Food Research International 65 (2014) 469–476

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species

Pedro J. García-Moreno [a,*], Irineu Batista [b], Carla Pires [b], Narcisa M. Bandarra [b], F. Javier Espejo-Carpio [a], Antonio Guadix [a], Emilia M. Guadix [a]

[a] Department of Chemical Engineering, University of Granada, 18071 Granada, Spain
[b] DivAv, IPMA, 1449-006 Lisbon, Portugal

ARTICLE INFO

Article history:
Received 27 January 2014
Received in revised form 20 March 2014
Accepted 29 March 2014
Available online 8 April 2014

Keywords:
Discards
Fish protein hydrolysates
Antioxidant activity
DPPH
Fe^{2+} chelating activity
Reducing power

ABSTRACT

In this study, five discarded species in the Mediterranean Sea, namely sardine, horse mackerel, axillary seabream, bogue and small-spotted catshark, were evaluated as raw material for obtaining fish protein hydrolysates exhibiting antioxidant activity. The DH of the hydrolysates ranged from 13.2 to 21.0%, with a protein content varying from 60.7 to 89.5%. The peptide profile of all hydrolysates was very similar, except for the hydrolysate of small-spotted catshark. Their lipid content was found to be between 4.6 and 25.3%. The highest DPPH scavenging activity was found for the hydrolysates of sardine and horse mackerel with EC_{50} values varying from 0.91 to 1.78 mg protein/mL. Sardine and small-spotted catshark hydrolysates exhibited the highest ferrous chelating activity with an EC_{50} value of 0.32 mg protein/mL. Moreover, sardine and bogue hydrolysates presented the highest reducing power. Finally, a total of six antioxidant peptides were theoretically identified within the structure of myosin and actin proteins from sardine and small-spotted catshark. The potential antioxidant activity exhibited by the hydrolysates suggests that it is feasible to obtain added-value products such as natural antioxidants from these discarded species.

1. Introduction

Marine discards are that portion of total fish catch which is not retained for sale and returned to the sea. It comprises non-target species with low commercial value, fish below minimum commercial size, fish caught in excess of individual quota and damaged fish which is not worthy for fishermen to keep on board (Kelleher, 2005). In the Alboran Sea, the portion of the West Mediterranean Sea lying between the Spanish southern coast and the north of Morocco, discards are mainly composed of commercial species such as sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*) and axillary seabream (*Pagellus acarne*) which are dumped at the sea due to high-grading practices, quota restriction and minimal commercial-size requirements. Other species such as bogue (*Boops boops*) and small-spotted catshark (*Scyliorhinus canicula*) are discarded due to their reduced commercial value (García-Moreno, Pérez-Gálvez, Morales-Medina, Guadix, & Guadix, 2013).

These practices represent an important underutilization of marine resources. Since discards are generally dead or dying when returned to the sea, they also cause significant environmental problems such as alterations on marine trophic chains (Bozzano & Sardà, 2002). In order to ensure the sustainability of EU fisheries, the EU Commission is in the process of implementing a reformed Common Fisheries Policy which aims to gradually implement a practice of zero-discards (EU, 2011). Nevertheless, technical measures should also be applied in order to successfully meet discard bans, since discards can be reduced (i.e. by improving the selectivity of the fishing gears) but cannot be completely eliminated. In this sense, it seems to be of special importance to develop up-grading processes which permit to obtain added-value products from this underutilized raw material.

In this context, discarded species in the Alboran Sea are good sources of protein, with protein contents ranging from 17 to 23% depending on the species (García-Moreno, Pérez-Gálvez, Morales-Medina, et al., 2013). Thus, enzymatic hydrolysis of their protein fraction is a convenient method for the production of bioactive compounds that could be utilised in the nutraceutical and pharmaceutical fields. In this regard, several fish protein hydrolysates have shown numerous bioactivities such as antioxidant, antihypertensive, antithrombotic, immunomodulatory, antimicrobial, among others (Je, Lee, Lee, & Ahn, 2009; Kim & Wijesekara, 2010).

Due to the increasing interest in finding antioxidants from natural sources which may have less potential hazard than synthetic ones, research on fish protein hydrolysates exerting antioxidant activity has gained an increased interest. Antioxidants are generally employed to prevent lipid oxidation in foods in order to avoid the formation of toxic compounds and undesirable odours and flavours (Lin & Liang, 2002). Furthermore, oxidative stress has also been involved in the

* Corresponding author. Tel.: +34 958 241329; fax: +34 958 248992.
E-mail address: pjgarcia@ugr.es (P.J. García-Moreno).

http://dx.doi.org/10.1016/j.foodres.2014.03.061
0963-9969/

occurrence of several diseases such as hypertension, cancer, diabetes, Alzheimer's and ageing (Hajieva & Behl, 2006).

In the last decade, several authors have reported a strong antioxidant activity for fish protein hydrolysates obtained from different species such as black scabbardfish (*Aphanopus carbo*) (Batista, Ramos, Coutinho, Bandarra, & Nunes, 2010), sardinelle (*Sardinella aurita*) (Bougatef et al., 2010), saithe (*Pollachius virens*) (Chabeaud, Dutournié, Guérard, Vandanjon, & Bourseau, 2009), yellowfin sole (*Limanda aspera*) (Jun, Park, Jung, & Kim, 2004), mackerel (*Scomber austriasicus*) (Wu, Chen, & Shiau, 2003), and herring (*Clupea harengus*) (Sathivel et al., 2003). However, there is little information about the production of fish protein hydrolysates with antioxidant activity from discarded species in the Alboran Sea.

Another important aspect to consider is the choice of the enzyme since it has a great impact on the release of antioxidant peptides by hydrolysis of fish protein (Laroque, Chabeaud, & Guérard, 2008). The endoproteases subtilisin (EC 3.4.21.62) and pancreatic trypsin (EC 3.4.21.4) have previously shown good results in the production of fish protein hydrolysates exhibiting antioxidant activity (Amarowicz & Shahidi, 1997; Rajapakse, Mendis, Byun, & Kim, 2005; Thiansilakul, Benjakul, & Shahidi, 2007). Nevertheless, only a few studies have addressed the effect of a combination of these enzymes (García-Moreno, Pérez-Gálvez, Espejo-Carpio, et al., 2013).

Therefore, the objective of this study was to investigate the potential of five discarded species in the Alboran Sea (sardine, horse mackerel, axillary seabream, bogue and small-spotted catshark) as raw material for the production of fish protein hydrolysates exhibiting antioxidant activity. For that purpose, the combined effect of subtilisin and trypsin as enzymatic treatment was evaluated.

2. Materials and methods

2.1. Raw material

Raw sardine, horse mackerel, bogue, axillary seabream and small-spotted catshark, was provided by the fishing harbour of Motril (Spain) in September 2011. All fish were kept in ice during the transportation and pressed in the same day.

2.2. Separation of protein fraction

The whole fish, included viscera and gonads, was preheated at 40 °C for 30 min (Digiterm 100, Selecta, Barcelona, Spain). Then, it was fed into an electric press (ESP-K, Sanahuja, Castellón, Spain) where it was subjected to three consecutive pressing steps until attaining a final pressure of 150 bar. The pressing stage permitted to reduce the moisture content and the volume of the protein rich material, which also implies a diminution of the handling and insulation costs. The cakes obtained from the pressing operation were grinded in a cutter (SK-3, Sammic, Sevilla, Spain) and then frozen at −20 °C prior to their use as substrate for protein hydrolysis. For small-spotted catshark, the grinding and homogenization of the press cake was not possible due to the high resistance of its skin. Thus, the muscle of this species was employed as substrate for protein hydrolysis. It was obtained by de-heading, degutting and removing the skin from the whole fish.

2.3. Hydrolysis procedure

For the enzymatic hydrolysis, two serine endoprotease enzymes were employed; one of bacterial origin (subtilisin, EC 3.4.21.62) and other from an animal source (pancreatic trypsin, EC 3.4.21.4), both provided by Novozymes (Denmark) as Alcalase 2.4 L and PTN 6.0S, respectively. The following hydrolysis conditions were studied: (a) 2 h hydrolysis with subtilisin followed by 2 h hydrolysis with trypsin; (b) 2 h reaction with trypsin followed by 2 h incubation with subtilisin and; (c) 4 h hydrolysis with simultaneous addition of both enzymes. The first enzyme utilized in treatments a) and b) was not inactivated prior to the addition of the second enzyme.

The protein content of the raw material for the hydrolysis was determined by using the Kjeldahl method (AOAC, 2006), with a nitrogen-to-protein conversion factor of 6.25. The results, expressed as % wet base, were as follows: sardine 19.2%, horse mackerel 20.2%, axillary seabream 23.4%, bogue 21.9% and small-spotted catshark 27.4%.

Then, a given mass of grinded press cake was homogenised with demineralised water until reaching a final volume of 200 mL. This suspension, having a protein concentration of 25 g/L, was then transferred into a jacketed reactor of volume capacity 250 mL. The experiments were conducted at pH 8 and 50 °C, while enzyme–protein ratio was set at 3% (w/w) for both enzymes. Protein was considered as substrate.

The degree of hydrolysis, defined as the percentage of the number of peptide bonds cleaved compared to the total number of peptide bonds in the substrate studied, was calculated as a function of the base consumption throughout the reaction employing an automatic titrator (718 Stat Titrino, Metrohm, Herisau, Switzerland) (Camacho, González-Tello, Páez-Dueñas, Guadix, & Guadix, 2001). According to this method, the degree of hydrolysis (DH) can be related to the amount of base (NaOH, 1 N) consumed to keep the pH constant during the reaction, as follows (Eq. (1)):

$$DH = B \cdot N_b/(\alpha \cdot m_P \cdot h_{TOT}) \times 100 \quad (1)$$

where B (mL) is the amount of base consumed, N_b (eq/L) is the normality of the base, α is the average degree of dissociation of the α-NH_2 amino groups released during the hydrolysis, which is dependent on the temperature and the pH, m_P (g) is the mass of protein in the substrate and h_{TOT} (meq/g) is the number of equivalents of peptide bonds per gramme of protein. At pH 8 and temperature of 50 °C, the 88.5% of the amino groups are dissociated, while h_{TOT} was assumed to be 8.6 meq/g of protein, as reported in literature (Adler-Nissen, 1986).

A set of 250 hydrolysates, originated from the five species and three enzymatic treatments studied and drawn at different times of reaction (0, 5, 10, 20, 30, 45, 60, 90, 120, 125, 130, 140, 150, 165, 180, 210 and 240 min), were evaluated in order to determine the influence of DH on the DPPH scavenging activity. The samples were heated in a boiling water bath for 15 min to inactivate the enzyme and were filtered in order to remove the solid remained. They were kept at −20 °C until the analyses were performed.

After completion of the hydrolysis, the final hydrolysates were also heated in a boiling water bath for 15 min and filtered. Then, they were lyophilized and stored at −20 °C until analyses were performed.

2.4. Characterization of the hydrolysates

2.4.1. Protein content

The protein content of the lyophilized hydrolysates was determined using a FP-528 LECO nitrogen analyser (LECO, St Joseph, MI, USA) calibrated with ethylenediaminetetraacetic acid according to the Dumas method (Saint-Denis & Goupy, 2004).

2.4.2. Lipid content and lipid classes

The lipid content of the lyophilized hydrolysates was determined according to the method described by Folch, Lees, and Stanley (1957). Lipid classes were determined by thin-layer chromatography (TLC) using hexane/diethyl ether/acetic acid (65:35:1, v/v/v) as the developing solvent system. The developed plates were sprayed with 10% phosphomolybdic acid in ethanol and heated at 120 °C for 5 min. The identification of the different classes was done by comparison with the standards from Sigma. For quantification purposes, the TLC plates were scanned (GS-800 densitometer, Bio-Rad, Alcobendas, Spain) and analyzed with Quantity One analysis software (Bio-Rad, Alcobendas, Spain).