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Peptide identification in a salmon gelatin hydrolysate with antihypertensive, dipeptidyl peptidase IV inhibitory and antioxidant activities

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ABSTRACT

Salmon gelatin (*Salmo salar*, SG) enzymatic hydrolysates were generated using Alcalase 2.4 L, Alcalase 2.4 L in combination with Flavourzyme 500 L, Corolase PP, Promod 144MG and Brewer's Clarex. The hydrolysate generated with Corolase PP for 1 h (SG-C1) had the highest angiotensin converting enzyme (ACE, IC_{50} = 0.13 ± 0.05 mg mL^{-1}) and dipeptidyl peptidase IV (DPP-IV, IC_{50} = 0.08 ± 0.01 mg mL^{-1}) inhibitory activities, and oxygen radical absorbance capacity (ORAC, 540.94 ± 9.57 μmol TE g^{-1} d.w.). The *in vitro* bioactivities of SG-C1 were retained following simulated gastrointestinal digestion. Administration of SG and SG-C1 (50 mg kg^{-1} body weight) to spontaneously hypertensive rats (SHR) lowered heart rate along with systolic, diastolic and mean arterial blood pressure. The SG-C1 hydrolysate was fractionated using semi-preparative RP-HPLC and the fraction with highest overall *in vitro* bioactivity (fraction 25) was analysed by UPLC-MS/MS. Four peptide sequences (Gly-Gly-Pro-Ala-Gly-Pro-Ala-Val, Gly-Pro-Val-Ala, Pro-Pro and Gly-Phe) and two free amino acids (Arg and Tyr) were identified in this fraction. These peptides and free amino acids had potent ACE and DPP-IV inhibitory, and ORAC activities. The results show that SG hydrolysates have potential as multifunctional food ingredients particularly for the management of hypertension.

1. Introduction

Gelatin is a coiled, partially hydrolysed form of collagen which has significant value in the food and pharmaceutical industries due to its versatility as an enhancer of a range of techno- functional properties. Moreover, gelatin is capable of forming highly stable three-dimensional gels (Gudipati, 2013). Gelatin has traditionally been extracted from bovine and porcine sources, specifically from skin and bone co-products of the meat processing industry. The global gelatin market is expected to grow at an annual rate of 8.25% between 2015 and 2022 (MRC, 2016). Currently, there is an increased interest in the utilisation of marine origin gelatin for religious (for halal and kosher food consumers) and disease risk reduction (specifically bovine spongiform encephalopathy) reasons (Gómez-Guillén et al., 2009; Karim & Bhat, 2008).

Food-grade gelatin is usually extracted using the so-called 'pH shift' process at temperatures in the region of 60 °C (Jaswir, Mirghani, Mohd Salleh, Hassan, & Yaakob, 2009; Montero & Gómez-Guillén, 2000; Zhou & Regenstein, 2005). Being rich in amino acids such as glycine (Gly), proline (Pro) and hydroxyproline (Hyp), gelatin has significant potential as a starting substrate for the generation of bioactive peptides (BAPs) for incorporation into functional foods (Grant, Weilbaecher, & Lichlyter, 2007; Ketnawa, Rungraeng, & Rawdkuen, 2017; Schrieber & Gareis, 2007). Fish gelatin hydrolysates/peptides have been shown to display a range of biological activities. These include antioxidant, anti-anaemia, immunoregulatory, Ca-binding, mineral chelating, ACE and DPP-IV inhibitory, antimicrobial and anti-hypertensive activities. Hydrolysates/peptides having these *in vitro* bioactivities have been reported for gelatin extracted from Atlantic salmon, cod, herring, hoki, Pacific whiting, pollack, snapper and sole (Harnedy & FitGerald, 2013a).

Bioactive peptides with multifunctional activities are of interest in the management of different diseases. Cardiovascular disease (CVD) and type 2 diabetes are major causes of death in developed countries, globally accounting for 17.5 million and 1.5 million deaths, respectively, in 2012 (World Health Organization, 2016). These diseases are often associated with each other. Furthermore, these diseases are linked with enhanced oxidative stress which can result in cell and tissue damage (Harnedy & FitGerald, 2013a). Studies with SHR have reported that gelatin hydrolysates from sea cucumber (Zhao et al., 2007), skate skin (Ngo et al., 2015), jellyfish (Zhuang, Sun, & Li, 2012) and squid

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skin (Lin, Lv, & Li, 2012) have the ability to reduce blood pressure, a controllable risk factor in the development of CVD. However, no studies with SHRs appear to have been performed with salmon gelatin hydrolysates.

Therefore, the objectives of this study were (a) to generate enzymatic hydrolysates from gelatin extracted from salmon (*Salmo salar*) processing co-products (consisting of skin, bone and residual meat), (b) to characterise the physicochemical and *in vitro* bioactive properties of the resultant hydrolysates, (c) to assess the hypotensive effects of the most potent hydrolysate and (d) to fractionate and identify the peptides within the most bioactive hydrolysate.

2. Materials and methods

2.1. Materials

Bovine lung was kindly provided by Gaelic Meats and Livestock Ltd. (Limerick, Ireland), Abz-Gly-p-nitro-Phe-Pro-OH, Abz-Gly-OH-HCl, H-Gly-Pro-7-amino-4-methyl coumarin (AMC), and Diprotin A (Ile-Pro-Ile) were obtained from Bachem (Bubendorf, Switzerland). HPLC-grade acetonitrile (ACN) and water were obtained from VWR (Dublin, Ireland). The synthetic peptides Gly-Gly-Pro-Ala-Gly-Pro-Ala-Val, Gly-Pro-Val-Ala, Pro-Pro and Gly-Phe and two free amino acids Arg and Tyr were obtained from Thermo Fisher Scientific (Ulm, Germany). Trinitrobenzenesulphonic acid (TNBS) reagent was from Medical Supply Co. Ltd. (Dublin, Ireland). Brewer's Clarex™ (*An*-PEP specific activity: 37×10^{-3} U/mg) was supplied as a gift from Dutch State Mines (DSM, Heerlen, Netherlands), Corolase PP was provided by AB Enzymes (Darmstadt, Germany), Alcalase 2.4 L and Flavourzyme 500 L were obtained from Sigma Aldrich Ltd. (Gillingham, UK) and Promod 144MG was kindly provided by Biocatalysts Ltd. (Parc Nantgarw, Wales, UK). Captopril™ and all other reagents were obtained from Sigma Aldrich Ltd. (Gillingham, UK).

2.2. Sample preparation

Representative samples of salmon trimmings (consisting of skin, bone and residual meat) were obtained from the Irish Seafood Producers Group (ISPG, Kilkieran, Connemara, Co. Galway) and the proteins therein were extracted as per Neves, Harnedy, O'Keeffe, and FitzGerald (2017) involving mincing the salmon trimmings, extracting the alkaline and acid soluble proteins and washing the pellets with distilled water. The pellet obtained following protein extraction from the salmon trimmings was used for gelatin extraction. The pellet obtained from 15 g of trimmings was suspended in 20 mL distilled water and the gelatin therein was extracted during heating at 70 °C over 16 h. The suspension was then centrifuged at 4000 × *g* for 15 min (Hettich Universal 320R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The supernatant containing gelatin was then freeze-dried (FreeZone freeze dryer system, Labconco, Missouri, USA) and stored at − 20 °C until required for analysis.

2.3. Generation of gelatin hydrolysates

Hydrolysates were generated according to the protocol outlined by Neves et al. (2017). Briefly, a 5% (*w/v*) gelatin suspension (600 mL) at 96% protein (*w*/w) was divided into six 100 mL aliquots. Four aliquots were equilibrated at 50 °C, adjusted to pH 7 and the food-grade enzyme preparations Alcalase 2.4 L, Alcalase 2.4 L in combination with Flavourzyme, Corolase PP or Promod 144MG were added at an enzyme:substrate (E:S) ratio of 1% (*w*/w or *v/w* depending on the enzyme format). One aliquot was equilibrated at 50 °C, adjusted to pH 4 with 1.0 M HCl and was supplemented with Brewers Clarex at an E:S of 1% (*v/w*). The remaining aliquot was used as a no enzyme control sample. During hydrolysis at 50 °C the suspensions were gently stirred and maintained at pH 7 or pH 4 using a pH stat (718 stat Titrino, Metrohm, Herisau, Switzerland) adjusted with 0.1 M NaOH or 0.5 M HCl. Samples were taken at 0, 1, 2 and 4 h during hydrolysis. All samples were inactivated by heating at 90 °C for 20 min, cooled to room temperature and then freeze-dried (FreeZone freeze dryer system, Labconco, Missouri, USA).

2.4. Characterisation of the hydrolysates

The degree of hydrolysis (DH) of the SG hydrolysates was determined using the 2, 4, 6 - trinitrobenzene sulfonic acid (TNBS) method following the procedure of Adler-Nissen (1979) as modified by Spellman, McEvoy, O'cuinn and FitzGerald (2003). Hydrolysate samples (0.125 mL) diluted in 1% (*w/v*) SDS were mixed with a 0.1% (*w/v*) TNBS solution (1 mL) and 0.2125 M sodium phosphate buffer at pH 8.21 (1 mL). The samples were incubated at 50 °C for 1 h in the dark before 2 mL of 0.1 N HCl was added to terminate the reaction. The samples were allowed to stand in the dark for 30 min and the absorbance was measured at 340 nm (Shimadzu UV mini 1240, Kyoto, Japan). All samples were analysed in triplicate ($n = 3$) and the amino nitrogen content of the hydrolysates (mg g^{-1} protein) was estimated by reference to an L-leucine standard curve in the range of 0 to 28 mg amino N L^{-1}.

The intact gelatin and its associated enzymatic hydrolysates were assessed using reverse-phase high performance liquid chromatography (RP-HPLC) and gel permeation high performance liquid chromatography (GP-HPLC) as described by Neves et al. (2017).

2.5. Simulated gastrointestinal digestion (SGID) of the hydrolysate

The SG hydrolysate generated with Corolase PP for 1 h (SG-C1) was subjected to SGID using the method of Walsh et al. (2004). Briefly, the freeze-dried hydrolysate was suspended in distilled water at 2% (*w/v*) protein equivalent and was incubated at 37 °C for 30 min. This suspension was initially incubated with pepsin at 37 °C, pH 2.0 at an E:S of 1% (*w*/w) for 90 min and then with Corolase PP at pH 7.5 at an E:S of 2.5% (*w*/w) for 150 min. The enzymes were then inactivated by heating at 90 °C for 20 min. The resulting solution was freeze-dried (FreeZone freeze dryer system, Labconco, Missouri, USA) and stored at − 20 °C.

2.6. Semi-preparative RP-HPLC

The SG-C1 was fractionated using semi-preparative RP-HPLC according to the protocol described by Nongonierma and FitzGerald (2013) with some modifications. The hydrolysate was resuspended at 10% (*w/v*) in HPLC grade water. Sample (0.5 mL) was injected onto a C18 semi-preparative column (250 × 15 mm I.D., 10 mm particle size, Phenomenex, Cheshire, UK) attached to a C18 guard column (Phenomenex, Cheshire, UK) coupled to an RP-HPLC system (Waters, Dublin, Ireland). Mobile phase A was HPLC grade water and mobile phase B was 80% (*v*/v) acetonitrile. Peptide separation was carried out at a flow rate of 5 mL min^{-1} using the following linear gradient: 0–10 min: 0% B; 10–20 min: 0–20% B; 20–30 min: 20% B; 30–40 min: 20–80% B; 40–50 min: 80% B; 50–60 min: 80–100% B. The absorbance of the eluent was monitored at 214 nm. Fractions (5 mL) were collected from min 6 to min 60 during the course of 14 separate runs. All fractions were evaporated to dryness using a solvent evaporator (GenVac, EZ2-plus, Genevac Ltd., Ipswich, UK).

2.7. Mass spectrometry analysis of fraction 25 of the SG-C1 hydrolysate

Fraction 25 from the semi-preparative RP-HPLC separation of the SG-C1 hydrolysate was analysed using UPLC-ESI-MS/MS. The peptide sequences therein were determined as described by Neves et al. (2017). In short, samples (2 μL at 0.1 mg mL^{-1} in mobile phase A (0.1% formic acid in H_2O)) were separated on an Aeris™ 1.7 μm PEPTIDE XB-C18 column (150 × 2.1 mm, Phenomenex, Cheshire, UK) using an ultra-performance