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ORIGINAL ARTICLE

Optimization of protein recovery from Atlantic salmon (*Salmo salar*) head and backbone by response surface methodology and characterization of functional properties and nutritional value

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Abstract

Atlantic salmon (*Salmo salar*) head and backbone by-products were hydrolysed using non-commercial protease enzyme (ERM 1) to produce protein. Response surface methodology was used to optimise conditions, including hydrolysis time, hydromodule and enzyme–substrate (E:S) ratio for maximum protein recovery. Highest protein recovery was obtained after 4 h hydrolysis, 1 L/kg hydromodule, and 0.39% of E:S ratio for the salmon head. Similarly, 3.75 h of hydrolysis time, 2.67 L/kg of hydromodule and 0.499% of E:S were found optimal for the salmon backbone. Total amino acid (TAA) composition revealed the presence of all essential amino acids in both hydrolysates. The sum of 16 TAAs was approximately both in salmon head and backbone samples 70 g/100 g, while FAAs were much higher in salmon head (13.4 g/100 g) then in the salmon backbone (8.8 g/100 g). The hydrolysates prevented the growth of *E. coli* K-12, but no significant effect on *Listeria innocua* (ATTC 33090) growth was seen. Fish hydrolysates showed nitrogen solubility indices above 90% at pH 5–8, with one exception of the salmon head hydrolysate at pH 5 with a value of 67.8%. Samples formed gels at 5 and 10% protein concentration. Gels were weak compared to gelatine gels.

Keywords Salmon head · Backbone · Protein hydrolysate · Solubility · Nutritional value · Antimicrobial properties

Introduction

Aquaculture is probably the fastest growing food production sector today. Consumers' awareness of the health effects of seafood in parallel with a decline in the supply of wild fish have created great interest in aquaculture and using fish processing by-products in food production. In 2022, global fisheries and aquaculture production surged to 223.2 million tonnes, a 4.4 percent increase from the year 2020. Production compromised 155.4 million tonnes of fish and 37.8 million tonnes of algae (FAO report 2024). The Atlantic salmon is an important food fish (*Salmo salar*) due to its high nutritional value and suitability for aquaculture. In the EU, the annual Atlantic salmon market was 957 million kg in 2018 (Mowi 2019). The fish processing industry produces large numbers of by-products, which usually constitute more than 50% of the original fish weight. Globally, these by-products are mainly used in animal feed production or are even wasted (Aspevik et al. 2016). Aquaculture by-products include backbones, belly fish fins, gills, heads, liver, roe, skin, viscera, and meat adhering to the bones. The ratio between the fish parts used directly for food and the sidestream varies by fish size and species, season, and zone. Salmon heads and backbones are an especially remarkable source of high-quality proteins and oils. Heads contain approx. 15% protein and 11% oil, while backbones contain approx. 30% protein.

Several studies have indicated the potential of enzymatic hydrolysis as a replacement for acid or alkali processing to produce fish protein hydrolysates with lower bitterness, better nutritional and functional properties, lower salt content, and higher homogeneity (Kristinsson and Rasco 2000a, b, c; Sanmartin et al. 2009). Enzymatic assisted processing has

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been used to produce protein hydrolysates with good techno-functional properties (Shadidi et al. 1995) and bioactive peptides (Cheng et al. 2015) and is also probably reproducible. He et al. (2012) observed the highest protein yields in Atlantic salmon processing co-products by Alcalase. A problem with hydrolysis is the development of a bitter taste due to the exposure of hydrophobic amino acid sidechains normally located in the interior of an intact protein. Kojizyme and Flavorzyme have been shown to reduce the bitterness of hydrolysates (Liaset et al. 2000; Zhang et al. 2019). However, the higher production costs and long production times of enzymatic treatments may reduce the success of industrial scale application. He et al. (2013) pointed to the need to focus the development of fish protein hydrolysates on yield and functionality instead of only optimising for a high yield. Fish protein hydrolysates have also shown antimicrobial effects (Cheng et al. 2015), which is interesting for food quality aspects.

The aim of this study was to produce functional protein hydrolysates from salmon head and backbones using a non-commercial protease enzyme (MeatCo, the Netherlands), and to gather multidisciplinary information on the hydrolysates. To this end, the operational parameters were optimised by response surface methodology to ensure process efficiency. The molecular weight distribution of protein fractions in head and backbone hydrolysates were analysed, as well as the amino acid and nucleotide profile. The hydrolysates were analysed for their techno-functional properties. Additionally, the antimicrobial effect of the hydrolysates and their purified solid phase extracts were tested against *Escherichia coli* and *Listeria innocua* strains.

Materials and methods

Salmon filleting by-products

Atlantic salmon head and backbone were provided by Hätälä Ltd, in Oulu in Finland. The by-products were transported under ice and stored below − 20 °C to ensure good microbiological quality. The backbones were thawed at 4 °C overnight and cut into three approximately equal pieces. The salmon heads were cut with a slicing machine (Metos, Italy) into 1 cm thick pieces after a similar thawing process to that described for the backbone.

Enzymatic hydrolyses

Salmon heads and backbones were hydrolysed using a non-commercial protease (ERM 1, MeatCo, the Netherlands). ERM 1 contains exclusively endo-proteinase with an activity of 840 UHb g^{-1} and is obtained from *Bacillus subtilis* cultures. The hydrolyses was carried out at 55 °C under continuous stirring for 60, 150, or 240 min after the addition of enzyme. We used different solid–liquid ratios (hydromodule 1–3 L/kg) and E:S ratios (0.05–0.5%). The enzyme was inactivated by heating to 85 °C for 15 min. The mixture was left to rest for 30 min to separate the solids consisting of the non-soluble protein and fishbone, and the liquid phase containing the hydrolysed protein and lipids. The oil fraction was separated by a separator (Frau CN 3/A, Italy) to obtain a high-quality protein fraction and prevent quick deterioration due to the oxidation of the oil. The protein hydrolysate was dehydrated by freeze-drying until a dry-matter content of > 96% was obtained.

Response surface methodology (RSM) for optimisation of processing

The Modde 12.1. (Umetrics, Sweden) was used to design the experiments based on three factors: hydrolysis time, hydromodule, and E:S ratio. The design included a total of seventeen test runs, with five replicates at the centre point to validate the model. The design is summarised in Table 1. The experiments were done in random order to avoid systematic error. The calculated protein recovery was used as a response function. The protein yield was calculated as follows:

$$\text{Protein yield}(\%) = \frac{\text{Protein concentration in the protein hydrolysate}(\%) \times \text{Weight of protein hydrolysate (g)}}{\text{Protein concentration in the raw material} \times \text{Weight of raw material (g)}}$$

The optimal processing parameters were determined using the software's optimiser function to achieve the highest possible protein yield. The samples of backbone and head protein hydrolysates from the experimental design were analysed only for the protein content to calculate the protein yield.

New test runs using the determined optimal conditions were performed to verify the model's validity. The samples were analysed for proximate composition, techno-functional

Table 1 Average proximate composition of the salmon head and backbone freeze dried protein hydrolysates (n = 2)

Sample	Energy kJ/100 g	Carbohydrate g/100 g	Moisture g/100 g	Protein g/100 g	Fat g/100 g	Ash g/100 g
Salmon head hydrolysate	1399 ± 1.41	1.35 ± 0.07	10.42 ± 0.007	81.1 ± 0.00	0.41 ± 0.01	7.75 ± 0.09
Salmon backbone hyrdolysate	1406.5 ± 24.75	2.75 ± 1.48	13.09 ± 1.53	76.7 ± 0.14	1.49 ± 0.04	5.96 ± 0.03

properties, molecular weight distribution, amino acid, and 5'-nucleotide composition and antimicrobial effects.

Proximate analysis

Protein, fat, moisture, ash, and carbohydrate content were determined or calculated using the procedures described in Pap et al. (2022).

The moisture content was determined by drying the samples at 105 °C until a constant final weight was reached. Measurements were made using a thermogravimetric analyser (LECO TGA 701, the Netherlands).

The nitrogen content of the samples was determined with an Kjeldahl method using a Kjeltec TM8400 analyser according to the method of the Association of Official Analytical Chemists (AOAC) 2001.11, SFS EN ISO 20483:2013 and EN ISO5983-2. A correction factor of 6.25 was used in protein content calculations.

The total fat content of the samples was determined using the SoxCap TM 2047 in combination with the Soxtec TM 2050 extraction system with a preparatory acid hydrolysis step and diethyl ether extraction (Foss A/B, Hillerød, Denmark) according to ISO 6492.

The total carbohydrate (TC) content was calculated with the following formula and expressed as g/100 g FW:

$$TC(\%) = 100 - \text{moisture}\ (\%) - \text{protein}\ (\%FW) - \text{crude fat}\ (\%FW) - \text{ash}\ (\%FW)$$

The ash content was analysed by weighing the samples before and after burning at 550 °C until a constant weight was reached. Measurements were performed with a thermogravimetric analyser (LECO TGA 701, the Netherlands).

Protein molecular weight distribution analysis

The molecular size distribution of the salmon backbone and head hydrolysates was analysed with size exclusion chromatography (SEC) using the Agilent 1290 UHPLC system with an ACQUITY UPLC BEH SEC column (125 Å, 1.7 µm, 150 mm) (Waters Co., Massachusetts, USA) (Pap et al. 2022). Prior to the analysis, the hydrolysates were pre-treated with solid phase extraction using Sep-Pak™ cartridges to diminish interfering non-protein compounds. The SEC column was equilibrated with a mobile phase of sodium phosphate buffer (100 mM, pH 6.8). Samples were diluted into double distilled water to a protein concentration of approx. 10 mg/mL and injected (2 µL) onto the column. Isocratic elution was performed with the 100 mM sodium phosphate buffer (pH 6.8) at a flowrate of 0.3 mL/min. Absorbance was monitored at 220 nm. The column was calibrated using BEH125 SEC Protein Standard Mix (Waters Co., Massachusetts, USA) containing thyroglobulin (660 kDa), ovalbumin (44.2 kDa), ribonuclease A (13.7 kDa), and Uracil (112 Da). The molecular weights of the protein and peptide fractions were estimated according to the calibration curve, and the amount of proteins and peptides in the fractions was estimated from the chromatogram by calculating the UV-absorbing area of each fraction.

The protein molecular weight was analysed using SDS-PAGE either with tris-tricine or tris–glycine buffered gels. The samples were solubilised either with Laemmli or tricine loading buffer (Bio-Rad, US) containing 10% β-mercaptoethanol. The proteins were visualised either with Sypro Ruby (Bio-Rad, US) (tris–glycine gel) or Coomassie Brilliant Blue (tris-tricine gel) stains.

Amino acid and 5'-nucleotide composition

Total amino acids, except cystine and methionine, were determined using the ISO 13903:2005; EU 152/2009 (F) method after acid hydrolysis; cystine and methionine were determined using the oxidative method. Tryptophan was determined using the EU 152/2009 LC-FLD method. All measurements were taken in an accredited laboratory (Eurofins, Finland).

An identical hot water extraction procedure was used for both free amino acids (FAAs) and 5'-nucleotides and the corresponding nucleosides. The samples were extracted as previously described by Ranogajec et al. (2010) and Rotola-Pukkila et al. (2015) with slight modifications. Finely ground, freeze-dried samples (0.5 g) were mixed with 20 mL of deionised water in centrifuge tubes, and the samples were heated for 1 min. in boiling water (100 °C). The tubes were kept in an ultrasound bath for 15 min and then centrifuged at 4000 rpm (2525 g) for 15 min (15 °C). The extraction was repeated twice with 15 mL and 10 mL of deionised water, and the supernatants were combined in a measuring flask and filled with water to 50 mL. The supernatants were stored at −40 °C if not analysed immediately.

Nucleoside, 5'-nucleotide, and FAA concentrations were determined by a Shimadzu Nexera X2 UHPLC (Kyoto, Japan) (Rotola-Pukkila et al. 2015). Nucleotides and nucleosides were separated using a Synergi Hydro-RP column (150 × 3 mm, 4 µm; Phenomenex). The peaks were detected by a diode array detector (DAD) at a wavelength of 254 nm. The analyses of FAAs were carried out with a Kinetex C18 Column (100 × 4.6 mm; 2.6 µm; Phenomenex). The fluorescence detector was set at a wavelength excitation of 340 and an emission of 450 nm.

Antimicrobial properties

The salmon backbone and head hydrolysates were tested for antimicrobial effect against *E. coli* K-12 and *Listeria innocua* (ATTC 33090). The hydrolysates were used for the analysis as such and after removal of non-protein compounds with solid phase extraction using Sep-Pak™ cartridges. Lyophilised hydrolysates, as well as samples from the solid phase extraction, were dissolved in water, the pHs were adjusted to 7.0–7.3 by adding NaOH and 0.5 M PBS (pH 7.3) and then filtered through a 0.22 µm filter. Sample aliquots were kept at − 20 °C until used. The hydrolysates are referred to as "Salmon backbone/head hydrolysate", and the Sep-Pak-treated samples as "SPE of salmon backbone/head hydrolysate".

The *E. coli* is a genetically modified bioluminescent test strain K-12 (pTetlux1) which produces light (bioluminescence) as a by-product of its metabolism. Measuring the amount of light is therefore in accordance with the growth of the bacteria (Nybond et al. 2013). It is well suited for the identification of antimicrobial compounds.

E. coli was cultivated to the log phase in BHI broth (Difco, Becton, Dickinson & Company, Sparks, MD 21152, USA), and 10^4 cells were added to a white microtiter plate per each well with the samples. The hydrolysates were tested with eight concentrations, ranging from 3.125 to 125 mg/mL, and the SPE fractions with five concentrations, ranging from 0.78 to 12.5 mg/mL. The growth of *E. coli* was continuously monitored for 30 h at 37 °C as bioluminescence emission with a luminometer (Luminoskan EL1, Thermo Scientific, Vantaa, Finland).

The growth of the *L. innocua* was monitored turbidometrically (A_{600nm}) by a plate reader spectrophotometer (Hidex Sense, Hidex Ltd, Turku, Finland). The *L. innocua* cells were first pre-grown to log phase in BHI broth and 2.5×10^7 cells per well were added in BHI broth to a microtiter plate with the samples. The tested sample concentrations with the SPE fractions samples were 0, 0.75, 1.5, 3.1, 6.25, and 12.5 mg/mL, and with the hydrolysates in concentrations of 0, 1.5, 3.1 6.25, 12.5, 25.0, 50, 100, and 125 mg/mL. Due to turbidity and to exclude potential bacterial contamination, samples in BHI without *L. innocua* were used as controls. The *L. innocua* was grown inside the device at 30 °C for 16 h, and the growth was continuously monitored.

Functional properties

The backbone and head protein hydrolysates produced in the optimal processing conditions were tested for their functionality in terms of the nitrogen solubility index (NSI, %) and gel hardness (GH, N).

The method was described earlier in Pap et al. (2022). Protein dispersions in 1% (w/v) were prepared in water, and the pH was adjusted to 8 with 0.5 M or 0.1 M NaOH. The dispersions were stirred at room temperature for 2 h. The suspension was divided into two parts. One part was stored at − 20 °C until further analysis. The rest was centrifuged at 3000 g for 10 min. The supernatants were filtered through a Whatman 42 filter, collected, and stored at − 20 °C until analysis. The nitrogen content of the suspension and the filtered supernatant was determined using the Kjeldahl protocol described above. The nitrogen solubility index was expressed as:

$$\text{NSI}\,(\%) = \frac{\text{concentration of nitrogen in supernatant}}{\text{total nitrogen concentration}} \times 100$$

Gel hardness was measured from protein hydrolysate gels with a protein content of 5% (pH 5.5, 6.0, 6.5) and 10% (pH 5.5) as described in Pap et al. (2022). The pH was adjusted with 0.1 M HCl, and the solution was stirred at room temperature at 150 rpm for one hour. The pH was measured and adjusted if necessary. The solutions were transferred to stirring bottles and filled with Milli-Q water to reach the 50 mL volume, and 1.9 mL of the protein solutions were transferred to Eppendorf tubes of 2.5 mL. One tube was filled with water as a control. The tubes were incubated for 45 min at 80 °C, transferred to an ice-bath, and left to rest for 16 h at 6 °C. The samples were tempered to room temperature for 30 min before the gel hardness was measured. The hardness was measured with a Lloyd texture analyser (LR10K MK4, Lloyd Instruments Ltd, UK) with a depression limit of 8 mm and a trigger of 0.001 kgf (kilogramforcemeter, 1 kgf = 9.81 Nm), and the gel hardness values were collected.

Results and discussion

Response surface methodology for salmon backbone and head protein hydrolysates production

To establish the statistical model between the processing factors and the response, the protein yield was calculated for each of the 17 test runs using the volume of the stock and protein concentration. The model was fitted with multiple linear regression, and an analysis of variance (ANOVA) was performed. In the salmon head hydrolyses data, three outliers were found in the analysis phase, and these were excluded in the refining stage to ensure the good fit of the model. No outliers were found in the case of the salmon backbone experiments. The experimental results and measured response is summarised in Supplementary Table 1.

The resulting regression coefficients for salmon backbone were as follows: $R^2 = 0.860$, R^2 adj. = 0.751, and $Q^2 = 0.457$. Similarly, the salmon head regression coefficients were

$R^2 = 0.916$, R^2 adj. $= 0.818$, and $Q^2 = 0.486$. The ANOVA showed that the model was statistically significant ($p < 0.05$), and no lack of fit was observed at $p = 0.003$ for salmon backbone and $p = 0.007$ for salmon head respectively. Based on these findings, the model was found to be statistically adequate to predict the optimal processing parameters to achieve the maximum protein yield. Earlier studies (Valencia et al. 2013) have shown that a D-optimal design of experiments can obtain reliable and accurate estimates of kinetic model parameters, in terms of sample variance and standard error of parameters, from far fewer data points (number of experiments) than the number required of traditional designs.

The E:S ratio played a significant role in the protein yield from salmon heads, as observed from the linear terms' coefficients. The interaction terms of the E:S ratio and the hydromodule, as well as the second potential of the E:S ratio, were also found to be significant. The major function of hydromodule was included in the model, because it is relevant in the interaction term. Including the hydromodule also improved the model's quality.

The polynomial equation to describe the model in the case of the salmon head is:

$$Y = 57.967 + 0.852X_1 + 5.938X_2 - 5.299X_2^2 + 2.97X_1X_2,$$

where Y (%) is the protein recovery, X_1 is the E:S ratio, and X_2 (L/kg) is the hydromodule.

The results for the backbone indicated that only the hydromodule (X_1), and the E:S ratio (X_2) were significant for reaching the highest protein recovery in the hydrolysation process.

The equation to describe the model in the case of the salmon backbone is:

$$Y = 55.47 + 3.9X_1 + 10.3X_2,$$

where Y (%) is the protein recovery, X_1 is the E:S ratio, and X_2 (L/kg) is the hydromodule.

The response contour plots of the optimisation procedure are depicted in Fig. 1. The optimal processing parameters for the highest protein yield were an E:S ratio of 0.386%, a hydromodule of 1 L/kg, and a hydrolysis time of 4 h for the salmon head. For the salmon backbone, an E:S ratio of 0.499%, a hydromodule of 2.67 L/kg, and a hydrolysis time of 3.745 h were determined as the optimal conditions. Protein recovery within these processing parameters was predicted to reach 64% in both by-products. This accords with the findings of Liaset et al. (2000), who found that a protein recovery of 67.6% was achieved with Alcalase hydrolysis for salmon backbone. Ovissipour et al. (2012) used response surface methodology with factorial design to minimise enzyme utilisation and model the degree of hydrolysis (DH). The optimal conditions to reach the highest degree of hydrolysis were: 60.4 °C, 90.25 min, and a protease (Alcalase 2.4 L) activity of 70.22 AU/kg protein. The protein recovery reached 70%, with low lipid content (1.43%), and the hydrolysates showed good nutritional properties.

Proximate analysis

Both hydrolysates from the salmon by-products had a similar proximate composition, as shown in Table 1.

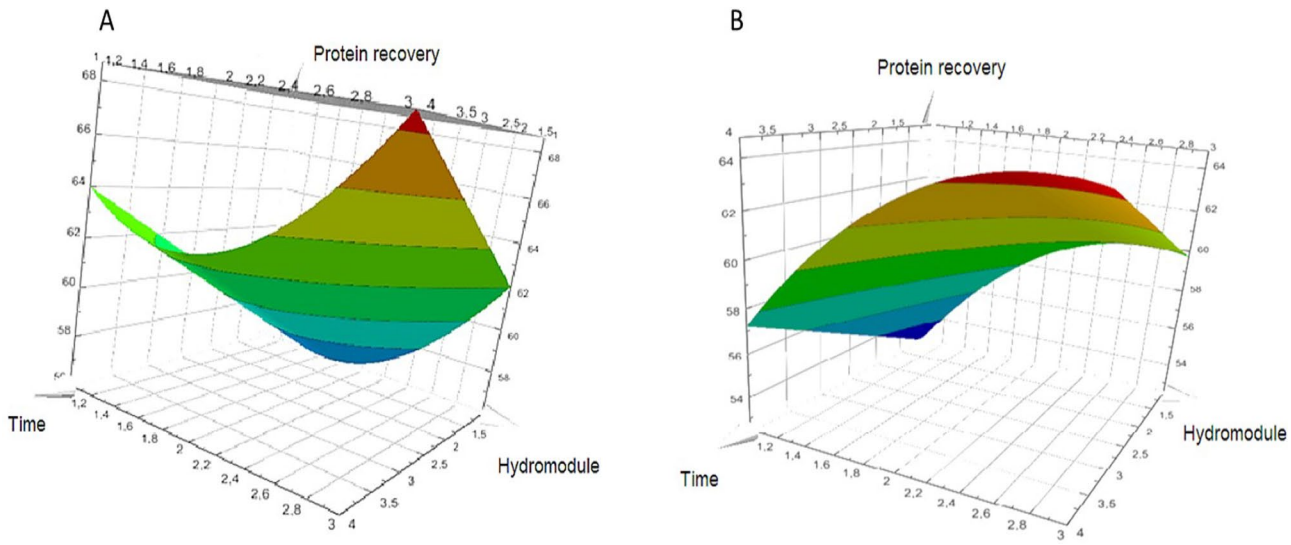


Fig. 1 The response surface plots of the enzymatic hydrolysis model of salmon head (**a**) and salmon backbone (**b**) showing the relationships between protein recovery, hydromodule and hydrolysis time

The backbone hydrolysate had higher residual fat content than the head hydrolysate. This phenomenon may be due to incomplete fat removal by the separator. The results showed that it was possible to produce protein hydrolysates with a high protein content (> 75%) from both salmon by-products with a low fat and carbohydrate content. The hydrolysates were shown to be good sources of protein supplementation in food products according to these results.

Molecular weight distribution of the hydrolysate

SDS-PAGE and SEC were used to analyse total protein and soluble protein fragmentation. The results are illustrated in Fig. 3. SDS-PAGE analyses showed that there are large proteins of approx. 100 kDa in backbone hydrolysates, whereas in head hydrolysates, the largest fragments were approx. 40 kDa. The lack of clearly visible protein bands in the salmon head sample indicates that the sample contained mainly small peptides that have migrated out of the gel. In the salmon backbone sample, most of the protein fragments were between 5–15 kDa. SEC analysis was carried out to study further the molecular weight distribution, especially the water soluble small fragments. The most abundant fractions in the salmon backbone hydrolysate were 5.0–8.0 kDa and < 2.0 kDa, which accounted 39% and 26% of the total protein, respectively. In the salmon head hydrolysate, the most abundant fraction were 8.0–10.0 kDa and 5.0–8.0 kDa accounting 38% and 24% of the total protein, respectively. The salmon backbone hydrolysate contained a higher proportion of small fragments than the salmon head hydrolysate, with MW < 2.0 kDa; the respective proportions were 26% for salmon backbone hydrolysate and 15% for salmon head hydrolysate. Small fragments with MW below 2.0 kDa contain free amino acids and small peptides, which are both putative flavour compounds.

The molecular weight distribution of fish hydrolysates varies depending on the enzymes and hydrolysis parameters used (e.g. Idowu et al. 2018; Gbogouri et al. 2004, Partanen et al. 2023). Atlantic salmon frame and head hydrolysates have shown abundant peaks at 3.5, 4.2, 11.9, and 13.2 kDa (Idowu et al. 2018 and Gbogouri et al. 2004) and are also rich in small peptides with MW less than 1 kDa (Idowu et al. 2018). Commercial, food grade fish protein hydrolysates have been reported to be rich in protein fragments of 1–10 kDa (Partanen et al. 2023). In the present study, most of the hydrolysate proteins are smaller than 10 kDa in MW, indicating a slightly higher hydrolysis degree than the previous results of Idowu et al. (2018), Gbogouri et al. (2004) and for commercial food grade fish protein hydrolysates (Partanen et al. 2023).

Amino acid and 5′-nucleotide composition

The results from the chromatographic separation and quantification of TAAs, FAAs, 5′-nucleotides, and corresponding nucleosides are shown in Table 2 and Fig. 2. A set of 16 amino acids (16 AAs) was used, in which the sums of TAAs and FAAs were compared. These 16 AAs were detected and quantified in both chromatographic AA analyses. Additionally, in the analysis of the FAAs, asparagine, glutamine, β-alanine, taurine, and GABA were also quantified. Instead, in TAA analysis, hydroxyproline, proline, cysteine + cystine, and ornithine were detected and quantified.

There was no difference in the sum of 16 TAAs (~ 70 g/100 g) between fish head and fish backbone hydrolysates (Fig. 2). However, the hydrolysed salmon head sample contained more FAAs than the backbone when both the sum of FAAs and individual amino acid concentrations were viewed. The sum of FAAs among the salmon samples was 52% higher in salmon head samples (13.4 g/100 g) than the salmon backbone samples (8.8 g/100 g). The sum of 16 FAAs in the salmon head comprised 19%, and in salmon backbone, 13%, of the sum of 16 TAAs (Fig. 2).

According to the results, glutamic acid, glycine, aspartic acid, alanine, arginine, and lysine were the six most dominant TAAs in both hydrolysates. Lysine is regarded as an essential amino acid, while glutamic acid, glycine, aspartic acid, and arginine are conditionally essential. In both sample types, the glutamic and aspartic acid content was higher, and leucine, isoleucine, phenylalanine, and tryptophan content lower, in TAA analysis than in FAAs analysis. In both processed salmon samples, leucine content was clearly the highest of all FAAs, accounting for 17–19% of the total amount of 16 FAAs. Phenylalanine, alanine, and glycine were also among the five most common FAAs present in both samples. Of these, leucine and phenylalanine are classified as bitter-tasting amino acids, and alanine and glycine as sweet. The bitter-tasting amino acids are also essential amino acids that increase the nutritional value of the hydrolysates.

Table 2 Nucleotide and nucleoside composition of the salmon head and backbone freeze dried protein hydrolysates (n = 3)

	Salmon head hydrolysate mg/100 g	Salmon backbone hydrolysate mg/100 g
5'-AMP	28.1 ± 2.6	39.7 ± 0.3
5'-CMP	42.5 ± 14.7	n.d
5'GMP	20.5 ± 2.0	n.d
5'-IMP	34.2 ± 2.1	n.d
Adenosine	2.6 ± 1.7	20.4 ± 0.5
Guanosine	159.8 ± 4.2	34.3 ± 1.0
Inosine	222.4 ± 1.5	508.2 ± 8.0
Uridine	40.8 ± 9.8	9.8 ± 2.1

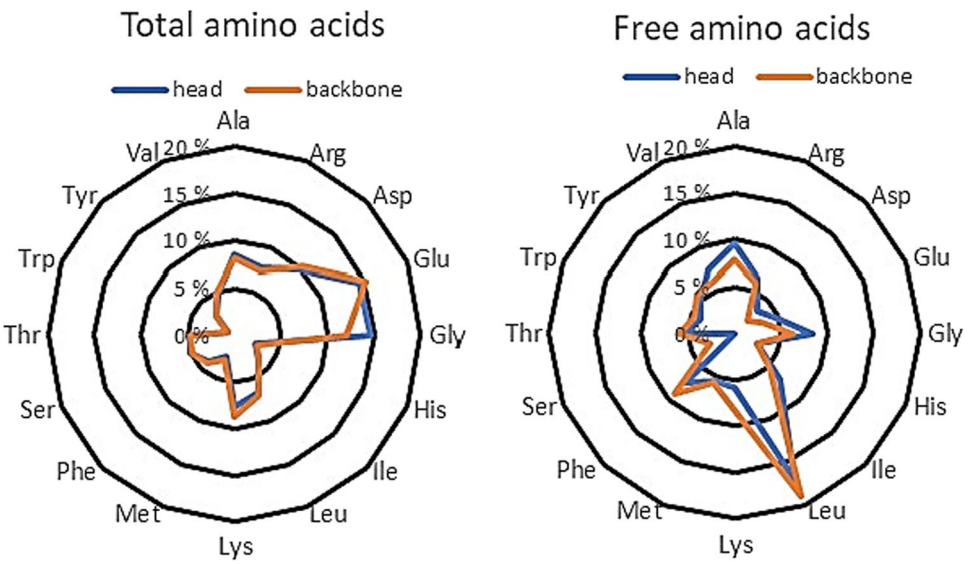


Fig. 2 The percentage of individual amino acids in the sum of 16 free or total amino acids ($n=3$)

These results are in accordance with previous studies. Lall and Anderson (2005) reported the whole-body amino acid composition of five salmonid fish species. According to their report, glutamic and aspartic acid concentrations were the highest of all amino acids in each of the five species studied.

Considering the amino acid composition from the taste perspective, the content of FAAs contributing to the umami taste was higher in the fish head sample (1.1 g/100 g) than in the fish backbone sample (0.4 g/100 g). On the other hand, the salmon head hydrolysate also contained more bitter and sweet amino acids. According to the analysis results, the concentration of free glutamic acid affecting the umami taste was relatively low, representing only 3–4% of the sum of 16 FAAs in both hydrolysates. On the other hand, the total amount of essential FAAs was above 60% in both samples.

In the analysis of 5′-nucleotides and nucleosides, detectable but low concentrations of 5′-nucleotides were measured in samples extracted from salmon heads (Table 2). The salmon samples mainly contained nucleosides, especially the samples extracted from the fish backbone. Nucleosides are degradation products of nucleotides that can be enzymatically degraded further into the corresponding sugar phosphates and bases (Solms and Wyler 1979). As nucleotides are shown to survive the analytical procedure, including hot water extraction and an ultrasound bath (Manninen et al. 2018), it is likely that the nucleotides possibly present in raw materials were largely degraded during the sample processing.

Antimicrobial properties of the salmon hydrolysates and purified peptides

The antimicrobial activity of the hydrolysates and their solid phase extracts (SPE) were tested. The SPE fractions did not inhibit the growth of *E. coli* in concentration area 0.75–12.5 mg/mL (Fig. 4). It was not possible to test the SPE fractions in higher concentrations since the yield from chromatographic purification was small. However, the hydrolysates both from backbones and heads showed clear dose dependent antibacterial effects in concentrations ranging from 3 to 166 g/L (Fig. 4). The concentrations 100 and 125 mg/mL showed more than two log inhibition values. The bioluminescence emission correlates well with the colony forming units during the logarithmic growth period in *E. coli*. This assay is specifically sensitive towards bacterial transcription and translation inhibitors (Nybond et al. 2013).

Like other salmonid fish, rainbow trout has an array of antimicrobial peptides which are small, usually cationic and amphiphilic molecules that can interact with negatively charged components of pathogenic microbes and can disrupt the cell membrane of the pathogen. These peptides are part of the innate and adaptive immune system of fish (Katzenbach 2015; Brunner et al. 2020). In rainbow trout the antibacterial peptides oncorhyncin II and III, hepcidin, cathelicidins 1 and 2, α-helix domain of IL-8 and histone H1 have been shown to possess antibacterial effect against *E. coli* (Fernandes et al. 2004; Álvarez et al. 2014; Chang et al. 2006; Santana et al. 2018; Richards et al. 2001). The antimicrobial peptides are this far reported to exist in skin and skin mucus, liver, intestine, gills, spleen and head kidney macrophages but, however, may be found also from other tissues. On the basis of the results in Fig. 3 some antimicrobial peptides against *E. coli* are present in tissues of head and backbone by products or may be formed in the hydrolysis. In healthy fish the antimicrobial peptides are commonly expressed in low levels. However, it is yet unknown whether commensal microbes influence antimicrobial peptide expression in salmonids (Brunner et al. 2020). Furthermore, fish

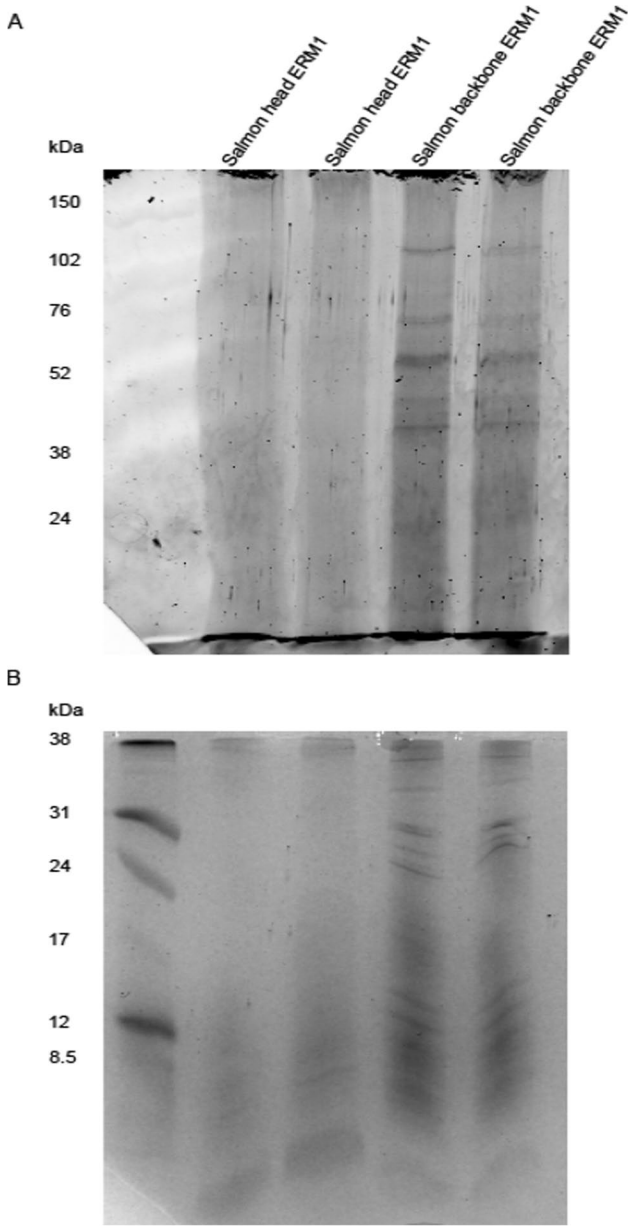


Fig. 3 Electrophoretic bands of the salmon head and backbone hydrolysates

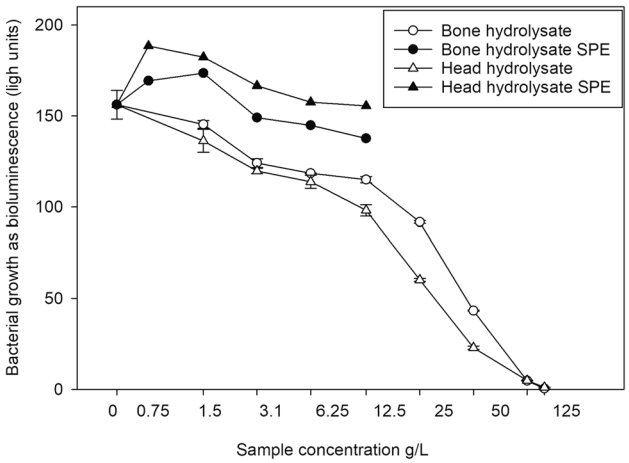


Fig. 4 The effects of Salmon backbone/head hydrolysates and Sep-Pak treated samples (SPE) of salmon backbone/head hydrolysates on metabolic activity of *E.coli* K-12 culture in BHR-broth after 8 h culturing at 37 °C measured as bioluminescence. The bioluminescence is generated as side effect of *E.coli* K-12 metabolism and correlates with the living cell number. The bioluminescence emission is expressed as relative light units (rlu). The error bars represent S.D

hydrolysates act as antioxidants against free radicals (Girgih et al. 2013) and they also have potential as antihypertensive pharmacological and anticancer agents (Halim et al. 2016).

The hydrolysates and their SPEs did not have any essential effects on growth of *L. innocua* (data not shown). *L. innocua* is a non-pathogenic species in Listeria family and therefore often used as safe and convenient model for the human pathogen *L. monocytogenes*.

Cheng et al. (2015) observed that the growth of inoculated *L. monocytogenes* slowed down in cold smoked salmon samples when the samples were either injected with or submerged in of salmine peptide solution (5 mg/g of salmon). Also, growth of other naturally occurring bacteria was slower. Besides, they also described that the salmine helped to reduce the growth speed of other naturally occurring bacterial flora in cold smoked salmon until day 22. Similar findings were not observed when an edible biofilm was produced from salmine. Our results with *L. innocua* suggest that hydrolysates and their SPEs did not contain significant amounts of salmine or that the effect may differ substantially between Listeria species. Salmine has been found especially in skin exudates and milt tissue. The proportion of skin was low in our samples.

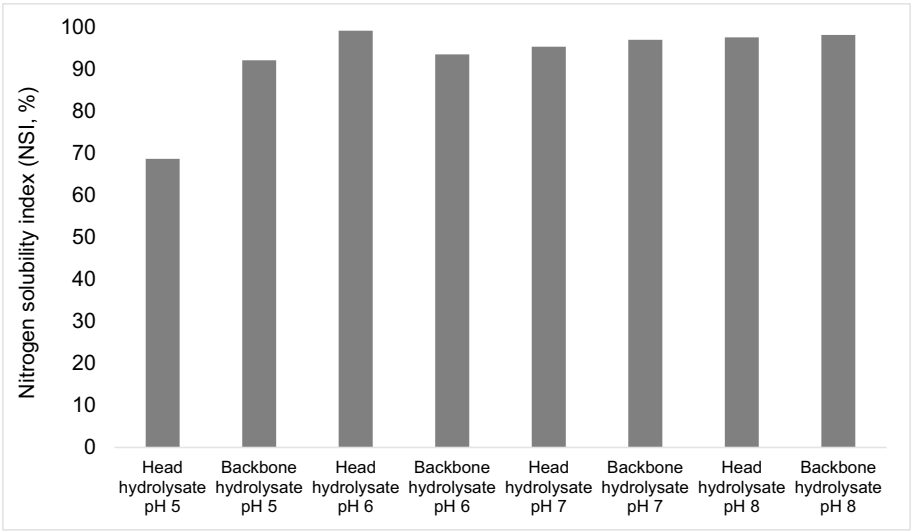
Functional properties

The samples were subjected to nitrogen solubility and gel hardness indices measurements at different pHs, and the results are illustrated in Fig. 5.

Nitrogen solubility index

The nitrogen solubility index is relevant for evaluating protein quality. It describes the proportion of nitrogen that is water soluble in comparison with total nitrogen content. The NSI values of the protein hydrolysates obtained in the optimal processing parameter conditions were determined at pH 5–8.

The measurements indicated that the salmon backbone protein hydrolysates showed high solubility at all investigated pHs, with an increasing trend with an increasing pH. All the solubility values were above 90%. Salmon head protein hydrolysates behaved differently in the investigated



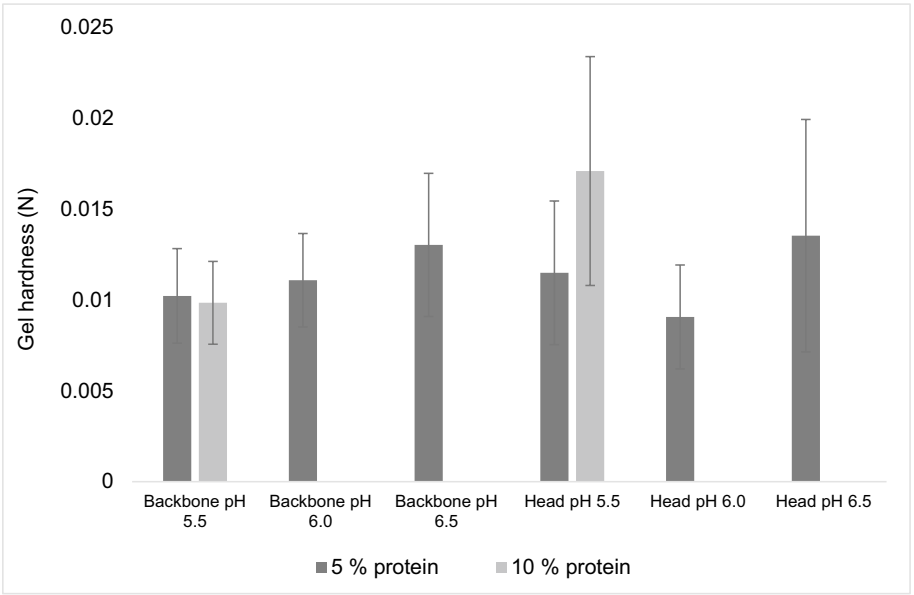


Fig. 5 Nitrogen solubility index of salmon backbone and salmon head protein hydrolysates as a function of pH (n = 1) and the breaking force of gels made of salmon backbone and head protein hydrolysates as a function of pH (n = 10)

pH range from the backbone hydrolysates. The lowest solubility of 68.5% was measured at a pH of 5, and the highest value of 99.20% at a pH of 6. The results are in accordance with the report of Gbogouri et al. (2004) for Atlantic salmon heads and with Kristinsson and Rasco (2000a) for salmon muscle protein hydrolysates, showing similar excellent solubility indexes at a pH of more than 6. The solubility of the protein hydrolysates is known to be highly dependent on the degree of hydrolyses (Kristinsson and Rasco 2000c). Small peptides have higher solubility properties due to their increased capacity to form hydrogen bonds with water (Sathivel et al. 2005).

Gel hardness

Gel hardness was evaluated at 5% protein concentrations for backbone and head samples at pH values between 5.5 and 6.5. Additionally, a sample with 10% protein content was evaluated as a reference at a pH of 5.5 (Fig. 4). In general, the gels produced from hydrolysates showed similar patterns to NSI; gel hardness increased with an increasing pH. On the other hand, head gels showed a more random pattern of gel hardness at different pH values. Both gels were weak compared with measurements with a 10% gelatine gel, which showed a gel hardness of 0.67 N in the same conditions. Abdollahi and Undeland (2019) found much higher

breaking forces (0.25–0.6 N) in gels made from salmon protein isolates produced in alkali conditions. In our study, we used proteolytic enzymes, which degraded the proteins and were therefore unable to form the network required for gel formation.

Conclusions

RSM was a viable tool to determine the optimal parameters for high protein yields from salmon head and backbone. The 240 min. hydrolysis using non-commercial protease enzyme resulted in high protein recovery, with a good solubility index, but weak gelling properties. The majority (> 80%) of the protein fragments were below 10 kDa. The results indicate that the salmon backbone hydrolysate contained a large number of small peptides, approximately two to ten amino acids (MW < 2.0 kD). Both hydrolysates contained all the essential amino acids, indicating high nutritional quality. However, there were few free umami taste-enhancing compounds, even though glutamic acid was the most common compound of TAAs. The total content of TAAs was 70 g/100 g in backbone and head hydrolysates and FAA content varied from 8.8 to 13.4 g/100 g in backbone and head hydrolysates. The salmon head and backbone hydrolysates effectively inhibited the growth of E. coli. However, the solid phase extraction diminished antibacterial efficacy, indicating that the activity was due to non-protein compounds. The effective antimicrobial characteristic of the protein hydrolysates against *E. coli* could be studied further in real model food formulations to evaluate the potential to improve food safety in practice. In addition, the investigation of the sensory properties will be important for the potential development of future food products for consumers.

Author´s contributions

NP Conceptualization; Methodology; data curation; Writing—original draft; SM Conceptualization; Methodology; Data curation; Writing and review; MN Conceptualization; Methodology, and Writing; PM Methodology, Data curation and Writing; AH Conceptualization; Methodology; Data curation, Writing and Project administration; MR-P Conceptualization; Methodology, Data curation; Writing; M S Conceptualization; Methodology, Data curation; writing; JM Methodology, data curation, writing, SK Methodology, data curation, writing-review and editing; AP Conceptualization; Methodology; Project administration; Funding acquisition; Data curation; Writing—review and editing.

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Data Availability Data available on request from the authors.

Code availability Modde 12.1. software, Umetrics, Sweden.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics approval Studies with human participants or live animal subjects were not included by any of the authors.

Consent to participate Not applicable.

Consent for publication Not applicable.

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