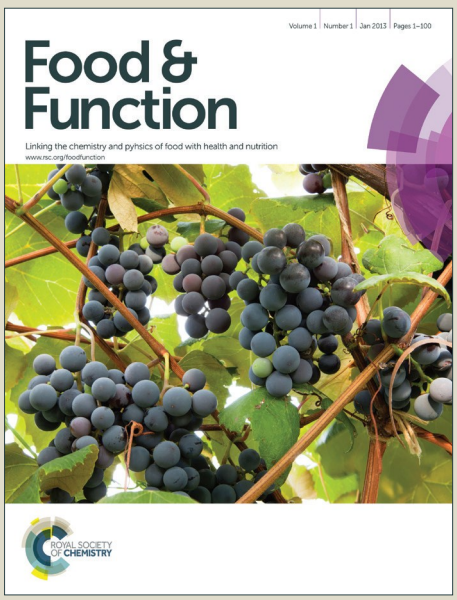
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Enhancement of ACE and prolyl oligopeptidase inhibitory potency of protein hydrolysates from sardine and tuna by-products by simulated gastrointestinal digestion

Oscar Martínez-Alvarez[1]; Irineu Batista[2]; Cristina Ramos[2]; Pilar Montero[1]

* Author to whom correspondence should be addressed [e-mail oscar.martinez@if.csic.es]

1. ICTAN (CSIC)**, Ciudad Universitaria, 28040 Madrid, Spain. Tel: +34-31-5492300; Fax: +34-91-5493627
2. Instituto Português do Mar e da Atmosfera (IPMA), Av. Brasília, 1449-006 Lisboa, Portugal

** This centre has implemented and maintains a Quality Management System which fulfils the requirements of the ISO standard 9001:2008

Short title: fish hydrolysates with potential applications in functional foods

Abstract

This work was focused on the study of the bioactive potential of three fish protein hydrolysates, one of them prepared from industrial sardine by-products (heads and viscera) and the others from tuna by-products (heads, and muscle and viscera). These protein hydrolysates exhibited moderate ability to inhibit Angiotensin Converting Enzyme (IC_{50} between 0.24-1.16 mg dry weight/ml) and Prolyl Oligopeptidase (IC_{50} between 3.30-9.57 mg/ml), being those obtained from tuna by-products the most effective. Overall, ACE- and PO-inhibiting activities were enhanced by sequential nanofiltration through 3 and 1 kDa MWCO membranes (IC_{50} between 0.02-0.16 mg/ml (ACE) and 1.10-4.21 mg/ml (PO)). The inhibitory properties of the hydrolysates were greatly improved by *in vitro* gastric digestion, and were barely affected by further intestinal digestion. The digested tuna hydrolysates proved to be the best source of PO- and ACE- inhibiting molecules (mainly that from heads (IC_{50} = 0.16 mg/ml (ACE) and 1.04 mg/ml (PO)) and could be potential new ingredients in food with interest in the prevention or treatment of cardiovascular and neurological diseases.

Keywords: hypertension, fish hydrolysate, Angiotensin-converting enzyme, Prolyl oligopeptidase, fish waste.

Introduction

Hypertension is a significant health problem worldwide. In fact, more than 30 percent of deaths that occur every year include hypertension as the primary or contributing cause. Changes in lifestyle habits are usually recommended to control high blood pressure, but at times this is not sufficient and medication (mainly angiotensin-I- converting enzyme or ACE inhibitors) is prescribed to lower blood pressure. ACE plays a crucial role in both blood pressure regulation and electrolyte balance in humans. It cleaves angiotensin-I into the potent vasopressor octapeptide angiotensin II (Ang II), inactivates the vasodilator peptide bradykinin and also degrades neuropeptides which interact with the cardiovascular system, such as enkephalins, neurotensin, and substance P.[1] Synthetic ACE-inhibitors commercialised to date cause undesirable side effects[2], hence natural, safe, and economical compounds are being investigated nowadays as alternatives to prevent and manage moderate hypertension. In this context, different protein hydrolysates from marine sources have been considered as interesting ACE-inhibitors, with a promising use in moderate hypertension management.[3-8]

Prolyl endopeptidase, also known as prolyl oligopeptidase (PO), is a serine protease that plays an important role in the degradation of biologically active peptide hormones and proline-containing neuropeptides.[9] Altered serum PO-activity has been linked to a variety of neurological disorders, such as Alzheimer's disease, amnesia, depression and schizophrenia.[10] Indeed, PO has been identified as a potential pharmaceutical target for different neurological diseases.[11] PO-inhibiting agents have been chemically synthesised and are also found in nature. In this regard, PO-inhibitors (mainly polyphenols and peptides) have been identified in plant extracts, green tea, red wine, sake and bovine

brain.[10] However, the occurrence of PO-inhibiting peptides in marine protein hydrolysates is scarcely documented. Sorensen et al.[12] used pepsin and trypsin to obtain cod, salmon and trout hydrolysates which showed PO-inhibitory activity, and recently Sila et al.[13] used different commercial enzymes to obtain PO-inhibitory hydrolysates from barbell skin gelatine.

Tuna and sardines are significant sources of food and play an important role in the economy of many countries. They are industrially processed as raw fish flesh or as canned food, generating huge amounts of waste which are usually discarded or processed into low-value market products, such as fish meal or fertilizer. This waste constitutes an important protein source and could be hydrolysed to obtain interesting PO- or ACE-inhibiting peptides with interest for seafood industries, granting not only the opportunity to valorise worthless by-products, but also the possibility to obtain new added-value products demanded by consumers. Bioactive peptides must reach the target site intact, in order to exert *in vivo* effects; this implies that, following oral administration, they must resist gastrointestinal digestion, pass across the small intestine and, still intact, reach the bloodstream. Nonetheless, only a very small number of these small peptides enter the blood in their intact form.[14] Although some PO-inhibiting hydrolysates have been obtained to date[10,12,13] the effect of simulated gastrointestinal digestion on this property has not been documented yet.

In the last years, ultrafiltration (UF) and/or nanofiltration (NF) have been used to fractionate and improve the bioactivity of seafood protein hydrolysates, as summarized by Bourseau et al.[15] While 4-100 kDa molecular weight cut-off (MWCO) membranes are used for UF of hydrolysates, 1-4 kDa MWCO membranes are used for NF. UF and NF

membranes are particularly useful to enrich seafood protein hydrolysates in bioactive peptides, and in this field most of works published have described the isolation of low molecular weight fractions including antioxidant and ACE-inhibiting peptides.[16] However, the use of NF membranes to concentrate PO-inhibiting peptides from seafood protein hydrolysates has not been reported to date.

The main objective of this work was to evaluate the effect of simulated gastro-intestinal digestion on the ACE- and PO-inhibitory abilities of three novel protein hydrolysates from tuna or sardine industrial by-products. Another objective of this work was to evaluate the effect of consecutive nanofiltration with 3 and 1 kDa MWCO membranes on the bioactivity of the hydrolysates.

Materials & Methods

Chemicals.

Angiotensin-Converting Enzyme (ACE, EC 3.4.15.1) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Prolyl oligopeptidase (PO, EC 3.4.21.26, *Flavobacterium meningosepticum* origin) was purchased from Seikagaku Corporation (Tokyo, Japan). Alcalase™ 2.4 L was acquired from Novozymes (Bagsværd, Denmark). Other chemicals were of reagent grade.

Protein hydrolysis

Sardine heads and viscera (*Sardina pilchardus*), tuna heads (*Thunnus thynnus*), and a mixture of tuna muscle debris and viscera, were used to prepare three different protein hydrolysates. These by-products were obtained from fish canning factories in Portugal. Raw materials were steamed at 80 ºC for 20 min to inactivate endogenous enzymes, and

later ground in a hammer mill. After cooling, 200 g of sample were mixed with 2000 ml of water and the pH was adjusted to 8 with sodium hydroxide. The slurries were then heated at 40 ºC and the enzymatic hydrolysis started by adding 1% (v/w) of Alcalase™ 2.4L. During the process of hydrolysis the pH was manually adjusted to the initial value by adding NaOH (2 N). After 4 hours, the reaction was stopped by boiling for 20 min. The hydrolysates were then sieved to remove the bony material and, subsequently, the liquid fraction was clarified by centrifuging. The soluble fractions obtained were ultrafiltered with a 10 kDa MWCO membrane, concentrated in a rotary vacuum evaporator, freeze dried and stored at low temperature (-20 ºC) for further use.

Degree of hydrolysis (DH)

The DH obtained for each sample was measured by using o-phthaldialdehyde (OPA) in the presence of beta-mercaptoethanol, following the method described by Nielsen et al.[17]

Molecular weight profile (MW)

The MW profile of each hydrolysate before and after *in vitro* gastrointestinal digestion was determined by SEC-HPLC according to Martínez-Alvarez et al.[18] The SEC column was a Superdex peptide PC 3.2/30 (GE Healthcare, Barcelona, Spain, fractionation range 7000-100 Da). The flow rate was 0.025 ml/min. The mobile phase was 30 % (v/v) acetonitrile with 0.1% TFA (v/v). Dry powders were previously dissolved in Milli-Q water (10 mg/ml), centrifuged at 9000 x g for 5 min and filtered at 0.45 µm before injection of 10 µl in the column. Absorbance was monitored at 214 nm. Standards used for the calibration were aprotinin (6,511 Da); vitamin B12 (1,345 Da); hippuryl histidyl leucine (429 Da) and glycine (75 Da). The void volume of the column was determined by injection of BSA (67,000 Da).

Amino acid composition

The freeze-dried samples were diluted with ultrapure water and hydrolysed with continuously boiling 6 N HCl containing 0.1% phenol with norleucine as internal standard, at 110 ºC for 24 h. After hydrolysis, HCl was removed under vacuum. The dried samples were reconstituted in the application buffer, and injected onto a Biochrom 20 amino acid analyser (Pharmacia, Barcelona, Spain). The amino acid content was expressed by the number of residues per 1000 residues.

Nanofiltration

FPH were nanofiltered to concentrate the bioactive peptides of interest. Nanofiltration was performed in a pilot scale ultrafiltration unit (Centramate Lab tangential flow system, Pall Corporation, New York, USA). The protein hydrolysates were dissolved in purified water (10 g/1000 ml) and fractionated successively through 3 and 1 kDa MWCO membranes (C-series Omega™ polyethersulfone (PES) membranes, Pall Corporation). Effective filtration area of each membrane was 0.1 m^2. Transmembrane pressure was 1.08 bar (3 kDa MWCO membrane) and 1.13 bar (1 kDa MWCO membrane). Filtrations were performed at tangential velocity of 300 ml min^{-1} at 20 ºC. Flux rate was constant throughout the filtration processes (117 L/m^2/h). At the end of the process, permeate (MW < 1 kDa) and retentate (MW 1-3 kDa) fractions were recovered and further freeze-dried and stored at low temperature (-20 ºC). The fractions including molecules higher than 3 kDa did not show inhibiting activity against PO and ACE.

ACE-inhibiting activity

ACE-activity was determined by using hippuryl-His-Leu (HHL) as substrate. The total reaction volume contained 25 µl of 5 mM HHL, 80 µl (2 mU) of ACE, and 10 µl of each assay sample at different concentrations of dry weight dissolved in 100 mM sodium-phosphate buffer (pH 8.3) containing 300 mM NaCl. In the control reaction mixture, 10 µl

of buffer solution were used instead of the assay sample. Substrate and hydrolysates were combined and incubated at 37 ºC for 4 min. ACE was also kept at 37ºC for 4 min. Both solutions were then combined and further incubated at 37ºC for 120 min in a water bath under constant stirring (160 oscillations/min). The enzyme reaction was stopped by adding 50 µl of 1 M HCl. The hippuric Acid (HA) released was then quantified by HPLC on an analytical C18 column (Tracer excel 120 ODSA 5 µm, Teknokroma, Barcelona, Spain). The injection volume was 50 µl. The sample was eluted at a flow rate of 0.8 ml/min. Water containing 0.1% (v/v) TFA (eluent A) and acetonitrile containing 0.1% (v/v) TFA (eluent B) were used as mobile phases. The column was developed with 20% B for 5 minutes, followed by a linear gradient of 60% B for the next 15 minutes. Elution was maintained isocratically at 60% B for 4 minutes and then returned to the initial eluent composition of 20% B for 2 minutes. Elution peaks of Hippuric Acid (HA) and HHL were monitored at 228 nm. ACE inhibition was calculated as follows:

ACE inhibition (%) = [1-(A*inhibitor*/A*control*)] x 100.

A*inhibitor* and A*control* express the relative areas (A) of the HA peak of the assays performed with and without ACE inhibitors, respectively. The half maximal inhibitory concentration (IC_{50}) was used to express the potency of the FPHs to inhibit ACE activity. The IC_{50} value was expressed in mg of dry weight/ml. The clinical inhibitor of ACE, Enalapril®, was used for comparative purposes. BSA was used as control and did not show any ACE-inhibitory activity. All analyses were carried out at six different concentrations in triplicate.

PO-inhibiting activity

PO-inhibiting activity of each hydrolysate was assayed by modifying the method described by Maruyama et al.[19] The hydrolysates were previously dissolved in 100 mM sodium

phosphate buffer (pH 7) containing 1 mM EDTA. Z-glycyl-L-proline-4-nitroanilide (Z-Gly-Pro-pNA, Sigma Chemical) was used as substrate of PO. The total reaction volume consisted of 35 µl of 7 mM Z-Gly-Pro-pNA, 635 µl of serial dilutions of the FPHs, and 10 µl of PO (10 mU). The control reaction mixture contained 635 µl of buffer solution instead of the assay sample. Substrate and sample were both mixed and incubated at 30ºC for 5 minutes in a water bath under constant stirring (160 oscillations min^{-1}). The reaction was initiated after adding the PO solution, which had been previously tempered at 30ºC. After 30 min of incubation, the reaction was stopped by adding 250 µl of 1 N HCl. Free nitroanilide liberated was quantified by HPLC on an analytical C18 column. The injection volume was 50 µl. Nitroanilide was eluted with an isocratic elution of 53% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min, while monitoring the absorbance at 410 nm. PO-inhibitory activity was calculated as follows:

PO inhibition (%) = [1-(A*inhibitor*/A*control*)] x 100.

A*inhibitor* and A*control* express the relative areas (A) of the nitroanilide peak of the assays performed with and without inhibitor, respectively.

Results were expressed as IC_{50} (the concentration of hydrolysate needed to inhibit 50% of PO activity). Z-Pro-Prolinal was used for comparative purposes. BSA was used as control and did not show relevant PO-inhibitory activity. The experiment was performed in triplicate.

Simulated gastrointestinal digestion (GID)

GID of FPHs was carried out in a thermally controlled water bath under constant stirring at 37 ºC, as described by Sannaveerappa et al.[20] with modifications. The protein hydrolysates were diluted (100 mg/ml) in 50 mM sodium phosphate buffer pH 6.75 with NaCl (9 mg/ml) and heated at 37ºC. Then, fifteen millilitres of pepsin solution (4.62 mg/ml) containing 49

mM NaCl, 12 mM KCl, 2.4 mM $MgCl_2$, 3.5 mM KH_2PO_4, 10 mM $CaCl_2$, and 95 µl of chloramphenicol (0.25 mg/ml) were added. The pH was adjusted to 5.5 with 3 M HCl and after 30 minutes of incubation an aliquot of 4 ml was removed (samples T1). The pH was then reduced to 3.8 with 3 M HCl. The samples were incubated for 30 minutes and then the pH was brought down to 2 with 3 M HCl. The digested sample was then incubated for 15 minutes, and afterwards an aliquot of 4 ml was withdrawn (samples T2). The pH was then raised to 5 by addition of 3 M NaOH. Subsequently, 2.4 mL of 0.1 M NaHCO3 with pancreatin (4 mg/ml) and bile salts (25 mg/ml) were added. An aliquot of 4 ml was withdrawn (samples T3) after 30 minutes of incubation. The pH was adjusted to 6.5 with 3 M NaOH. After 60 minutes of incubation, an aliquot of 4 ml was withdrawn (samples T4). All pooled aliquots were heated at 90ºC for 5 minutes and immediately stored on ice. The aliquots were then ultra-filtered with Amicon ultra centrifugal filters (10 kDa MWCO, Millipore, MA, USA), and the eluates were freeze-dried and kept at -20º C for further assays.

Statistical analysis

Statgraphics Plus 2.1 computer program (STSC Inc., Rockville, MD, USA) was used for statistical processing. ANOVA was used to compare the ACE and PO-inhibiting capacities of each hydrolysate (IC_{50} values). A value of $P < 0.01$ was considered statistically significant.

RESULTS AND DISCUSSION

Degree of hydrolysis (DH)

The hydrolysate generated from sardine by-products showed the highest DH (57.0 ± 4.0 %). Lower values of DH (approx. 13-15%) have been reported in a sardine hydrolysate obtained with subtilisin and trypsin.[21] Tuna hydrolysates showed the lowest DH, attaining

32.3±7.1 and 30.7±5.9 % for the hydrolysates obtained from heads, and muscle and viscera, respectively. The differences in the DH attained when hydrolyzing tuna or sardine protein could be ascribed to previous autolysis occurring in sardine by-products.

Amino acid composition

The FPHs showed similar amino acid composition (Table 1). They were rich in essential (392-432/1000) and hydrophobic (486-520/1000) residues. The FPHs were abundant in acidic or amide- containing residues as well (Asp+Asn, Glu+Gln), Gly, Ala, Leu and Lys. Low amounts of Hyp (9-20 per 1000 residues) and Hyl (3-4 per 1000 residues) were also found in all FPHs. Simulated GID affected the amino acid composition of tuna and sardine hydrolysates. Specifically, the relative amount of essential amino acids in tuna hydrolysates increased from 386 to 395 per 1000 residues (muscle and viscera hydrolysate) and from 370 to 375 (tuna heads hydrolysate). Similarly, the relative amount of hydrophobic residues increased from 471 to 481 in tuna muscle and viscera hydrolysate, and from 505 to 509 in the tuna heads hydrolysate. In contrast, the relative amount of both amino acid groups decreased after digestion of sardine hydrolysate. Thus, the relative amount of essential and hydrophobic amino acids changed from 420 to 413 and from 525 to 518 respectively.

Molecular weight profile

The hydrolysates were mainly composed by LMW-peptides as a consequence of the extensive hydrolysis achieved by Alcalase at optimal conditions (Figure 1a,b,c). Sardine hydrolysate was abundant in peptides of MW around 861 Da (5-7 residues), 224 and 152 Da (2 residues), as depicted in Figure 1a. During the simulated gastric digestion of this hydrolysate (T0-T2), pepsin hydrolysed the highest MW peptides and new peptides were released (490 and 254 Da, Figure 1d). Further simulated intestinal digestion (T2-T4) hydrolysed these new peptides and released smaller peptides of MW around 224 Da and

322 Da (presumably di- and/or tripeptides). Some peptides around 861 Da showed hydrolysis resistance throughout the simulated GID.

The MW profile of tuna hydrolysates proved to be similar (Figure 1b,c). This could be due to the use of similar raw materials and the same enzyme when preparing tuna hydrolysates. Additionally, it is worth mentioning that the DH obtained of both samples was not significantly different. Tuna hydrolysates were mainly composed by peptides of MW around 1107 Da (6-9 residues), around 843 Da (5-7 residues), and around 353-170 Da (2-3 residues); that is, in general terms, tuna hydrolysates included peptides showing higher MW than those of the sardine hydrolysate. It would be ascribed to the lower DH value of tuna hydrolysates. Simulated GID affected the MW profile of tuna hydrolysates (Figure 1e,f). Thus, some of the highest and lowest MW peptides (1107 and 170 Da) seemed surprisingly resistant to GID, whilst the peptides around 843 and 353 Da gradually disappeared and were not found at the end of the digestion.

ACE-inhibiting abilities of FPHs

The hydrolysates from tuna by-products exerted the highest ACE-inhibiting activity (IC_{50} = 0.27±0.07 mg/ml for tuna heads and 0.24±0.07 mg/ml for tuna muscle and viscera, Figure 2), although moderate when compared to the control pharmacological inhibitor Enalapril® (IC_{50} = 6.95±0.08 μM, or 2.61±0.03 μg/ml). The similar MW profile and ACE-inhibiting capacity of tuna hydrolysates suggest that they could contain similar ACE-inhibitory peptides. Other authors have also reported ACE-inhibiting activity of tuna hydrolysates. Thus, Je et al.[22] found a maximal ACE-inhibition of approximately 36% in tuna liver hydrolysates. Lee et al.[23] prepared an Alcalase hydrolysate of tuna frame protein that showed 45% of ACE inhibition at 2 mg/ml. The ACE-inhibiting activity of our lab-prepared tuna hydrolysates was higher than others reported in literature for other marine

hydrolysates. Hence, Byun and Kim[24] obtained IC_{50} values between 5.37 and 0.63 mg/ml for different hydrolysates of Alaskan Pollack skin after partial purification. Alemán et al.[25] evaluated the ACE-inhibiting ability of different squid gelatine hydrolysates and obtained IC_{50} values between 1.06-0.34 mg/ml. Wu et al.[26] reported ACE-inhibitory activity of shark meat hydrolysate (IC_{50} = 0.4 mg/ml) higher than that depicted in Figure 2 for our tuna hydrolysates. Other hydrolysates from various non-marine proteins (e.g. milk and other sources) have showed IC_{50} values in the range of 0.18–246.7 mg/ml.[27,28]

The ACE-inhibiting ability of protein hydrolysates from different sardine species has been previously reported *in vitro* and also *in vivo*. [5,21,29-31] However, to our knowledge, ACE-inhibiting ability of Alcalase hydrolysate from heads and viscera of any sardine species has not been reported to date. The hydrolysate prepared from sardine heads and viscera showed moderate ACE-inhibiting ability (IC_{50}=1.16±0.04 mg/ml, Figure 2), although it was lower than that (2.3 mg/ml) reported by Bougatef et al.[32] for sardinelle viscera hydrolysate prepared with alkaline protease. Bordenave et al.[33] prepared an autolysate of sardine by-products (including heads and viscera), and found higher ACE-inhibiting ability than that reported in our work (0.04 mg/ml produced 33% inhibition of ACE-activity).

LMW peptides exerting potent ACE-inhibiting activity are of interest as nutraceuticals because some of them could directly pass through the intestine without being decomposed by digestive enzymes.[34,35] In our work, the FPHs were nanofiltered consecutively through 3 and 1 kDa MW cut-off membranes, and the ACE-inhibiting ability of the obtained retentates (molecules between 1-3 kDa) and permeates (molecules lower than 1 kDa) was assessed. The results showed that NF significantly enriched the ACE-inhibitory activity of the crude hydrolysates (Table 2). As an exception, NF of sardine hydrolysate in a two-step process through 3 and 1 kDa MWCO membranes yielded two fractions (permeate and

retentate) that displayed similar ACE-inhibiting activity (IC_{50}=0.024 mg/ml), although it was more than 50-fold higher than that of the starting material (IC_{50}=1.16 mg/ml). This suggests that peptides of very different MW were responsible for this activity. Other authors have also found ACE-inhibiting peptides in the range 200-2000 Da in different sardine hydrolysates.[1,36]

NF of tuna hydrolysates in a two-step process through 3 and 1 kDa MWCO membranes resulted in an increment of their ACE-inhibiting ability. Permeates were mainly composed by peptides of MW around 843 and 170 Da (Figure 1), suggesting that some of these were responsible for this activity. He et al.[37] were also able to increase the ACE-inhibiting ability of a seafood protein hydrolysate by UF (from 0.98 mg/ml to 0.22 in terms of IC_{50}). Nonetheless, it is worth noting that fraction of MW between 1-3 kDa obtained by NF of the tuna heads hydrolysate showed similar ACE-inhibiting potency than that of the unfiltered hydrolysate (Table 2) and significantly higher than that of the fraction of MW lower than 1 kDa. It suggests that this hydrolysate contains ACE-inhibiting molecules of MW mainly lower than 1 kDa (Figure 1).

Different authors have obtained small peptides possessing strong ACE-inhibiting potency by hydrolysis of fish protein. Li et al.[3] and Kim et al.[38] observed that ACE-inhibiting peptides derived from seafood generally contain 2–8 residues and have MW usually lower than 1 kDa. Di- and tripeptides showing strong ACE-inhibiting activity have been isolated from anchovy, sardine and bonito hydrolysates[39], Atlantic krill[40] and Alaskan Pollack skin gelatine[24], respectively.

PO-inhibiting ability of FPHs

The PO-inhibiting ability of FPHs was moderate, although it was lower than that reported by Sila et al.[13] for other fish protein hydrolysates. The PO inhibitor Z-Pro-Prolinal was used

for comparative purposes (IC_{50} = 25.4±8.3 μg/ml). The tuna hydrolysates showed the highest inhibitory capacity for PO (IC_{50}=4.59±0.47 and 3.30±1.05 mg/ml for hydrolysates of tuna muscle and viscera and tuna heads, respectively), being that significantly better than that of the sardine hydrolysate (IC_{50}=9.57±1.48 mg/ml, Figure 3). NF of the hydrolysate of sardine heads in a two-step process with 3 and 1 kDa MWCO membranes greatly improved the PO-inhibitory activity, suggesting that LMW peptides were the main responsible for this activity (Table 2). Nonetheless, there were not significant differences between the PO-inhibiting activity of permeate (MW < 1 kDa) and retentate (MW 1-3 kDa) fractions, indicating that molecules of MW higher than 1 kDa were also responsible for this activity. NF of the tuna heads hydrolysate did not improve the PO-inhibitory activity. In contrast, the fraction including molecules of MW lower than 1 kDa obtained by NF of the hydrolysate of tuna muscle and viscera showed significant higher PO-inhibiting ability when compared with the unfiltered hydrolysate or with the retentate (MW 1-3 kDa).

Effect of simulated gastrointestinal digestion of FPHs on their potential bioactivity

It is known that *in vitro* inhibitory potency of protein hydrolysates does not always correlate with its effect *in vivo*.[3] Therefore, it is necessary to evaluate the ACE- and PO-inhibitory activity of protein hydrolysates after simulated GID in order to have relevant information on their possible biological effect after oral administration. The results contradictory results obtained after NF of FPH aimed us to digest the unfiltered hydrolysates instead of those nanofiltered. In global terms, the inhibiting potency of FPHs against ACE and PO augmented after simulated GID (Figures 2 and 3). The digested hydrolysate (T4) of tuna heads showed the strongest ACE- and PO-inhibiting activity (IC_{50} = 0.16 and 1.04 mg/ml respectively), while the sardine hydrolysate showed the weakest. Both PO- and ACE-inhibiting activities were improved by simulated gastric digestion

(evolution from T0 to T2), and decreased slightly or remained constant during further simulated intestinal digestion (from T2 to T4).

The enhancement of both PO- and ACE-inhibiting abilities of the sardine hydrolysate produced by pepsin digestion coincided with the release of small peptides of MW around 490 and 254 Da (Figure 1d, T2). Some of these peptides, contained in the BIOPEP DATABASE created by Minkiewicz et al.[41], could be LVYP (490 Da) or YA (252 Da). Peptides resistant to gastric digestion were further and completely digested by pancreatin. At the same time, new peptides were released (around 322 and 224 Da) and the ACE-inhibitory capacity decreased. This indicates that peptides of MW around 490 and 254 Da inhibited ACE more intensively than those liberated by pancreatin. Nonetheless, it is worth mentioning that the PO-inhibiting capacity remained constant throughout pancreatin digestion. It suggests that some peptides showing resistance throughout intestinal digestion (around 861 Da) could also be responsible for the PO-inhibiting ability of the sardine hydrolysate.

The amount of peptides of MW around 1107 and 843 Da found in tuna hydrolysates decreased gradually during GID. On the contrary, dipeptides in the hydrolysates of MW around 170 Da seemed to resist GID. The enhancement of PO-inhibiting activity after gastric digestion suggests that gastrin was responsible for the liberation of new PO- and ACE-inhibiting dipeptides. These peptides could be GP (170 Da), GV or VG (174 Da), reported as ACE-inhibitors in BIOPEP DATABASE. The peptides PG and GP (172 Da) could also be responsible for the PO-inhibiting activity. Both peptides show PO-inhibitory effect[19, 42] and are able to cross the blood-brain barrier and potentiate memory consolidation processes in the central nervous system[43]. GP and PG are abundant in actin chains, myosin heavy chains and fish collagen[44] and could be liberated during simulated GID of tuna

hydrolysates. Nonetheless, the fact that ACE-inhibiting activity significantly decreased after gastrin digestion, unlike PO-inhibiting activity that remained constant, indicates that different molecules were responsible for both activities.

The results globally show that tuna and sardine derived hydrolysates contain populations of inhibiting peptides resistant to degradation by digestive enzymes in the gastrointestinal tract, as reviewed by Li et al.[3]. As well, they contain others that are digested by pepsin releasing new peptides with PO- and ACE-inhibiting potency higher than that of the undigested hydrolysates. In general, these peptides (mainly di- or tripeptides) seem to be resistant to further intestinal digestion. It has been demonstrated that di- or tri-peptides, especially those with C-terminal proline or hydroxyproline residues, are generally resistant to degradation by digestive enzymes[14]. In this regard, tripeptides containing C-terminal Pro-Pro are resistant to proline-specific peptidases, being absorbed in their intact form into the bloodstream and consequently produce biological effects following oral administration.[45] These ACE- and PO-inhibitory peptides (especially the smallest) could directly pass through the intestine and show *in vivo* activity even at a low dosage, as documented by Li et al.[3].

CONCLUSIONS

To summarise, sequential NF with 3 and 1 MWCO membranes almost always improves the PO- and ACE-inhibiting activities of the fish protein hydrolysates prepared in this work. Nonetheless, PO- and ACE-inhibiting peptides were found in retentate (1-3 kDa) and permeate (<1kDa), suggesting that molecules of very different MW are responsible for these properties. As well, it can be concluded that simulated GID of the hydrolysates (mainly that from tuna heads) increases their PO- and ACE-inhibiting potency, and therefore they could be considered as prodrug-type inhibitors. Some of these peptides

cannot support pancreatic digestion and they should be protected by encapsulation in order to reach the intestine in their intact form. In contrast, others seem to be resistant to pancreatic digestion. It should be interesting to know the *in vivo* effect of FPHs, mainly those from tuna by-products, alone and/or incorporated in fishery products ready for consumption. The interesting hypotensive effect of different FPHs reported *in vitro* suggests that the protein hydrolysates prepared from sardine and tuna by-products could have a relevant therapeutic effect on mild hypertensive subjects. Also, the *in vivo* effect of the hydrolysates on animals with neurological disorders requires further study. To conclude, these hydrolysates are potential functional food additives and represent a healthier and natural alternative to synthetic drugs.

ACKNOWLEDGMENTS

This research was financed by EU under the SEAFOODplus European Integrated Programme (Seafoodplus CT-2004-506359), by the Spanish Ministry of Economy and Competitiveness (project AGL2014-52825-R) and also by the Spanish National Research Council (CSIC) under project ref. 200970I113.

REFERENCES

1. L. Vercruysse, J. Van Camp and G. Smagghe, *J. Agric. Food Chem.*, 2005, **53**, 8106-8115.
2. Z. J. Qian, J. Y. Je and S. K. Kim, *J. Agric. Food Chem.*, 2007, **55**, 8398-8403.
3. G.-H. Li, G.-W. Le, Y.-H. Shi and S. Shrestha, *Nutr. Res.*, 2004, **24**, 469-486.
4. A. Alemán and O. Martínez-Alvarez, *Nat. Prod. J.*, 2013, **3**, 105-114.
5. T. Kawasaki, E. Seki, K. Osajima, M. Yoshida, K. Asada, T. Matsui and Y. Osajima, *J. Hum. Hypertens.*, 2000, **14**, 519-523.
6. H. Fujita, T. Yamagami and K. Ohshima, *Nutr. Res.*, 2001, **21**, 1149-1158.
7. K. Majumder and J. Wu, *Int. J. Mol. Sci.*, 2014, **16**, 256-283.

8. I. Wijesekara and S.-K. Kim, *Mar. Drugs*, 2010, **8**, 1080-1093.
9. J. Gass and C. Khosla, *Cell. Mol. Life Sci.*, 2007, **64**, 345-355.
10. J. Wilson, M. Hayes and B. Carney, *Food Chem.*, 2011, **129**, 235-244.
11. L. Polgár, *Cell. Mol. Life Sci.*, 2002, **59**, 349-362.
12. R. Sorensen, E. Kildal, L. Stepaniak, A. H. Pripp and T. Sorhaug, *Nahrung*, 2004, **48**, 53-56.
13. A. Sila, O. Martinez-Alvarez, A. Haddar, M. C. Gómez-Guillén, M. Nasri, M. P. Montero and A. Bougatef, *Food Chem.*, 2015, **168**, 478-486.
14. V. Vermeirssen, J. Van Camp and W. Verstraete, *Brit J Nutr*, 2004, **92**, 357-366.
15. P. Nielsen, D. Petersen and C. Dambmann, *J. Food Sci.*, 2001, **66**, 642-646.
16. P. Bourseau, A. Chabeaud, L. Vandanjon, A. Masse, P. Jaouen, J. Fleurence and J.-P. Berge, in Added Value to Fisheries Waste, ed. J.-P. Bergé, 2009, ch. 12, *http://archimer.ifremer.fr/doc/00000/7408/*.
17. P. Nielsen, D. Petersen and C. Dambmann, *J. Food Sci.*, 2001, **66**, 642-646.
18. O. Martínez-Alvarez, R. Ravallec, B. Cudennec, L. Guimas, C. Delannoy and M. Fouchereau-Peron, *J. Funct. Foods*, 2012, **4**, 767-775.
19. S. Maruyama, S. Miyoshi, T. Osa and H. Tanaka, *J. Ferment. Bioeng.*, 1992, **74**, 145-148.
20. T. Sannaveerappa, S. Westlund, A. S. Sandberg and I. Undeland, *J. Agric. Food Chem.*, 2007, **55**, 10977-10985.
21. P. J. García-Moreno, F. J. Espejo-Carpio, A. Guadix and E. M. Guadix, *J. Funct. Foods*, 2015, **18**, 95-105.
22. J. Y. Je, K. H. Lee, M. H. Lee and C. B. Ahn, *Food Res. Int.*, 2009, **42**, 1266-1272.
23. S.-H. Lee, Z.-J. Qian and S.-K. Kim, *Food Chem.*, 2010, **118**, 96-102.
24. H. G. Byun and S. K. Kim, *Process Biochem.*, 2001, **36**, 1155-1162.
25. A. Alemán, E. Pérez-Santín, S. Bordenave-Juchereau, I. Arnaudin, M. C. Gómez-Guillén and P. Montero, *Food Res. Int.*, 2011, **44**, 1044-1051.
26. H. Wu, H. L. He, X. L. Chen, C. Y. Sun, Y. Z. Zhang and B. C. Zhou, *Process Biochem.*, 2008, **43**, 457-461.

27. M. M. Mullally, H. Meisel and R. J. Fitzgerald, *Int. Dairy J.*, 1997, **7**, 299-303.
28. D. H. Lee, J. H. Kim, J. S. Park, Y. J. Choi and J. S. Lee, *Peptides*, 2004, **25**, 621-627.
29. H. Matsufuji and T. Matsui, *Biosci.. Biotechnol.. Biochem.*, 1994, **58**, 2244-2245.
30. T. Matsui and H. Matsufuji, *Biosci., Biotech., Biochem.*, 1993, **56**, 922-925.
31. L. Otani, T. Ninomiya, M. Murakami, K. Osajima, H. Kato and T. Murakami, *Biosci., Biotech., Biochem.*, 2009, **73**, 2203-2209.
32. A. Bougatef, N. Nedjar-Arroume, R. Ravallec-Ple, Y. Leroy, D. Guillochon, A. Barkia and M. Nasri, *Food Chem.*, 2008, **111**, 350-356.
33. S. Bordenave, I. Fruitier, I. Ballandier, F. Sannier, A. Gildberg, F. Batista and J. M. Piot, *Prep. Biochem. Biotech.*, 2002, **32**, 65-77.
34. T. Matsui, K. Tamaya, E. Seki, K. Osajima, K. Matsumoto and T. Kawasaki, *Clin. Exp. Pharmacol P.* 2002, **29**, 204-208.
35. O. Masuda, Y. Nakamura and T. Takano, *J. Nutr.*, 1996, **126**, 3063-3068.
36. A. Yasuo, *Trends Food Sci. Tech.*, 1993, **4**, 139-144.
37. H. L. He, X. L. Chen, C. Y. Sun, Y. Z. Zhang and B. C. Zhou, *J. Pept. Sci.*, 2006, **12**, 726-733.
38. S.-K. Kim, D.-H. Ngo and T.-S. Vo, in *Advances in food and nutrition research*, ed. K. Se-Kwon, Academic Press, 2012, vol. Volume 65, pp. 249-260.
39. F. Hong, L. Ming, S. Yi, L. Zhanxia, W. Yongquan and L. Chi, *Peptides*, 2008, **29**, 1062-1071.
40. H. Akimasa, M. Hiroko, S. Ken Ichi and S. Seizo, *J. Food Sci.*, 2009, **74**, H116-H120.
41. P. Minkiewicz, J. Dziuba, A. Iwaniak, M. Dziuba and M. Darewicz, *J. AOAC Int.*, 2008, **91**, 965-980.
42. J. A. García-Horsman, P. T. Männistö and J. I. Venäläinen, *Neuropeptides*, 2007, **41**, 1-24.
43. I. P. Ashmarin, E. P. Karazeeva, L. A. Lyapina and G. E. Samonina, *Biochemistry (Moscow)*, 1998, **63**, 119-124.

44. O. Martínez-Alvarez, in *Marine Proteins and Peptides: Biological Activities and Applications*, 2013, pp. 351-367.
45. G. Vanhoof, F. Goossens, I. De Meester, D. Hendriks and S. Scharpe, *FASEB J.*, 1995, **9**, 736-744.

TABLES

- Table 1: Comparative amino acid profiles of protein hydrolysates of sardine and tuna before and during simulated GID. Essential amino acids are indicated with *. Hydrophobic residues are indicated with ^. Results are expressed as number of residues per 1000 residues.

	Tuna (muscle and viscera)					Tuna (heads)					Sardine (heads)				
	undigested	**T1**	**T2**	**T3**	**T4**	**undigested**	**T1**	**T2**	**T3**	**T4**	**undigested**	**T1**	**T2**	**T3**	**T4**
Asp+Asn	91	104	103	105	100	87	88	87	87	87	90	91	92	90	90
Thr*	50	47	46	45	45	49	48	49	48	47	48	47	46	46	46
Ser	55	57	58	56	57	56	58	58	57	57	60	60	59	59	60
Glu+Gln	116	132	132	131	126	110	117	114	114	115	109	113	110	112	110
Gly^	116	125	118	122	122	147	148	146	149	148	123	124	124	123	124
Ala ^	94	105	107	107	108	100	109	105	106	110	98	109	108	110	107
Cys ^	5	4	5	5	5	2	3	3	3	3	3	4	5	5	4
Val* ^	49	37	38	37	39	46	40	42	42	39	62	57	57	57	57
Met*^	28	24	25	24	26	23	24	24	23	25	27	26	25	25	27
Ile*^	35	26	27	27	27	33	28	30	29	28	41	38	37	38	37
Leu* ^	74	68	71	69	71	69	66	68	66	68	77	79	75	78	76
Tyr	25	23	23	24	24	24	22	23	23	23	26	18	25	19	25
Phe*^	32	32	34	34	35	32	29	29	29	31	36	38	37	38	37
Hyl	3	3	3	2	3	4	6	8	7	4	4	2	3	2	5
His*	53	49	51	49	51	33	33	34	33	35	22	23	23	23	22
Lys*	71	70	62	61	63	62	60	60	59	60	71	71	71	71	70
Arg*	41	34	35	34	37	43	41	41	43	42	41	41	42	41	41
Pro^	54	51	50	50	49	59	58	58	57	58	53	51	52	51	49
Hyp	9	9	12	16	13	20	22	22	24	21	10	8	9	13	11
Essential amino acids	432	386	389	382	395	392	370	377	373	375	425	420	413	416	413
Hydrophobic residues	486	471	475	475	481	512	505	505	504	509	520	525	520	524	518

- Table 2. IC_{50} values (mean ± SD) for both ACE and PE-inhibitory molecules included in sardine and tuna protein hydrolysates before and after successive fractionation through MW cut-offs membranes of 3 kDa (retentate) and 1 kDa (permeate).

HYDROLYSATE	***FRACTION***	***ACE IC_{50} (mg dry weight /ml)***	***PO IC_{50} (mg dry weight /ml)***
Sardine (heads)	Hydrolysate	1.16±0.04[b]	9.57±1.48[b]
	1-3 kDa	0.02±0.00[a]	4.68±0.29[a]
	< 1 kDa	0.02±0.00[a]	4.22±0.27[a]
Tuna (heads)	Hydrolysate	0.24±0.05[b]	3.30±1.05[a]
	1-3 kDa	0.10±0.00[c]	4.20±0.15[a]
	< 1 kDa	0.08±0.00[ac]	4.02±0.69[a]
Tuna (muscle and viscera)	Hydrolysate	0.27±0.08[a]	4.59±0.47[b]
	1-3 kDa	0.31±0.06[a]	35.22±3.64[c]
	< 1 kDa	0.16±0.01[b]	1.10±0.02[a]

Results were expressed as mg dry weight/ml and mg protein/ml. Different letters (a, b, c) indicate significant differences between fractions derived from the same raw material.

FIGURES

Figure 1: MW profile of FPH before (T0) and during simulated GID (T1-T4). a, b, c) MW profile of hydrolysates of sardine heads and viscera, tuna heads and tuna muscle and viscera, respectively (T0); d, e, f) MW profile of hydrolysates of sardine heads and viscera, tuna heads and tuna muscle and viscera, respectively, during its simulated GID. Stage T1 is shown in black, T2 in blue, T3 in red and T4 in brown.

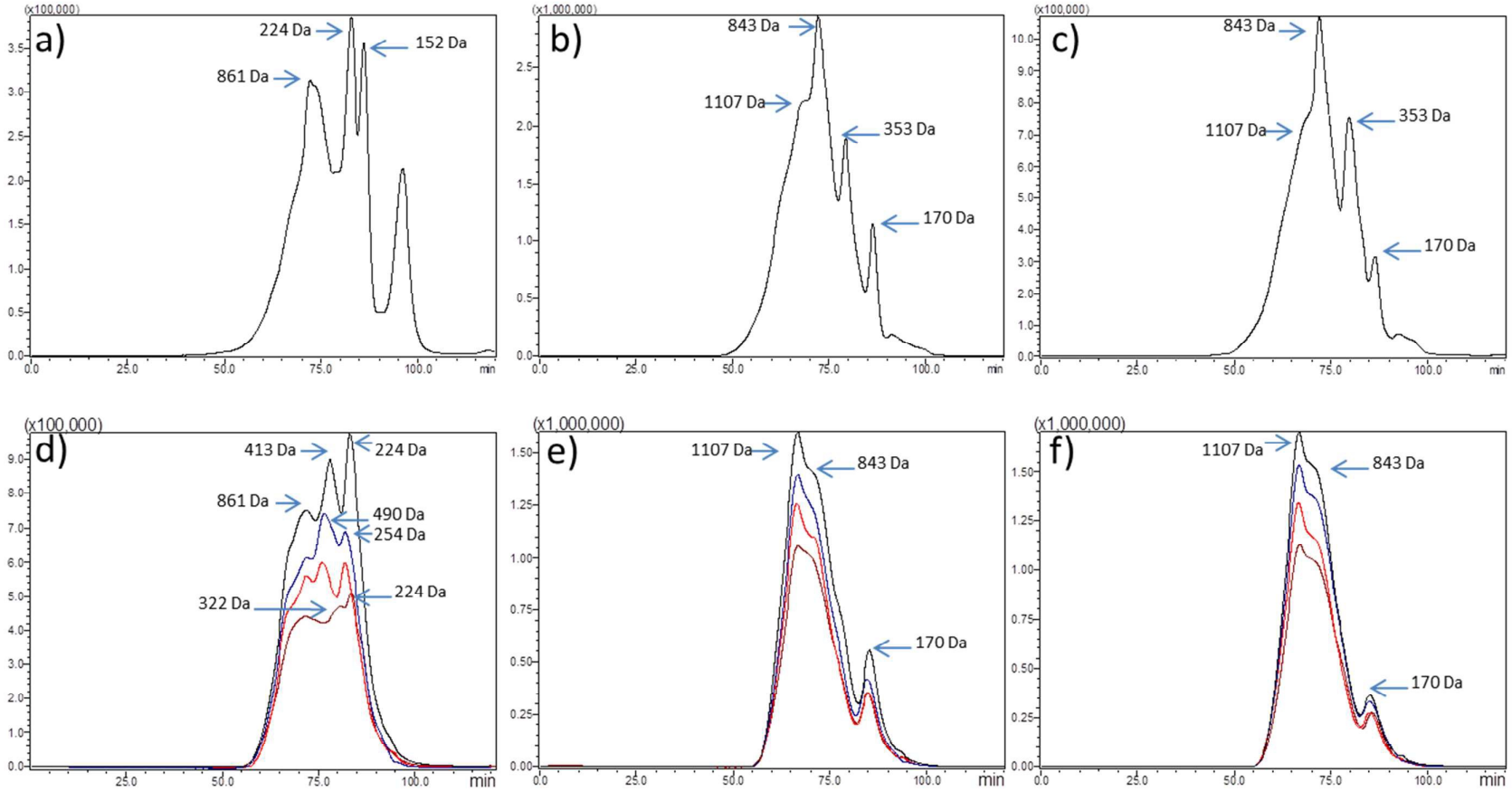


Figure 2. Evolution of ACE-inhibiting activity of the protein hydrolysates along simulated gastrointestinal digestion. Results are expressed as IC_{50} (mean ± SD) in mg dry weight/ml. Different letters (a, b, c) indicate significant differences during the GID ($P \leq 0.05$). Different letters (x, y, z) indicate significant differences in the activity of the FPHs at the same step of GID.

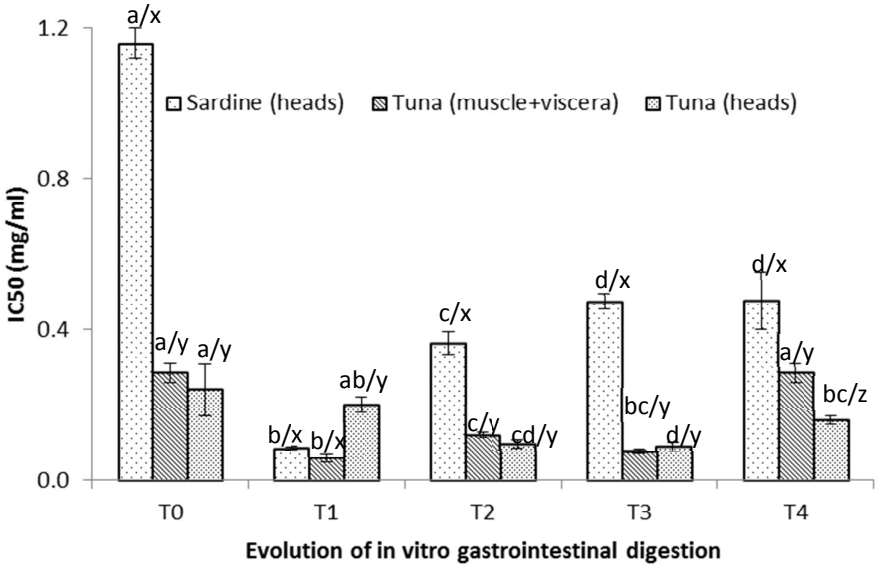


Figure 3. Evolution of PO-inhibiting activity of the protein hydrolysates along simulated gastrointestinal digestion. Results are expressed as IC_{50} (mean ± SD) in mg dry weight/ml. Different letters (a, b, c) indicate significant differences during the GID ($P \leq 0.05$). Different letters (x, y, z) indicate significant differences in the activity of the FPHs at the same step of GID.