



Research article

Induced oxidation and addition of antioxidant before enzymatic hydrolysis of heads of rainbow trout (*Oncorhynchus mykiss*) – effect on the resulting oil and protein fractionKristine Kvangarsnes^{a,*}, Sophie Kendler^b, Turid Rustad^b, Grete Hansen Aas^a^a Faculty of Natural Sciences, Department of Biological Sciences Ålesund, Norwegian University of Science and Technology (NTNU), PO Box 1517, 6025 Aalesund, Norway^b Faculty of Natural Sciences, Department of Biotechnology and Food Science, Norwegian University of Science and Technology (NTNU), N-7491 Trondheim, Norway

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ABSTRACT

The effect of freshness of heads of rainbow trout (*Oncorhynchus mykiss*) for hydrolysing fish protein was investigated. To simulate storage and transportation, hydrogen peroxide and iron was added to minced heads to induce oxidation in the raw material prior to one week of storage. The effect of antioxidant in retarding oxidative changes during hydrolysis or to the raw material was investigated by adding butylated hydroxy toluene (BHT) prior to hydrolysis or storage. Enzymatic hydrolysis was carried out using bromelain and papain. The oil fraction was separated from the water soluble proteins, and the soluble phase was freeze dried. Both the oil fraction and protein fraction from enzymatic hydrolysis was affected by oxidative state of raw material. FFA was significantly higher in those FPH made from raw material added pro-oxidants, addition of antioxidant did not affect the level of FFA. The solubility of proteins in dried fish protein hydrolysates (FPH) decreased significantly when using oxidized raw material. Although addition of antioxidant improved the solubility, it was still significantly lower compared to those FPHs not added pro-oxidants. The FPH with decreased solubility also had higher levels of carbonyl groups which indicate protein oxidation. However, the oxidative state of raw material did not affect fatty acid composition in oil fraction or the amino acid composition in the FPH.

1. Introduction

Aquaculture production of seafood is increasing globally. Norway is one of the leading seafood nations, producing 1 452 928 tonnes of farmed salmonids in 2019. Farming of Atlantic Salmon is the most important species with a production of 1 364 044 tonnes in 2019, but rainbow trout is important with a production of 83 489 tonnes (Statistics Norway, 2020). Production and processing of salmon and rainbow trout produces large amounts of rest raw material (RRM). In 2018, about 417 900 tonnes rest raw material was produced from salmon and rainbow trout (Sustainability in Aquaculture, 2020). Both salmon and rainbow trout are often headed before export. In Norwegian supermarkets and retail stores, fish are usually sold as gutted whole fish or fillets.

Even though the RRM contain valuable protein and lipid fractions, consisting of amino acids and fatty acids essential for human health, it is usually considered as a by-product.

Today, these by-products are normally processed into low market-value products such as feed for livestock or pet food (Srikanya et al., 2017). The world population is increasing, leading to an increased demand for high quality protein for human consumption. Thus, there is a huge potential in utilizing the rest raw material from food processing for value added products. Producing fish protein hydrolysates (FPH) can be a suitable method to meet the challenge of increased demand for protein.

Production of fish protein hydrolysate (FPH) and industrial use of FPH have increased rapidly in recent years (Ölmez and Alak, 2019). In order to increase the industrial production of FPH, more knowledge is needed on the relationship between raw material quality, process conditions and FPH properties. The main purpose of hydrolysis is to extract protein, peptides and amino acids, but also fish oil is extracted (Carvajal et al., 2015). Both the chemical composition and quality of the oil depends on the production process and oxidative state of the raw material (EFSA, 2010). There are several aspects which are used to determine oil quality. It can be defined both by degradation, hygienic and oxidative

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state. Quality of RRM will vary due to conditions (temperature and time) during storage and transportation. In order to use the oil fraction and peptides for human consumption, it is important that omega 3 fatty acids and essential amino acids are retained during storage and processing.

Fish protein hydrolysates have applicable physicochemical and functional properties which include water holding capacity, oil absorption capacity, protein solubility, gelling activity, foaming capacity and emulsion ability (Chalamaiah et al., 2012; Chalamaiah et al., 2010). There are some challenges related to sensory quality, especially when it comes to bitterness (Aspevik et al., 2016). Using papain and bromelain has been observed to give a hydrolysate with less bitterness compared to Alcalase (Šližytė et al., 2014).

Fatty fish contain high amounts of unsaturated fatty acids, thus; they are prone to undergo oxidation. Lipid oxidation lead to development of unpleasant flavour and loss of nutritive values (Ahmed et al., 2016; Kolakowska et al., 2006b). Oxidation of fatty acids leads to unstable free radicals and hydroperoxides. These compounds decompose to e.g. ketones, aldehydes, alcohols, hydrocarbons, acids, and epoxides. In addition to affecting flavour, these compounds can bind to protein and form insoluble lipid-protein complexes (Halldorsdottir et al., 2014).

Fish muscle is also liable to undergo protein oxidation. These oxidative modifications might be triggered by interaction with lipid oxidation intermediates leading to formation of carbonyl groups as well as reduction in sulfhydryl groups. These modifications cause changes in the physical and functional properties of proteins such as a loss in solubility as well as fragmentation and aggregation.

Along with high contents of polyunsaturated fatty acids, fish contain pro-oxidants such as haemoglobin and iron (Hultin, 1994). These substances are prone to react with each other before, during and after enzymatic hydrolysis, thereby generating negative oxidation products (Halldorsdottir et al., 2014). Halldorsdottir et al. used a cod model system to study oxidative processes during enzymatic hydrolysis with added pro-oxidants. The authors observed that oxidation developed rapidly during enzymatic hydrolysis when pro-oxidants were present. Also, heating and shifts in pH may cause oxidation during enzymatic hydrolysis. In this study, rainbow trout was chosen due to availability. Other studies have shown that rainbow trout is relatively stable, i.e. no initial visual sensory changes was observed until 14 days of storage at 2 °C (Kolakowska et al., 2006b). This is also confirmed in another study on salmon heads stored at 4 °C. PV, free fatty acids (FFA) and total volatile nitrogen (TVN) remained low during storage for two weeks (Kvangarsnes et al., 2018).

Antioxidants are widely used to inhibit lipid oxidation in the food industry, and one of the most common synthetic antioxidants is butylated hydroxytoluene (BHT) (Yehye et al., 2015). BHT is a phenolic compound that scavenge free radicals and has been shown to have good effectiveness in inhibiting oxidation during enzymatic hydrolysis using papain and bromelain (Carvajal et al., 2014).

RRM is generated in slaughtering plants. Many of these slaughtering plants do not have their own facilities for processing the rest raw material. This makes it necessary to collect and store RRM before transport. In some cases, transportation involves long distances. During handling, storage and transport, RRM will be exposed to increased temperature and eventually also be mixed with other RRM fractions. We hypothesize that storage, transportation and handling of the raw material will affect the oil fraction but also the solubility properties of the protein fraction from enzymatic hydrolysis. Adding an antioxidant prior to storage, may have a positive effect on the quality of oil fraction as well as solubility of proteins. Our hypothesis is that the antioxidant will not affect the hydrolysis process. To simulate the effect of storage and transportation on the quality of the raw material, oxidation was induced by pro-oxidants. The aim of this study has been to investigate the effect of oxidative status of raw material on the quality of oil and the properties of the proteins resulting from enzymatic hydrolysis of rest raw material.

2. Material and methods

2.1. Mincing of heads

Heads of trout (*Oncorhynchus mykiss*) were donated from a local fish farm in November 2018 and handed over at slaughter day. The heads were transported to the laboratory in insulated industrial boxes filled with ice. The mean weight of the heads was 200 ± 40 g and they were collected from fish weighing between 1-2.7 kg. Immediately after weighing, the heads were minced using a mincer with 4.5 mm hole size (Hobart A 200 N). The fish mince was divided into batches of 1 kg, and immediately frozen and stored at -80 °C until further analysis or hydrolysis.

2.2. Preparation of raw material with different quality

The raw material was thawed at 4 °C overnight and divided into three different experimental groups, each of 2.5 kg minced heads. The study design is illustrated in Figure 1. One group was hydrolysed immediately

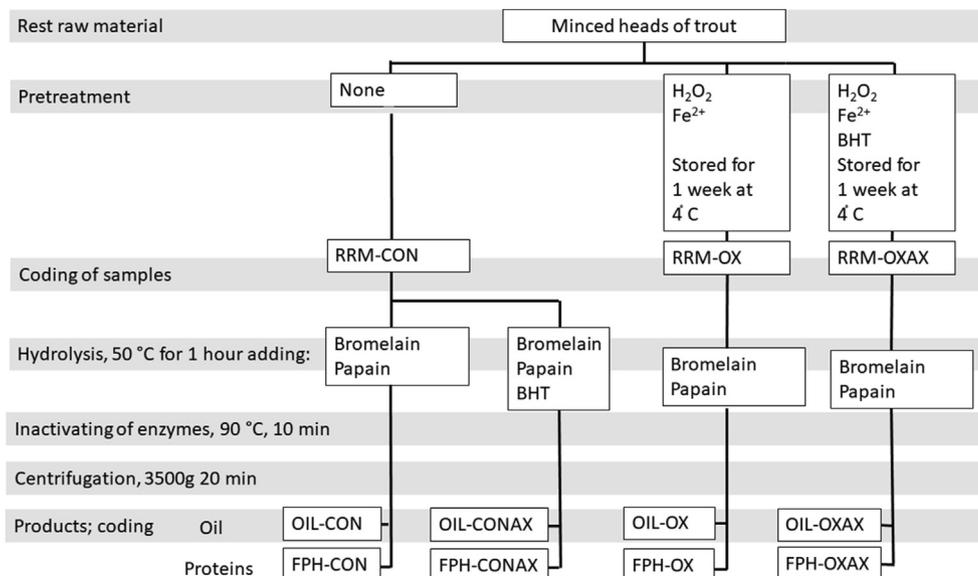


Figure 1. Experimental design of preparation of raw material of different freshness, and the resulting oil and protein hydrolysate from enzymatic hydrolysis.

after thawing (RRM-CON). In the second group (RRM-OX) oxidation was induced by adding iron sulphate (3.5 g) and cumene hydrogen peroxide (4.4 ml, 80%). The mince with added pro-oxidants were stored for at 4 °C for one week, in a plastic bucket with a lid. During storage, the sample was stirred several times. The last batch, RRM-OXAX was prepared in the same way as RRM-OX, except that 200 ppm BHT was added along with the pro-oxidants. This third group was also stored for one week at 4 °C, with stirring during storage. Before hydrolysis, RRM was visually described.

2.3. Proximate analysis of raw material

Total nitrogen was determined by using the Kjeldahl method, quantity of protein was calculated as $6.25 \times N$ (NMKL, 2003). Content of water was determined gravimetrically after drying at 105 °C until constant weight. Ash content was determined by drying to constant weight at 550 °C (AOAC, 1990).

2.4. Extraction of lipids and analysis of raw material

The extraction was performed according to the method of Bligh and Dyer (1959). Methanol and chloroform used in the extraction contained butylated hydroxytoluene (BHT) to minimize oxidation (100 mg/l). Lipid content was calculated as g/100 g. After extraction, the oil was covered with nitrogen and stored at -80 °C until further analysis.

2.4.1. Oxidation parameters

Peroxide values were determined by using a micro-procedure of the original method (AOCS, 2011). Sample size was 0.5 g, reagents 10 % of the amounts recommended for the standard procedure (Crowe and White, 2001). Starch was used as indicator.

Thiobarbituric acid reactive substances (TBARS) were determined according to Ke and Woyewoda (1979). Determination of TBARS was performed by using 200 µl of chloroform phase from the Bligh and Dyer extraction.

2.4.2. Free fatty acids

Free fatty acids in samples were determined according to Bernárdez et al. (2005). About 150 mg of oil was dissolved in 5 ml of isooctane, and 1 ml of cupric acetate-pyridine reagent was added. Tubes were vortexed for 30 s and centrifuged at 2000 g for five minute. The upper layer was measured at 715 nm.

2.5. Extraction of proteins and analysis from raw material

Sarcoplasmic (water soluble) proteins and myofibrillar (salt soluble) proteins were extracted as described by Anderson and Ravesi (1968) and Licciardello et al. (1982). About two grams of minced muscle was homogenized in 40 ml of buffer (50 mM KH_2PO_4 , pH7), and centrifuged at 4 °C for 20 min at 4100 g. The supernatant containing the sarcoplasmic proteins was decanted through glass wool and the volume was made up to 50 ml with buffer. To collect the myofibrillar proteins, the sediment was re-homogenized in 40 ml of buffer (50 mM KH_2PO_4 , 0.6M KCl, pH7), centrifuged, decanted through glass wool, and volume was made up to 50 ml with buffer.

The protein content in the extracts was determined in triplicate in suitable dilutions of both fractions by the method of Bradford (1976). Diluted colour reagent (5 ml) was added to blank (distilled H_2O), standards and samples, and absorbance at 595 nm was measured after five minutes.

Total thiol groups content was determined spectrophotometrically using the Ellman reagent (DTNB) (Ellman, 1959; Standal et al., 2018). To 100 µl of water soluble, salt soluble samples and blanks (distilled water), 800 µl urea and 100 µl DTNB were added. Samples were mixed, incubated at room temperature for 30 min and centrifuged for 3 min at 11

300 g at room temperature. The absorbance was measured at 412 nm with a blank as reference.

Carbonyl groups were determined using an immunoassay method developed by Buss et al. (1997). The ELISA kit, STA-310 Oxiselect TM was purchased from Cell Biolabs, Inc (San Diego, CA, USA). BSA standards (bovine serum albumin) and protein samples were adsorbed onto a 96-well plate for 2 h at 37 °C. The protein carbonyls present in the sample or standard were derivatized with DNP hydrazine and probed with an anti-DNP antibody, followed by an HRP conjugated secondary antibody.

2.6. Enzymatic hydrolysis

Enzymatic hydrolysis was performed in four different batches (Figure 1). Hydrolysis experiments were performed in 4 L closed glass vessels placed in a water bath at 52 °C. Warm (50 °C) distilled water was added to fish mince in a 1:1 ratio. The mixture was stirred at 150 rpm with an overhead stirrer. When temperature of the mixture was 50 °C, the enzymes papain and bromelain were added at levels of 0.05 % (w/w) (0.1% in total). After 60 min of hydrolysis, bones were removed by filtering the hydrolysate through a sieve before the enzymes were inactivated by heating at 90 °C for 10 min in microwave oven. The mixture was cooled down before being transferred to one litre centrifugation bottles and then centrifuged at 3500 g at 20 °C for 30 min. Three phases were created; oil phase on the top, liquid protein phase in the middle and sludge at the bottom. The oil phase was collected, weighed and frozen at -80 °C until analysis. The liquid protein phase was collected and freeze dried (72 h), before storage at -80 °C until analysis.

2.7. Analysis of lipids extracted during enzymatic hydrolysis

The yield of oil obtained in enzymatic hydrolysis was calculated as a percentage of weight of oil fraction relative to oil fraction in raw material.

2.7.1. Oxidation parameters

PV and TBARS were performed in the oil separated after enzymatic hydrolysis, the procedure is described in chapter 2.4.1.

2.7.2. Free fatty acids

Free fatty acids were determined by the procedure described in chapter 2.4.2.

2.7.3. Fatty acid distribution

Lipids were saponified with NaOH and esterified with BF_3 according to AOCS Official Method Ce 1b-89 (AOCS, 2005).

Due to an error in the gas chromatograph, RRM and oil fraction were analysed on different chromatographs. RRM was analysed on a Perkin Elmer Autosystem XL Gas Chromatograph equipped with a CB WAX 52 capillary column (25 m \times 0.25 mm) connected to a flame ionization detector (FID). Hydrogen was used as the carrier gas. The temperatures of injector and detector were 250 °C and 270 °C respectively. The oven temperature was programmed as follows: 90 °C, hold time 1.5 min. 90–150 °C at 45 °C/min, 150–225 at 4.5 °C/min and was held isothermally at 225 °C for 4 min.

The oil fraction was analysed on a Perkin Elmer Perkin Elmer GC, equipped with a Carbowax 20 M column, capillary column (25 m \times 0.25 mm) connected to a flame ionization detector (FID). Helium was used as the carrier gas. The temperatures of injector and detector were 220 and 270 respectively. The oven temperature was programmed as follows: 90 °C, hold time 2 min. 90–150 °C at 30 °C/min, 150–225 °C at 2.5 °C/min and was held isothermally at 225 °C for 15 min.

2.8. Proximate analysis and yield of FPH

The dry matter and ash content in FPH were determined as described in chapter 2.3.

Yield was calculated as a percentage of the weight of the hydrolysates obtained relative to the wet weight of the frames.

2.8.1. Determination of soluble proteins

Protein extracts were prepared by dissolving about 0.5 g of samples in 10 ml distilled water. The solutions were homogenized and centrifuged. These extracts were used to determine water soluble and acid soluble proteins.

Water soluble proteins were determined by the Lowry method (Lowry et al., 1951), and measured in triplicates. Bovine serum albumin (BSA) was used as a standard. The absorbance of the incubated standards and samples was determined using a GENESYS 10 UV-VIS Spectrophotometer (Thermo Fisher Scientific Inc., USA) at a wavelength of 750 nm.

Acid soluble proteins were determined according to Hoyle and Merritt (1994). The water soluble extract was filtered and then 2 ml were mixed with 2 ml of 20 % trichloroacetic acid and incubated at room temperature for 30 min. After incubation, the samples were filtered and the content of acid soluble peptides were determined by the Lowry method in triplicates. The absorbance was measured at 750 nm using a GENESYS 10S UV-VIS Spectrophotometer (Thermo Fisher Scientific Inc.).

2.8.2. Determination of degree of hydrolysis

The degree of hydrolysis (DH) was measured by formol titration as described by Taylor (1957). Samples of 1.5 g of protein hydrolysate was diluted in 50 g distilled water. The pH of the solution was adjusted to 7.0 by 0.1 M NaOH and 10 ml of formaldehyde with a pH of 8.5 was added. The mixture was left for 5 min at room temperature and titrated to pH 8.5 using 0.1 M NaOH. Total nitrogen in the sample was determined using the Kjeldahl method according to the Application Note from Büchi Switzerland (2013).

2.8.3. Protein oxidation in FPH

Carbonyls were determined using the protein extract from the determination of water-soluble proteins and performed in the same way as for raw material (chapter 2.5).

Thiols were determined using the protein extract from the determination of soluble proteins and performed in the same manner as for raw material (chapter 2.5).

2.8.4. Determination of molecular size

Molecular size was determined using a Hitachi HPLC system connected to a DAD-detector. FPH samples were dissolved in doubly distilled water (100 mg/ml, filtered through a 0.22 µm membrane disk) and injected on a 10/300 separation column. Fractions were eluted at a flowrate of 0.3 ml/min using 30 % ACN. Elution was monitored at 280 nm. Integration was done using a standard curve of seven standards with a molecular weight ranging from 238-12 327Da.

2.8.5. Determination of total amino acid composition

About 50 mg of freeze-dried raw material and 50 mg of hydrolysate samples were weighed into glass tubes and 1 ml 6 M HCl was added. The analysis was performed in triplicates. The glass tubes were placed into a heating cupboard for approximately 22 h at 105 °C. After cooling to room temperature, and neutralizing with NaOH, the samples were filtered and diluted to a volume of 10 ml with distilled water. Aliquots of suitably diluted samples were filtered before pipetted into vials and provided for HPLC. Determination was carried out on a Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific Inc., USA) connected to a fluorescence detector. A NOVA-PAK C18 column was used for separation of amino acids. The mobile phases were methanol and 0.08 M sodium acetate in gradient mode, with a flowrate of 0.9 ml/min.

2.8.6. Determination of free amino acids by HPLC

The determination of free amino acids in the freeze-dried raw material and hydrolysates was performed according to Osnes and Mohr (1985).

Two parallels of each sample were made by transferring 1 ml of water-soluble protein extract (0.5 g sample in 10 ml doubly distilled water) into EPPENDORF tubes and added 0.25 ml of 10 % sulphosalicylic acid. The samples were shaken to mix the solutions evenly. Next, they were placed into a cold room at 4 °C. After 30 min, the samples were centrifuged with an EPPENDORF 5415 R centrifuge (Eppendorf AG, Germany) for 10 min at 10 000 g. The supernatant was diluted and filtered. 0.25 ml of each filtered sample was pipetted into vials and analysed by HPLC in the same way as total amino acids described in chapter 2.8.5.

2.9. Statistical analysis

All data are presented in Dataset 1. Data were analysed by one-way analysis of variance (ANOVA) with Tukey HSD multiple comparison test using STATA statistical program (stata corporation v16). Welch ANOVA followed by Homer HSD was performed when homogeneity of variances was not met. Results are presented as mean ± standard deviation of three replicates unless otherwise specified.

3. Results and discussion

3.1. Description of raw material

Before enzymatic hydrolysis, RRM-CON was red in colour, and had a fresh, neutral odour. RRM-OXAX was brown, slightly grey in colour. The odour was described as more sour and fishy compared to RRM-CON. The colour of RRM-OX was grey and the odour was even more sour and fishy compared to RRM-OXAX. Both RRM-CON, RRM-OXAX and RRM-OX was evenly minced, with visible bony parts.

3.2. Proximate composition of raw material and yield of oil

The proximate chemical composition of raw material is presented in Table 1. RRM-CON contained 14.3% protein, 58.4 % water, 4.5 % ash and 20.1 % lipid. These results differ significantly from those found by Li et al. (2019). Their results showed that heads of rainbow trout contained 62 % moisture, 2 % ash, 29 % protein and 6 % lipids, while Kolakowska et al. found 65 % moisture, 15 % protein and 16 % lipids in rainbow trout offal (Kolakowska et al., 2006a). Kolakowska et al. reported that rainbow trout offal contained less protein and more lipids than fillets with skin. Whole rainbow trout has been reported to contain 73–75 % moisture, 19–20 % protein, 1–4 % lipids and 1 % ash (Gokoglu et al., 2004; Testi et al., 2006). The high content of lipids in heads found in our study compared to lipids in rainbow trout fillet shows that it is a good source for extraction of lipids.

After one week of storage, the proximate composition in the raw material changed. The most noticeable is the decrease in moisture from 58.4% in RRM-CON to 51.9 % in RRM-OX. The lower water content is difficult to explain, there are several factors which could lead to changes in water content. The oxidation trials were done in buckets with lids, no drip loss is possible. Water is consumed during hydrolysis of proteins, which may explain part of the loss of water. The storage trial done by Kolakowska et al. (2006a) also showed changes in proximate analysis with a decrease in content of water and protein. They observed the lowest content of water at day zero. The highest lipid content was observed at day three, followed by a decrease during storage. The higher content of lipids in stored fish offal was explained by autolytic changes. However, in our study, the content of lipids in RRM-OX (18.6 %) was lower than content of lipids in RRM-CON (20.1 %).

Table 1. Proximate composition of raw material. Means and standard deviations are shown.

	RRM-CON	RRM-OX	RRM-OXAX
Lipids % (n = 1)	20.1	18.6	22.0
Crude protein % (n = 3)	14.28 ± 0.14	14.95 ± 0.58	16.28 ± 0.85
Water % (n = 3)	58.44 ± 0.39	51.91 ± 0.52	55.48 ± 2.15
Ash % (n = 3)	4.46 ± 0.03	4.82 ± 0.28	4.98 ± 0.47

3.3. Lipid oxidation and lipolysis in raw material

Lipid oxidation of raw material was monitored by measuring PV and TBARS in oil extracted from the raw material (Table 2).

PV was not detected in raw material with no added pro-oxidants (RRM-CON). Also, it had the lowest value of TBARS (0.06 nmol MDA/kg oil). RRM-OX contained the highest value of PV (7.26 meq O/kg oil) and had a TBARS value of 0.33 nmol MDA/kg oil. Both these values were significantly higher compared to RRM-CON. When antioxidant was added along with pro-oxidants (RRM-OXAX) both PV and TBARS were significantly lower compared with RRM-OX with a PV of 3.02 meq O/kg oil and TBARS of 0.19 nmol MDA/kg oil. However, PV and TBARS were significantly higher in RRM-OXAX compared to RRM-CON. Oxidation of unsaturated fatty acids takes place in the presence of pro-oxidants like heme proteins during storage. A positive correlation between PV and amount of non-heme iron has been observed in earlier studies (Yarnpakdee et al., 2012).

Content of free fatty acids was lowest in RRM-CON and increased significantly in both stored samples (RRM-OX and RRM-OXAX). Hydrolysis of glycerol-fatty acid esters catalyzed by lipase is an important change that occurs in fish muscle lipids during post-mortem storage (Pacheco-Aguilar et al., 2000). Released free fatty acids are more prone to oxidation, compared to TG. This coincided with increased lipid oxidation as evidenced by the increases in PV and TBARS value.

3.4. Proteins in raw material

Adding pro-oxidants also affected the solubility, carbonyls and thiol groups of proteins – both in the water soluble and the salt soluble part of proteins. Results are shown in Table 3.

RRM-CON showed the highest content of soluble proteins and thiol groups, while content of carbonyl groups was lowest. RRM-OX had the lowest proportion of soluble proteins and thiol groups, and the highest concentration of carbonyl groups. This indicate that carbonyl groups and disulfides are formed during oxidation. When antioxidant was added to the raw material along with pro-oxidants, this resulted in a higher proportion of soluble proteins, thiols and a lower percentage of carbonyls compared to RRM-OX. However, these differences were not significant. Even if these results showed that BHT was able to decrease oxidation, further studies should be done. The level of antioxidant concentrations should be optimized, and also other antioxidants should be tested.

Regarding salt soluble proteins, the same trend is visible. The highest solubility and the lowest content of carbonyls were observed in raw material not stored (RRM-CON). The lowest solubility and highest content of carbonyls were observed in the group with added pro-oxidants.

3.5. Yield of oil

Yield of oil fraction after enzymatic hydrolysis compared to raw material was 89.6, 92.0, 93.7 and 93.2% for OIL-CON, OIL-CONAX, OIL-OX and OIL-OXAX respectively. This high yield of lipids obtained by enzymatic hydrolysis might be explained by absence of emulsion. Use of bromelain and papain in combination in enzymatic hydrolysis of by-products of herring has been shown to give higher lipid yield compared to Alcalase (Šližytė et al., 2014). They explained that the size of the peptides produced by Alcalase result in higher emulsifying properties.

3.6. Hydrolysis and oxidation in oil separated after enzymatic hydrolysis

Table 4 shows the lipid oxidation parameters and the FFA in the oil. The highest amount of peroxide was found in oil (OIL-CON) made of RRM-CON. In RRM-CON, no PV was detected, indicating a formation of PV during hydrolysis. In comparison, when BHT was added along with the enzymes (OIL-CONAX), the PV was 2.72 meq O/kg oil. OIL-OXAX had the lowest PV (1.9 meq O/kg oil), while the PV in OIL-OX was 4.55 meq O/kg oil. A study done by Hathwar et al. (2011) also showed positive effects by adding antioxidant in enzymatic hydrolysis of fish visceral waste. This was also observed in hydrolysis of by-products of herring (Carvajal et al., 2014). Both OIL-OXAX and OIL-OX had lower PV than the respective values in oil extracted from raw material (7.26 and 3.02 meq O/kg oil). This is probably due to the degradation of PV to form secondary oxidation products.

Regarding TBARS, the highest values were found in oil with no added antioxidant, with a value of 0.27 nmol MDA/kg oil and 0.24 nmol MDA/kg oil for OIL-CON and OIL-OX respectively. When antioxidant was added either in the hydrolysis tank (OIL-CONAX) or when additionally, adding pro-oxidants (OIL-OXAX) the value decreased to 0.11 nmol MDA/kg oil and 0.10 nmol MDA/kg oil respectively. TBARS is one of the secondary lipid oxidation products that arise from the decomposition of hydroperoxides. The value of TBARS was higher in OIL-CON and OIL-CONAX compared to RRM-CON, suggesting that some of the PV are degraded during enzymatic hydrolysis. In contrast, TBARS in OIL-OX and OIL-OXAX was lower than in the corresponding raw material (RRM-OX and RRM-OXAX). A possible reason for this is that also other secondary degradation products than TBARS are formed during hydrolysis. TBARS could also have been reacting with the proteins.

The amount of FFA was less than 0.7 % for oil made of raw material not stored. When oxidation was induced (OIL-OX and OIL-OXAX), FFA increased to about 1.6 %. It is noticeable that adding BHT prior to storage (OIL-OXAX) resulted in a higher level of FFA compared with the FFA level in sample without BHT (OIL-OX).

Table 2. Free fatty acids (FFA), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) in raw material. Mean values and standard deviations are shown (n = 3).

	RRM-CON	RRM-OX	RRM-OXAX
FFA %	0.81 ± 0.20 ^a	1.63 ± 0.09 ^b	1.87 ± 0.09 ^b
PV meq O/kg oil	-	7.26 ± 0.07 ^b	3.02 ± 0.13 ^c
TBARS nmol MDA/kg oil	0.06 ± 0.01 ^a	0.33 ± 0.05 ^b	0.19 ± 0.0 ^c

Values with a different letter within a row are significantly different (p < 0.05).

Table 3. Soluble proteins, carbonyl groups and thiols in water soluble and salt soluble proteins of raw material..

	Water soluble (sarcoplasmic proteins)			Salt soluble (myofibrillar proteins)		
	RRM-CON	RRM-OX	RRM-OXAX	RRM-CON	RRM-OX	RRM-OXAX
Soluble proteins % (n = 9)	4.58 ± 0.16 ^a	2.98 ± 0.17 ^b	3.62 ± 0.39 ^c	1.63 ± 0.14 ^a	1.18 ± 0.06 ^b	1.34 ± 0.03 ^c
Carbonyls, nmol carbonyls/mg protein (n = 6)	2.40 ± 0.59 ^a	10.30 ± 0.86 ^b	8.86 ± 2.44 ^b	5.43 ± 1.15 ^a	19.56 ± 4.97 ^b	13.91 ± 2.66 ^c
Thiols nmol/mg protein (n = 6)	64.33 ± 5.34 ^a	46.11 ± 2.59 ^b	52.30 ± 5.33 ^{ab}	59.98 ± 4.43 ^a	51.23 ± 3.83 ^b	62.61 ± 2.92 ^a

Values with a different small letter within each group of protein (sarcoplasmic or myofibrillar) in a row on the same storage day are significantly different ($p < 0.05$).

Table 4. Free fatty acids (FFA), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) in oil fraction. Mean value and standard deviation is shown (n = 3).

	OIL-CON	OIL-CONAX	OIL-OX	OIL-OXAX
FFA %	0.69 ± 0.02 ^a	0.63 ± 0.02 ^a	1.58 ± 0.06 ^b	1.63 ± 0.05 ^b
PV meq O/kg oil	6.23 ± 0.38 ^a	2.72 ± 0.25 ^b	4.55 ± 0.36 ^c	1.90 ± 0.19 ^b
TBARS nmol MDA/kg oil	0.27 ± 0.03 ^a	0.11 ± 0.01 ^b	0.24 ± 0.02 ^a	0.10 ± 0.01 ^b

Values with a different letter within a row are significantly different ($p < 0.05$).

3.7. Fatty acid distribution in raw material and oil from enzymatic hydrolysis

Table 5 displays the fatty acid composition of total lipids in raw material and oil after enzymatic hydrolysis. Contents of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were mostly similar for both raw material and fraction obtained by enzymatic hydrolysis, with only small variances between groups.

Also, the FA composition of oil resulting from hydrolysis was almost identical to that in raw material. About 20 percent of fatty acids are

omega 3 fatty acids. In both raw material and oil obtained after enzymatic hydrolysis, the dominant fatty acids were C16:0 and C18:1. The content of the omega 3 fatty acids EPA and DHA was about 5 % and 8 %, respectively.

In the raw material, the fatty acid distribution was not affected by addition of pro-oxidants and BHT. Similarly, no changes were observed during a storage study on salmon heads for up to two weeks (Kvangarsnes et al., 2018). Furthermore, there were no changes in fatty acid distribution regarding oil from enzymatic hydrolysis. This shows that oxidation of the raw material does not affect fatty acid distribution. This correlates with a study done by Gbogouri et al. (2006), did not find differences in

Table 5. Fatty acid distribution in raw material and oil fraction after enzymatic hydrolysis. Means and standard deviations are shown, n = 3.

	RRM-CON	RRM-OX	RRM-OXAX	OIL-CON	OIL-CONAX	OIL-OX	OIL-OXAX
C14:0	4.36 ± 0.01	4.34 ± 0.02	4.35 ± 0.01	3.67 ± 0.01	3.67 ± 0.01	3.68 ± 0.01	3.72 ± 0.02
C16:0	13.49 ± 0.02	14.02 ± 0.01	13.36 ± 0.01	12.11 ± 0.01	12.00 ± 0.02	12.25 ± 0.06	12.31 ± 0.01
C16:1 n7	6.00 ± 0.01	5.90 ± 0.01	6.04 ± 0.02	5.35 ± 0.02	5.39 ± 0.01	5.36 ± 0.02	5.36 ± 0.01
C18:0	3.05 ± 0.02	3.27 ± 0.02	3.02 ± 0.01	2.89 ± 0.01	2.84 ± 0.01	2.93 ± 0.01	2.92 ± 0.01
C18:1 n9	33.36 ± 0.03	33.02 ± 0.10	33.39 ± 0.04	31.82 ± 0.02	31.88 ± 0.04	31.72 ± 0.19	31.75 ± 0.03
C18:1 n7	3.47 ± 0.01	3.43 ± 0.01	3.45 ± 0.01	3.22 ± 0.03	3.23 ± 0.03	3.25 ± 0.04	3.25 ± 0.01
C18:2 n6	11.32 ± 0.01	11.10 ± 0.01	11.40 ± 0.01	10.70 ± 0.02	10.77 ± 0.01	10.70 ± 0.04	10.72 ± 0.01
C18:3 n3	4.79 ± 0.01	4.69 ± 0.02	4.83 ± 0.02	4.54 ± 0.01	4.58 ± 0.02	4.53 ± 0.01	4.52 ± 0.01
C18:4 n3	-	-	-	1.03 ± 0.01	1.03 ± 0.01	1.02 ± 0.01	1.03 ± 0.01
C20:1 n9	3.61 ± 0.04	3.55 ± 0.01	3.56 ± 0.02	3.67 ± 0.02	3.64 ± 0.01	3.66 ± 0.01	3.63 ± 0.01
C20:2 n6	-	-	-	0.58 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.56 ± 0.01
C20:4 n6	0.46 ± 0.01	0.55 ± 0.05	0.49 ± 0.01	0.51 ± 0.04	0.51 ± 0.03	0.52 ± 0.04	0.53 ± 0.04
C20:3 n3	0.39 ± 0.08	0.30 ± 0.01	0.31 ± 0.01	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.28 ± 0.01
C20:4 n3	-	-	-	1.06 ± 0.01	1.04 ± 0.01	1.03 ± 0.01	1.02 ± 0.01
C20:5 n3	5.04 ± 0.02	5.05 ± 0.02	5.11 ± 0.02	4.97 ± 0.01	5.01 ± 0.01	5.02 ± 0.01	4.99 ± 0.02
C22:0	-	-	-	2.75 ± 0.01	2.70 ± 0.01	2.69 ± 0.01	2.66 ± 0.01
C22:1n9	0.41 ± 0.01	0.42 ± 0.01	0.41 ± 0.01	0.41 ± 0.01	0.40 ± 0.01	0.40 ± 0.01	0.39 ± 0.01
C22:5 n3	2.09 ± 0.02	2.09 ± 0.02	2.11 ± 0.01	2.14 ± 0.01	2.14 ± 0.01	2.13 ± 0.01	2.08 ± 0.01
C22:6 n3	8.16 ± 0.04	8.29 ± 0.06	8.18 ± 0.01	8.30 ± 0.03	8.29 ± 0.03	8.27 ± 0.02	8.30 ± 0.01
SFA	20.90^a ± 0.02	21.62^b ± 0.04	20.72^c ± 0.01	21.42^a ± 0.01	21.21^b ± 0.04	21.54^{ac} ± 0.08	21.61^c ± 0.01
MUFA	46.84^a ± 0.05	46.31^b ± 0.09	46.84^a ± 0.03	44.47^a ± 0.01	44.55^a ± 0.03	44.39^a ± 0.14	44.38^a ± 0.04
PUFA	32.26^a ± 0.04	32.06^b ± 0.13	32.43^a ± 0.02	34.11^a ± 0.02	34.24^b ± 0.06	34.07^a ± 0.06	34.02^a ± 0.04

Values in bold with a different letter within the same material (raw material or oil fraction) in a row are significantly different ($p < 0.05$).

fatty acid profile of oil recovered from salmon heads by solvent extraction and enzymatic hydrolysis.

Unsaturated fatty acids are prone to oxidation, and the hydrolysis takes place at 50 °C, followed by treatment at high temperature for ten minutes. This might lead to unwanted reactions in the polyunsaturated fatty acids present in the oil. However, no significant changes in fatty acid composition were observed.

3.8. Characterisation of hydrolysates

The characterisation of FPH are shown in Table 6. The crude protein content of FPH extracted from trout heads was found to be 79.7, 88.5, 85.9 and 83.1 % for FPH-CON, FPH-CONAX, FPH-OXAX and FPH-OX respectively. These variations reflect the differences in raw material with the highest protein content in RRM-CONAX.

A positive correlation between protein in raw material compared to FPH was observed. The high protein content found in our study, demonstrates its potential use as protein supplements for human nutrition.

The yield of protein in FPH from heads of trout varied from 6.25-6.85 g/100 g. This is in agreement with other studies using bromelain and papain. Gajanan et al. (2016) found a yield of FPH from threadfin bream frame waste from 4.6-9.7 %. Slizyte et al. (2016) obtained a yield of FPH of 7.5 from defatted backbones of salmon.

The hydrolysate made from RRM-CON and RRM-CONAX showed the highest amount of water-soluble proteins, with a mean value of 83.5 % (Table 6). The hydrolysates made from RRM-OX and RRM-OXAX showed significantly lower ($p < 0.05$) amounts of soluble proteins (62.6 and 66.8.6 % respectively). RRM used to make FPH-OX and FPH-OXAX did have significantly higher carbonyl groups and significantly lower level of thiol groups compared to RRM used to prepare FPH-CON and FPH-CONAX. These chemical modifications are known to affect the solubility of proteins and might be one of the causes for the lower solubility observed in FPH made of oxidized raw material.

FPH-CON, FPH-CONAX and FPH-OXAX contained about 28 g/100 g acid soluble peptides while FPH-OX contained 25 g/100 g. However, these values are not significantly different. Acid soluble peptides are believed to consist of small peptides with an average molecular weight of 3-4 amino acid residues and free amino acids (Greenberg and Shipe, 1979). Gbogouri et al. (2004) and Dong et al. (2008) reported that when the amount of the smallest peptides increases, the solubility increases as well. Smaller peptides are expected to have more polar residues which increase the ability to form hydrogen bonds with water. This is explained by new carboxylic and amine groups which are liberated when proteins are cut into smaller peptides. These groups expose more charged and polar groups to the surrounding water.

The degree of hydrolysis (DH) ranged from 17.4 % in FPH-CON and FPH-OX, to 18.2 % in FPH-OXAX. A higher degree of hydrolysis generally results in a hydrolysate with a higher proportion of smaller peptides,

resulting a hydrolysate with higher solubility (Pacheco-Aguilar et al., 2008; Ölmez et al., 2019). An increase in solubility has been positively correlated with DH (Liu et al., 2014). In our study, FPH-OXAX have higher DH compared to FPH-CON, but lower solubility. However, the difference in DH between the four hydrolysates is small. In addition, DH is not the only factor that determines the solubility of proteins in a protein hydrolysate. Other factors are also important, such as hydrophobicity.

Bitterness in hydrolysates is also linked to DH. According to Dauksas et al. (2004), DH levels between 4 and 40 give the highest risk of bitterness in hydrolysates, while Adler-Nissen (1984) claims that low DH's between 3 and 5 % significantly lower the bitterness. To get hydrolysates with only low levels of bitterness, it is therefore suggested to either have a very low DH with large peptides or a high DH, with only small peptides.

Carbonyls were lowest in FPH-CON (2.21 nmol/mg protein). Surprisingly, FPH-CONAX contained more carbonyl groups than FPH-CON. As oxidation occurred in raw material, carbonyl content increased to 5.14 in FPH-OX. Carbonyl groups are formed by the oxidation of amino acid side chains. Particularly the side chains of lysine, proline, arginine, threonine and asparagine are reported to form carbonyl residues due to metal-catalysed reactions (Lund et al., 2011). These chemical modifications cause changes in the physical and functional properties of proteins resulting in a loss in solubility as well as fragmentation and aggregation. This could explain the low concentration of carbonyls in FPH-CON.

Oxidative stress also leads to formation of disulphide bonds, leading to a reduction in number of thiol groups. The number of thiol groups are relatively low in the hydrolysates, ranging from 1.08 – 3.3 nmol/mg protein. There were no significant differences among the four hydrolysates. FPH-CONAX had the lowest number of thiol groups. To the best of our knowledge, the effect of oxidation on FPH has not been studied earlier.

3.9. Molecular weight distribution

Compared to FPH without added pro-oxidants (FPH-CON), hydrolysates made of stored raw material (FPH-OXAX and FPH-OX) had a higher concentration of peptides below 2000 Da (Figure 2). About 15 % of peptides in FPH-CON, 20 % in FPH-OXAX and FPH-OX were below 500 Da, containing di and tripeptides. There was no significant difference in molecular weight distribution between FPH-OXAX and FPH-OX. According to Maehashi et al. (2008), peptides with a molecular size between 100 and 6000 Da and a high hydrophobicity often exhibit bitter taste. Klompong et al. (2007) reported that interfacial activities such as emulsifying and foaming properties decrease with increasing DH. These differences are probably caused by endogenous enzymes in the raw

Table 6. Proximate composition in raw material and freeze dried fish protein hydrolysate. Means \pm standard deviations are shown.

	FPH-CON	FPH-CONAX	FPH-OX	FPH-OXAX
Crude protein %, n = 3	79.70 \pm 0.33 ^a	88.53 \pm 0.29 ^b	83.09 \pm 0.68 ^c	85.90 \pm 0.21 ^d
Water %, n = 3	2.74 \pm 0.27	3.06 \pm 0.04	3.73 \pm 0.65	3.86 \pm 0.22
Ash %, n = 3	9.06 \pm 0.83	9.23 \pm 0.12	9.41 \pm 0.21	10.10 \pm 0.40
Water soluble proteins (g/100 g hydrolysate), n = 3	83.59 \pm 1.71 ^a	83.44 \pm 0.72 ^a	62.61 \pm 0.49 ^b	66.82 \pm 1.74 ^c
Acid soluble proteins (g/100 g hydrolysate), n = 2	28.44 \pm 0.94	28.75 \pm 2.15	25.00 \pm 2.28	27.99 \pm 3.02
Carbonyl groups (nmol carbonyls/mg protein), n = 3	2.27 \pm 0.17	3.66 \pm 0.90	5.14 \pm 0.81	2.75 \pm 1.51
Thiol (nmol/mg protein), n = 2	3.00 \pm 0.30	1.08 \pm 0.01	2.90 \pm 0.03	3.26 \pm 0.11

Values with a different letter in a row are significantly different ($p < 0.05$).

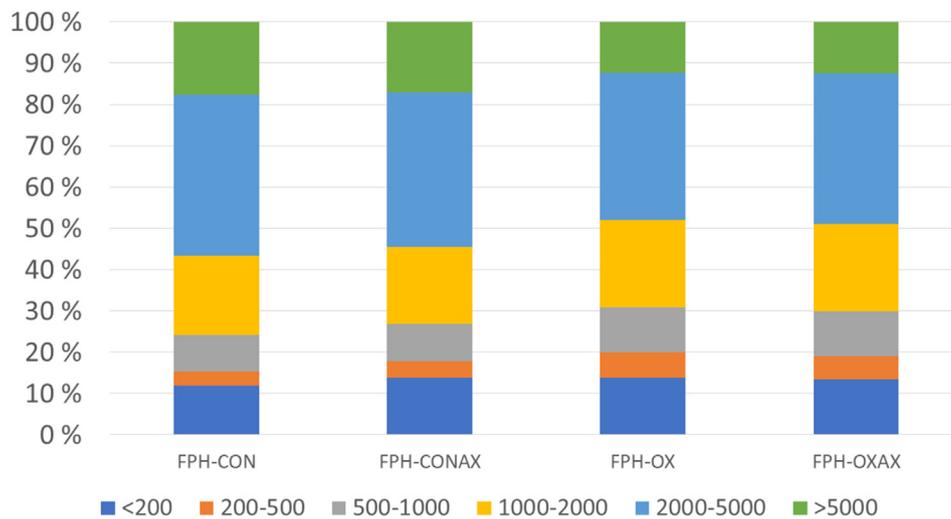


Figure 2. Molecular weight distribution of peptides (%), n = 3.

Table 7. Amino acid composition in FPH, % of amino acids. Mean values ± standard deviations are shown, n = 3.

	FPH-CON	FPH-AX	FHP-OX	FPH-OXAX
Aspartic acid	10.02 ± 0.11	9.88 ± 0.09	10.04 ± 0.20	10.23 ± 0.26
Glutamic acid	14.00 ± 0.22	14.01 ± 0.23	14.20 ± 0.14	14.63 ± 0.51
Asparagine	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.01
Histidine*	2.55 ± 0.16	2.66 ± 0.01	1.73 ± 0.81	1.72 ± 0.64
Serine	6.24 ± 0.08	6.48 ± 0.18	5.95 ± 0.20	5.98 ± 0.17
Glutamine	0.87 ± 0.19	0.65 ± 0.13	0.48 ± 0.16	0.69 ± 0.25
Glycine/Arginine*	20.36 ± 0.17	19.91 ± 0.06	19.68 ± 0.33	20.73 ± 0.42
Threonine*	5.10 ± 0.03	5.01 ± 0.08	5.16 ± 0.07	5.27 ± 0.14
Tyrosine	2.05 ± 0.06	2.06 ± 0.06	0.71 ± 0.94	1.25 ± 0.97
Alanine	9.65 ± 0.09	9.38 ± 0.06	9.88 ± 0.18	10.15 ± 0.32
Methionine*	3.18 ± 0.14	3.18 ± 0.09	3.15 ± 0.13	3.10 ± 0.23
Valine*	4.16 ± 0.04	4.26 ± 0.02	4.62 ± 0.10	4.46 ± 0.06
Phenylalanine*	3.52 ± 0.04	3.57 ± 0.02	5.59 ± 2.82	3.58 ± 0.10
Leucine*	7.02 ± 0.03	7.24 ± 0.13	7.70 ± 0.17	7.39 ± 0.09
Isoleucine*	3.19 ± 0.03	3.26 ± 0.06	3.60 ± 0.13	3.46 ± 0.11
Lysine*	8.08 ± 0.07	8.48 ± 0.08	7.50 ± 0.13	7.35 ± 0.26
Hydrophobic¹	30.72 ± 0.04^a	30.84 ± 0.26^a	34.53 ± 2.33^b	32.14 ± 0.60^b
Essential¹	36.81 ± 0.14	37.62 ± 0.26	39.04 ± 1.37	36.34 ± 0.17

Values in bold in the same row followed by different letters differ significantly (p < 0.05).

* Essential amino acids.

¹ Glycine/arginine left out from calculations.

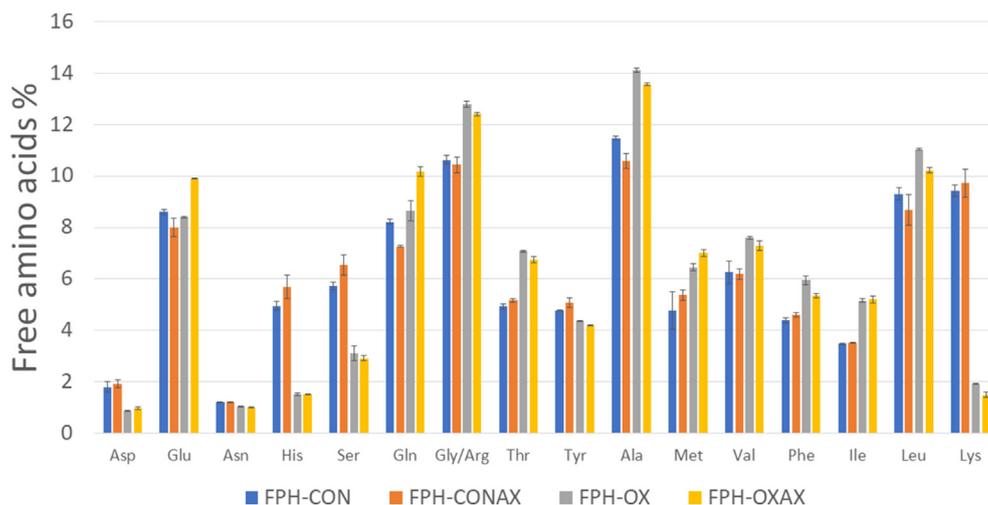


Figure 3. Free amino acids, % of total free amino acids. Means ± standard deviations are shown, n = 3.

material. Endogenous enzymes cause some degradation during storage. This is also reflected by the molecular weight distribution of the raw material where RRM with added pro-oxidants and stored have a higher proportion of smaller molecules compared to RRM-CON (results not shown). These enzymes will cause some degradation of proteins during storage of raw material before hydrolysis, and this is probably the reason for this difference in molecular weight distribution in the FPH.

3.10. Amino acid composition

The amino acid profiles of the hydrolysates generated from the rainbow trout heads are presented in Table 7. The content of essential amino acids ranged from 36.30–38.26 %, with the highest amount in FPH-OX.

The most abundant amino acids were Glutamic acid (14.0–14.6%), Aspartic acid (10.02–10.2%), Alanine (9.65–10.2%), Lysine (7.35–8.08%), Leucine (7.02–7.79%) and Serine (5.98–6.24%). These results are in the same range as Li et al. (2019) found in heads of rainbow trout. Glycine and arginine are merged in our method and have a content of 19.8–20.7% of the total amino acids in our study. In contrast, Li found a content of 4.8 % Glycine and 6.2% arginine, which give a sum of 11%. This is around 50% of the value found in our study. Bone and skin are rich in collagen, having a high content of glycine. Li found a higher content of protein in the heads compared to our results. These results also indicate that the ratio of collagen to crude protein is higher in our raw material.

The percentage of trout protein hydrolysate residues with hydrophobic side chains was significantly higher for those FPH made of stored RRM. The balance of hydrophilic and hydrophobic forces between peptides and water is important for their solubility (Gbogouri et al., 2004). The smaller peptides from myofibrillar proteins are expected to have proportionally more polar residues, with a higher ability to form hydrogen bonds with water thus increase the solubility. This increased content of hydrophobic amino acids in our study might partly explain the reduced solubility of the proteins.

3.11. Free amino acids

The free amino acids in the different FPH ranged from 29.49–36.77 mg/g. The free amino acids present in the highest amount were Glutamic acid (Glu), Glutamine (Gln), Glycine/Arginine (Gly/Arg), Alanine (Ala), Leucine (Leu) (Figure 3). A trend of increased amount of free amino acids in minced heads with added pro-oxidants could be observed. However, the amount of Aspartic acid (Asp), Histidine (His), Serine (Ser) and Lysine (Lys) showed significantly lower amounts in FPH made of RRM added pro-oxidants (FPH-OX and FPH-OXAX) compared to the control hydrolysate (FPH-CON). His, Ser and Lys have been shown to exhibit antioxidative effects. Also, Asp has high antioxidative effects due to the presence of electrons that can be donated during interactions with free radicals (He et al., 2013). The antioxidant effect of the FPH was not investigated in this study.

4. Conclusion

By using oxidized raw material before enzymatic hydrolysis, we have shown that the composition and oxidative state of raw material influences the properties of both the resulting oil fraction and protein hydrolysates. FFA was higher in oil fraction made from oxidized raw material. PV and TBARS were in the same range in oil made from raw material with or without pro-oxidant. Addition of antioxidant prior to hydrolysis decreased both PV and TBARS. All produced oil fractions contained about 30 percent of high valuable polyunsaturated fatty acids. These amounts were not affected by the state of raw material.

Use of oxidized raw material resulted in FPH with significantly decreased solubility. Use of antioxidant did not seem to affect the enzymatic hydrolysis, however it improved the solubility of FPH made of RRM where oxidation was induced. There were no large differences in

the amino acid composition between the different FPH, indicating that the oxidative state of raw material did not affect the amino acid composition.

Bromelain and papain were effective in enzymatic hydrolysis of heads of rainbow trout. Results showed that to obtain FPH with high solubility, it was important to have raw material with high quality. Nevertheless, raw material that does not have high enough quality for human consumption, may be used for making FPH for feed and pet food.

Declarations

Author contribution statement

Kristine Kvangarsnes: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sophie Kendler: Performed the experiments; Wrote the paper.

Turid Rustad: Conceived and designed the experiments; Wrote the paper.

Grete Hansen Aas: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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