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Three differently generated salmon protein hydrolysates reveal opposite effects on hepatic lipid metabolism in mice fed a high-fat diet

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ABSTRACT

This study investigates the effects of salmon peptide fractions, generated using different enzymatic hydrolyzation methods, on hepatic lipid metabolism. Four groups of mice were fed a high-fat diet with 20% casein (control group) or 15% casein and 5% of peptide fractions (treatment groups E1, E2 and E4) for 6 weeks. Weight gain was reduced in mice fed E1 and E4-diets compared to control, despite a similar feed intake. Reduced plasma and liver triacylglycerol levels in E1 and E4-mice were linked to reduced fatty acid synthase (FAS) activity and hepatic expression of lipogenic genes. By contrast, plasma and liver lipids increased in the E2 group, concomitant with increased hepatic FAS activity and Δ9 desaturase gene expression. Shotgun lipidomics showed that MUFAs were significantly reduced in the E1 and E4 groups, whereas PUFAs were increased, and the opposite was observed in the E2 group. In conclusion, bioactive peptides with distinctive properties could potentially be isolated from salmon hydrolysates.

1. Introduction

The role of dietary protein is to provide the body with essential amino acids for protein synthesis and energy. Beyond this nutritional role, ingested proteins have a wide range of biological functions affecting protein, glucose and lipid metabolism, transportation, immune function, blood pressure and hormonal functions (Chou, Affolter, & Kussmann, 2012). It has been increasingly clear that the dietary source of protein can affect cellular energy metabolism, and that hydrolyzed peptides can have potent and specific bioactive potential (Erdmann, Cheung, & Schroder, 2008). Health benefits from fish consumption have been attributed to the *n*-3 polyunsaturated fatty acids, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, recent studies have drawn attention towards proteins from marine sources, which are considered valuable bioactive components as their amino acid composition and protein profile differ from terrestrial sources (Kim, Ngo, & Vo, 2012; Larsen, Eilertsen, & Elvevoll, 2011). According to Kelleher, 7 million tons of fish byproducts were discarded as processing waste in 2005 (Kelleher, 2005), which constituted 50% of the total catch being used for human consumption (Rustad, 2003). Fish byproducts can be hydrolyzed enzymatically, using various techniques, liberating potentially bioactive peptides incorporated in the parental molecule, according to molecule size, stability in water, refined protein and protein mix. Rodent studies on fish protein hydrolysates have shown that marine proteins exhibit cholesterol-lowering (Shukla et al., 2006), antihypertensive (Je, Park, Kwon, & Kim, 2004; Kim & Mendis, 2006), immunomodulating and antioxidant effects (Ahn, Cho, & Je, 2015), in addition to reparative properties in the intestine (Fitzgerald et al., 2005), and increased insulin sensitivity (Pilon et al., 2011). How fish protein hydrolysate may affect lipid metabolism in liver is less clear. The liver is the major site of lipid metabolism and both fatty acid oxidation and liponeogenesis are carried out here. These processes are dependent on rate-limiting enzymes, e.g., carnitine palmitoyltransferase (CPT)-1 and 2, acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS). Several desaturases are involved in *de novo* fatty acid synthesis. The Δ9 desaturase, encoded by the gene *Scd1*, generates monounsaturated fatty acids. Along with elongases, the Δ5 and Δ6 fatty acid desaturases, encoded by the genes *Fads1* and *2*, respectively, are important in the biosynthesis of essential polyunsaturated fatty acids (PUFAs). Regulation of these enzymes will influence fatty acid composition

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and thus important cellular functions, including cell signaling (Nakamura & Nara, 2004).

In this study, we investigated the effect of three different salmon peptide fractions (designated E1, E2, and E4), generated using different enzymatic hydrolyzation and microfiltration methods, on hepatic lipid metabolism in male C57BL/6J mice. We show that different peptide compositions, generated from the same species of fish, varied in their beneficial effects on body weight and lipid metabolism.

2. Materials and methods

2.1. Animals and diets

The animal experiments were carried out with ethical permission obtained from the Norway State Board for Biological Experiments and followed the Norwegian Research Councils ethical guidelines. Nine to ten weeks old male C57BL/6J mice were housed, 3 per cage, at constant temperature (22 ± 2 °C) and humidity (55 ± 5%), and exposed to a 12 h light–dark cycle with unrestricted access to food and tap water. After 1 week of acclimatization to these conditions, they were divided into 4 groups and fed either a high-fat (HF) diet containing 24% (w/w) fat (21.3% lard and 2.7% soy oil) and 20% casein (control group, $n = 9$), or the HF diet supplemented with protein hydrolysate E1, E2 or E4 from salmon byproducts (a generous gift from Marine Bioproducts, Storebø, Norway) (15% casein and 5% peptide, group E1, E2 and E4, $n = 6$) *ad libitum* for 6 weeks. The different salmon peptide fractions were produced as follows: for fractions E1 and E2, fish material (spine) was treated enzymatically with alkaline protease and a neutral protease and the resulting protein hydrolysate was subjected to a second enzymatic treatment. Fraction E1 was treated with an Acid Protease A, while fraction E2 was treated with the proteolytic enzyme Umamizyme from *Aspergillus oryzae*. The final hydrolysate was then filtered, using micro- and ultra-filtration, and the size distribution of the peptides analyzed. For both fractions, more than 50% of the final preparation consisted of peptides in the range 200–1200 Da and approximately 25% of the preparation consisted of peptides below 200 Da. Salmon backbones, including heads, were hydrolyzed with proteolytic Alcalase 2.4 L (Novozymes, Denmark) and subjected to micro- and ultra-filtration and constituted peptide fraction E4. Nearly 60% of the final preparation consisted of peptides in the range below 1200 Da.

Amino acid composition of the control and peptide diets is given in Supplementary Table 1. Diets were packed airtight and stored at −20 °C until used to prevent lipid oxidation. Mice were housed in groups of three per cage at a constant temperature of 22 ± 2 °C and a dark/light cycle of 12/12 h. Body weights of the mice were measured approximately every seventh day and food intake was measured 3 times during the study. At sacrifice, animals were fasted overnight, anesthetized with 2% isoflurane (Schering-Plough, Kent, UK) and blood was collected by heart puncture. The blood was centrifuged, EDTA–plasma separated and frozen prior to further analysis. Livers were collected and immediately frozen in liquid nitrogen and stored at −80 °C prior to further analysis.

2.2. Lipid and fatty acid analysis

Liver lipids were extracted according to Bligh and Dyer (1959), solvents were evaporated under nitrogen and samples re-dissolved in isopropanol before analysis. Lipids from liver extracts or plasma were then measured enzymatically on a Hitachi 917 system (Roche Diagnostics, Mannheim, Germany), using kits for analyzing total TAG (GPO–PAP kit, Roche Diagnostics), cholesterol (CHOD–PAP kit, Roche Diagnostics), and total phospholipids (Diagnostic Systems GmbH, Holzheim, Germany). Fatty acid composition was analyzed in extracted liver lipid, using gas chromatography, as described previously by Grimstad et al. (2012).

2.3. Lipidomic analysis

Liver samples were stored at −80 °C prior to analysis and lipidomics analysis was performed on 50–100 mg of liver tissue from each mouse from two groups, E1 and E2, in addition to the control group. The tissue samples were pulverized with a CP02 CryoPrep Dry Pulverization System (Covaris), and resuspended in ice-cold methanol containing 0.1% butyl-hydroxytoluene (BHT) at a concentration of 100 mg/mL.

For lipidomics analysis, lipids were extracted from liver homogenates, using a modified Folch lipid extraction procedure (Ekroos, Chernushevich, Simons, & Shevchenko, 2002). Samples were spiked with known amounts of deuterium-labeled or heptadecanoyl-based synthetic internal standards, serving for quantification of the endogenous lipid species, as previously described (Bergan et al., 2013). The samples were stored at −80 °C prior to mass spectrometry analysis.

Molecular glycerophospholipids, glycerolipids, cholesteryl esters sphingomyelins and triacylglycerols (TAGs) were analyzed by shotgun analysis on a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, ABSCIEX) equipped with a robotic nanoflow ion source (NanoMate HD, Advion Biosciences) (Stahlman et al., 2009). In shotgun lipidomics a 5 μL volume was infused at a concentration of 0.5 μg liver/μL. For TAG analysis, the concentration was diluted to 0.05 μg/μL. The analyses were performed in both positive and negative ion modes, using multiple precursor ion scanning (MPIS) and neutral loss (NL)-based methods (Ekroos et al., 2002, 2003). Sphingolipids were analyzed by reverse phase ultra-high pressure liquid chromatography (Rheos Allegro UHPLC, Flux Instruments AG), using an Acquity BEH C18, 2.1 × 50 mm column with a particle size of 1.7 μm (Waters, Milford, Massachusetts, USA) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, ABSCIEX). A 25 min gradient, using 10 mM ammonium acetate in water with 0.1% formic acid (mobile phase A) and 10 mM ammonium acetate in acetonitrile:2-propanol (4:3, v/v), containing 0.1% formic acid (mobile phase B), was used. The column temperature was set to 60 °C and the flow rate to 500 μL/min. 10 μL samples were injected. Sphingolipids were monitored, using multiple reactions monitoring (MRM) as described by Merrill, Sullards, Allegood, Kelly, and Wang (2005).

The MS data files were processed, using Lipid Profiler™ and MultiQuant™ software for producing a list of lipid names and peak areas. The individual lipids measured can be found in Supplementary Table 2. Masses and counts of detected peaks were converted into a list of corresponding lipid names. Lipids were normalized to their respective internal standard (Bergan et al., 2013) and tissue weight to retrieve their concentrations. Data were analyzed, using the software Tableau Desktop 7.0 and the percentage differences between the groups (E1 vs. controls and E2 vs. controls) were estimated in pairwise comparisons, using a Hodges–Lehmann estimator, and the significances were calculated using Wilcoxon rank-sum t-test.

2.4. Hepatic enzyme activities

Liver samples were homogenized and a post-nuclear fraction was prepared as previously described (Berge, Flatmark, & Osmundsen, 1984). The activity of CPT-1 was measured in the presence and absence of malonyl-CoA, as previously described (Vik et al., 2014). The assay conditions for CPT-2 were identical to CPT-1, apart from some changes in the reaction mix; BSA and KCN were