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PARTIAL PURIFICATION OF PROTEOLYTIC ENZYMES AND CHARACTERIZATION OF TRYPSIN FROM MERLUCCIUS HUBBSI BY-PRODUCTS

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ABSTRACT

Proteolytic enzymes have been detected and partially purified from Merluccius hubbsi viscera. Crude proteinase extract exhibited its maximal activity at pH 7.94, 59.52ºC and 33.93 minutes using azocaseín as a substrate. The molecular weight was estimated to be 25 kDa by SDS-PAGE. The best ratio of crude extract to cold acetone for the partial purification of protease was found in 1:1.25 with a 94.02% of recovery. Results relative to the substrate specific BAPNA, indicated that the recovered protease was a trypsin. The kinetic trypsin constant Km and kcat were 0.38 mM and 0.97/s respectively, while the catalytic efficiency was 2.54/mMs. The stability results obtained with surfactants suggest that this enzyme can be incorporated as an ingredient in detergent formulations.

Keywords: Purification; Alkaline proteases; Merluccius hubbsi; Trypsin; Viscera

INTRODUCTION

The argentine hake (Merluccius hubbsi) is the most important economic resource for the fisheries of the South-West Atlantic Ocean. This demersal specie is distributed from 22◦to 55◦S in the Southwestern Atlantic Ocean at depths between 50 and 500 m (Cousseau & Perrotta, 2000). Of two main stocks, the southern or Patagonian stock (between 41°-55° S) is the most abundant (accounting for 85% of total hake biomass in the Argentine Sea) and economically more important (Aubone et al. 2000; Santos & Villarino, 2013). In recent years, catches of M. hubbsi exceeded 250,000 tons (MinAgri, 2015). This specie is mainly marketed as frozen fillet and lesser proportion headless and gutted (H&G). By-products generated from this fish processing industry constitute 40% of original raw material (Gbogouri et al. 2004). These by-products are commonly used for the manufacture of fishmeal and other products with negligible market value (Klomklao et al. 2013). Besides, this material is discarded many times without undergoing further treatment, collaborating with environmental pollution and ecological problems (Castro Ceseña et al. 2012; Klomklao et al. 2013), with important economic impact (Hordur & Rasco, 2000).

Currently, there is a great interest regarding the revalorizing of fishery by-products through the manufacture of nutritional and functional compounds (Hayes et al. 2008, Kim & Wijesekara 2010; Manni et al. 2010; Ngo et al. 2009; Klomklao et al. 2013). The fish viscera, one of the most important byproducts of fishing industry, are recognised as a potential source of digestive enzymes, especially proteases (Shahidi & Kamil, 2001; Klomklao et al. 2010a; Klomklao et al. 2010b). This is due to the highly specific nature of enzymes and their high catalytic activity at low concentration under lower temperatures and more alkaline pH values than their equivalents in mammals (Simpson & Haard, 1987; Menzefricke, 1997; Macouzet et al. 1999; Simpson, 2000; Shaidi & Kamil,2001; Macouzet et al. 2005). These properties contribute to avoid undesirable side effects in diverse production process (Simpson & Haard, 1987; Menzefricke, 1997; Gerday et al. 2000; Gudmundsdóttir & Pálsdóttir, 2005). Another important property observed, is their salt tolerance, which results an additional advantage in making savoury products, such as fish sauce (Caviccholi et al. 2002; Klomklao et al. 2006; Blanco et al. 2014). Therefore, recovery and characterization of enzymes from fish by-products has led to the emergence of some interesting new applications of these enzymes in food processing (Shahidi & Kamil, 2001). Between the most important proteolytic enzymes in the viscera of fish and aquatic invertebrates are the pepsin and the trypsin (Simpson, 2000; Castillo Yañez et al. 2005; Klomklao and Songklanakarin, 2008; Rawdkuen et al. 2012; Ferraro et al. 2013). Both enzymes are widely used in protein coagulation, selective tissue degradation, meat tenderization, additive in laundry detergent production, and wastewater treatment, among others (Shaidi and Kamil, 2001; Shaidi, 2007; Ferraro et al. 2013; Blanco et al. 2014).

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There have been many reports about the purification of proteolytic enzymes from fish viscera by various separation techniques (Rawdkuen et al. 2012). Typically, reports of extraction and purification of proteases, describe methods with salt as ammonium sulphate and column isolation (Heu et al. 1991; Ishida et al. 1995, Siringan et al. 2006; Klomklao et al. 2007; Blanco et al. 2014). Others, mentioned heat treatments followed by salt precipitation and gel filtration (Klomklao et al. 2011), salt and organic precipitation using t-butanol (Rawdkuen et al. 2012; Ketnawa et al. 2013), vacuum filtered and acetone as solvent (Klomklao et al. 2013; Klomkalo et al. 2014) and cold acetone precipitation (Michail et al. 2005). Also, Castro Ceseña et al. (2012) reported a centrifugation at low temperature followed by acetone precipitation step.

The use of enzymes in technological processes requires special attention to the reaction conditions. To optimize reaction conditions it is necessary to identify the variables that significantly influence the system under study, and how they affect. One of the popularly used methods is the one-factor at a time, but it requires many experiments and time. The design of experiments allows simultaneously studying the effects of all factors of interest in a given process and the possible interaction between them (Montgomery, 2005).

Nowadays, there is no information regarding the proteolytic enzymes of the viscera of hake and their possible application. Thus, the aim of this research was to partially purify and characterize trypsin-like proteinases from Merluccius hubbsi viscera.

MATERIALS AND METHODS

REAGENTS

Hydrochloric acid (HCl), sodium hydroxide (NaOH), Tris–HCl Tris (hydroxymethyl) aminomethane hydrochloride, sodium chloride (NaCl), azocasein, trichloroacetic acid (TCA), calcium chloride (CaCl2), dimethyl sulfoxide (DMSO), acetone, ethylenediamine tetraacetic acid (EDTA), Na-benzoyl-DL-arginine-p-nitroanilide (BAPNA), and bovine albumin serum (BSA Sigma A9647) were purchased from Sigma. All chemicals used were of analytical grade.

BIOLOGICAL SAMPLES

Samples of Merluccius hubbsi were collected during the research cruises of the National Institute for Fisheries Research and Development (INIDEP). Specimens were obtained in coast of Buenos Aires Province (34º S - 41º S). Six adult individuals of commercial size (39-42 cm total length) were selected. The viscera were separated and placed in polyethylene bags vacuum-sealed. Then, the samples were frozen at -80 ºC until used for enzyme extraction.

PREPARATION OF CRUDE ENZYME EXTRACTS

For enzyme extraction, viscera were thawed and processed using food blender homogeniser. Thereafter, were homogenized in chilled distilled water (1:4 w/v) in three times using Omni Mixer Homogeneizer. The homogenate was centrifuged at 10,000g for 30 min at 4 ºC (Multipurpose centrifuge, Hanil Industrial, Korea). The lipid phase was discarded and the supernatant collected was referred to as 'crude enzyme extract'.

The proteins of crude enzyme extract were evaluated using the Lowry method (1951), with bovine albumin as protein standard. The absorbance was measured at 500 nm in Uv Spectrophotometer Shimazdu.

PARTIAL PURIFICATION

The partial purification of protein extract was carried out following the procedure developed by Michail et al. (2005) with slight modifications. The crude enzyme extract was first subjected to acetone precipitation. Acetone, which was precooled to about 15 ºC, was slowly added to the crude enzyme extract preparations until the ratio enzyme solutions and acetone was 1:0.75. Then, the mixture was stirred for 10 min. The precipitates obtained were separated from the supernatant after centrifuging at 10,000g for 10 min at 4 ºC. Precooled acetone was added to the supernatants until the ratios between enzyme solutions and acetone were 1:1 and 1:1.25. The solutions were stirred for another 10 min before being centrifuged at 10,000g for 10 min at 4 ºC. The precipitates collected were dissolved in TRIS-HCl buffer, pH 8.

ENZYME ACTIVITY ASSAYS

Total proteolytic activity was evaluated according to Castro Ceseña et al. (2012) with some modifications: 0.5 % (w/v) azocaseín in 50 mM TRIS-HCl was used as a substrate. Aliquots (15 µL) of crude protein extract and the substrate, were mixed and incubated in Eppendorf tubes during 30 min. The reaction was stopped by adding 500 µL of 10 % trichloroacetic acid (TCA), and then the sample was stored for 10 min at 0-4 °C. The tubes were centrifuged for 5 min at 10,000g. The supernatant was separated, and the absorbance at Abs 366 nm was measured. The supernatant was separate. In this fraction, absorbance at Abs 366 nm was measured. Blank of activity was determined using TCA before the substrate. The substrate hydrolysis units per mg of protein were calculated using the following equation:

U/ (mg protein) = (ΔAbs/ min) (final reaction vol) / (mg protein)

EFFECTS OF TEMPERATURE, PH AND TIME ON PROTEINASE ACTIVITY USING EXPERIMENTAL DESIGN

The effect of temperature on protease activity was determined by incubating enzyme reaction mixtures at different temperature ranging from 40 to 80 °C. Also, the reaction was evaluated at different pH; using azocasein 0.5 % (w/v) dissolved in 50 mM Tris-HCl at pH range between 6.0-10.0. At specific time intervals, the enzyme solutions were withdrawn and assayed. A factorial experimental design was used to optimize the studied. The

Box–Behnken method-3^3 (three factors each, at three levels) experimental design model were used. Actual values of the factors were selected at three levels, coded as −1, 0 and +1 for low, middle and high values respectively (Table 1). The software Design Expert 7.0 (Stat-Ease, USA) was used for the design of experiment. 17 runs of experiments were performed in duplicate.

An Analysis of Variance (ANOVA) was conducted to determine the significant effects of process variables on the response. By applying multiple regression analysis to the experimental data, a polynomial equation was generated, representing the enzymatic activity as a function of variables. The analysis was performed using coded units. Desirability functions were used to the fitting model optimization by maximizing the enzyme activity. The response surface charts were used to represent the enzyme activity as a function of pH and temperature with the time held at the optimum level.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was carried out in gels with 30 % of acrylamide/bisacrylamide (37.5:1) solution according to the procedure of Laemmli, (1970). Aliquots of the crude extract and purified extracts were denatured with an equal volume of denaturalization buffer (0.125M 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 during 2 h. After 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. Trypsin 25 kDa was used as molecular weight marker.

TRYPSIN SPECIFIC ACTIVITY

Trypsin activity was evaluated according to Castillo Yañez et al. (2005), using BAPNA as substrate with slight modifications: 0.010 of crude protein extract was mixed with 10 µL of 0.5 M Tris–HCl (pH 8), 0.1 ml of 0.2 M CaCl2; and 40 µL of 0.02 M BAPNA in DMSO. The final reaction volume was adjusted to 1 ml with distilled water. Assays were performed at 30 ºC. One activity unit was defined as the production of 1 µmol/min of free p-nitroanilide released, which was measured by monitoring the change in absorbance at Abs 410 nm for 10 min. The substrate hydrolysis units per mg of protein were calculated using the following equation:

U/ (mg protein) = (ΔAbs/ min) (1000) (final reaction vol) / (8800) (mg protein)

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

EFFECT OF EDTA AND SDS ON TRYPSIN ACTIVITY

Enzyme extract was incubated with the metalloprotease inhibitor EDTA. A mixture of 10 µL of 0.5 mg/ml inhibitor solution and 10 µL of enzyme extract was incubated for 30 min at room temperature. The residual enzyme activity was measured at 30 ºC and pH 8 using BAPNA as substrate. Controls and blanks were assayed under the same conditions. The activity of the enzyme without inhibitors was considered as 100 %.

The effect of SDS surfactant on enzyme activity was studied by incubating 10 µL of 1 mg ml^{-1} SDS solution and 10 µL of enzyme extract for 60 min at 40 ºC. The activity of the enzyme without surfactant was considered as 100 %. The residual enzyme activity was measured at 30 ºC and pH 8 using BAPNA as substrate

KINETIC PARAMETERS

The Michaelis–Menten constant (Km), maximum velocity (Vmax) and catalysis constant (kcat) were evaluated at 30 ºC by varying BAPNA substrate concentration between 0.02 and 2 mM. Kinetic parameters including Vmax and Km were evaluated by plotting the data on a Lineweaver–Burk double-reciprocal graph (Lineweaver and Burk, 1934). Values of turnover number (Kcat) were calculated from the following equation: Vmax / [E] = Kcat, where [E] is the active enzyme molar concentration and Vmax 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 duplicate and the results were expressed as mean value ± standard deviation. The data were subjected to analysis of variance (ANOVA), and the differences between means were carried out using Duncan's multiple range tests, being statistically different at significance level of 5 %.

RESULTS AND DISCUSSION

EXPERIMENTAL DESIGN AND ANOVA

Initially, protease activity was performed on crude extract in order to detect the optimal conditions to achieve the maximum enzyme reaction in the range of the parameters studied. Applying multiple regression analysis to the experimental data, an equation model that represents the enzyme activity as a function of significant variables was generated. The fitted model, expressed in coded significant variables, is represented by:

Proteolityc activity (U/mg proteína) = $- 0.551 + 0.096\,A + 3.17 \times 10^{-3}\,B + 7.36 \times 10^{-3}\,C – 2.50 \times 10^{-6}\,AB -1.875 \times 10^{-5}\,AC - 3.0 \times 10^{-6}\,BC - 6.023 \times 10^{-3}\,A^2 - 2.530 \times 10^{-5}\,B^2 - 1.030 \times 10^{-4}\,C^2$

Analysis of variance (ANOVA) was performed to check the significance of the fit of the second-order polynomial equation for the experimental data. The results are summarized in Table 2. The value of Prob > F less than 0.05 indicated model terms was statistically significant and that the established model could adequately represent the real relationship among the selected parameters. The model F-value 72.33 implied the model was significant.

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The lack of fit F-value 4.99 implied the lack of fit was not significant relative to the pure error. The P-value was also used to check the significance of each coefficient, being the smaller the P-value, the more significant coefficient. The coefficients of time and the quadratic function of the pH, temperature and time were statistically significant, because the P-values of them were very small ($P < 0.05$). The most significant factor was the reaction time (P-value < 0.001). The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the determination coefficient (R^2) was 0.9894. This implies that 98.94 % of the variation could be explained by regression model, this model had a high credibility. In addition, the value of adjusted determination coefficient (R^2 Adj= 0.9757) was also high and supported the high applicability of the model. The value of predicted determination coefficient (R^2 Pred= 0.8622), which gave information about the suitability of the model for predicted a response value, was in reasonable agreement with the R^2 Adj. Furthermore, the value of the coefficient of variation (CV = 8.05%) was also relatively low indicating high precision and reliability of the experiments (Yetilmezsoy et al. 2009).

OPTIMIZATION

The numerical optimization indicates the maximum activity predicted by the model was obtained at 59.52 °C, pH 7.94 during 33.93 minutes. In order to verify the optimized results, and test the suitability and validity of the model for predicting the optimal response value, the reaction was carried out under the optimal condition in triplicate. The result showed that the experimental value (DS = 0.0146) was in agreement with the predicted values (DS = 0.0139). Moreover, the experimental value under the optimal condition was better. It was indicated that the model was powerful and suitable for estimation of the experimental value.

The response surface curve for pH vs. temperature, at time fixed at the optimum value is presented in Figure 1. It provided a visualization of the interactions between these two variables on the responses. Increasing and decreasing pH and temperature than optimal values leads to reduce the proteolytic activity. So, the proteases recoveries from M. hubbsi by-products are very stable a wide broad pH range, showing high activity between pH 6 and 10 in the range of temperature and time studied. In the viscera of fish, the most important intestine enzymes are alkaline proteases (Simpson, 2000). Within industrial processes, the use of alkaline proteases has increased remarkably, since they are stable and active under drastic conditions, such as at temperatures of 50-60 °C, high pH and the presence of surfactants or oxidizing agents (Joo et al. 2001). These results suggest that M. hubbsi visceras enzymes would be a potential source of alkaline proteases for certain industrial applications. One of the most important variables within the selection of proteases for detergent industry is the optimum pH (Banerjee 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 & Saleemudin, 1998; Gupta et al. 2002).

Table 1 - Experimental design for enzymatic activity of Merluccius hubbsi byproducts

Factors	Name	Levels		
		-1	0	1
pH	A	6	8	10
Temperature °C	B	40	60	80
Time (min)	C	20	30	40

PROTEOLYTIC ACTIVITY UNDER OPTIMAL CONDITIONS

The results obtained of the crude extract and applied purification methods (cold acetone) are shown in Table 3. As can be seen, the proteolytic activity (U/mg) achieved using cold acetone in a ratio of crude extract to acetone of 1:1 and 1:1.25 was higher than crude extract used. These results indicate that cold acetone is a good effective agent for the initial step of protease purification. The results showed that the ratio 1:1 provided a 1.02-fold purification. The procedure used in this paper with a ratio of crude extract to acetone of 1:1.25 produced more than 90 % of enzyme activity recovery.

Popova & Pishtiyski, (2001) reported the effectiveness of cold acetone as a purification agent for proteolytic enzymes, previously. Also, these results are in accordance with others studies of partial purification of proteolytic enzymes from sardine by-products (Castro Ceseña et al. 2012) and precipitation techniques of proteolytic enzymes from trout (Salmo gairdnerii) heads (Michail et al. 2005). Many different protein and enzyme purification techniques have been reported previously. Ketnawa et al. (2013) studied the recovery of alkaline protease from fish viscera by phase partitioning. Klomklao et al. (2005) reported partitioning of spleen proteinase from yellowfin tuna by aqueous two-phase systems and magnesium sulfate showed high yield.

Bezerra et al. (2005) have obtained alkaline protease from the viscera (intestine) of Nile tilapia by using heat treatment, ammonium sulphate fractionation and Sephadex G-75 gel filtration.

Also, insufficient partial purification by ammonium sulfate was observed by Wang et al. (2002). Maehashi et al. (2002) and Olivas-Burrola et al. (2001) besides reported that cold acetone was a much better purification agent. So, the results obtained in the present studies confirmed that cold acetone is the most effective agent for the initial step of protease purification.

Under optimal conditions, the enzyme activity of the 1:1 and 1:1.25 purified cold acetone extracts were 0.0149 and 0.0208 respectively. These results suggest that the lipids presents in crude extract obstructs the protease activity. Similarly, Matsushita et al. (1970) reported that lipid oxidation inhibits protease activity. Likewise, Castro Ceseña et al. (2012) concluded that acetone purification

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remove lipids content by monitoring protein and lipids content after the process. In addition, lipid separation should be performed as soon as possible after filleting, because the lipids accelerate protein oxidation, producing a significant detriment to protein quality (Kanner & Rosenthal, 1992).

Table 2 - Analysis of variance and regression coefficients of the second-order polynomial equations obtained from experimental data

Source	Sum of square	Degree of freedom	Mean square	F Value	p-value
Model	4.22 x 10^{3}	9	4.69 x 10^{-1}	72.33	< 0.0001
A-pH	9.46 x 10^{-3}	1	9.46x 10^{-3}	1.46	0.2662
B-Temperature	288 x 10^{-3}	1	2.88 x 10^{-3}	0.44	0.5264
C-Time	5.83 x 10^{-1}	1	5.83 x 10^{-3}	89.98	< 0.0001
AB	4.0 x 10^{-5}	1	4.0 x 10^{-5}	6.17 x 10^{-3}	0.9396
AC	5.63 x 10^{-4}	1	5.63 x 10^{-4}	0.087	0.7768
BC	1.44 x 10^{-3}	1	1.44 x 10^{-3}	0.22	0.6517
A^2	2.44 x 10^{3}	1	2.44	377.12	< 0.0001
B^2	4.31 x 10^{-3}	1	4.31 x 10^{-1}	66.51	< 0.0001
C^2	4.46x 10^{-1}	1	4.46 x 10^{-1}	68.83	< 0.0001
Residual	4.54x 10^{-2}	7	6.48 x 10^{-3}		
Lack of Fit	3.58 x 10^{-2}	3	1.19 x 10^{-2}	4.99	0.0774
Pure Error	9.57 x 10^{-3}	4	2.39 x 10^{-3}		
Cor Total	4.26 x 10^{3}	16			

R^2 = 0.9900, R^2Adj = 0.9771, RP^2red = 0.8825, CV = 7.82%

Table 3 - Summary of proteolytic activity and trypsin activity from Merluccius hubbsi viscera

Fraction	Proteolytic Activity (U/mg)	Activity (U)	Purified fold	Recovery (%)
Crude extract (CE)	0.0146 ± 0.0040 a	0.181± 0.047 b	1	100
Acetone: CE 1:1	0.0149 ± 0.0005 a	0.099± 0.003 a	1.02	54.82
Acetone: CE 1: 1.25	0.0208 ± 0.0040 b	0.170± 0.003 b	1.42	94.02
	Trypsin Activity (U/mg)			
Crude extract (CE)	0.0978 ± 0.003 a	1.211± 0.04 b	1	100
Acetone: CE 1:1	0.135 ± 0.002 b	0.898 ± 0.016 a	1.38	74.15
Acetone: CE 1: 1.25	0.138 ± 0.001 b	1.127± 0.008 b	1.41	93,12

Results are mean values ± standard deviation (n = 2), Different letters in the same column indicate significant differences ($P < 0.05$), ("a" means the lowest value, "b" means the highest value)

Table 4 - 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	91	95.65
SDS	1	107.36	107.98

Table 5 - Kinetic constants of Merluccius hubbsi viscera trypsin and other trypsin

Fish specie	Km mM	Kcat/s	Km/kcat	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)

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Cuttlefish (S. officinalis)	0.064	2.32	36.3	Balti et al. (2009)
Sardinelle (Sardinella aurita)	0.0208	0.170	1.42	Khaled et al. (2008)
Montery sardine (S. sagax cearula)	0.051	2.12	41	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	Heu et al. (1995)
Anchovy (E. encrasicholus) A	0.830	1.55	186	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)
Hake (Merluccius hubbsi)	0.38	0.97	2.54	Present study

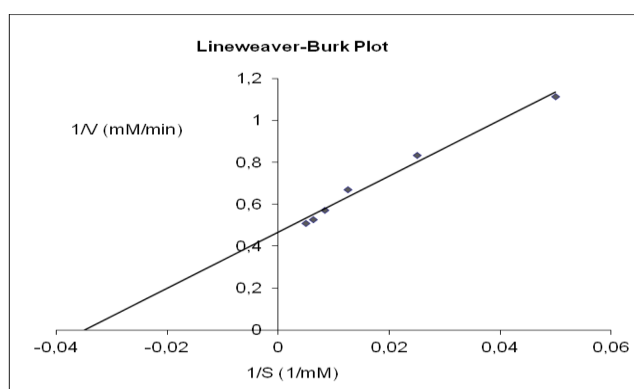

Figure 1-Effect of temperature and pH on the proteolytic activity of Merluccius hubbsi viscera at optimal fixed time

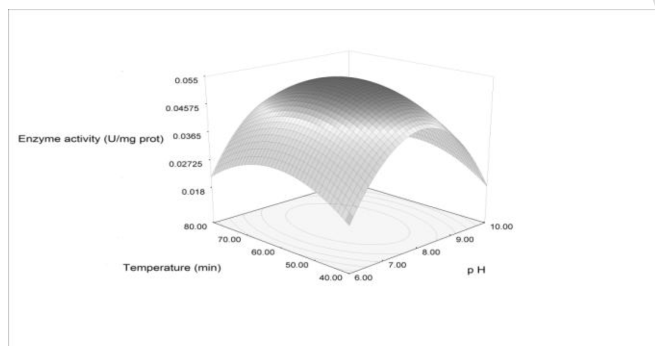

Figure 2- Lineaweaver –Burke plot of trypsin activity from Merluccius hubbsi viscera crude extract

SDS-PAGE

The protein patterns of crude enzyme extract and the acetone purified fraction of 1:1.25 ratio were analyzed. The protein fractions detected in the crude extracted by-product were from 20.6 to 80 kDa.

A protein band with molecular weight of approximately 25 kDa was identified and it was present in crude and purified extracts. This was located within the range of 23 to 30 kDa, as trypsin according to the reported for other fish species by Simpson, (2000). Kurtovic et al. (2006) identified a trypsin band of 28 kDa molecular weight related to pyloric caecum salmon (Oncorhynchus tshawytscha). Castillo Yañez et al. (2005) identified a 25 kDa band of trypsin from pyloric caeca of Monterey sardine (Sardinops sagax caerulea). Molecular weights of other-trypsins present in viscera of marine organisms, were 24 kDa for skipjack tuna (Katsuwonus pelamis) (Klomklao et al. 2007) and 25 kDa in sardine (Sardina pilchardus) (Bougatef et al. 2007).

TRYPSIN SPECIFIC ACTIVITY

The initial specific trypsin activity of the crude extract was 0.0978 U/mg (Table 3). This result is in agreement with the trypsin activity (0.11 U/mg) from Thunnus albacores yellowfin tuna spleen (Klomklao et al. 2006). Likewise, Blanco et al. (2014) reported a similar data (0.137 U/mg) in the homogenate of trypsin from Scyliorhinus canicula pancreas of small-spotted catshark. Conversely, Castillo Yañez et al. (2005) informed a higher value (0.5 U/mg) in the trypsin from pyloric caeca of sardine Sardinops sagax caerulea. Acetone precipitation increased the trypsin activity in the viscera purified extracts, being 0.135 U/mg and 0.138 U/mg in the acetone: crude extract 1:1 and 1:1.25 fraction extracts respectively. Castro Ceseña et al. (2012), found a similar behaviour using acetone precipitation in viscera from sardine by-products (Sardinops sagax caerulia). The retain activity in both extract purified were more than 70 %. This result is in accordance with the 68 % of activity retain in trypsin from the intestine of grey triggerfish (Balistes capriscus) achieved by Jellouli et al. (2009) using purification with 60-80 % of acetone. The results obtained in the present work, might indicate that hake viscera may be rich in trypsin enzyme and its purification in two steps with acetone could be advantageous to be used in different industrial areas.

EFFECT OF METALLO-PROTEASE INHIBITOR AND SURFACTANT ON TRYPSIN ACTIVITY

The effect of EDTA on crude extract and on the purified extract wherein better precipitation was achieved (1: 1.25 crude extract: cold acetone) was investigated (Table 4).

The trypsin activity was slightly affected by the metallo-protease inactivator EDTA. The activity retained in crude extract was more than 90 %. This is in accordance with the 86 % residual activity results obtained by Castillo

Yañez et al. (2005) in the trypsin from pyloric caeca of sardine. Klomklao et al. (2006) reported a higher inhibition in trypsins from yellowfin tuna spleen. The metallo-protease inactivator EDTA (Jellouli et al. 2009) did, not affect trypsin activity from the intestine of grey triggerfish. Blanco et al. (2014) reported only 1 % of the inhibition of the trypsin activity with EDTA in pancreas of small-spotted catshark. So, the high activity retention in the presence of EDTA is very useful for application as a detergent additive because chelating agents are components of most detergents (Jellouli et al. 2009).

The effect of SDS surfactant on trypsin activity was evaluated in crude and purified extract (crude extract: cold acetone 1:1.25). In both extracts, it was verified an increased activity compared to the control (Table 5). These results are in agreement with the increment obtained by Blanco et al. (2014) in pancreas of small-spotted catshark. Other studies described an opposite effect on trypsin isolated from the intestine of sardinelle (Khaled et al. 2008) and triggerfish (Jellouli et al. 2009). The enzymes that resist the denaturation ability of SDS have been reported previously (Rao et al. 1989). Also, Sweadner, (1991) demonstrated that trypsin retains activity in presence of SDS if a trypsin inhibitor it is present. Moreover, the stability achieved in trypsin activity after incubated with SDS, suggest that this enzyme can be used as an additive in chemical formulations that include this surfactant such as detergents.

KINETIC STUDY

Kinetic constants Km and kcat for trypsin in hake viscera were calculated using Lineweaver–Burk plots (Figure 2). The Km and Vmax values for the crude extract were 0.38 and 0-029 mM/min respectively. As can be seen in Table 5, the Km values achieved for trypsin activity vary in other fish species using BAPNA as a substrate. The value obtained in the present study is in agreement with the reported from pyloric caeca of bigeye snapper Pricanthus macracanthus (Hau & Benjakul 2006). The Km informed for trypsins from carp (C. carpio) (Cohen et al. 1981), pyloric caeca of Monterey sardine (S. sagax cearula) (Castillo-Yañez et al. 2005) and intestine of grey triggerfish (Balistes capriscus) were lower than the result obtained in hake viscera. However, the data reported by other authors were higher than the value achieved in the present work, demonstrating that trysin from Merluccius hubbsi viscera has a superior affinity for BPANA.

The catalytic efficiency (kcat/Km) of Merluccius hubbsi viscera trypsin, 2.54/s mM, was close to those of trypsins from the digestive tract of anchovy (Engraulis encrasicholus) (Martinez et al. 1988), sardinelle (Sardinella aurita) (Khaled et al. 2008) and pancreas of small-spotted catshark (Scyliorhinus canicula) (Blanco et al. 2014), using the same substrate.

Trypsin (EC 3.4.21.4) is an enzyme family member of the serin proteinase 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). Trypsin has many biochemical and bio-industrial applications; its high specificity allows controlled protein hydrolysis. Trypsins from marine origin are of great interest because they exhibit higher catalytic activity than its mammalian homologues and retain a high degree of activity at lower temperatures, doing them more suitable for biotechnological processes and food processing applications (Macouzet et al. 1999; Macouzet et al. 2005). Also marine trypsins tend to be more stable at alkaline pH, having less stability at acidic pH while mammalian trypsins show increased stability at acidic pH (Simpson & Haard 1987; Simpson, 2000).

Various enzymes which can function at extreme conditions compared to enzymes from terrestrial sources are available in marine organisms with novel application for the food industry (Shahidi & Kamil 2001; Trincone, 2011; Zhang & Kim 2012). Anusha et al. 2008 investigated the potential use of endogenous enzymes presents in Pacific hake muscle, to produce fish protein hydrolysates with antioxidative properties. Mazorra-Manzano et al. (2012) reported that a fish protein hydrolysate from Pacific whiting (Merluccius productus) muscle was produced by autolysis of a minced homogenate. Jellouli et al. (2009) considering the high activity and stability in high alkaline pH, activity at low temperature, stability in the presence of surfactants and oxidising agents of alkaline trypsin from the intestine of grey triggerfish, it could be consider an application in laundry detergents. Blanco et al. (2014), found a similar application for the trypsin isolated from pancreas of small-spotted catshark.

CONCLUSIONS

Enzymes are product of great interest in biotechnological process. The properties of marine enzymes to maintain their activity at low temperature might be very advantageous in food industry, in order to avoid undesirable chemical reactions. The wide range of pH from the alkaline proteases is useful in detergents industry. The pH and temperature environmental factors are important for the proteolytic activity.

The waste from hake (Merluccius hubbsi) could be considered as a good source of proteolytic enzymes, which have shown a sufficient stability under the pH and temperature studied.

Based on SDS-PAGE and activity for the specific substrate BAPNA the fraction enzyme recovery and purify from the Merluccius hubbsi viscera was trypsin. The enzyme was similar in many biochemical characteristics to trypsin in other fish, but was slightly different from others trypsins in some kinetic parameters.

Cold acetone precipitation was indicated as a good precipitation method, with a high percentage of recovery of proteolytic activity. Also, the enzyme activity under optimal conditions was higher in purified extract with respect to crude extract. Thus, the cold acetone step resulted very important in order to eliminate the initial lipids content that obstruct the proteolytic activity.

The characteristics of the isolated trypsin are interesting from a technological perspective.

Considering the high activity in high alkaline pH, stability in the presence of EDTA and surfactants, trypsin

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obtained from Merluccius hubbsi viscera may find application in laundry detergents. So, in this work, is proposed a process that increases the value of the by-products hake.

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