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Bioactive peptides from Atlantic salmon (*Salmo salar*) with angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory, and antioxidant activities

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ABSTRACT

The pH shift method was utilised for the recovery of proteins from salmon trimmings (ST), yielding 93% (w/w) protein. ST protein (STP) hydrolysates were generated with different enzyme preparations. STP incubated with Corolase PP for 1 h (STP-C1) had the most potent angiotensin converting enzyme (ACE) and dipeptidyl peptidase IV (DPP-IV) inhibitory and oxygen radical absorbance capacity (ORAC) activities. Analysis of fractions of STP-C1 using UPLC-MS/MS identified sixteen peptides/amino acids. Tyr-Pro had the highest ACE inhibitory activity (ACE IC_{50} = 5.21 ± 0.94 μM). The highest DPP-IV inhibitory activity was found with the amino acid Tyr (DPP-IV IC_{50} = 75.15 ± 0.84 μM). Val-Pro had the highest ORAC activity (19.45 ± 2.15 μmol of TE g^{-1}). To our knowledge, the peptides Gly-Pro-Ala-Val, Val-Cys, and Phe-Phe have not been previously identified to have the activities tested in this study. These results indicate that STP hydrolysates are potential sources of bioactive peptides.

1. Introduction

In recent years the incidences of obesity and associated diseases, such as hyperglycaemia, type-II diabetes and cardiovascular diseases (CVD) such as hypertension, have increased dramatically. In 2012 an estimated 17.5 million people died from CVDs (WHO, 2013) while 1.5 million died from diabetes mellitus (WHO, 2014). These two conditions are interlinked with 75% of people suffering from type-II diabetes also being affected by hypertension (Schutta, 2007). Different antioxidants and enzyme inhibitors are employed as part of the control strategy for the management of diabetes, hypertension and oxidative stress. Peptides from marine sources, such as fish, shellfish and algae, have been reported to have biological activities such as antioxidant along with angiotensin converting enzyme (ACE) and dipeptidyl peptidase (DPP-IV) inhibitory properties. This may result in their potential use as functional food ingredients for the management of hypertension and type-II diabetes (Harnedy & FitzGerald, 2013a).

Large quantities of marine processing co-products, such as viscera, heads, trimmings and fish frames, are generated annually (Kim & Mendis, 2006). For example, in 2007, an estimated 2778 t of co-products were generated from farmed fish processing in Ireland (Harnedy & FitzGerald, 2013a). These co-products corresponded to approximately 25% of total production. Currently, the co-products are discarded or used as fertilizer, animal feed or for fish oil extraction (Arvanitoyannis & Kassaveti, 2008). Due to negative ecological impact of returning discard to the sea and new EU regulations on waste prevention and discards, the quantity of fish processing co-products needs to be reduced. Conversion of marine co-products into functional components would not only add value to the marine processing sector, but may also provide an answer to the environment restrictions and disposal costs associated with marine processing co-products (Harnedy & FitzGerald, 2012).

According to the Food and Agriculture Organization of the United Nations (FAO, 2012), salmon (*Salmo salar*) makes up a large portion of all fish processed globally, not only farmed but also wild catch. Co-products from salmon processing include trimmings (ST, containing bone, skin and muscle), heads (containing the gills), frames and viscera (liver, kidneys and roe). These co-products can contain significant quantities of high quality protein (10–23% (w/w)) that may act as candidate substrates for the generation of bioactive peptides (Harnedy & FitzGerald, 2012). On average, the total protein content of salmon flesh is 21.5% wet weight (w.w, Harnedy & FitzGerald, 2011). Peptides from different marine sources have been shown to have bioactivities

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such as antioxidant, anti-fungal, Ca-binding, ACE inhibitory, appetite suppressant, anti-hypertensive and HIV-1 protease inhibitory properties (Harnedy & FitzGerald, 2011, 2012). The most commonly used methods of extraction of proteins from fish meat are the pH shift and the surimi methods. The pH shift process consists of homogenization of the sample in an acidic or alkaline solution followed by recovery of the proteins by centrifugation and precipitation of the supernatant. This method can vary in several parameters, such as weight:volume ratio during extraction, number of sequential extractions employed, homogenization method, pH of extraction and stirring time (Raghavan & Kristinsson, 2009, Thorkelsson et al., 2008, Vareltzis & Undeland, 2008). The surimi process for protein recovery leads to lower yields of protein than the pH shift method as the sarcoplasmic proteins are lost during this process. This process consists of three washes with cold water with 0.2% (w/v) NaCl included in the last wash, followed by refining and dewatering the slurry (Kristinsson, Theodore, Demir, & Ingadottir, 2005). The higher yield of proteins recovered using the pH shift method makes it more advantageous than the surimi process for the extraction of proteins from fish sources.

Some bioactivities, such as ACE and DPP-IV inhibitory and antioxidant activities, have been linked with peptides generated from Atlantic salmon proteins through enzymatic hydrolysis (Gu, Li, Liu, Yi, & Cai, 2011). Although peptides, such as Ala-Pro and Val-Arg, have been identified to date from ACE inhibitory fractions of Atlantic salmon protein hydrolysates (Gu et al., 2011) many other bioactive peptides have yet to be identified.

The objectives of this study were (a) optimization of the method for extraction of proteins from ST, (b) generation of bioactive peptides through enzymatic hydrolysis of the extracted proteins using four different proteolytic preparations, (c) determination of the *in vitro* bioactivity of the hydrolysates, (d) fractionation of the STP hydrolysate with the highest bioactivities and (d) identification and characterization of the peptides in the separated fractions with ACE and DPP-IV inhibitory activity as well as antioxidant properties.

2. Materials and methods

2.1. Materials

ProtoGel®, ProtoGel® Resolving Buffer (4×), ProtoGel® Stacking Buffer (4×), Tris–Glycine-SDS PAGE Buffer (10×) and Protein Loading Buffer Blue (2×) were obtained from National Diagnostics (Yorkshire, UK). Quick Start Bradford 1x Dye Reagent® was supplied by Bio-Rad (Dublin, Ireland). Bovine lung was kindly provided by Gaelic Meat and Livestock Ltd. (Limerick, Ireland), Kjeldahl catalyst tablets were obtained from Sigma-Aldrich (Wicklow, Ireland). H-Gly-Pro-AMC and Diprotin A were obtained from Bachem (Bubendorf, Switzerland). HPLC-grade acetonitrile (ACN) and water were obtained from VWR (Dublin, Ireland). Corolase® PP was provided by AB Enzymes (Darmstadt, Germany), Alcalase® 2.4 L and Flavourzyme® 500 L were obtained from Novozymes A/S (Bagsvaerd, Denmark), Promod 144MG was kindly provided by Biocatalysts Ltd (Parc Nantgarw, Wales, UK). All other reagents were obtained from Sigma-Aldrich (Wicklow, Ireland).

2.2. Sample preparation

Representative samples of ST were obtained from the Irish Seafood Producers Group (ISPG, Kilkieran, Connemara, Co. Galway). The trimmings were minced using a 1.5 cm diameter perforated disk (Breville Meat Mincer, Breville, Oldham, UK) and stored at −20 °C.

2.3. Protein extraction

The method used for extraction of the protein from minced ST is outlined in Supplementary Fig. 1. The effects of weight to volume ratio, number of sequential extractions, homogenization, extraction pH and stirring time on the yield of protein recovered were assessed.

Minced ST was suspended at 1:5 (w/v) in distilled water and then homogenised at 16,000 rpm for 30 s (Ultraturrax® T25 Basic, IKA®, Staufen, Germany). The pH was adjusted to pH 2.5, 3.0, 3.5 or 4.0 using 1 M HCl, and to pH 10.0, 10.5, 11.0 or 12.0 using 2 N NaOH. These solutions were then stirred gently at room temperature for 5, 10, 15, 30 or 45 min. The soluble protein extract was obtained following centrifugation at 4000×*g* for 15 min at room temperature (Hettich Universal 320R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The extract (supernatant) was than freeze-dried (Labconco Benchtop Freeze Dry System, Kansas City, USA) and stored at −20 °C until use.

2.4. Generation of protein hydrolysates and monitoring the extent of hydrolysis

STP hydrolysates were generated according to the protocol described by CunhaNeves, Harnedy, and FitzGerald (2016), with the enzyme preparations Alcalase 2.4 L, Flavourzyme 500 L, Corolase PP and Promod 144MG. The extent of hydrolysis was determined using the 2, 4, 6 – trinitrobenzene sulfonic acid (TNBS) method following the procedure of Adler-Nissen (1986) as modified by Spellman, McEvoy, O'cuinn, and FitzGerald (2003) with an Npb value of 7.501 (Kristinsson & Rasco, 2000).

2.5. Protein determination

The protein content of each extract was determined using the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA).

2.6. Kjeldahl nitrogen quantification

The protein content of the starting ST and extracted protein sample was quantified using a CunhaNeves et al. (2016) modification of the Kjeldahl procedure (IDF 1993) as performed by using the salmon protein Nbp of 7.501 (Kristinsson & Rasco, 2000).

2.7. Physicochemical characterization of the STP extracts and hydrolysates

SDS-PAGE was performed using a Mini-Protean® Tetra Cell system (Bio-Rad, Hemel Hempstead, UK) according to the method of Laemmli (1970). Reverse phase-high performance liquid chromatography (RP-HPLC) was carried out according to the method of Flanagan and FitzGerald (2002) and gel permeation HPLC (GPC) was carried out as previously described (Spellman, O'Cuinn, & FitzGerald, 2005).

2.8. Simulated gastrointestinal digestion (SGID) of STP hydrolysates

SGID of the STP hydrolysates was carried out using the method of Walsh et al. (2004). Briefly, the freeze-dried STP hydrolysate was resuspended in distilled water at 2.0% (w/v) on a protein basis at 37 °C for 30 min. The suspension was first incubated with pepsin at 37 °C, pH 2 at an E:S of 2.5% (w/w) for 90 min and then inactivated by heating to 90 °C for 20 min. Then the suspension was incubated with Corolase® PP at pH 7.5 at an E:S of 1% (w/w) for 150 min followed by inactivation by heating to 90 °C for 20 min. The solution was freeze-dried and stored at −20 °C.