Process Biochemistry 41 (2006) 1217–1222

Process Biochemistry

www.elsevier.com/locate/procbio

Short communication

Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines

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Received 2 June 2005; received in revised form 29 November 2005; accepted 29 November 2005

Abstract

Antiproliferative activity of 18 fish protein hydrolysates was measured on 2 human breast cancer cell lines grown in vitro. Three blue whiting, three cod, three plaice and one salmon hydrolysates were identified as significant growth inhibitors on the two cancer cell lines. Preliminary analysis of hydrolysates composition evidenced they contained a complex mixture of free amino acids, peptides with various sizes ranging up to 7 kDa and in a lower proportion, lipids and sodium chloride. RP-HPLC fractionation of fish hydrolysates is currently undertaken to purify anticancer peptides, lipids or other bioactive trace compounds responsible for this antiproliferative activity.

Keywords: Breast cancer; Fish protein hydrolysate (FPH); Antiproliferative activity

1. Introduction

Fish consumption is associated to health benefits because of a rich content in proteins of high nutritional value, minerals, vitamins and distinctive lipids [1]. Estimates for 2003, based on reports from major fishing countries, indicate that fish provided more than 2.6 billion people with at least 20% of their average animal protein intake [2]. However, fish stocks are decreasing and the annual world fish catch is stabilized. Moreover, a rational valorization of by-products is extensively investigated, as the yearly average discards are estimated to 7.3 million tonnes [2]. Fish proteins show interesting rheological properties [3], and can be valorized for numerous applications such as production of surimi or protein enrichment of fillets. Fish protein hydrolysates (FPH), obtained by controlled enzymatic hydrolysis, are among the best protein hydrolysates in term of nutritional properties (balanced amino acid composition, high digestibility [4]), but are mainly used for animal nutrition because of their bitter flavour and fishy odour.

The identification of growth factors [5], immunomodulating [6], antimicrobial [7], antithrombotic [8], hypotensive [9], and anticancer peptides [10] in hydrolysates from milk [5,6,8,9,11], wheat [12], soybean [10,13] and egg [7,14] is a scientific evidence that enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides. Also, research on fish or FPH demonstrated they constitute a source of promising health benefits molecules for nutritional or pharmaceutical applications [15–20]. Isolation of potent anticancer compounds from fish tissue [21–25] is also a strong argument to consider that by-products could constitute a source of anticancer or chemopreventive molecules.

In this context, a preliminary study was designed to determine whether FPH exert an antiproliferative activity on cancer cells grown in vitro.

Abbreviations: Ara-C, cytosine-beta-D-arabinofuranoside; DAD, diode array detector; FPH, fish protein hydrolysate; SEC, size exclusion chromatography

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doi:10.1016/j.procbio.2005.11.024

2. Materials and methods

2.1. Fish protein hydrolysates origin

Twenty-one FPH were prepared by three European fish processing companies: Coopérative de Traitement des Produits de la Pêche (CTPP), Boulogne/mer, F-62203-France; Marinova (Danish Fish Protein), Hoejmark, DK-6940-Denmark and Primex, Reykjavik, IS-101-Iceland, from Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), plaice (*Pleuronectes platessa*), blue whiting (*Micromesistius poutassou*), Atlantic emperor (*Lethrinus atlanticus*), pollack (*Pollachius pollachius*) and Portuguese dogfish or siki (*Centroscymnus coelolepis*).

2.2. Fish protein hydrolysates preparation and analysis

Fish proteins were purified from fresh filleting by-products or headed and gutted by-catches using the pH-shift extraction method [26], based on the solubility of myofibrillar and sarcoplasmic proteins at extreme acid and alkaline pH. Fish muscle was minced, prewashed with water and centrifuged (3000 × *g*, 10 min). The resulting pellet was dissolved in water (1:6, w:v) and pH was adjusted with NaOH 1 M at 10.8, to solubilize myofibrillar and sarcoplasmic proteins. Proteins were then efficiently separated from lipids, membranes, skin and bones by centrifugation (3000 × *g*, 10 min). Two phases were obtained: a solid phase containing membranes, skin and bones (pellet) and a soluble phase containing solubilized proteins. The soluble phase was carefully collected and pH was adjusted to 5.6 with HCl 1 M, to precipitate proteins, which were collected by centrifugation (3000 × *g*, 10 min). The pellet was dissolved in two parts water at pH 7.5 and hydrolyzed at 55–57 °C for 106 min with Protamex 98 g/1000 kg and Alcalase 20 mL/1000 kg (Novo Nordisk). The enzymes were inactivated by heating the resulting suspension to 90 °C for 10 min. The suspension was centrifuged (3000 × *g*, 10 min) to pellet inactivated proteases and non-hydrolyzed fish proteins. The supernatant, containing soluble peptides, was collected and freeze dried.

Peptide, lipid and sodium chloride content of FPH were analyzed by the certificated laboratory IFL, 101-Reykjavik, Iceland, following the Kjeldahl (ISO 5983), Volhard (AOAC 937-09) and Soxhlet methods (AOCS Official Method), respectively. Molecular weight distributions of FPH peptides were determined using SEC in FPLC mode on a Superdex Peptide HR 10/30 column (Pharmacia, fractionation range: 7000–100 Da) with a Waters 600 automated gradient controller pump and a Waters 996 DAD. The mobile phase (isocratic) consisted of MilliQ water-TFA 0.1% and acetonitrile (70:30) at a flow rate of 0.5 mL min^{-1}. Hydrolysates were dissolved in mobile phase to 5 g L^{-1} and sterile filtered. Sample size was 40 μL (200 μg of hydrolysate) and peptides were detected at 220 nm. Millenium software was used to collect, plot and process the chromatographic data. Standard peptides (Sigma, France) were used to calibrate the column.

2.3. Anticancer activity of fish hydrolysates

2.3.1. Cell culture

Two human breast carcinoma cell lines, MCF-7/6 and MDA-MB-231 kindly provided by Dr. Mareel (Laboratoire de Cancérologie Expérimentale, Hôpital Universitaire, Gand, Belgium) were grown at 37 °C in a 5% CO_2–95% air humidified atmosphere, in DMEM-Ham's F12 medium (1:1, v:v, Gibco), supplemented with 10% heat inactivated (56 °C, 30 min) fetal calf serum (FCS, Dutscher) to which were added penicillin 100 U mL^{-1} and streptomycin 100 μg mL^{-1}.

2.3.2. Antiproliferative activity of fish hydrolysates

In order to perform a screening of the antiproliferative activities of FPH on cancer cells grown in optimal conditions (cell culture medium containing FCS growth factors), a relatively high concentration of fish hydrolysate (1 g L^{-1}) was assayed. To avoid pH variation of the cell culture medium during hydrolysate solubilization, a 10 g L^{-1} fish hydrolysate stock solution was prepared in PBS 0.1 M pH 7.4, sterile filtered and five fold diluted in cell culture medium (FPH concentration of 2 g L^{-1}). The same dilution was prepared replacing FPH stock solution by PBS (control culture medium) or SDS 10% in PBS (lysis medium, SDS 2%). Using a multichannel pipettor, 50 μL of each medium were pipetted into separate columns of the microplate and equilibrated at 37 °C. Cancer cells grown in flask to confluence were rinsed three times with PBS at 37 °C, trypsinized, and centrifugated (3000 × *g*, 3 min). Cells were washed with fresh medium and resuspended in culture medium at a concentration of 10^5 cells mL^{-1}. The cell suspension (50 μL) was then added to each well to deposit 5000 cells and adjust the final fish hydrolysate (1 g L^{-1}) or SDS (1%) concentrations. The microplate was then incubated at 37 °C for 72 h. At the end of the incubation, 15 μL of MTS (soluble tetrazolium salt) solution was added to each well, and the plate was incubated for a further 4 h to allow MTS metabolism to formazan by the succinate-tetrazolium reductase only active in viable cells. A solubilization/stop solution (100 μL) was added to stop the succinate-tetrazolium reductase activity, kill the cells and solubilize formazan cristals for 12 h at 37 °C. Optical densities were read on a plate reader (VERSAmax, Molecular Devices France, 35762 Saint Grégoire) at 570 nm. The data were analyzed to calculate the percentage of growth inhibition induced by the presence of FPH in cell culture medium determined by the equation:

$$\text{Growth inhibition}\,(\%) = 100 - \left(\frac{(\text{OD}_{\text{FPH}} - \text{OD}_{\text{lysis}})}{(\text{OD}_{\text{Control}} - \text{OD}_{\text{lysis}})} \times 100\right)$$

where OD_{FPH}, OD_{lysis} and $OD_{control}$ are optical densities, respectively measured for cells grown in culture medium containing FPH 1 g L^{-1}, SDS 1% or PBS only. Antiproliferative activity of FPH was compared with that of reference anticancer molecules (Sigma–Aldrich) with various cellular targets, namely actinomycine D (intercalating agent), Ara-C (nucleoside analogue), cyclophosphamide (alkylating agent), etoposide (topoisomerase II inhibitor), kenpaullone and roscovitine (cyclin-dependent-kinases inhibitors). Anticancer compounds were dissolved in DMSO to give 10^{-3} M stock solutions from which further dilutions were made in culture medium to get final concentrations of 10^{-9} and 10^{-6} M. For control experiments, culture cell medium contained a DMSO concentration equivalent to that found in the 10^{-6} M anticancer molecule assay.

2.4. Statistics

Antiproliferative data are given as mean percentage ± S.E.M of 24 assays from triplicate independent experiments. Statistical significance of growth inhibition induced by FPH was calculated following χ^2 test with $\alpha = 0.05$.

3. Results

3.1. Fish hydrolysates composition

Table 1. shows the composition of FPH. Most hydrolysates were homogeneous dry powders containing more than 70% peptide-nitrogen material, except Salmon 3, 4 and 5 with 58% lipids. Because of weak solubility in cell culture medium, these three hydrolysates were discarded. Cod 1–3, Plaice 1–3, Pollack, Salmon 1 and Salmon 2 hydrolysates contained a relatively high sodium chloride content compared to the other hydrolysates. SEC performed on standard peptides allowed to establish a near linear correlation between the retention time (R_t) and the logarithm of the molecular weight: log (MW) = $-0.0996 \cdot R_t$ (min) + 5.52. Peptides contained in cod, plaice, and blue whiting hydrolysates showed a large MW distribution (Fig. 1), ranging from ∼100 Da to 7 KDa, indicating the presence of free amino acids and peptides ranging up to an approximate maximal length of 70 amino acids.

3.2. Antiproliferative activity of fish hydrolysates on human breast cancer cell lines

As depicted in Fig. 2, Blue whiting 3–5, cod, plaice and Salmon 2 hydrolysates exerted a significant antiproliferative

Table 1
Fish hydrolysates weight composition

Fish hydrolysate	Peptide-nitrogen	Lipids	Water	NaCl
Blue whiting 1	96.2	0.4	2.5	0.2
Blue whiting 2	96.4	0.4	2.5	0.2
Blue whiting 3	96.0	0.4	2.6	0.2
Blue whiting 4	95.9	0.4	2.7	0.2
Blue whiting 5	96.3	0.5	2.5	0.2
Cod 1	77.1	0.04	3.9	20.2
Cod 2	76.6	0.05	4.8	20.0
Cod 3	75.5	0.04	4.0	20.9
Emperor	88.0	0.02	3.6	7.6
Plaice 1	74.0	0.4	3.8	20.9
Plaice 2	70.6	0.6	2.9	21
Plaice 3	72.8	0.1	4.4	20.5
Pollack	85.4	0.06	2.4	11.3
Pollack egg	82.5	ND	5.0	ND
Salmon 1	73.0	0.09	3.0	21.4
Salmon 2	72.9	0.5	3.4	18.9
Salmon 3	37.0	58.1	2.3	0.5
Salmon 4	37.0	58.2	2.2	0.5
Salmon 5	37.0	58.4	2.3	0.5
Siki 1	96.8	0.04	9.1	ND
Siki 2	97.1	ND	9.0	ND

ND: non-determined. Total percentage can be superior to 100% when a source of nitrogen different from proteins is present in the hydrolysate (e.g. urea in siki hydrolysates).

activity at 1 g L^{-1}. Blue whiting 3–5, containing a very low NaCl concentration and 96% peptide, respectively, induced a growth inhibition of 24.5, 22.3 and 26.3% on MCF-7/6, and 13.5, 29.8 and 29.2% on MDA-MB-231. These values were in the range of those measured in the presence of etoposide, roscovitine or kenpaullone 10^{-6} M (data not shown) which exerted a moderate antiproliferative activity on cancer cells. Antiproliferative activity of FPH were dependent on cell lines investigated. MDA-MB-231, classed as a highly invasive breast cancer cell line, was usually less sensitive to a fish hydrolysate treatment than MCF-7/6. This observation is in accordance with previous data reported when the two cells lines were grown in the presence of antiproliferative thiazolocarbazoles analogues of ellipticine [27].

4. Discussion

4.1. Relationship between antiproliferative activity and fish hydrolysates composition

4.1.1. Influence of sodium chloride content

FPH usually contain a moderate NaCl content due to salting for conservation or pH adjustements during the pH shift process. The most salted FPH (Plaice 2 and Salmon 1), respectively, contained 21 and 21.4% (w:w) sodium chloride. Consequently, presence of FPH at a final concentration of 1 g L^{-1} in cell culture medium, compared with the control growth conditions, corresponded to a maximal supply of 0.22 g NaCl/L of cell culture medium. Considering that DMEM and Ham's F12 are isotonic cell culture media (9 g inorganic salts L^{-1}, 4750 mg NaCl L^{-1}), the increase in Nacl concentration associated to the presence of FPH was limited and appeared insufficient to explain the significant antiproliferative activity of FPH.

4.1.2. Presence of anticancer peptides in fish hydrolysates

Addition of FPH to a final concentration of 1 g L^{-1} in cell culture medium, compared with the control growth conditions (PBS in cell culture medium), constitutes a supply of nitrogen sources, amino acids, peptides and vitamins that could be used by cancer cells as growth promoting factors, as previously observed in models of gastro-intestinal cell injury and repair [28]. Conversely, the presence of Blue whiting 3–5, cod, plaice and Salmon 2 hydrolysates in the cell culture medium induced a significant inhibition of cancer cells growth. To identify the nature of molecules responsible for this activity and understand if the process used to prepare FPH could explain and influence the biological activity, a tentative of correlation between activity and FPH composition was made. Blue whiting 3–5 were of particular interest as they contained 96% of peptide-nitrogen material, a content 20% higher than for the rest of the hydrolysates. This observation suggests that the antiproliferative activity could be related to the presence of specific peptides exerting a direct cytotoxicity on cancer cells, as previously reported for valorphin, a haemoglobin derived peptide [29], or for an anchovy hydrophobic peptide, able to induce apoptosis in human U937 lymphoma cells through the increase of caspases activity [30–31]. The hypothesis of a binding competition between fish peptides and FCS growth factors on cell membrane receptors cannot also be excluded, and fish peptides could then act as antagonists of FCS growth factors receptors. No clear correlation between degree of hydrolysis and antiproliferative activity was determined. An identical enzymatic hydrolysis process applied to different batches of a given fish source resulted in comparable global composition and molecular weight profiles as observed for the three cod hydrolysates, the three plaice hydrolysates or Blue whiting 1–3 hydrolysates. The cod hydrolysates (as well as the plaice hydrolysates), displaying equivalent global composition and hydrolysis degree, exerted equivalent antiproliferative activity. Conversely, in spite of comparable global compositions and molecular weight profiles, Blue whiting 3 exerted a high antiproliferative activity, while Blue whiting 1 and 2 had no activity. This variation of activity remains to be clearly explained as the only difference in between these hydrolysates is the blue whiting batch, and no modification of the process (temperature, enzyme) can account for the variation in their activity.

4.1.3. Presence of other bioactive compounds in fish hydrolysates

The lipid quantitative content was not identified as a relevant marker to predict the antiproliferative activity. For example, the lipid content of all blue whiting hydrolysates was quantitatively equivalent but the antiproliferative activity of Blue whiting 3–5 was much greater than that of Blue whiting 1 and 2. This analysis does not exclude that the presence of peculiar bioactive lipids in hydrolysates, as well as trace elements of minerals or other bioactive compounds such as fin chondroitine sulfate, or

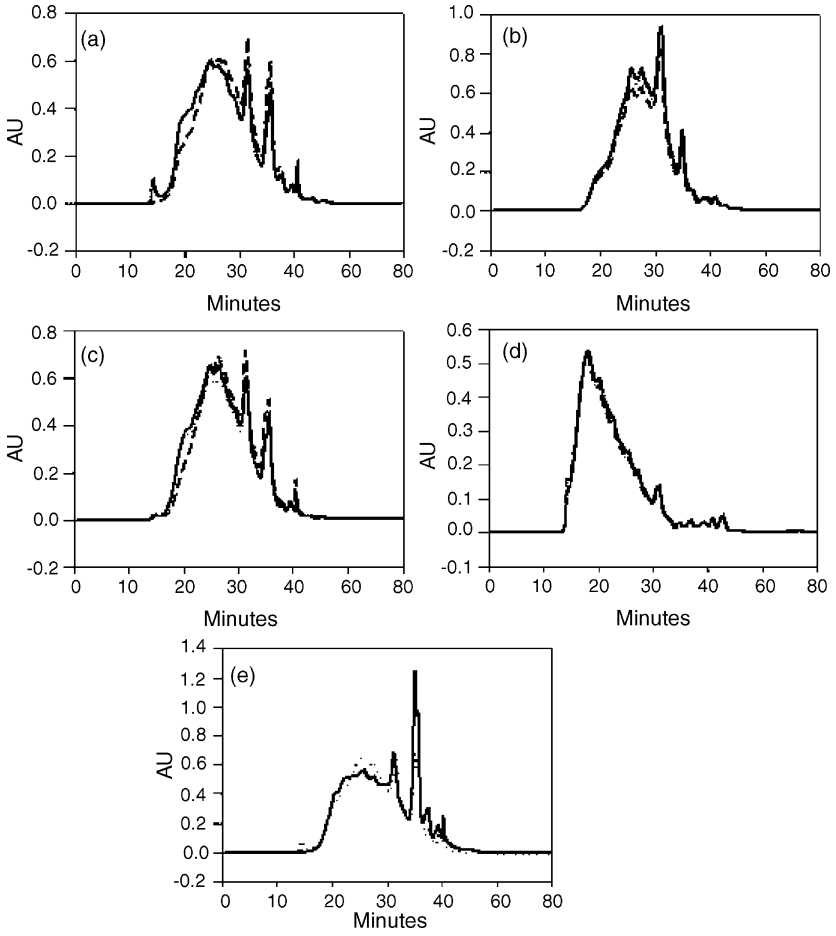

Fig. 1. Molecular weight distribution of material absorbing at 220 nm in fish hydrolysates. Samples were run as described in Section 2: (a) Cod 1 (full line), Cod 2 (dotted line) and Cod 3 (broken line); (b) Blue whiting 4 (full line) and Blue whiting 5 (dotted line); (c) Plaice 1 (full line), Plaice 2 (dotted line), Plaice 3 (broken line); (d) Blue whiting 1 (full line), Blue whiting 2 (dotted line) and Blue whiting 3 (broken line); (e) Salmon 1 (full line) and Salmon 2 (dotted line).

arsenobetaine in plaice [32], could influence the antiproliferative activity.

4.2. Concluding remarks

Anticancer molecules isolated from marine organisms belong to diverse structural classes including polyketides, terpenes, steroids and peptides [33]. These molecules are usually obtained from fixed animals such as corals, sponges and ascidians, which protect themselves from predation by synthesizing potent cytotoxic molecules. However, fish tissues also constitute a potential source of anticancer molecules to be explored. For example, squalamine, an aminosterol isolated from the liver of the dogfish shark *Squalus acanthias* [22], was demonstrated to be a potent inhibitor of angiogenesis and tumour growth in several animal models [23,24]. Alkylglycerols, natural etherlipids abundant in shark liver oil, were recently described as inhibitors of tumor vascularization [25]. To our knowledge, the only anticancer peptide described from a fish source is a 440.9 Da anchovy hydrophobic peptide, able to induce apoptosis in human U937 lymphoma cells through the increase of caspase-3 and caspase-8 activity [30–31]. The present study demonstrates that some FPH, obtained by controlled enzymatic hydrolysis of muscle proteins, exert a significant antiproliferative activity on human cancer cell lines in vitro. These preliminary data suggest that FPH could represent an interesting source of anticancer peptides or lipids to be explored. Preliminary study of the global fish hydrolysate composition dit not allow to correlate the antiproliferative activity to the presence of any peculiar molecules, particularly peptides of defined molecular weight. Moreover, slight variations in the hydrolysis process led to high variations in bioactivity, thus confirming the need to accurately control the hydrolysis process to ensure repeatability of FPH bioactivity. Industrials and scientists searching for bioactive compounds in fish hydrolysates are very aware of the rules and regulations to follow in order to ensure consumer's safety and efficacy of new products. In this view, the demonstration of bioactive properties in in vitro screening tests does by no means prove that fish peptides (or native proteins) exert the same beneficial effects when consumed by humans. Gastric and intestinal protein digestion generates a huge variety of short chain peptides among which only di- and tripeptides are absorbed into intestinal epithelial cells by active transporters located in the apical membrane of enterocytes [34]. Further studies will be necessary to determine whether fish or shellfish ingestion can

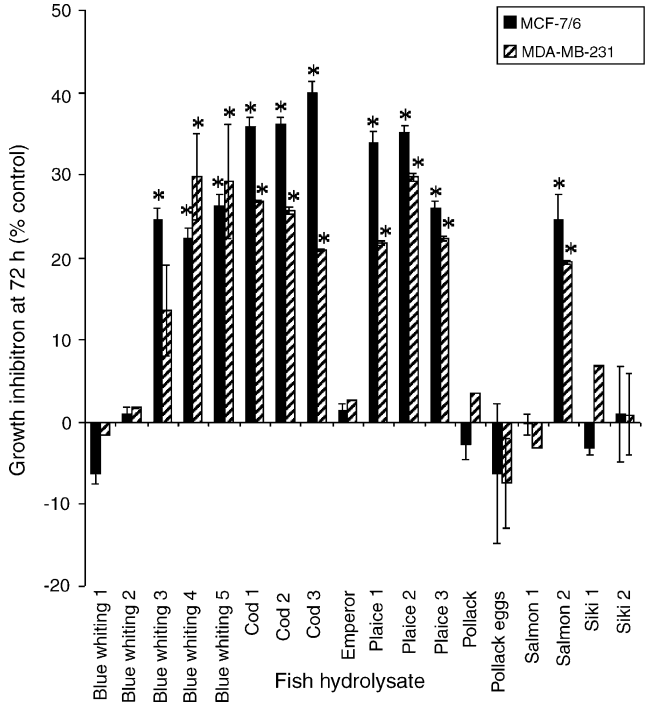

Fig. 2. Effect of 18 fish hydrolysates on growth of MCF-7/6 and MDA-MB-231 cells cultured for 72 h in cell culture medium containing 1 g L^{-1} of hydrolysate. Results are expressed as percentage of growth inhibition relative to control. Each value was the mean of 24 measures from 3 independent experiments. Statistical significance (*) of growth inhibition was calculated following χ^2 test with $\alpha = 0.05$.

modulate health via the presence of bioactive peptides in the bloodstream, acting on cellular pharmacological targets.

Acknowledgements

This study was performed within the SEAFOODplus European Integrated Programme (http://www.seafoodplus.org) as part of the ≪Propephealth≫ project 4.1. High-added value functional seafood products for human health from seafood by-products by innovative mild processing. LBCB is the coordinator partner of the Intereg IIIB VALBIOMAR project (http://www.valbiomar.org) for biotechnological valorization of marine resources. L.P. is thankful to the Conseil Général de Charente Maritime for a post-doctoral research grant.

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