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Simultaneous double cationic and anionic molecule separation from herring milt hydrolysate and impact on resulting fraction bioactivities

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ABSTRACT

The increase in fish consumption, over the last twenty years, is linked to by-product production, rich in valuable compounds such as proteins, vitamins, minerals or lipids (omega-3). Milt herring hydrolysate, containing different molecules, has been fractionated by electrodialysis with ultrafiltration membrane (EDUF) in a new configuration using four ultrafiltration membranes, allowing a simultaneous and double fractionation of anionic and cationic molecules. Four fractions were recovered, each were concentrated in compounds of low molecular weight (< 800 Da). Furthermore, free amino acids present at high concentrations and representing 20% of the initial hydrolysate were demonstrated for the first time to be concentrated as well as peptides in some specific recovery fractions. Anionic fractions presented a high population of acidic amino acids (Glu, Asp) whereas cationic fractions were concentrated in basic amino acids (Arg, Lys). In addition, it was demonstrated that EDUF allowed the modulation of biological activities, such as an enhancement of 27.5% in glucose uptake by L6 cells reported for the final hydrolysate and an increase of 214% of the antioxidant activity of one anionic fraction. EDUF appeared as an effective way to generate fractions with valuable bioactivities which could be used for the prevention of some diseases such as metabolic syndrome.

1. Introduction

Beneficial effects of seafood and specifically fish have been well known since many years, due to their good balance in essential amino acids and fatty acids [1]. The increasing fish consumption [2] by the world population, generates a large quantity of by-products representing up to 50% of the initial product depending on the type of fish [3]. These by-products pooled the head, skin, milt, viscera or bones of fish and contains high value molecules such as protein, gelatin, peptide, polyunsaturated fatty acids (PUFAs), vitamins or pigments (astaxanthin) [3]. Optimizing the utilization of these fish by-products would be an ecological, economic and social issue. Enzymatic hydrolysis is one of the major way used to promote fish protein by producing bioactive peptides (BPs). Several studies have reported the improvement of some biological activities by fish peptides such as antioxidant, antibacterial, antihypertensive or anticancer properties [4–8]. Recently, Chevrier et al. [9] reported that small peptides from salmon protein enhance glucose uptake by L6 myocytes and prevents glucose intolerance, adipose tissue inflammation and dyslipidemia in mice fed with a high-fat and high-sucrose diet. This suggests that fish peptides could be used in the prevention of type 2 diabetes and metabolic syndrome.

However, fish hydrolysates are a complex mixture of peptides with different molecular weights and amino acids composition [10,11]. Moreover, peptide bioactivities are linked to both weight and amino acid (AA) composition, so purification and concentration steps are required [10,11]. Indeed, Picot et al. [10] reported an increase of calcitonin-gene-related-peptide like activity by the permeate fraction after ultrafiltration of a fish hydrolysate. They also reported that antioxidant activity was mainly depending on amino acid composition [10]. Nowadays, pressure driven and chromatographic methods are principally used by industrials. However, pressure driven technics separate compounds only according to their molecular weights and are subjected to membrane fouling which limits peptide selectivity [10–12]. Rather, chromatographic processes using physicochemical properties, are very selective but they consume solvent and can only treat small volume of

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product. Recently, electrodialysis with ultrafiltration membranes (EDUF) was used for the separation of several marine protein hydrolysates [13–15]. This green technology allows separation and concentration of bioactive peptides according to their charges under electric field and their molecular weights (MW) [16]. This double selection enables a higher selection of peptides. In addition, different molecular weight cut off (MWCO) of ultrafiltration membranes (UFs) could be used during the process to increase selectivity. Roblet et al. [14] reported the *in vitro* improvement of glucose uptake by salmon peptide after electroseparation by EDUF. The authors also demonstrated the capacity of EDUF to remove inhibiting peptides and/or to concentrate antidiabetic peptides in the final fraction. Furthermore, EDUF is an ecoefficient process using no solvent except water and electricity as power.

In this context, the aims of this work were to (1) characterize the composition of milt herring hydrolysate and its EDUF fractions; (2) evaluate the double and simultaneous separation of compounds of herring milt hydrolysate by their charges and molecular weights and (3) test valuables biological efficiencies of this fishery by-product on different *in vitro* experiments (glucose uptake and antioxydante activity) in order to increase the value of herring milt hydrolysate.

2. Materials and methods

2.1. Material

2.1.1. Chemicals

Sodium sulphate (Na_2SO_4) was purchased from Laboratoire MAT (Québec, QC, Canada), potassium chloride (KCl) from ACP Inc (Montreal, QC, Canada), sodium chloride (NaCl) from VWR international (Montreal, QC, Canada), calcium chloride ($CaCl_2$), HEPES-Na, magnesium sulfate ($MgSO_4$), 2-déoxy-D-glucose, sodium phosphate monobasic, fluorescein and 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma Aldrich (Oakville, ON, Canada) acetonitrile optima® LC/MS, 1.0 M NaOH solutions from Fisher Scientific (Montreal, QC, Canada), and trifluoroacetic acid from J.T. Baker (Philipsburg, NJ, USA). Polyvinylidene fluoride (PVDF) filters were obtained from Chromatographic Specialities Inc (Brockville, ON, Canada). HiClone™ Bovine Growth Serum (BGS) was purchased from GE Healthcare Life Sciences (Logan, UT, USA), Alpha-Minimal Essential Medium (α-MEM) was obtained from Invitrogen (Burlington, ON, Canada). D-2deoxy-[^{3}H] glucose was obtained from Perkin Elmer (Woodbridge, ON, Canada) and insulin from CHUL's pharmacy (Québec, QC, Canada). Pierce® BCA Protein Assay and micro BCA Protein Assay from Pierce Biotechnology (Rockford, IL, USA). L6 skeletal muscle cell line, derived from neonatal rat thigh skeletal muscle, was provided by Dr. A. Klip, Hospital for Sick Children (Toronto, ON, Canada).

2.1.2. Milt herring hydrolysate

The initial hydrolysate (IH) was obtained from Ocean NutraSciences (Matane, QC, Canada). Briefly, herring milt was hydrolyzed by a mix of enzymes (duration, temperature and enzymes are confidential). The liquid was then filtrated on ultrafiltration (UF) membranes (MWCO confidential), the retentate was dried by atomization to obtain a fine powder. The chemical composition of the herring milt hydrolysate is presented in Table 1.

2.1.3. Electrodialysis material

The electrodialysis cell used for the peptide separation was a MP type cell (100 cm^2 of effective area) manufactured by Electrocell AB (Taby, Sweden) configured with one Neosepta AMX-SB anionic membrane (AEM, Astom ltd, Tokyo, Japan), one Neosepta CMX-SB cationic membrane (CEM, Astom ltd Ltd, Tokyo, Japan), 2 ultrafiltration membranes with a MWCO of 50 kDa (Synder Filtration, Vacaville, CA, USA) with an average thickness and conductivity of 0.19 mm ± 0.003 mm and 6.32 mS/cm ± 0.43 mS/cm, and 2 ultrafiltration membranes with a MWCO of 20 kDa (Synder Filtration, Vacaville, CA, USA) with an average thickness and conductivity of 0.19 mm ± 0.003 mm and 4.93 mS/cm ± 1.10 mS/cm. The 4 UF membranes were made of polyether sulphone (PES).

2.2. Electrodialysis cell configuration

The separation by electrodialysis was performed by four UF membranes and two ion-exchange membranes as indicated in Fig. 1. The cell configuration used specially designed spacers to allow such a six recirculating chambers including the electrolyte. The UF membrane with a MWCO of 20 kDa placed near the anode was named UF 1 while the one with a MWCO of 50 kDa placed at the left of the feed solution was named UF 2. The UF membrane with a MWCO of 20 kDa placed near the cathode was named UF 4 while the one with a MWCO of 50 kDa placed at the right of the feed solution was named UF 3. The ED configuration presented in Fig. 1 consisted of six compartments. The central one was the initial hydrolysate (IH) solution composed of dry milt herring hydrolysate, dissolved to 4% of protein (W/V) in water overnight at 4 °C. Four compartments containing a KCl solution (2 g/L), were used for peptide recovery. Two anionic peptide recovery compartments were formed: the KCl solution placed between the AEM and UF 1 was named anionic recovery compartment 2 (A_{RC2}) while the KCl solution located between the UF 1 and the UF 2 was named A_{RC1}. In the same way, two cationic peptide recovery compartments were formed: the KCl solution placed between the CEM and UF 4 was named cationic recovery compartment 2 (C_{RC2}) while the KCl solution located between the UF 4 and the UF 3 was named C_{RC1}. The last compartment contained the electrolyte solution which circulated between each electrode. It was the first time that such a complex configuration was used for peptide electroseparation.

The solutions were circulated using six centrifugal pumps and the flow rates were controlled by flow meters at 3 L/min for the A_{RC1}, C_{RC1} and feed solution, at 0.6 L/min for the A_{RC2} and C_{RC2} solutions. The electroseparation was performed at a constant electric field strength of 5.6 V/cm during a period of 4 h at a controlled temperature ranging between 10 °C and 15 °C. The pH of the feed and KCl solutions was adjusted at 7 with 1.0 M HCl or NaOH and kept constant throughout the treatment in order to maintain the peptide electromigration [13]. The conductivity of the 4 recovery solutions was also adjusted with KCl powder at 3000 µs per cm and maintained constant during the treatment to maintain a linear migration of peptides [13]. After four repetitions, each compartment was lyophilied and analyzed to check for the reproducibility before pooling the same fraction for the four repetitions. 2 mL samples of feed and peptide recovery compartments were collected before applying voltage and at 30, 60, 120, 80 and 240 min. Following each treatment, the stack was rinsed with 0.1 N HCl solution and 0.1 N NaOH solution.

Table 1
Chemical composition of the herring milt hydrolysate.

Composition in dry powder (%)	Total nitrogen	Peptides	Nucleic acids	Lipids	Ashes
Initial Hydrolysate (g/100 g dry weight)	79.27 ± 0.17	48.28 ± 0.44	27.30 ± 3.57	18.48 ± 1.27	11.55 ± 0.20