*Sardinops sagax caerulia*) was described by Castro Ceseña and coworkers (2012), also using acetone purification. The yield of trypsin activity was increased to 138.3% in the 1 : 1.25 acetone: crude extract fraction. Ketnawa and coworkers (2013) reported that alkaline proteases from the viscera of farmed giant catfish yielded 142.46% using sodium citrate-t-butanol as a purifying agent. In addition, Rawdkuen and coworkers (2012) reported that when t-butanol-ammonium sulfate was applied in the same alkaline proteases, a yield of approximately 163% was obtained. The precipitation of trypsin from the pancreas of small-spotted catshark with ammonium sulfate resulted in 8% recovery (Blanco et al., 2014). This loss of trypsin activity could be explained by the denaturation effect caused by high salt concentration (Klomklao et al., 2004). Moreover, Olivas-Burrola and coworkers (2001) reported that cold acetone was a better purification agent than ammonium sulfate. From the results obtained, we concluded that *E. anchoita* by-products are rich in trypsin and their two-step purification with acetone could be advantageous, as it results in an increase in trypsin-specific activity.

Effect of metallo-protease inhibitor and surfactant on trypsin activity

The effect of EDTA on BAPNA hydrolysis by the crude and purified extracts (1 : 1.25 crude extract: cold acetone) was examined (Table 2).

Trypsin activity was only slightly affected by the metallo-protease inactivator EDTA. The retained activity

Table 2. Effect of EDTA and SDS on trypsin activity

	Final concentration [mM]	Activity retain [%]	
		Crude extract (CE)	Acetone: CE 1:1.25
NONE	0	100	100
EDTA	0.5	92.04	89.11
	0.25	93.92	90.77
SDS	1	108.13	107.53
	0.5	101.33	101.23

in the crude extract was more than 90%. This is in accordance with the 86% residual activity results obtained by Castillo-Yañez and coworkers (2005) in the trypsin from the pyloric caeca of sardines. Klomklao and coworkers (2006) reported a higher inhibition of trypsin from the yellowfin tuna spleen. Likewise, trypsin-like enzymes purified from the pyloric caeca and intestines of anchoita (*E. encrasicholus*) were readily inhibited by EDTA (Martinez et al., 1988). Trypsin activity from the intestines of gray triggerfish was not affected by the metallo-protease inactivator EDTA, as reported by Jellouli and coworkers (2009). Blanco and coworkers (2014) reported only 1% inhibition of the trypsin activity with EDTA in the pancreas of small-spotted catshark. Therefore, retaining the high activity of trypsin in the presence of EDTA is very useful for its application as a detergent additive, because chelating agents are components of most detergents (Jellouli et al., 2009).

The effect of the SDS surfactant on trypsin activity was evaluated in crude and purified extracts (crude extract: cold acetone 1:1.25). Both extracts exhibited a similar increase in activity compared with the control (Table 2). These results are in agreement with the increment obtained by Blanco and coworkers (2014) in the pancreas of small-spotted catshark. Other studies described an opposite effect on trypsins isolated from the intestines of sardinella (Khaled et al., 2008) and of triggerfish (Jellouli et al., 2009). Enzymes that resist the denaturation ability of SDS have been reported earlier (Rao et al., 1989). In addition, Sweadner (1991) demonstrated that trypsin retained its activity in the presence of SDS, if a trypsin inhibitor was present. Moreover, the achieved stability in trypsin activity after incubation with SDS suggests that this enzyme can be used as an additive in chemical formulations that include this surfactant, such as detergents.

Kinetic study

Kinetic constants K_m and K_{cat} of the *E. anchoita* by-products were determined using the Lineweaver-Burk plots (Fig. 5).

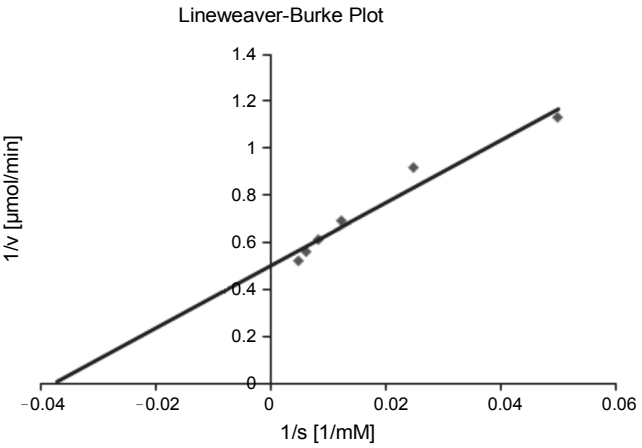

Fig. 5. Lineweaver-Burke plot of trypsin from *Engraulis anchoita* viscera and heads

The K_m and V_{max} values of the crude extracts were 0.36 and 0.027 mM/min, respectively. As can be seen in Table 3, the K_m values achieved for trypsin activity with the use of BAPNA as a substrate vary in other fish species. The value obtained in our study is in agreement with the one reported from the pyloric caeca of bigeye snapper (*Pricanthus macracanthus*) (Hau and Benjakul, 2006). The K_m values reported for the trypsins from carp (*C. carpio*) (Cohen et al., 1981), the pyloric caeca of Monterey sardine (*S. sagax cearula*) (Castillo-Yañez et al., 2005) and the intestines of gray triggerfish (*Balistes capriscus*) were lower than the results obtained in the case of *E. anchovy* by-products. However, the data reported by other authors were higher than the values achieved in our study, demonstrating that the trypsin from *E. anchoita* viscera has a superior affinity for BAPNA.

Table 3. Kinetic constants of *Engraulis anchoita* viscera and heads trypsin and other trypsin

Fish species	K_m mM	K_{cat}/s	K_{cat}/K_m s^{-1} mM^{-1}	Reference
Spotted catshark (*Scyliorhinus canicula*)	0.104	0.23	2.21	Blanco et al. (2014)
Grey triggerfish (*Balistes capriscus*)	0.068	2.76	40.6	Jellouli et al. (2009)
Cuttlefish (*S. officinalis*)	0.064	2.32	36.3	Balti et al. (2009)
Sardinelle (*Sardinella aurita*)	1.67	3.87	2.31	Khaled et al. (2008)
Monterey sardine (*S. Sagax cearula*)	0.051	2.12	41.0	Castillo-Yañez et al. (2005)
Bigeye snapper (*Priacanthus macracanthus*)	0.312	1.06	3.4	Hau and Benjakul (2006)
Anchovy (*E. japonica*)	0.049	1.55	31.0	Heu et al. (1995)
Anchovy (*E. encrasicholus*) A	0.830	1.55	1.86	Martinez et al. (1988)
Anchovy (*E. encrasicholus*) B	0.660	3.2	4.84	Martinez et al. (1988)
Carp (*C. carpio*)	0.38	3.1	79.5	Cohen et al. (1981)
Anchoita (*Engraulis anchoita*)	0.36	0.76	2.11	Present study

The catalytic efficiency (k_{cat}/K_m) of *E. anchoita* trypsin by-products, 2.11/s mM was close to that of trypsins from the digestive tract of anchovy *E. encrasicholus* (Martinez et al., 1988), sardinella (*Sardinella aurita*) (Khaled et al., 2008) and the pancreas of small-spotted catshark (*Scyliorhinus canicula*) (Blanco et al., 2014), using the same substrate.

Trypsin (EC 3.4.21.4) is a member of the serine proteinase family that specifically hydrolyzes proteins and peptides at the carboxyl side of arginine and lysine residues constituting important roles in biological processes (Cao et al., 2000). Trypsins have many biochemical and bio-industrial applications; their high specificity allows for a controlled hydrolysis of proteins. Trypsins of marine origin are of great interest because they exhibit a higher catalytic activity than their mammalian homologues and retain a high degree of activity at lower temperatures, making them more suitable for biotechnological processes and food processing applications (Macouzet et al., 1999; Macouzet et al., 2005). Moreover, marine trypsins tend to be more stable at an alkaline pH, with less stability at acidic pH, whereas mammalian trypsins show increased stability at acidic pH (Simpson and Haard, 1987; Simpson, 2000).

Various enzymes that can function at extreme conditions compared with enzymes from terrestrial sources are disposed in marine organisms, and can have novel applications in the food industry (Shahidi and Kamil, 2001; Trincone, 2011; Zhang and Kim, 2012). Considering their high activity and stability in high alkaline pH and, at low temperatures, and the stability in the presence of surfactants and oxidizing agents, alkaline trypsin from the intestine of gray triggerfish could be considered for applications in laundry detergents (Jellouli et al., 2009). Blanco and coworkers (2014) found a similar characteristic for the trypsin isolated from the pancreas of small-spotted catshark.

Conclusions

The viscera and heads from *Engraulis anchoita* could be considered as a good source of proteolytic enzymes, which display proper characteristics and sufficient activity under the pH and temperature conditions studied. The isolated proteolytic enzyme fraction was classified as trypsins on the basis of their pH optima, molecular weight, substrate specificity, and the effect of inhibitors tested.

Cold acetone precipitation has been indicated as a good precipitation method, with a high percentage of recovery of the proteolytic activity. In addition, the enzyme activity under optimal conditions was higher in a purified extract in comparison with crude extract. After the final purification step, the trypsin activity was purified 1.83-fold with a specific activity of 0.175 U/mg and 138.03% recovery.

Considering the high activity and stability in high alkaline pH, its activity at low temperature and stability in the presence of SDS, the extracts obtained from the viscera and heads of *E. anchoita* might find applications in laundry detergents.

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