Journal of Agriculture and Environmental Sciences
December 2015, Vol. 4, No. 2, pp. 8-17
ISSN: 2334-2404 (Print), 2334-2412 (Online)

Published by American Research Institute for Policy Development
DOI: 10.15640/jaes.v4n2a2
URL: http://dx.doi.org/10.15640/jaes.v4n2a2

Characterization of Proteolytic Enzymes from *Sparus aurata L.* Viscera, Obtained by Supercritical Carbon Dioxide, Mechanical and n-hexane Extraction Methods

Daniela L. Lamas[1]

Abstract

The oil in gilthead sea bream (*Sparus aurata L.*) was extracted using an environmental friendly solvent, supercritical carbon dioxide (SC-CO2) at a flow extraction process, mechanical method and n-hexane solvent extraction. The SC-CO2 extraction was carried out at temperature 45 °C and 25 MPa of pressure. The flow rate of CO2 (10Kg/h) was constant at the entire extraction period of 240 min. The extracted residue after oil extraction was used for activity measurement of digestive enzymes. In addition, an untreated viscera crude portion was used as a control. Crude protein extract exhibited its maximal activity at pH 8 and 60ºC of temperature using azocaseín as a substrate. Results relative to the substrate specific BAPNA, indicated that a protease recovery was a trypsin type enzyme. All methods achieved higher activity than crude extract. The supercritical fluid extraction was found to be the best for the partial purification of protein phase and retains the highest proteolytic activity. Mechanical extraction was found to be the slightest protein recovery method. Stability results obtained have shown the great potential of these specific compounds for use as nutraceuticals or as ingredients for functional foods. In addition, the stability against surfactants suggests that this enzyme can be incorporated as an ingredient in detergent formulations.

Keywords: alkaline enzymes, protein purification, proteolytic activity, *Sparus aurata* L., fish viscera.

1. Introduction

Gilthead sea bream (*Sparus aurata L.)* is widely farmed around the Mediterranean area with a high annual production (FAO, 2015). It is commonly used as a fresh fish. Because of its commercial importance, the viscera are considered an important by-product.

The most common and major outlet for this raw material is fishmeal, oil production and small quantities are used for pet food. However, the large amounts of by-products generated have great potentials for the extraction of biologically desirable high added value compounds (Ferraro et al., 2013). One of the concerns of the fishing industry is focused on an adequate destiny and/or use for these residues, in order to reduce or avoid environmental aggressions.

Thus, nowadays, as opposed to the traditional conversion into low value products, novel approaches involve the identification and recovery of useful biochemical compounds. They include proteins, enzymes, ω3 fatty acids, aminoacids, biopolimers and biomaterials (Shaidi & Kamil, 2001; Mireles DeWitt & Morrissey, 2002; Ferraro et al., 2013). The most important proteases in the viscera of fish and aquatic invertebrates are aspartic protease (pepsin) and serine proteases (trypsin, chymotrypsin, collagenase, and elastase) (Simpson, 2000; Klomklao et al., 2012). Proteases are by far the most studied enzymes for industrial bio-processing. Almost half of all industrial enzymes are proteases, mostly used in the detergent, leather, and food industries (Klomklao et al., 2005, Ketnawa et al., 2014).

[1]CONICET- UNMdP- INIDEP - Instituto Nacional de Investigación y Desarrollo Pesquero -. Paseo Victoria Ocampo N °1, Escollera Norte (7602); Mar del Plata - Buenos Aires, Argentina, Ph: +54 (223) 4862586, e-mail: dlamas@inidep.edu.ar

The use of alkaline proteases from marine digestive organs has increased remarkably, since they exhibit unique properties of activity and stability. These characteristics might be due to the adaptation of marine organisms to extreme environmental conditions as wide ranges of temperatures and pHs, availability of oxygen fluctuating and the presence of surfactants and heavy metals due to the adaptation of marine organisms to extreme environmental conditions (Haard, 1992; Diaz-Lopez & Garcia-Carreño, 2000, Joo et al., 2001; Ketnawa et al, 2013).

There have been many reports about the purification of proteolytic enzymes from fish viscera by various separation techniques. Typically, reports of extraction and purification of proteases describe salt and organic precipitation, chromatography, or phase separation by an aqueous two-phase system. Most of these operations are time consuming, difficult to scale up, involve expensive reagents, and require technical skill (Rawdkuen et al., 2012). For higher efficiency of enzyme isolation, lipid removal is needed from the sample (Azzaduzaman & Chun, 2015). Different methods are commonly used to obtain a purified oil lipid phase. Mechanical extraction offers quite low initial and operational costs. The most used method to extract the lipids is by means of non-polar solvents. The selection of the solvent is very important in order to obtain extracts of good quality. moreover, solvent must be non-inflammable, non-toxic and non-volatile. In addition, removal lipids with solvent extraction cause protein denaturation.

The considerable concern with environment pollution has focused the studies on finding alternative extraction methods (Delgado Vargas et al., 2003). The extraction with supercritical carbon dioxide (SC-CO2) has become an important separation technique in the area of nutraceutical supplements and functional foods (Reverchon & De Marco, 2006; Parajó et al., 2008). Supercritical fluid extraction (SFE) technology is a promising alternative to the traditional processing methods. This extraction technique is especially indicated when thermolabile compounds are present, in addition to avoiding the use of toxic solvents (Reverchon & De Marco, 2006; Parajó et al., 2008; Sahena et al., 2009a). The major advantage of supercritical fluid extraction over the conventional extraction is that this technique does not require subsequent processing steps to separate the solvent, since CO2 is a gas at normal temperature and pressure, easy to separate from the extract (Reverchon & De Marco, 2006; Parajó et al., 2008; Sahena et al., 2009a; Sanchez-Camargo et al., 2012).

The use of supercritical fluids in the food industry is widely established (Brunner, 2005; Catchpole, et al., 2009, Sahena et al., 2009b). The first commercial supercritical extraction was for the decaffeination of green coffee beans (Palmer & Ting, 1995). Commercial applications at present include decaffeination of coffee and tea, extraction of natural colours, antioxidants, nutraceuticals, and hops, as well as extraction of lipids and cholesterol from egg yolks, milk fat, beef and pork (Sahena et al., 2009b).

In this work, different oil methods extraction were applied on dried viscera from *Sparus aurata* as an indirect technique for extraction and purification of protein part. In addition to that, proteolytic activity of crude extract was compared to proteolytic activity of other extracts obtained by different methods in terms of yield reaction. Thus, this investigation aims to partially purify and characterize alkaline trypsin-like proteinases from *Sparus aurata* L. viscera.

2. Materials & methods

2.1 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.

2.2 Sample preparation and storage

Gilthead sea bream *Spaurus aurata* L. were caught and delivered to the commercial market (El Arbol, Burgos, Spain) within 12 hours, in an ice bath. Then, the samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the research laboratory within 30 min. Before excise the viscera sample from each fish, the biometric parameters (total weight, total length) were measured.

The internal organs were separated and viscera were collected, rinsed with distilled water and then stored in sealed plastic bags at - 20ºC until used for protein and enzyme extraction. After crushed, the samples were dried in a freeze-drier for about 72 h. These samples were then stored at −80 °C and processed for oil extraction by SC-CO2, mechanical pressure and organic solvent.

The supercritical CO2 extraction experiments were carried out in a semi-pilot SFE plant. The extraction was performed at 45°C of temperature, 25MPa of pressure and solvent flow of 10 kg CO2/h.

The sample of the *Spaurus aurata* L. by-product (20g) was placed in the extractor that was later pressurized up to the extraction pressure, with carbon dioxide (liquid CO2 ≥ 99.9%). Then, the solvent was circulated during 240 min.

The mechanical extraction was performed over 20g of freeze dried raw material using a Mixer Homogeneizer, followed by the centrifugation during 10 min at 2400*g*. The supernant was collected as a lipid phase and the remaining residue was dried.

Soxhlet using hexane was carried out for the solvent extraction at 45°C during 240 min. Around 20g of freeze dried raw material was loaded into the beaker with hexane. After extraction, the solvent was filtered and evaporated. The remaining residue was dried during 6h.

2.3 Characterization of fish by-products

Fish by-products samples were characterized by determining their ash, moisture, protein and fat content in order to establish their profitability as raw materials for protein extraction (Table 1). Ash, moisture and protein content were determined using the AOAC Official Methods (2000). Total fat content was determined by Soxhlet extraction using petroleum ether as solvent. Soxhlet extraction was performed over freeze-dried samples in 140 min distributed in three stages: extraction (120 min), rinsing (10 min) and drying (10 min).

Table 1: Proximate composition of the *Sparus aurata* L. by-products studied.

Raw material	Moisture	Protein	Lipid	Ash	Total
Viscera *Sparus aurata*	74.33±5.30	23.45±5.80	2.13±0.52	0.09±0.02	100

Results are mean values ± standard deviation (n=3).

2.5 Protein determination

For enzyme extraction, all the residues obtained 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 supernatant collected was referred to as 'crude enzyme extract', 'SFE enzyme extract', 'hexane enzyme extract' and 'mechanical enzyme extract'. Protein concentration of all the extracts was measured by the method of Lowry et al., (1951) using bovine serum albumin as a standard.

2.4 Quantification of proteolytic activity.

Total proteolytic activity was assayed according to Castro Ceseña et al., (2012) with some modifications: 0.5 ml of 0.5 % (*w/v*) azocaseín in 50 mM TRIS-HCl used as a substrate and 0.015 ml of the protein extract were mixed. The reaction was initiated by adding the sample and was stopped with 0.5 ml of 10% trichloroacetic acid (TCA), incubating at 0°C for 10 min. The reaction mixture was centrifuged in Eppendorf tubes for 5 min at 10,000*g*, at 4°C. The supernatant was separated from the undigested substrate, and the absorbance at Abs 366nm of the released dye was measured.

The blank was prepared by adding TCA before the substrate. Protease activity was expressed as the number of protease units per mg of protein.

2.6 pH and temperature profile

Proteolytic activity was assayed over the pH range of 6 .0–10.0 (50 mM Tris–HCl buffer) for 30 min. For temperature profile study, the activity was assayed by incubating enzyme reaction mixtures at different temperatures ranging from 40 - 80 °C for 30 min.

2.7 Enzyme activity time stability

The effect of time on enzyme stability was evaluated by measuring the residual activity after incubation at pH 8, at 60°C of temperature. The analysis was carried out on crude extract for various times (0, 10, 20, 30, 60, 120, 180 and 240). The remaining activity was determined using azocaseín as a substrate.

2.8 Trypsin activity

Trypsin activity was evaluated according to Castillo-Yañez et al. (2005), using BAPNA as substrate with slight modifications: 0.005 ml of the *S. aurata* protein extract was mixed with 0.1 ml of 0.5 M Tris–HCl, pH 7.5, and 0.1 ml of 0.2 M CaCl2. Then, 0.04 ml of 0.02 M BAPNA in dimethyl sulfoxide (DMSO) was also added to the mixture. The final reaction volume was adjusted to 1 ml with distilled water. Assays were performed at 30 °C. 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 410nm.

2.9 Effect of EDTA and SDS on trypsin activity

Crude enzyme extract was incubated with the metalloprotease inhibitor EDTA. A mixture of 0.010 mL of 0.5 mg/ml inhibitor solution and 0,010 ml 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. Control was 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 0,010 ml of 1 mg ml-1 SDS solution and 0,010 ml 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

2.10 Statistical analysis

The proximate analyses of the raw material were carried out by triplicate. Protein content, enzyme activity and trypsin activity experiments were performed in duplicate. 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 %.

3. Results and discussion

3.1 Proteolytic activity of crude extract, pH and temperature profile

Temperature and pH profiles of crude extract proteolytic activity from the viscera of *Sparus aurata* L. are depicted in Fig. 1. The highest proteolytic activity was found in the 55 to 60ºC range. All the range studied, shown measurable activity. However, a sharp decrease in activity at temperature above 70º C was observed, most likely due to thermal denaturation.

The effect of pH on the enzyme activity from the viscera of *Sparus aurata* L. was determined over a pH range of 6.0–10.0. The protein extract was highly active between pH 7.0–9.0 with a relative activity of more than 50% in all the pHs range studied. The maximum proteolytic activity was found at pH 8.

Therefore, the proteases recovered from gilthead sea bream by-products are very stable in a wide pH range, showing high activity between pH 6 and 10 in the range of temperature 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 the viscera's 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).

The time stability of proteolytic activity from the viscera is shown in Fig. 2. The activity was stable during the first hour. However, a marked decrease in the activity was noticeable at 180 minutes.

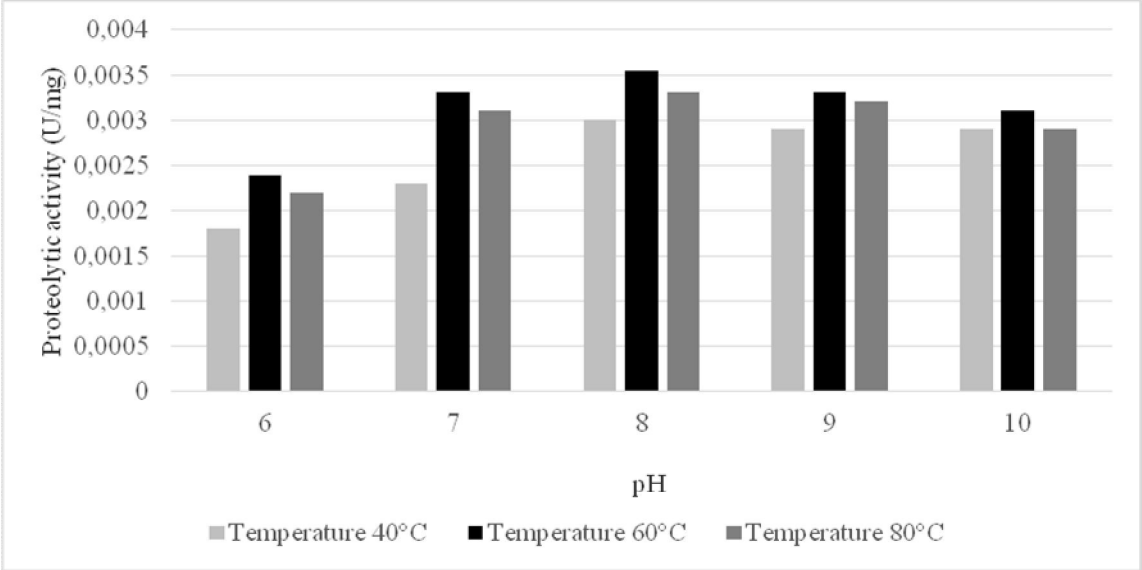

Fig. 1: pH and temperature profile of proteolytic activity of the crude protein extract from *Sparus aurata* L. viscera.

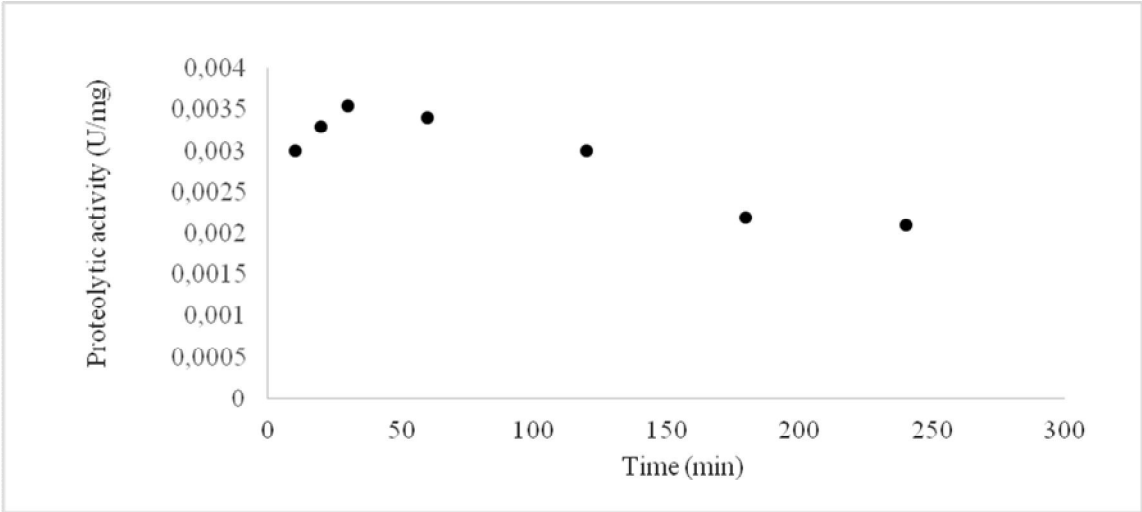

Fig. 2: Time stability of proteolytic activity of the crude protein extract from *Sparus aurata* L. viscera.

3.2 Protein and proteolytic activity of protein purified extracts

The extraction percentages of protein phase from viscera, obtained with the different oil extraction methods are illustrated in Fig. 3. These percentages are obtained by the magnitude of protein phase retained after oil extraction of the samples over time. The extraction time of at least about 240 min can be considered sufficient to extract oil from the samples.

The protein content in all the studied fractions and the proteolytic enzyme activities measured are summarized in Table 2. The viscera maintain 112.17% of proteolytic activity after SCO2 extraction. Also the samples maintain 100.54% after hexane extraction. Proteolytic activity of the viscera was 100.30% after mechanical extraction. No significant differences were found between the hexane and mechanical extracts, but these results indicated that using SCO2, the denaturation of digestive enzyme was minimized. However, there was a loss of enzyme activity after SCO2 extraction compared to crude extract if it is observed that this method achieved the highest protein fraction recuperation. This has been attributed to the interactions between CO2 and the enzyme (Kamat et al., 1995a; Kamat et al., 1995b; Habulin & Knez, 2001; Knez & Habulin, 2002).

These results are close with the data reported by Park et al., (2008), about digestive enzyme activities determined in the untreated and treated SCO_2 viscera's mackerel. Lipid separation should be performed as soon as possible after filleting, because the lipids accelerate protein oxidation, producing a significant detriment on protein quality (Kanner & Rosenthal, 1992).

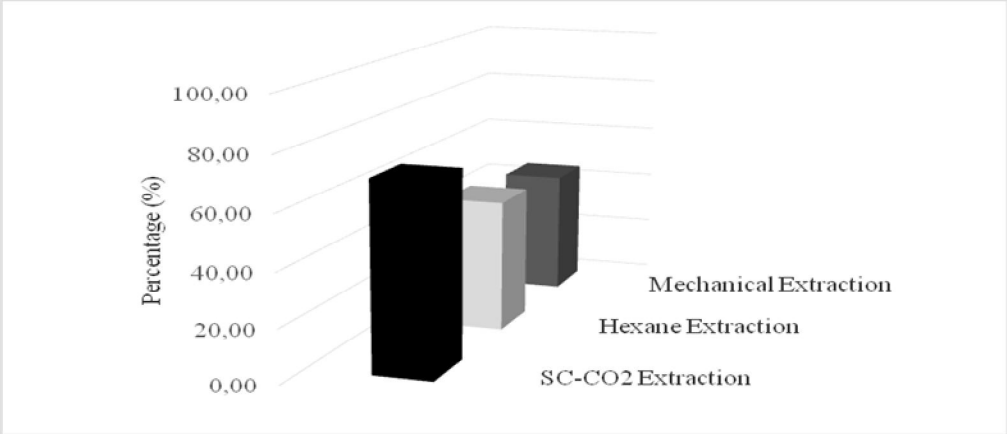

Fig. 3: Percentages of protein phase content recovery in the residue obtained by the different oil extraction methods.

3.3 Trypsin Specific activity

The initial specific trypsin activity of the crude extract was 0.0106 U/mg (Table 2) using BAPNA as a substrate. It has been reported previously the digestive trypsin gene in gilthead seabream using real time PCR by Cerezuela et al., (2013). The activity result obtained is lower than those reported by others studies. The trypsin activity from spleen of yellowfin tuna *Thunnus albacores* was found to be 0.11 U/mg (Klomklao et al., 2006). Castillo Yañez et al., (2005) informed a higher value (0.5 U/mg) in the trypsin from pyloric caeca of sardine *Sardinops sagax caerulea.* This might be due to the different species, and the purified steps used in these investigations.

All the methods analyzed increased the trypsin activity. This increment was more than 60 % in every extract purified. The results obtained in the present work indicate that the viscera may be rich in enzymes type trypsin; therefore, its concentration in purified extracts after remove the lipid phase could be advantageous. Asaduzzaman & Chun, (2015) reported a similar behaviour in trypsin activity of water soluble extracts after SC-CO2 treated, from mackerel muscle.

Table 2: Summary of proteolytic and trypsin activity from *Sparus aurata* L. viscera.

Fraction	Proteolytic activity (U/mg)	Protein (mg/mL)	Activity (U)	Purified fold	Recovery (%)
Crude extract (CE)	0.0036± 0.0004a	104.50± 5.20c	0.3711± 0.0104[a]	1	100
SFE protein extract	0.0057± 0.0008b	73.03± 3.87b	0.4163± 0.0122b	1.605	112.17
Hexane protein extract	0.0073± 0.0007c	51.11± 3.22[a]	0.3731± 0.0097[a]	2.056	100.54
Mechanical protein extract	0.0078± 0.0007c	47.72± 2.38[a]	0.3722± 0.0094[a]	2.196	100.30
	Trypsin activity (U/mg)				
Crude extract (CE)	0.0106± 0.0010[a]	104.50± 5.20c	1.108± 0.0091b	1	100
SFE protein extract	0.0116± 0.0011[a]	73.03± 3.87 b	0.853± 0.0102[a]	1.094	77.00
Hexane protein extract	0.0137± 0.0014b	51.11± 3.22[a]	0.700± 0.0075[a]	1.292	63.21
Mechanical protein extract	0.0149± 0.0012b	47.72± 2.38[a]	0.711± 0.0056[a]	1.406	64.20

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)

3.4 Effect of metallo-protease inhibitor and surfactant on trypsin activity

The effects of EDTA and SDS on activity proteolytic of crude and purified extracts are presented in the Table 3. Proteolytic activity was slightly affected by the metallo-protease inactivator EDTA. The activity retained in the crude extract was more than 97 %. This is in accordance with the 99% of residual activity obtained by Blanco et al., (2013) in pancreas of small-spotted catshark (*Scyliorhinus canicula*). Trypsin activity from the intestine of grey triggerfish (*Balistes capriscus*) was not affected by the metallo-protease inactivator EDTA (Jellouli et al., 2009). The high activity retained in the presence of EDTA is very useful for application as a detergent additive since chelating agents are components of most detergents (Jellouli et al., 2009).

Klomklao et al., (2006) reported a higher inhibition in trypsins from yellowfin tuna Spleen (*Thunnus albacores*) being the residual activity 88.2 % and 70.01% respectively. Also, Castillo Yañez et al., (2005) reported a 14% of inhibition in the trypsin activity from pyloric caeca of sardine *Sardinops sagax caerulea*.

For the effect of SDS surfactant on trypsin activity, the crude extract results increased compared to the control. These results are in agreement with the increment obtained by Blanco et al., (2013) in pancreas of small-spotted catshark (*Scyliorhinus canicula*), using the same SDS concentration. Whereas, other authors described the opposite effect on trypsin isolated from the intestine of sardinelle (Khaled et al., 2008) and triggerfish (*Balistes capriscus*) (Jellouli et al., 2009).

The SDS is considered a denaturating surfactant, furthermore, enzymes that resist the binding and unfolding ability of SDS has been reported previously (Rao et al., 1989). Also, Sweadner (1991), demonstrated that trypsin retains its activity in presence of SDS if a trypsin inhibitor it is present. Moreover, the stability achieved in trypsin activity after the incubation with SDS, suggest that this enzyme can be used as an additive in chemistry formulations that include this surfactant.

Table 3: Effect of EDTA and SDS on trypsin activity

	Final concentration mM	Activity retain %
		Crude extract (CE)
NONE	0	100
EDTA	0.5	97.17
SDS	1	102.83

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). Trypsins have 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, making 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. These enzymes present 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 disposed in marine organisms with novel application for the food industry (Shahidi & Kamil, 2001; Trincone, 2011; Zhang &Kim, 2012).

Considering the high activity and stability in alkaline pH, at low temperature and in the presence of surfactants, alkaline trypsin from the intestine of grey triggerfish (Balistes capriscus) was studied to be used in laundry detergents (Jellouli et al., 2009). Blanco et al., (2013), found a similar application for the trypsin isolated from pancreas of small-spotted catshark (*Scyliorhinus canicula*).

4. Conclusions

Viscera are a great source of nutritionally valuable compounds, including lipids, proteins, enzymes, and other biomaterials. Marine enzymes are compounds of great interest to be used in biotechnological process because their eco-friendly properties. The viscera from Sparus aurata L. could be considered as a good source of proteolytic enzymes, which have shown a sufficient stability under the pH and temperature studied.

Based on activity for the specific substrate BAPNA, the recovered and purified enzyme was identified as trypsin.

Conventional and alternative oil extraction methods were used as indirect protein purified techniques including solvent extraction, mechanical extraction and supercritical fluid extraction. The methods assayed were indicated as a good first protein purification step, since all of them were very usefull in order to eliminate the initial lipids content that obstruct the proteolytic activity. However, a isolation after the extraction of the lipids must be studied to achieve a pure trypsin fraction.

Characteristics of the isolated trypsin are interesting from a technological perspective, especially the maximum activity at pH 8.0, the high neutral pH activity, stability against EDTA and the recovery of the activity in SDS presence.

Considering the high activity in alkaline pH, the stability in the presence of EDTA and surfactants, Spaurus aurata L. viscera trypsin might find application in laundry detergents.

Acknowledgements

The author gratefully acknowledges the Erasmus Mundus SUDUE Consortium and Burgos University for a partial financial support. Also, the acknowledgment to El Arbol Market (Burgos, Spain) for provides the samples.

References

Anwar, A., Saleemudin, M., (1998). Alkaline proteases A Review. Bioresource Technology. 6, 175-183.

Asaduzzaman A. K. M., & Chun B.S. (2015). Characterization of digestive enzymes from de-oiled mackerel (*Scomber japonicus*) muscle obtained by supercritical carbon dioxide and n-hexane extraction as a comparative study. Journal of Food Science and Technology. 52, (6), 3494-3503.

AOAC, (2000). In: Horwitz, W. (Ed.), Official Methods of Analysis of AOAC International, 17th ed. AOAC International.

Banerje, U.C., Sani, R.K., Azmi, W., & Soni, R. (1999). Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. Process Biochemistry 35, 213-219.

Blanco, M., Simpson, B.K., Perez-Martín, R.I., & Sotelo, G.C. (2014). Isolation and partial and characterization of trypsin from pancreas of small-spotted catshark (*Scyliorhinus canicula*) Journal of Food Biochemistry 38, 196-206.

Brunner, G. (2005).Supercritical fluids: technology and application to food processing,
Journal of Food Engineering 67, 21–33.

Cao, M.J., Osatomi, K., Suzuki, M., Hara, K., Tachibana, K., & Ishihara, T. (2000). Purification and characterization of two anionic trypsins from the hepatopancreas of carp. Fish Sci. 66, 1172–1179.

Castillo-Yañez, F.J., Pacheco-Aguilar, R., García-Carreño, F.L., & Navarrete del Toro, M., (2005). Isolation and characterization of trypsin from pyloric caeca of Monterrey sardine (*Sardinops sagax caerulea*). Comparative Biochemistry and Physiology, Part B. 140, 91–98.

Castro Ceseña, A.B., Sánchez-Saavedra, M.P., & Márquez-Rocha, F.J. (2012). Characterisation and partial purification of proteolytic enzymes from sardine by-products to obtain concentrated hydrolysates. Food Chemistry. 135, 583-589.

Catchpole, O.J., Tallon, S.J., Eltringham, W.E., Grey, J.B., Fenton, K.A., Vagi, E.M.,
Vyssotski, M.V., MacKenzie, A.N., Ryan, J., & Zhu, Y. (2009). The extraction and fractionation of specialty lipids using near critical fluids. Journal of Supercritical Fluids. 47, 591–597.

Cerezuela, R., Meseguer, J., & Esteban, M.Á. (2013). Effects of dietary inulin, Bacillus subtilis and microalgae on intestinal gene expression in gilthead seabream (*Sparus aurata* L.). Fish Shellfish & Immunology. 34, (3), 843-848.

Delgado-Vargas, F., & Paredes-Lopez, O. (2003). Carotenoids, in: F. Delgado-Vargas, O. Paredes-Lopez (Eds.), Natural Colorants for Food and Nutraceutical Uses, CRC Press, LLC, NW, (Chapter 7).

Díaz López M., & García Carreño F.L. (2000). Applications of fish and shellfish enzymes in food and feed products. Food Science. 97, 571-618.

FAO http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en (2015)
(February 2015).

Ferraro, V., Carvalho, A.P., Piccirillo, C., Santos, M.M., Castro, P.M.L., & Pintado, M.E. (2013). Extraction of high added value biological compounds from sardine, sardine-type fish and mackerel canning residues. Materials Science & Engineering C. 33, 3111–3120.

Gupta, R., Beg, Q.K., & Lorenz, P., (2002). Bacteria alkaline proteases: molecular approaches and industrial applications. Applied Microbiology & Biotechnology 59, 15-32.

Haard, N. F. (1992). A review of proteolytic enzymes from marine organisms and their application in the food industry. Journal of Aquatic Food Product Technology. 1, 17–38.

Habulin, M., & Knez, Z. (2001). Activity and stability of lipases from different sources in supercritical carbon dioxide and near-critical propane. Journal of Chemical Technology & Biotechnology. 76, 1260-1266.

Jeloulli, K., Bougatef, A., Daassi, D., Balti, R., & Nasri, M. (2009). New alkaline trypsin from the intestine of grey triggerfish (Balistes capriscus) with high activity at low temperature: Purification and characterisation. Food Chemistry. 116, 644–650.

Joo, H.S., Park, G.C., Kim, K.M., Paik, S.R., & Chang, C.S. (2001). Novel alkaline protease from the polychaeta, Periserrula leucophyrna: Purification and characterization. Process Biochemistry. 36, 893–900.

Kamat, S., Critchley, G., Beckman, E.J., & Russell, A.J. (1995a). Biocatalytic synthesis of acrylates in organic solvents and supercritical fluids. III. Does carbon dioxide covalent l y modify enzymes? Biotechnology & Bioengineering. 46, 610-620.

Kamat, S.V., Beckman E.J., & Russel A.J. (1995b). Enzyme activity in supercritical fluids. Critical Reviews in Biotechnology. 15, 41-71.

Kanner, J., & Rosentha, I. (1992). An assessment of lipid oxidation in foods. Pure & Applied Chemistry. 64, (12), 1959-1964.

Ketnawa, S., Benjakul, S., Ling, T.C., Martínez-Alvarez, O., & Rawdkuen, S. (2013). Enhanced recovery of alkaline protease from fish viscera by phase partitioning and its application. Chemistry Central Journal. 7, 79–88.

Ketnawa, S., Benjakul, S., Martínez-Alvarez, O., & Rawdkuen, S. (2014). Three-phase partitioning and proteins hydrolysis patterns of alkaline proteases derived from fish viscera. Separation & Purification Technology. 132, 174–181.

Khaled, H.B., Bougatef, A., Balti, R., Triki-Ellouz, Y., Souissi, N., & Nasri, M. (2008). Isolation and characterisation of trypsin from sardinelle (Sardinella aurita) viscera. Journal of Science & Food Agriculture. 88, 2654–2662.

Klomklao, S., Benjakul, S., Visessanguan, W., Simpson, B.K., & Kishimura, H. (2005). Partioning and recovery of proteinases by tuna spleen by aqueous two phase system. Process Biochemistry. 40, 3061–3067.

Klomklao, S, Benjakul, S., Visessanguan, W., Simpson, B.K., Kishimura, H., & Saeki, H. (2006). Trypsins from yellowfin tuna (Thunnus albacores) spleen: Purification and characterization. Comparative Biochemistry & Physiology Part B. 144, 47-56.

Klomklao, S., Benjakul, S., & Simpson, B.K. (2012). Seafood enzymes: biochemical properties and their impact on quality, in: B.K. Simpson (Ed.), Food Biochemistry & Food Processing, Wiley-Blackwell, USA, pp. 271–273.

Knez, Z., & Habulin M. (2002). Compressed gases as alternative enzymatic reaction solvents: A short review. Journal of Supercritical Fluids. 23, 29-42

Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry. 193, 256–275.

Macouzet, M., Simpson, B.K., & Lee, B.H. (1999). Cloning of fish enzymes and other fish protein genes. Critical Reviews in Biotechnology. 19, 179–196.

Macouzet, M., Simpson, B.K., & Lee, B.H. (2005). Expression of a cold-adapted fish trypsin in *Pichia pastoris*. Fems Yeast Research. 5, 851–857.

Mireles DeWitt C., & Morrissey, M. T. (2002). Parameters for the recovery of proteases

from surimi wash water. Bioresource Technology. 81, 241-247.
Palmer, M.V. & Ting, S.S.T. (1995). Applications for supercritical fluid technology in food processing. Food Chemistry. 52, 345–352.
Parajó, J.C., Dominguez, H. Moure, A., & Diaz-Reinoso, B. (2008). Obtaining antioxidants by supercritical fluid extraction applied, in: M.A.A. Meireles (Ed.), Extracting
Bioactive Compounds for Food products, Theory and Aplications, CRC Press Taylor & Francis Group, LLC, USA, pp. 288–315.
Park, J.Y., Back, S.S., & Chun, B.S. (2008). Protein properties of mackerel viscera extracted by supercritical carbon dioxide. Journal of Environmental Biology. 29, (4), 443-448.
Rao, Y.K., Bahadur, P., & Ghosh, S. (1989). Stability and kinetic behavior of some enzymes in surfactant environment. Indian Journal of Biochemistry & Biophysics. 26, 390–39.
Rawdkuen, S., Vanabun, A., & Benjakul, S. (2012). Recovery of proteases from the viscera of farmed giant catfish (*Pangasianodon gigas*) by three-phase partitioning. Process Biochemistry. 47, 2566–2569.
Reverchon, E., & De Marco,I. (2006). Supercritical fluid extraction and fractionation of
natural matter. Journal of Supercritical Fluids. 38, 146–166.
Sahena, F., Zaidul, I.S.M., Jinap, S., Saari, N., Jahurul, H.A., Abbas, K.A., & Norulaini, N.A. (2009a). PUFAS in fish: extraction, fractionation, importance in health. Comprehensive
Reviews in Food Science & Food Safety 8, 59–74.
Sahena, F., Zaidul, I.S.M., Jinap, S., Karim, A.A., Abbas, K.A., Norulaini, N.A.N., &
Omar, A.K.M. (2009b). Application of supercritical CO(2) in lipid extraction—a review, Journal of Food Engineering 95, 240–253.
Sánchez-Camargo A.P., Martinez-Correa, H.A., Paviani C.L., & Cabral, F.A. (2011). Supercritical CO2 extraction of lipids and astaxanthin from Brazilian redspotted shrimp waste (*Farfantepenaeus paulensis*) Journal of Supercritical Fluids. 56, 164–173.
Shahidi, F., & Kamil, J.Y.V.A. (2001). Enzymes from fish and aquatic invertebrates and their application in the food industry. Trends in Food Science & Technology. 12, 435–464.
Simpson, B.K. (2000). Digestive proteases from marine animals. In Seafood Enzymes. Edited by Haard N. F, Simpson B. K. New York, NY: Marcel Dekker, 191–213.
Simpson, B.K., & Haard, N.F. (1987). Trypsin and trypsin-like enzyme from the stomachless cunner. Catalytic and other physical characteristics. Journal of Agricultural & Food Chemistry. 35, 652–656.
Sweadner, K.J. (1991) Trypsin inhibitor paradoxically stabilizes trypsin activity in sodium dodecyl sulphate, facilitating proteolytic fingerprinting. Anal Biochemistry. 194, 130–135.
Trincone, A. (2011). Marine biocatalysts: enzymatic features and applications. Mar Drugs. 9, 478–499.
Zhang, C., & Kim, S.K. (2012). Application of marine microbial enzymes in the food and pharmaceutical industries. Advances in Food & Nutrition Research. 64, 423–435.