Sensory and physicochemical properties of enzymatic protein hydrolysates

Influence of raw material, protease, and downstream processing

Silje Steinsholm

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

The research activity of this thesis has been carried out at the Norwegian Institute of Food, Fisheries, and Aquaculture Research (Nofima) and at the University of Bergen (UiB) under the principal supervision of Dr. Tone Aspevik (Nofima) and co-supervision of Dr. Åge Oterhals (Nofima) and Dr. Jarl Underhaug (UiB). The experimental work on enzymatic protein hydrolysis was performed at Nofima, Bergen. The chemical analyses were performed by Nofima BioLab (accredited according to ISO 17025) and sensory evaluations by the sensory panel at Nofima, Ås (in accordance with ISO 8586). NMR analyses to determine hydrolysate metabolite composition and critical micelle concentration, were done at the Norwegian NMR Platform, NNP.

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Bergen, January 2021

Silje Steinsholm

Abstract

The world aquaculture, fisheries and poultry sectors generate large amounts of residual raw materials, such as heads, backbones, and carcasses. Almost 85% of the residual materials from Norwegian aquaculture and fisheries were utilized in 2019, but over 150 000 metric tons were wasted. This is not compatible with the aim of a circular bioeconomic food production where all the biomass should be utilized. Furthermore, the majority of utilized raw materials were used as low-value feed ingredients. The residual raw materials are excellent food-grade sources of protein and have high potential for further upgrading. However, the materials are not directly applicable for human consumption, but through enzymatic protein hydrolysis, the proteins will be cleaved into more water-soluble peptides and made accessible for use and valorisation.

Enzymatic protein hydrolysates may be utilized within human consumption as protein enrichment of food products and/or as a functional ingredient. However, the sensory properties of protein hydrolysates are considered a major limitation for hydrolysate inclusion in foods. Peptides, free amino acids, minerals, and other water-soluble molecules will follow the hydrolysate phase and contribute to the overall sensory profile. Increased knowledge of the flavour development in protein hydrolysates is imperative when producing products destined for human consumption. Furthermore, the potential amphiphilicity of the peptides generates surface-active properties which is important to understand for their use as functional ingredients in food applications.

The main objective of this study was to assess hydrolysate properties, important for food formulations, of products based on species generating a substantial fraction of residual raw materials. Sensory profiles of the hydrolysates were evaluated using a trained panel and combined with metabolite composition, based on 1H NMR. Both enzyme specificity and new membrane filtration technology were assessed to reduce the sensory properties of the hydrolysates. Furthermore, the effects of hydrolysis parameters on important physicochemical properties, i.e. emulsion activity index (EAI), emulsion stability index (ESI) and critical micelle concentration (CMC) were evaluated.

In Paper I, the use of nuclear magnetic resonance (NMR) spectroscopy as a new tool in sensory assessment of protein hydrolysates were evaluated. Hydrolysates were produced based on muscle tissue from cod, salmon, and chicken with two different enzymes (Bromelain and FoodPro PNL) and hydrolysis times (10 and 50 min). Metabolite composition of the 12 hydrolysates were determined by NMR and the sensory profiles assessed by a trained sensory panel. The results showed that raw material had a major effect on attribute intensity and metabolite variation. The formation of bitter taste was not affected by raw material, indicating a comparable release of bitter peptides independent of substrate. Partial least squares regression on ^{1}H NMR and sensory data provided models for 11 of the 17 evaluates attributes, and significant metabolite-attribute associations were identified based on the obtained models. The study confirmed a potential for prediction of sensory properties based on ^{1}H NMR data.

In Paper II, the effect of hydrolysis parameters on emulsion and surface-active properties were assessed. Direct protein extracts from salmon and cod backbones were compared to hydrolysates based on two different enzymes (Bromelain and FoodPro PNL) with increasing hydrolysis time. EAI, ESI, and CMC were measured for all products. Protein hydrolysis was found to have a negative impact on ESI and CMC, while the ESI generally increased. The direct protein extracts had comparable EAI to that of the commercial emulsifier casein but considerably lower ESI values. The study emphasized the complexity of functional properties in protein hydrolysates and the challenges of achieving high protein yield simultaneously with high surface-activity.

In Paper III, the effect of membrane filtration on sensory properties were evaluated. Heads and backbones from cod and salmon were hydrolysed for 50 min with either Bromelain or FoodPro PNL. The hydrolysates were purified by microfiltration and further refined by nanofiltration and diafiltration. Sensory profiles and metabolite compositions were assessed prior to, and after each nanofiltration step. Metabolite composition were determined and quantified by ^{1}H NMR and sensory profiles were evaluated by a trained sensory panel. The results showed a substantial reduction in metabolite concentration by nanofiltration, with a concomitant reduction in the intensity of several sensory attributes. Bitterness, however, increased as small peptides associated

with bitter taste (MW range 0.5−2 kDa) were rejected by the membrane. About 19-24% of the raw material protein were recovered in the nanofiltered product and the main loss was attributed to the removal of bones and solids in the crude hydrolysates. Considerable amounts of protein were also retained in the microfiltration retentate, emphasizing the need for process optimization.

In Paper IV, the sensory, nutritional, and chemical quality properties of protein hydrolysates based on backbones, heads, and viscera from salmon and mackerel were assessed. The hydrolysates were produced using FoodPro PNL and hydrolysed for 50 min. All products were high in essential amino acids and had low biogenic amines content. The raw material fractions caused most of the variation in sensory properties, where viscera products had highest attribute intensities. Mackerel was perceived as the most taste intense of the species, mostly due to high ash content giving strong salty taste of the mackerel hydrolysates. This illustrated the importance of salt removal when producing products for human consumption.

List of Publications

I **Sensory assessment of fish and chicken protein hydrolysates. Evaluation of NMR metabolomics profiling as a new prediction tool**

Steinsholm, S., Oterhals, Å., Underhaug, J., Måge, I., Malmendal, A. & Aspevik, T. *Journal of Agricultural and Food Chemistry.* **2020**, 68(12): 3881-3890. DOI: 10.1021/acs.jafc.9b07828

II **Emulsion and surface-active properties of fish solubles based on direct extraction and after hydrolysis of Atlantic cod and Atlantic salmon backbones**

Steinsholm, S., Oterhals, Å., Underhaug, J. & Aspevik, T. *Foods.* **2021,** *10(1), 38.* DOI: 10.3390/foods10010038

III **Reduction in flavor-intense components in fish protein hydrolysates by membrane filtration**

Steinsholm, S., Oterhals, Å., Thoresen, L., Underhaug, J., Kousoulaki, K. & Aspevik, T. Submitted to *Journal of Food Science*.

IV **Sensory and chemical properties of protein hydrolysates based on salmon and mackerel side stream materials**

Aspevik, T., Thoresen, L., Steinsholm, S., Carlehög, M. & Kousoulaki, K. Accepted by *Journal of Aquatic Food Product Technology.* DOI: 10.1080/ 10498850.2020.1868644

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Abbreviations

ANOVA	Analysis of variance
CFF	Crossflow filtration
CMC	Critical micelle concentration
DM	Dry matter
DMA	Dimethylamine
DSS	2,2-dimethyl-2-silapentane-5-sulfonate
EAI	Emulsion activity index
ESI	Emulsion stability index
FAO	Food and Agriculture Organization
f_N	Nitrogen factor
ISO	International Organization for Standardization
K	Kelvin
MANOVA	Multivariate analysis of variance
MF	Microfiltration
MW	Molecular weight
MWCO	Molecular weight cut-off
MWD	Molecular weight distribution
NF	Nanofiltration
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PLSR	Partial least squares regression
PR	Protein recovery
QDA	Qualitative descriptive analysis
SEC	Size exclusion chromatography
SDS	Sodium dodecyl sulphate
TMA	Trimethylamine
TMAO	Trimethylamine oxide
TR1	Taste receptor related to sweet and umami tastes
TR2	Taste receptor related to bitter taste

U	Units of proteolytic activity
UF	Ultrafiltration
WHO	World health organization

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1. General introduction

Food production has a substantial environmental impact, and the consensus is that waste in the food chain should be nil (Springmann *et al.* 2018). The European domestic animal, aquaculture, and fishing industries estimates a biomass loss of 40% prior to distribution (FAO 2011, Pérez Roda *et al.* 2019). In industrialized countries, most of the loss can be attributed to retail and consumer waste, but processing lines, such as filleting, may contribute substantially to the loss. The filleting process generates residual raw materials that can be defined as parts of the animal that is not considered the main product. Bones, heads, skin, viscera, carcasses, connective tissue, and trimmings are rich in protein of food quality with high potential value (Aspevik *et al.* 2017). The ratio of fillet to residual fractions varies depending on species. The Norwegian aquaculture sector, mainly Atlantic salmon, generated 29% residuals from the filleting lines in 2019. The white fish (cod, saithe, etc.) and pelagic sector (mackerel and herring) generated 44 and 15% residuals, respectively (Myhre *et al.* 2020). This corresponds to 460, 300 and 200 thousand metric tons, of which a total of 145 000 metric tons were unutilized. The chicken production in Norway is small compared to that of fish. However, the yearly production is steadily increasing, and yields approx. 50% residuals (56000 metric tons residuals in 2012) (Lindberg *et al.* 2016). Residual raw materials are similarly generated from other livestock, but due to a danger of transmissible spongiform encephalopathy, there are limitations on the use of residuals from ruminants (Aspevik *et al.* 2017).

Residuals from filleting industries are increasingly utilized, but in 2019 only 13% of residuals from the fish industry in Norway were used for human consumption, either directly or as nutritional supplements (Myhre *et al.* 2020). About 70% were rendered into products for feed formulations where silage constitutes the main product (44% of available residuals), and mostly based on residuals from aquaculture (Myhre *et al.* 2020). Silage is usually produced by addition of formic acid to the raw material (Raa & Gildberg 1982). This inhibits bacterial growth and activates endogenous viscera enzymes, resulting in an acidic ($pH < 4$), protein-rich liquid containing high levels of free amino acids, but unsuitable for human consumption (Aspevik *et al.* 2017, Olsen

& Toppe 2017). Fish meal, based on thermal protein coagulation by cooking, pressing (separating the soluble protein, or stickwater, from the solids), and drying (Schmidtsdorff 1995), is also a substantial product category (18% of available residuals). Residuals from the pelagic fish sector constitutes most of the raw material basis for fish meal production. Poultry residuals have traditionally been processed into feed, fertilizer, and pet food, but also some food products such as mechanically deboned meats (Lasekan *et al.* 2013); however, there is increasing interest in transforming more of this raw material into products for human consumption. It would be more economically and environmentally sustainable to increase the ratio of food ingredients generated from the residual raw material (Stevens *et al.* 2018). Enzymatic protein hydrolysis is a promising method for producing food-grade products from filleting residuals (Panyam & Kilara 1996, Aspevik *et al.* 2017). The conversion into water-soluble peptides and free amino acids facilitates recovery of proteins otherwise attached to e.g. bones, heads, and carcasses, post filleting. The composition of an enzymatic protein hydrolysate is determined by substrate, choice of enzyme, and processing conditions (Kristinsson & Rasco 2000b).

Protein hydrolysates are highly nutritious, like the raw materials, but there are limitations to their inclusion in food formulations. Particularly the sensory properties of hydrolysates can limit consumer acceptance, and taste-neutral products are highly desired. The sensory properties of a protein hydrolysate are determined by both the peptide composition and flavor-active metabolites. Bitter taste is associated with the formation of small peptides containing hydrophobic amino acids during hydrolysis (Kim & Li-Chan 2006). Bitterness, and the reduction of bitter taste intensity, is much studied in relation to utilization of protein hydrolysates, as it is considered a major limiting factor (Fu *et al.* 2019, Idowu & Benjakul 2019). Peptides of eight amino acids, or less, contribute to bitter perception, and the intensity of bitterness is determined by the number and position of the hydrophobic amino acids (Ishibashi *et al.* 1988, Tamura *et al.* 1990). The other tastes and flavors of a protein hydrolysate can mainly be attributed to non-protein water-soluble molecules (Temussi 2012, Aspevik *et al.* 2016b, Paper I). The functional properties of a protein hydrolysate are dependent on the surface-activity of the peptides, which is highly reliant on the balance of hydrophobic

and hydrophilic amino acids (Dexter & Middelberg 2008). Given good functionality, such as emulsion or foam forming properties, the hydrolysates may be used as functional additives in food formulations to influence appearance and miscibility (Wilding *et al.* 1984, Wouters *et al.* 2016).

The residual materials are rich in essential amino acids (Liaset & Espe 2008), and the inclusion of hydrolysates based on such materials to a food product will increase total amino acid content. The recommended protein requirements for sustaining the body's nitrogen balance is disputed, but WHO recommends 0.83 g protein / kg body weight a day (WHO 2007). The trend for average dietary protein intake in the US is increasing (Shan *et al.* 2019) and illustrates the need for new protein sources. There is also a focus on increasing dietary protein to the growing elderly population to reduce sarcopenia (Gilmartin *et al.* 2020). This, along with the rising focus on "clean" labelled foods and circular bioeconomy (Ozturk & McClements 2016, Springmann *et al.* 2018) unfolds new opportunities for applying enzymatic protein hydrolysates in a sustainable food industry.

1.1 Objectives

The main objective of the research activity was to expand the knowledge of properties essential for utilization of enzymatic protein hydrolysates, based on residual raw materials from the filleting industries, as food ingredients.

Sub-objectives:

- Elucidate effects of hydrolysate metabolite composition on sensory attributes (Paper I).
- Evaluate the effects of different raw materials on sensory and physicochemical properties of protein hydrolysates (Paper I, II, IV).
- Evaluate the use of NMR metabolomics as a tool in sensory profiling of protein hydrolysates (Paper I).
- Characterize functional properties of direct protein extracts and hydrolysates relevant for food formulations (Paper II).
- Assess the effect of crossflow membrane filtration on the intensity of sensory attributes of hydrolysates (Paper III).

2. Enzymatic protein hydrolysis

Protein hydrolysis is the cleaving of peptide bonds in a reaction with H_2O (Figure 1). This causes conversion of proteins into smaller peptides and free amino acids. In enzymatic protein hydrolysis this reaction is catalysed by enzymes, more specifically proteases. The liberation of amino acid side chains and terminal ends increases the water-solubility of the peptides, thus facilitating their recovery as a protein hydrolysate. There are alternatives, such as acidic or alkaline hydrolysis. However, alkaline hydrolysis may generate toxic compounds, such as lysinoalanine, and both alkaline and acidic hydrolysis methods are non-specific, cleaving peptide bonds randomly, as opposed to enzymatic hydrolysis where the specificity of the enzyme directs the cleaved site (Aspevik *et al.* 2017).

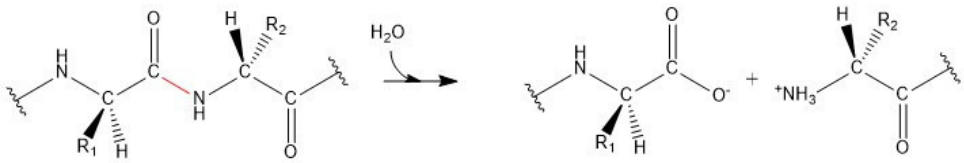

Figure 1. The amide bond between two amino acids residues is cleaved by a water molecule, catalysed by an enzyme, in protein hydrolysis.

Enzymatic protein hydrolysis can be performed with both endogenous and added enzymes. The former usually involves the inclusion of the viscera fraction from the residual products, which contain digestive proteases (Gildberg 1993). This study, however, focuses on the effect of added exogenous enzymes in protein hydrolysis.

2.1 Production of enzymatic protein hydrolysates

The main steps in traditional enzymatic protein hydrolysis are illustrated in Figure 2. The raw material is sliced or ground and then mixed with water prior to heating to the optimal temperature for enzyme activity. A water to raw material ratio of 1:1 (w/w) is typically used. Using more water may increase the yield but then requires additional energy for its removal during downstream processing. If the protein concentration is too high, i.e. low water activity, the hydrolysis reaction may be inhibited as the amount

of water necessary to hydrate the peptides are too low (Butre *et al.* 2014). Also, substrate inherent protease inhibitors released during the reaction may influence enzyme efficiency if not sufficiently diluted (Hjelmeland 1983, Wubshet *et al.* 2019).

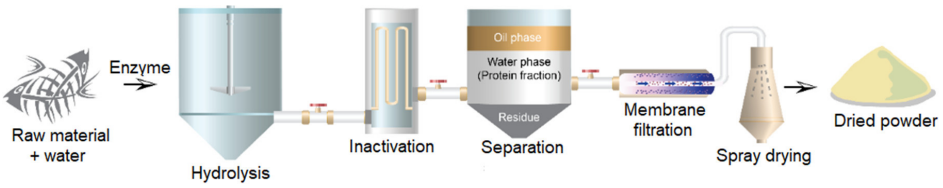

Figure 2. Illustration of the main steps in an industrial protein hydrolysis process (Kristoffersen 2019).

The protease is added when the slurry of water and raw material have reached the optimum temperature of the chosen protease, usually between 40 and 60°C. After a predefined hydrolysis time, the reaction is terminated by heating to > 85°C. At this stage, the water-soluble components, i.e. the hydrolysate, are separated from the lipid and solid phase. The two latter phases also have nutritional value. Particularly the oil fraction, when fatty fish is used as raw material, is valuable, as it may be high in omega-3 fatty acids. The solid phase contains insoluble proteins and minerals, which have potential for further refining (Liaset & Espe 2008). Process parameters are frequently the focus of studies on protein hydrolysis. Substrate (raw material), enzyme (specificity and efficiency), enzyme-to-substrate ratio, and hydrolysis conditions (pH, temperature, hydrolysis time) all influence the hydrolysis process (Wubshet *et al.* 2019). The hydrolysis reaction is complicated by several factors. The raw material may have batchwise variations in composition, the reaction components are both substrate and products throughout the process, and peptide bonds are cleaved both in sequence and parallel reactions. In addition, the potential presence of protease inhibitors can influence the process (Qi & He 2006). Downstream processing can be applied to alter the hydrolysate depending on desired properties. Common downstream steps are membrane filtration to purify or fractionate the peptides, concentration, and drying (Petrova *et al.* 2018).

2.1.1 Quantification and characterization of hydrolysate peptides

There are many methods for analysis of the hydrolysis process (Spellman *et al.* 2003). It is desirable to obtain as high a process yield as possible whilst maintaining the peptide properties appropriate for the intended product application. Protein recovery (PR) is the amount of protein recovered in the hydrolysate divided by the amount of protein in the substrate.

$$PR\ (\%) = \frac{Protein\ in\ hydroysate\ (g)}{Protein\ in\ substrate\ (g)} \quad (1)$$

A parameter for determining the efficiency of hydrolysis, degree of hydrolysis (DH), is the percentage of peptide-bonds cleaved in the process (Nielsen *et al.* 2001). It may be analyzed by a variety of methods, such as pH-STAT, OPA, and TNBS, all based on distinct analytical principles that will give different results, dependent on both the method used and the peptide composition of the product (Morais *et al.* 2013, Aspevik *et al.* 2016a). Further, the DH does not provide information about the peptide chain lengths nor peptide distribution, both important parameters in assessment of hydrolysate properties.

The molecular weight distribution (MWD) of proteins and peptides in a hydrolysate can be determined by HPLC size exclusion chromatography (SEC) (Wubshet *et al.* 2017). The peptides move through a stationary phase which separates the compounds based on size, as smaller peptides are adsorbed into the packed column material, decelerating their elution compared to compounds of higher molecular weight (MW). Peptides and proteins of known MW are used to create a calibration curve for determination of elution time based on MW of the hydrolysate components. The chromatogram area within a specified retention time range provides an estimate of the relative amount of peptides within the MW size group (Wang-Andersen & Haugsgjerd 2011). SEC provides a good estimation of the peptide population in enzymatic protein hydrolysates and is especially beneficial for monitoring changes as an effect of different process parameters (Figure 3). A limitation to the methodology is the potential of protein and peptide properties affecting the retention time, such as folding of larger peptides increasing the apparent molecule size (the hydrodynamic volume) (Barth *et*

al. 1998). In addition, the optimal UV wavelength varies between amino acids which adds to the uncertainty of the measurement (Wang-Andersen & Haugsgjerd 2011, Fekete *et al*. 2014).

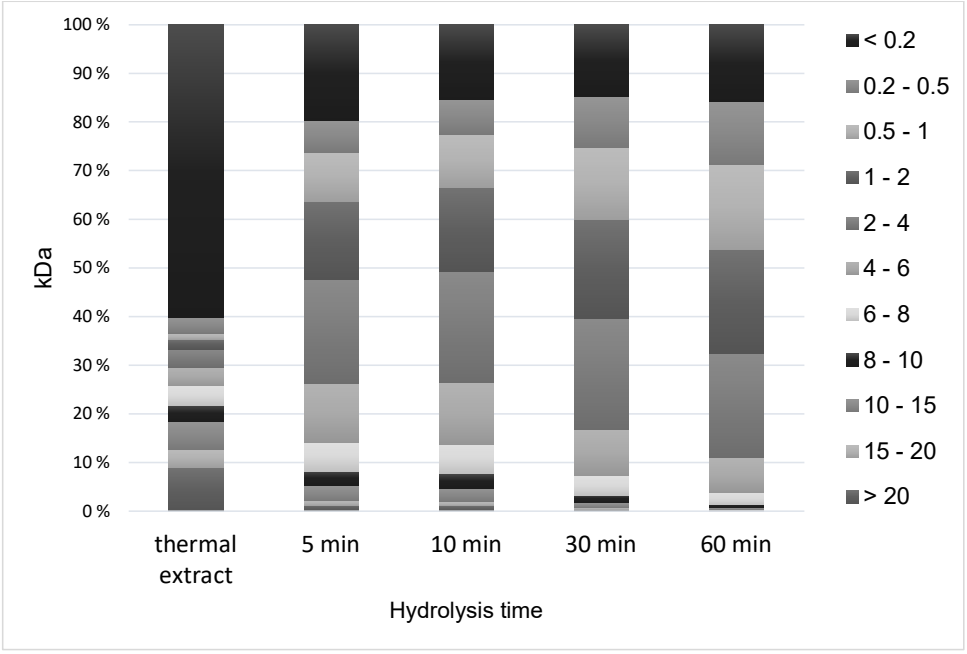

Figure 3. The molecular weight distribution (MWD) as an effect of hydrolysis time determined by size exclusion chromatography. The presented example is based on data from Paper II and shows the MWD of direct thermal extract and FoodPro PNL hydrolysates of cod backbones.

2.2 The raw materials

For inclusion of protein hydrolysates in a food product, the raw material must meet certain quality criteria. Although most residuals from the fish and meat filleting industry are highly nutritious, not all are considered food grade. When the raw materials do have food grade quality and are handled as such, they may be used to processing of food products. Raw material considered of non-food quality or the processing and handling of the raw materials do not meet food grade criteria, they are defined as animal by-products (ABPs) according to EU regulation No. 1069/2009. Products based on ABPs can not be used for human consumption but may have

potential for feed applications depending on risk category. Category 3 APBs, originating from slaughterhouses, fisheries, etc., may be used for pet and animal feed, whereas category 1 (e. g. pets and zoo animals) and 2 (e. g. carcasses from dead livestock) are considered high risk products.

Fish and meat processing residuals contain high levels of proteins, fat, and bones, all of which may influence a hydrolysis process and final product quality. The protein contents and amino acid composition of the raw material determines the nutritional properties of the protein hydrolysate, protease cleavage sites and accessibility (Archer *et al.* 1973, Wubshet *et al.* 2019). The structure of a folded protein may block the accessibility of proteases to specific peptide-bonds, depending on type of protein. The two main food protein structures are globular and fibrous, of which the latter type has generally less impact on reaction rate (Adler-Nissen 1986). Muscles of animals and fish are very similar on a cellular level, where the fibres are mostly composed of the same amino acid sequences independent of specie. The main variations between sea and land animals are related to strength of connective tissue, muscle function based on their ability to thermoregulate, and the structural arrangement. 25-30% of proteins in the muscle cell are readily soluble sarcoplasmic, globular, proteins (Foegeding *et al.* 1996). Variations in the type of proteins may constitute a difference in enzyme accessible proteins at reaction initiation, which will likely affect product MWD.

The protein fraction of a food source is typically calculated by measuring the nitrogen contents (N) and multiplying by a conversion factor based on the assumption of nitrogen in the protein. Historically, a nitrogen-to-protein conversion factor of 6.25 has been applied, which estimates the wt% of N in protein to be 16% (Mariotti *et al.* 2008, Sriperm *et al.* 2011), despite the fact that Jones (1931) explicitly showed that nitrogen contents varied considerably among different sources of pure protein. The variation is mainly due to varying amino acid composition, as amino acids have different number of nitrogen atoms and molecular mass, and to variations in non-protein nitrogenous compounds, such as nucleic acids and ammonia. In Paper I, conversion factors for cod, salmon and chicken were determined to be 5.3, 5.2, and 5.3 respectively, which differ considerably from the factor of 6.25. Correct determination of protein concentration is

imperative for calculation of enzyme:substrate ratio, particularly when comparing effects of process factors. Thus, substrate specific N to protein conversion factors should be determined prior to hydrolysis of new raw materials.

The lipid content may influence both the hydrolysis process and the product. The lipids, particularly poly-unsaturated ones, are susceptible to oxidation and are a major potential source of quality deterioration (Ladikos & Lougovois 1990). The removal of remnant lipids from fish hydrolysates have been found to reduce both fishy odour and give a product of lighter colour (Hoyle & Merritt 1994), both of which are important factors in regard to consumer acceptance. In addition, the proximate composition of bones from various fish species varies (Toppe *et al.* 2007), which is likely to influence the content of ash, collagen protein, and lipids in the hydrolysates. For wild-caught fish, proximate composition may be influenced by seasonal variations (Aubourg *et al.* 2005, Jafarpour *et al.* 2020), and in agri- and aquaculture based raw material, the feed regime affects the nutritional value of the end-product (Berge *et al.* 2004, Wubshet *et al.* 2018).

Microbial proliferation is a major cause for potential spoilage for all fish and meat products. Some products of microbial activity can be hazardous, such as biogenic amines formed by bacterial decarboxylation of amino acids. Histamine, putrescine, and cadaverine are important biogenic amines, particularly in seafood. The compounds are derived from histidine, ornithine, and lysine, respectively (Biji *et al.* 2016). Different fish raw materials have varying tendency to produce these compounds, and mackerel species have been tied to toxic levels of histamine (Sone *et al.* 2019). Water-soluble metabolites in the raw material will be a part of the final hydrolysate. Some of these are vulnerable to autolytic or microbial degradation. The degradation products of trimethylamine oxide (TMAO), namely trimethylamine (TMA) and dimethylamine (DMA), have considerable negative effects on sensory properties of fish hydrolysates. Paper IV demonstrated that levels of biogenic- and volatile amines not only vary between different raw material species, but also between fractions (heads, backbones, viscera) within the species. To keep levels of deterioration products within acceptable limits, food-grade post-harvest handling of the raw materials is key for obtaining

consumable protein hydrolysates. It is crucial to minimize the levels of potential hazardous components, and unpalatable flavours, when the hydrolysates are intended for human consumption.

2.3 Proteases

The enzymes used for protein hydrolysis belong to the family of proteases. There is an increasing availability of commercial food grade proteases (Bio-Catalyst 2015). The choice of protease will influence both process costs and sensory- and physicochemical properties of the final hydrolysate (Kristinsson & Rasco 2000b, Himonides *et al.* 2011, Aspevik *et al.* 2016a). The hydrolysis reaction can be catalyzed by both endo- and exopeptidases. The former cleaves peptides somewhere within the chain of amino acids, leading to smaller peptides, whereas the latter will cleave at one of the terminal ends, resulting in a peptide and a free amino acid (Barrett 2001).

The important features of proteases are their hydrolytic activity and specificity, which together determine the hydrolytic efficiency of the protease. The specificity determines where the enzymes will cut the peptide bonds within the protein, directed by the amino-acid sequence, as illustrated in Figure 4 for trypsin and pepsin. The extent of the enzyme specificity is also a source of variation. Some proteases are less specific in their cleavage site, resulting in a hydrolysate with a larger variation in the peptide population. The activity of the protease determines the reaction rate of the protease and is influenced by extrinsic factors such as pH and temperature. Enzymes are classified and given a European commission (EC) number based on the reactions they catalyze (NC-IUBMB 1992). Proteases are classified by the numbers 3 (hydrolases), 4 (proteases), and 11-19 (exopeptidases) or 21-99 (endopeptidases) (Brenda 2017). Although enzymes with the same EC number are expected to have the same activity, this is not always the case. The purity of the commercial enzyme products may vary, and traces of other enzymes may be present (Aspevik *et al.* 2016a).

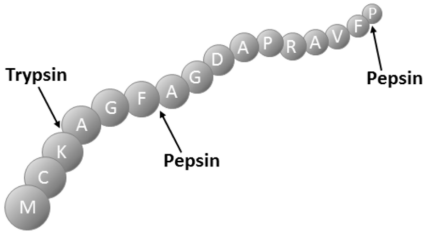

Figure 4. An illustration of the difference in cleaving sites with the use of two different proteases, trypsin and pepsin, on a peptide segment as determined by the PeptideCutter software (NCBI). The different letters are shorthand forms of the different amino acids present in the peptide chain

In the majority of available research, hydrolysis is performed on one type of raw material and enzymes are compared at equal enzyme-to-substrate ratio, not based on the specific activity of the enzymes. This will result in products of different degrees of hydrolysis and MWD, which greatly influence the sensory and physicochemical properties of the final products (Kristinsson & Rasco 2000b, Aspevik *et al*. 2016b).

2.4 Downstream processing

Downstream processing can be defined as every process post hydrolysis, or, as in the case of this work, additional processes after the initial, traditional, phase separation where solids and lipids are removed from the water phase by coarse sieving and centrifugation. The crude hydrolysate can be subjected to a variety of downstream processing techniques including filtration steps, evaporation, drying, and stabilization of the hydrolysate. Evaporation is commonly used to reduce the water content prior to drying. This decreases volume to a manageable size, reduce the cost of a potential drying step and may enhance the stability of the product given sufficiently low water content (Petrova *et al.* 2018).

2.4.1 Filtration technology

Filtration technology is commonly applied in food and biotechnology industries to purify, fractionate, desalt, and concentrate the product (Bourseau *et al.* 2009). This method facilitates a physical separation of components based on molecular properties,

such as size. The hydrolysate is filtered through a membrane that acts as a barrier, and the material (ceramic or polymeric) and molecular weight cut-offs (MWCO) can be selected based on desired product composition. The process results in a permeate, the filtered fraction, and a retentate consisting of all components retained by the membrane (Figure 5).

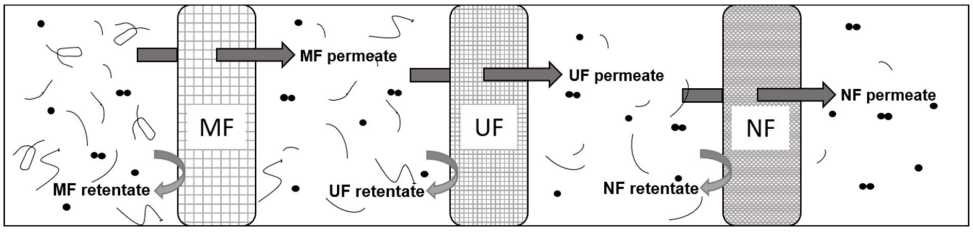

Figure 5. Illustration of a filtration cascade where high molecular weight molecules and suspended particles are retained by microfiltration (MF). Ultrafiltration (UF) membranes have a wide selection of cut-offs for fractionation of desired molecules. Nanofiltration (NF) retains all but the smallest solutes and ions.

Cross flow filtration (CFF), or tangential flow filtration (6), is preferred to dead-end filtration when working with protein hydrolysates. In the latter method, formation of a filter cake on the retentate side of the membrane will cause a decrease in permeation, and eventually lead to complete blockage. In CFF, a transmembrane pressure causes solutes to cross the membrane as the filtration feed flows by. The retentate may be circulated back to the feed tank, as depicted in Figure 6, facilitating increased concentration factor, or collected in a separate container. The process can be continuous, where feed is added throughout the process, or batchwise.

Microfiltration (MF), usually ranging between 0.1 to 100 µm in pore size, can be used to purify the hydrolysate after the initial phase separation where the majority of lipids and solids are removed (Castro-Munoz & Fila 2018). In the case of protein hydrolysates, cut-offs ≤ 0.3 µm have been reported for the removal of suspended solids (Beaulieu *et al.* 2009). MF may also function as a measure to reduce the number of bacterial colony forming units (CFU). Ultrafiltration (UF) membranes facilitate fractionation of hydrolysate peptides around 350 to 1 kDa. This may be relevant in cases where size-specific peptide populations are of interest (Picot *et al.* 2010).

Permeates from MF or UF can be further purified and concentrated by nanofiltration (NF; Figure 5). With a cut-off range around 120 to 2000 Da, free amino acids, small peptides, metabolites, and/or monovalent salts, permeate the membrane along with water, leaving a retentate of higher peptide concentration.

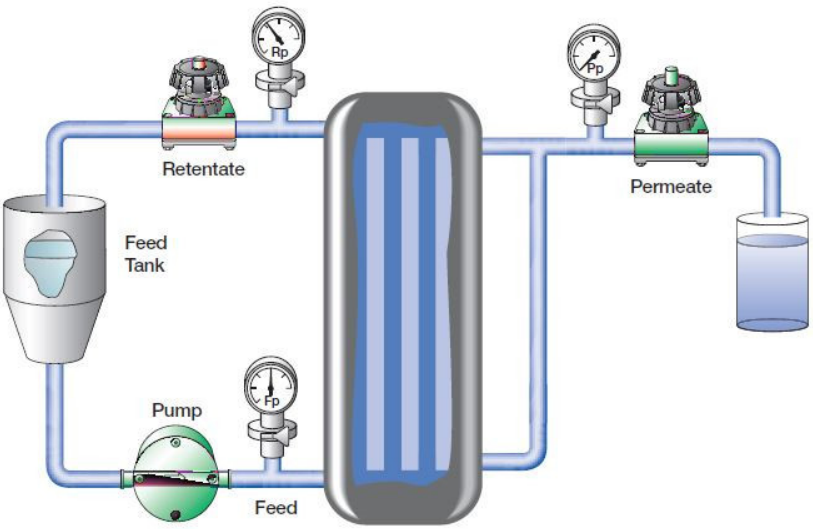

Figure 6. Illustration of a typical cross flow filtration where the retentate is recirculated into the feed tank (Pall Centramate user guide).

After filtration of a protein hydrolysate, both permeate and retentate will have nutritional value and potential for various applications, depending on the fraction properties. The retentate of MF consists of high MW molecules and the insoluble fraction in the filtration feed. These are likely to have physicochemical properties beneficial in food formulations, such as emulsion capabilities (see chapter 4), or potential for application within the feed industry (Wei *et al.* 2017). Challenges concerning this fraction may be potential microbes rejected by the membrane, and the suspended solids of low solubility. The NF permeate will usually contain very little dry matter which would be impractical to collect. The amount of nutrients in the permeate should be minimized to reduce product loss, but the reduction in salt content is important for the nutritional value. Depending on MWCO, free amino acids and

potentially small peptides may be filtered through along with the monovalent salts resulting in product loss. To achieve an economical large-scale commercial production line, optimization studies should be performed to get as high PR as possible. Membrane technology is an advantageous processing step for valorization of residual raw material as it enables product modifications to suit the intended application, though its use must be balanced against optimizing protein recovery.

3. Sensory properties

Sensory evaluation is the measurement of human responses perceived through the senses of sight, smell, taste, touch, and hearing. In the case of food products, perception of smell, tastes and, flavours are the dominating attributes for assessment of sensory properties. Sensory evaluation of a food product can be determined by a simple test of discriminating between two or more products, a form of hedonic consumer test or a full unbiased descriptive analysis of sensory attributes relevant to the products in question (Lawless & Heymann 2010d). Basic tastes and flavours are differentiated by sensory mechanism, which can be a combination of taste, olfaction, and somatosensation (Simon *et al.* 2006).

3.1 Perception of flavours and basic tastes

The definition of what should be considered basic tastes, and the distinction between basic and primary taste are disputed by some (Beauchamp 2019), but five sensory attributes are generally recognized as the basic tastes and will be the definition used for this thesis. The basic tastes are sweet, sour (acidic), salt, bitter, and umami (Simon *et al.* 2006). The definition of taste, or gustation, is that its perception is evoked by a physiological chemosensory response to a specific molecule or ion binding to a taste receptor cell (TRC; Figure 7). Several of these cells are located in each taste bud within the papilla of the oral cavity. The nature of how humans perceive tastes correlates with the nutritional value of the component. Bitterness and sour taste may indicate toxicity or spoiled food. Bitter taste is elicited by a vast variety of compounds, peptides being just one example, while sour taste is stimulated by acids. Salty taste is a response of mainly sodium, but also other minerals, important for maintaining bodily functions. Sweet taste, as a response to sugars, suggests a presence of carbohydrates. Umami taste, also called meaty taste, is the newest recognized basic taste, with the specific stimulants being sodium glutamate and inosine compounds (Bachmanov & Beauchamp 2007, Roper 2007). Bitter, sweet, and umami tastes are induced when the appropriate compounds bind to the G-protein coupled receptors T1Rs (sweet and umami) and T2Rs

(bitter). Upon binding to the receptor, a cascade of signal transductions occurs (Figure 7b), evoking taste perception.

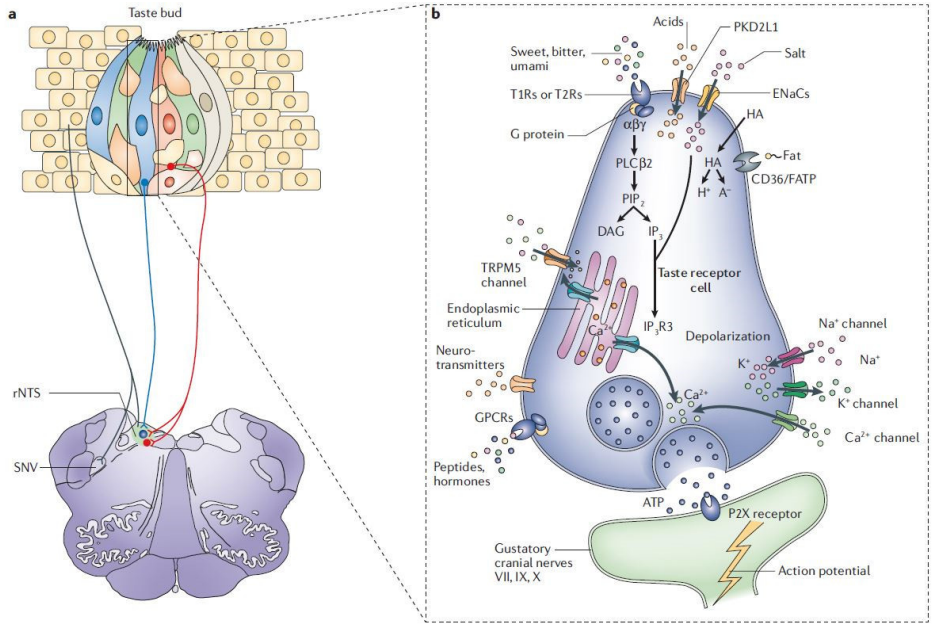

Figure 7. a) Illustration of a taste bud and receptor cells with neurons. Different colours indicate different types of cells **b)** a generic taste response cell (TRC) showing the different signal transductions by the various taste stimuli. Reprinted from Simon *et al*. (2006) with permission of Springer Nature.

Salty taste is the least understood basic taste, but in general it is induced by cation (and anions to a lesser extent) permeating through channels on the TRC, where sodium (Na^+) has its own dedicated response cells (Roper 2007, Nomura *et al.* 2020). Sour taste is mediated by a proton-selective channel (Zhang *et al.* 2019). In general, the basic tastes can all be detected simultaneously due to their individual ways of signal transduction. However, it is possible that a strong attribute intensity of one may influence, or mask, the perception of another.

Flavour can be defined as a combination of taste and odour/smell. Olfactory receptors located in the nasal cavity are stimulated by volatile compounds released from food in the mouth. The receptors are true nerve cells with G-protein coupled receptors, and each odour has its own type of cell (Lawless & Heymann 2010b). Texture, appearance,

and expectations can also influence flavour perception (Stevenson 2012, Spence 2017), and together with the effect of all potential compounds leading to a signal transduction, gives a vast foundation for consumer acceptance or rejection of foods.

3.2 Assessment of sensory properties

Traditionally, sensory properties are assessed by human responses. An emerging trend is the use of analytical methods for detection of chemical compounds and extrapolating sensory attribute intensity based on modelled compound-attribute association. This chapter will give an overview of a generic descriptive analysis as a method for objective attribute assessment based on sensory panels and some emerging alternative techniques for sensory profiling.

3.2.1 Sensory profiling by human senses

When aiming for objective assessment of sensory attributes, it is necessary to use a highly trained sensory panel. The amount of training may vary depending on the type of method and products involved, but a highly trained expert panel requires continuous training to maintain necessary assessment standards. A panel will often consist of 8-12 assessors guided by a panel leader (Lawless & Heymann 2010c).

There is a vast array of methods for attribute assessment, but one highly informative method is quantitative descriptive analysis (QDA). A QDA provides detailed information of the product(s) in question. Relevant sensory attributes included in an analysis is determined in advance. The attributes are by no means arbitrary, as vocabulary consensus between panellists and avoidance of ambiguous terminology is important for generation of quality results. In addition, if a relevant attribute is omitted from the analysis it may cause displacement in intensity of another attribute. Once attributes have been determined, the assessors should be calibrated to the same intensity scale. The test should be performed in a controlled environment (light, temperature) with randomized sample serving and an unstructured line for attribute intensity scaling (Lawless & Heymann 2010a). Analysis of variance (ANOVA), and some multivariate data analysis methods, are commonly applied to evaluate the individual attributes and

sensory profile results (Lea *et al.* 1997). Although QDA can provide information on the intensity of many product attributes, and the differences between products analysed in the same experiment, some limitations exist. Comparing results between different experiments, panels, and laboratories are difficult and should be done with caution (Murray *et al.* 2001). In general, only comparisons of the relative differences are possible.

There is a limit to the number of samples that a sensory panel can assess in one experiment, as the human palate is easily fatigued. To circumvent this, and to facilitate a rapid and reproducible method, new analytical methods for sensory profiling are increasingly used (Hatzakis 2019, Zaukuu *et al.* 2019). The methods ascertain attribute intensity based on determined chemical compounds in the product (Stroble *et al.* 2009).

3.2.2 Sensory profiling by NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy can be used to evaluate quality parameters, nutritional aspects, metabolomic fingerprinting etc. The method has also been tested for sensory profiling (Hatzakis 2019). ^{1}H NMR spectroscopy of food products can detect and quantify many components with high accuracy and reproducibility, without the need for separation or purification prior to analysis (Hatzakis 2019). Protons gives different signals depending on the ambient molecular environment. In short, NMR uses the nuclear spin to ascertain information on the atom's placement within a molecule. When irradiating the nuclei with electromagnetic waves, energy transitions where energy absorption is the dominating one, signals are created (Friebolin 2011). Changes in the spectra can be compared to differences in attribute intensity determined by a sensory panel, which provides the basis for prediction models. This methodology, sometimes termed "magnetic tongue", has been attempted for canned tomatoes (Malmendal *et al.* 2011), olive oil (Lauri *et al.* 2013), and coffee bean extracts (Wei *et al.* 2014). All the studies were able to provide prediction models and define sensory descriptors for several of the assessed attributes, but not all. The use of NMR spectroscopy in sensory profiling provides a rapid and reproducible method for determining or predicting many sensory attributes important for consumer acceptance of hydrolysates. There are limitations to the methodology,

some dependent on the type of product, others related to the type and number of components in the type of product. In more conventional NMR techniques, only the soluble fraction of the samples can be measured. In the case of the canned tomatoes, this implies a sample variation between those presented to the sensory panel and those analysed by NMR. In addition, it is possible that some compounds are not detected or impossible to assign in the spectra due to overlapping peaks, weak signals, or poor signal to noise ratio.

The predictive power of NMR data is possible through applying multivariate data handling, which enables a unique method for evaluating the changes in hydrolysate composition with the concomitant changes in sensory profiles. Pre-processing of NMR data prior to multivariate data analysis should also be considered with care. Both scaling and normalization influence the information extracted from the data (Craig *et al.* 2006). With unit variance scaling (autoscaling) the compounds will become equally important and is a good method for comparing correlations, but information on covariance will be lost. Pareto scaling reduces the relative importance of compounds giving considerably higher signals than others, but the original measurement is fairly well maintained (van den Berg *et al.* 2006), which is necessary when elucidating covariation between NMR spectra and sensory data. Normalization can be used to eliminate variations due to dilution effects (Craig *et al.* 2006). However, in sensory profiling considerations as to whether the dilution effect is desired or not, should be made. For example, if a sample is diluted to certain concentrations when presented to the sensory panel, it may be that the same dilution is desired in the spectra, for comparative reasons.

3.2.3 Electrochemical methods

When addressing alternative methods for sensory assessment of foods, the electronic nose (e-nose) and electronic tongue (e-tongue) should be mentioned. These methods are emerging as new tools in quality and sensory analysis (Zaukuu *et al.* 2019). They aim to mimic the human senses through methods of signal transduction as a response to a chemical detected through changes in current or voltage (Reis de Araujo *et al.* 2017). The E-tongue method has been found to correlate well with a sensory panel in

determination of bitterness in dairy protein hydrolysates (Newman *et al.* 2014), and said to have determined a variety of attributes in fish meat (Mabuchi *et al.* 2019), although this was not correlated with a sensory panel. As with the NMR method, the interpretation of responses to the signals must initially be determined through appropriate modelling. Although both methods may give rapid, reliable, and cost-efficient analyses, they cannot fully replace a human sensory panel.

3.3 Tastes and flavours of protein hydrolysates

Protein hydrolysates are basically a mixture of water-soluble peptides and compounds that all influence the sensory profile to some extent. The peptides have different MW and amino acid composition, depending on the enzyme applied for hydrolysis, while the remaining compounds, including metabolites, minerals, vitamins, nucleotides etc., are dependent on the type of raw material and its quality (Adler-Nissen 1986). This section will describe some hydrolysate attributes, their development and effect on the sensory profile.

Of all the hydrolysate's tastes and flavours, bitterness has by far been the attribute in focus (Aspevik *et al.* 2016b, Liu *et al.* 2016, Fu *et al.* 2019, Idowu & Benjakul 2019), as it has been considered a major limitation for human consumption of protein hydrolysates. Bitter taste is mainly ascribed to liberation of peptides containing hydrophobic amino acids (Asao *et al.* 1987, Kim & Li-Chan 2006). In a protein, the hydrophobic moieties tend to be folded within the protein structure, while the hydrophilic amino acids are in contact with the ambient environment. Upon proteolysis, the structures of the proteins are broken, and the peptides have limited, to none of the flexibility needed for folding, thus exposing the hydrophobic amino acids (Adler-Nissen 1984). The position of the hydrophobic amino acid within the peptide is not arbitrary for the contribution to bitterness. Ishibashi *et al.* (1988) indicated that a hydrophobic amino acid is needed as a binding unit for the peptide, and either a basic amino acid or another hydrophobic amino acid functions as a stimulation unit. However, Matoba & Hata (1972) proposed that hydrophobic amino acids in a peptide contribute to bitterness independent of the sequence, and that increased amount of the

amino acids heightens the bitter intensity. The MW of bitter-tasting peptides is somewhat disputed (Fu *et al.* 2019, Idowu & Benjakul 2019), but more recent studies indicate an association between the bitter attribute and peptides with MW 0.5 to 1 kDa (Aspevik *et al.* 2016b, Fu *et al.* 2018). Restricted hydrolysis and proper choice of enzyme may facilitate production of hydrolysates with low bitterness intensity. Restricted hydrolysis, however, will result in low PR and potentially poor raw material utilization.

Other basic tastes, and flavours, are generally not thought to be associated with any specific group of peptides, and mostly correlated to different metabolites. The existence of umami tasting peptides have been disputed (Tamura *et al.* 1989, Maehashi *et al.* 1999, Temussi 2012), but L-glutamic acid is often included, in the list of metabolites enhancing the umami perception (Farmer 1994). The sensory attributes not associated with peptides are mostly reliant on raw material, which is in agreement with results in Paper I, which also found umami intensity to be a function of the raw material (Maehashi *et al.* 1999). The same applies for most of the other sensory attributes. The concentration of flavour-eliciting metabolites in the hydrolysate is dependent on the composition of the raw material. The presence of some attributes is more self-explanatory than others, such as marine or fish flavour in fish protein hydrolysates. Many ascribe this attribute to TMA and DMA, however different fish species have varying contents of these metabolites (Pena-Pereira *et al.* 2010), and TMAO, from which TMA and DMA are derived. Despite this, the fish flavour may be similar, supporting the contribution of other metabolites such as alcohols and carbonyls (Josephson & Lindsay 1986). Though the formation of tastes and flavours in protein hydrolysates is complex and influenced by many factors, it is highly dependent on the ingoing raw material and hydrolysis parameters.

4. Surface-active properties of peptides

The food industry utilizes a variety of surface-active ingredients to improve product texture and appearance for processing purposes or to suit consumer preferences (Dickinson & Miller 2001, Kralova & Sjoblom 2009). However, with the recent demand for more natural additives (Ozturk & McClements 2016), the utilization of protein hydrolysates as functional ingredients becomes increasingly relevant. There are already many natural protein products applied in food formulations, such as milk and egg proteins, but new sources are in demand.

4.1 Protein hydrolysates as emulsifiers

Emulsions of two immiscible phases, commonly oil and water, are found in numerous food products. They can be in the form of oil-in-water (O/W) emulsions, where small droplets of lipids are homogenously distributed in a continuous aqueous media, or water-in-oil (W/O), where oil is the continuous media (McClements 2005). Such colloidal systems are thermodynamically unstable and require surface-active components for stabilization through reduction of surface-tension. Depending on the surface-activity of the emulsifier, the structures are susceptible to gravitational separation or flocculation, where particles collide without disrupting particle surfaces and may float to the uppermost layer of the aqueous phase, or precipitate. Droplet aggregations often lead to coalescence, in which droplets merge to form larger particles and may eventually end in full phase separation (Dickinson 1994, Wouters *et al.* 2016).

Proteins and peptides from fish and meat residues can function as emulsifiers given a certain balance of hydrophobic and hydrophilic moieties, amino acid sequence, flexibility, solubility (Figure 8), that are required for surface-activity (Damodaran 2005, Dexter & Middelberg 2008). The amphiphilicity of peptides, given by the balance of hydrophobic and hydrophilic amino acids, determines the capability of adsorbing to the interphase between the emulsified liquids (Damodaran 1996). The diffusion rate, the ability to move between different adsorption sites, is affected by the

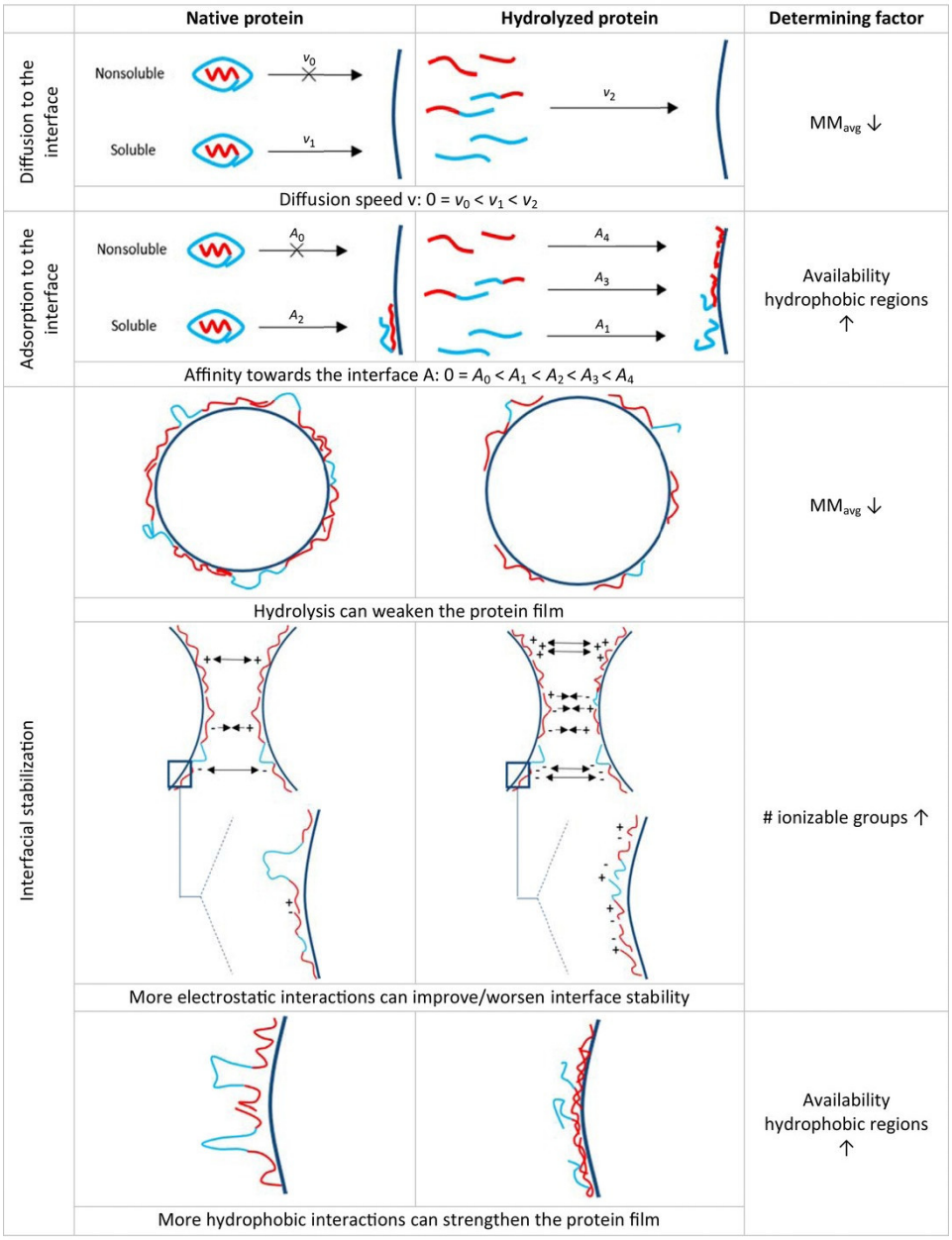

Figure 8. Illustration of how proteins and their hydrolysed moieties may diffuse and adsorb at an interphase, and the stabilizing effect thereof. Reprinted from Wouters *et al.* (2016) with permission of John Wiley & Sons, Inc.

molecular weight, where smaller peptides are more mobile than big bulky protein and peptides (Wouters *et al.* 2016). The stabilization of emulsions depends on the formation

of an elastic film on the droplet surface and electrostatic interactions that may facilitate droplet repulsion (Figure 8) (Dickinson 1994). The protein and peptide solubility, which is considerably increased by hydrolysis, is imperative for emulsifying capabilities. However, extensive hydrolysis creates peptides with low flexibility and unsuitable amino acid balance for emulsifying capabilities (Kristinsson & Rasco 2000c, van der Ven *et al.* 2001, Elavarasan *et al.* 2014, Schroder *et al.* 2017). Some studies have found restricted hydrolysis to give peptides with good emulsion capabilities (Liceaga-Gesualdo & Li-Chan 1999), and it has been suggested that peptides of at least 20 amino acids are necessary for good surface-activity (Lee *et al.* 1987). The correlation between MWD and emulsion capacity is, however, disputed (Kristinsson & Rasco 2000a, van der Ven *et al.* 2001).

There are many methods to evaluate emulsifier effectiveness, and there are several factors that will influence the outcome. Oil type, pH, ionic strength, and temperature all affect emulsions, as do ingredient interactions. If there is a specific type of food formulation, it is beneficial to imitate the specific conditions in the emulsion evaluation assay (McClements 2007). Emulsion activity index (EAI) measures the obtainable interfacial area of emulsion per unit weight of protein. It gives a measure of the emulsifier performance, rather than the property of the emulsion system, given by emulsion capacity (EC) methods (Pearce & Kinsella 1978). An emulsion stability index (ESI) can be calculated based on the reduction in EAI over a defined time period.

4.2 Critical micelle concentration

At a certain concentration, surface-active, amphiphilic compounds may start to self-associate and form micelles. In an aqueous solution the compounds will adhere to the interphase between water and air, lowering the surface-tension. When the interphase is covered and maximum reduction in surface-tension is reached, surplus surfactants will start to aggregate (Figure 9). This is known as the critical micelle concentration (CMC) or critical aggregation concentration (McClements 2016). The CMC indicates the minimum concentration of a product needed for maximum reduction of surface-tension (Soderman *et al.* 2004). A low CMC implies good surface activity, thus little product

is needed to obtain reduction in surface-tension. The ability of peptides to form micelles is associated to their surface-activity, which again is dependent on the amino acid sequence. Hence, the enzyme specificity and degree of hydrolysis is likely to influence the CMC value of hydrolysate peptides. Various methods can be used to assess the effect of surfactant concentration, such as fluorescence, conductivity, surface tension, and ^{1}H NMR spectroscopy (Al-Soufi *et al.* 2012, Liu *et al.* 2020). Aspevik *et al.* (2016b) showed that CMC of protein hydrolysates can be measured by NMR spectroscopy, and that a low degree of hydrolysis gave the lowest CMC-values, implying that restricted hydrolysis is essential for optimum surface-activity.

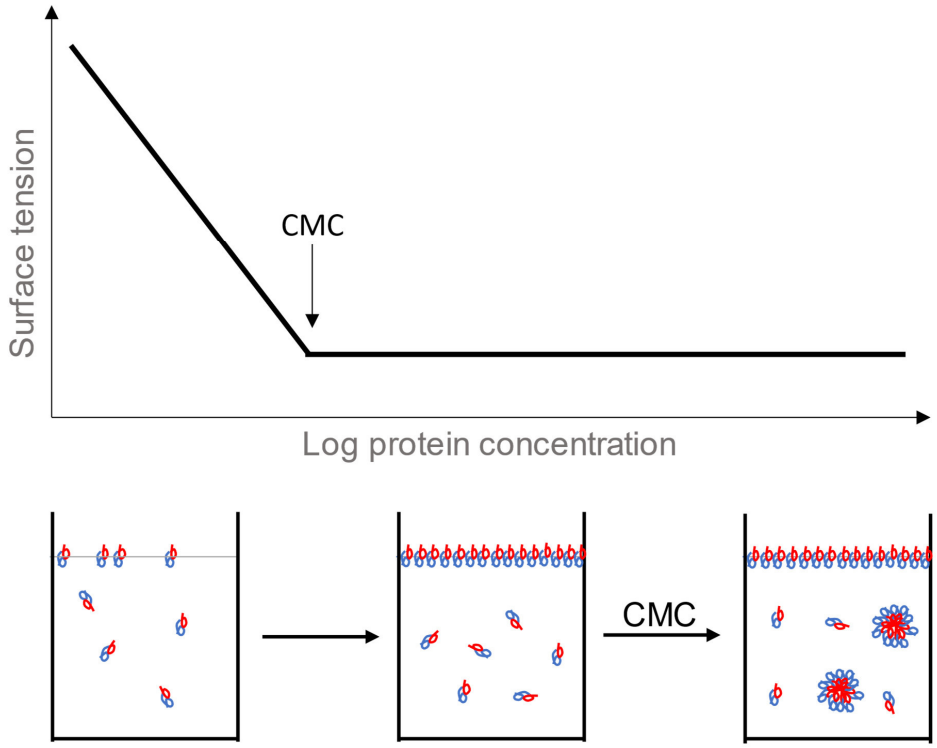

Figure 9. Illustration of the reduction in surface tension as an effect of increased log protein concentration. The amphiphilic peptides/proteins adhere to the interphase between air and water lowering the surface-tension, until the compounds start to self-associate upon maximum reduction in surface-tension, i.e., when the interphase is fully covered.

5. Protein hydrolysates in foods

Protein hydrolysates contain essential amino acids, reflected by the raw material source (Liaset & Espe 2008, Paper IV), and can with advantage be supplementary components in food formulations. The required MWD and amino acid composition will vary depending on its intended use. In the case of specialized nutritional support to consumers with certain ailments, such as restricted tolerance or restricted uptake of common protein sources, the requirements may be very specific. This is a fairly well-known application of protein hydrolysates and is mostly focused on more conventional proteinaceous residuals such as whey and casein (Clemente 2000). When considering utilization of residuals from the fish and meat filleting industry for food applications, the objective is often either as protein enrichment or as a functional ingredient. The increasing trend of preference for high protein diets, increased focus on the importance of protein in a health food regiment and sustainable food industries, may facilitate consumer acceptance of side stream materials in food products.

5.1 Considerations for food formulations

In addition to the nutritional benefits of protein hydrolysates in foods, there are other considerations to be made. Bioactivity of small peptides present in the hydrolysates, such inhibition of angiotensin-converting enzyme and diabetes type 2 related enzymes, are also well documented (Li-Chan 2015). Though not the scope of the current study, the bioactive properties may influence consumer acceptance in a positive manner, but any potential effect on the consumer should be extensively investigated to assert safety upon ingestion (Li-Chan 2015). Also, the levels of amino acids, both free and as part of peptides needs to be determined to ensure they are within recommended limits (Schaafsma 2009). The salt content is also an important factor, as high levels of dietary sodium is associated with high blood pressure related deceases (WHO 2012). In addition, any other compound with potential toxicological effects should be considered, such as the biogenic amines described in section 2.2. High levels of histamine ingestion can cause food poisoning like symptoms and respiratory

difficulties (Lehane & Olley 2000), while putrescine and cadaverine are potentiators for the toxicity of histamine (Hernández-Jover *et al.* 1997).

The functional properties of the products (section 4) can be exploited, but they can also complicate food formulation. Protein hydrolysates contain considerable amounts of small peptides and free amino acids (< 0.5 kDa), which have plasticization effects that may alter the mechanical properties through changing the glass transition temperature of any formulated product (Oterhals & Samuelsen 2015). The use of protein hydrolysates as food ingredients may thus influence the physical and rheological properties (Franco-Miranda *et al.* 2017, Ahmad *et al.* 2019), making extensive formulation studies necessary. A major challenge resides in obtaining a standardized commercial large-scale production of hydrolysates. The raw material composition may be inconsistent, causing batchwise variations in the hydrolysate. In addition, the processing costs need must be commensurate with the value of the final product, whether it being in the form of a nutraceutical or a food product.

5.2 Commercial products

Despite the challenges associated with production and commercializing of protein hydrolysates, there are several such products available based on marine raw materials, most of which are sold as nutraceuticals (Hayes 2019). A fillet hydrolysate from Pacific whiting (Seacure®) claims to have positive intestinal health effects, and several fish protein hydrolysates of undefined species are sold as antidepressants (Gabolysat PC 60®, Stabilium®, Procalm®). Hydrolysates based on collagen are generally sold as bone and skin supplements, while others, such as Velyron® have ACE-1 inhibitory properties. There is also a newly EFSA approved blood pressure reducing supplement based on shrimp shell hydrolysate (PreCardix®), with a tripeptide as the active ingredient, from Norway. Products based on Atlantic salmon, Amizate® (Nesse *et al.* 2014), ProGo®, and Biomega® peptides are marketed as a dietary supplements. The mentioned products are supplied as tablets, capsules, or powder, but it does imply consumer acceptance for food formulations with protein hydrolysates.

6. Experimental and analytical approaches

6.1 Protein hydrolysate production

The hydrolysis reactions in this study were performed both in lab- (Papers I and II) and pilot scale facilities (Papers III and IV), but the hydrolysis parameters were generally the same in all the studies (Table 1). Bromelain (EC 3.4.22.32, Enzybel, Waterloo, Belgium) and FoodPro PNL (EC 3.4.24.28, DuPont, Wilmington, DE) were used at approximately the same enzyme to protein ratio (10 U / g protein). Enzyme activity was determined by a nonspecific protease activity assay based on micromoles tyrosine equivalents released from casein per min (Cupp-Enyard 2008). FoodPro PNL and Bromelain were chosen as the former has previously been found to be cost effective and resulting in hydrolysates of relatively low bitterness (Aspevik *et al.* 2016a), and the latter to be efficient in hydrolysis of connective tissue and has broad specificity (BRENDA 2019).

For the most part, different parts of fish were used as raw materials (Table 1). In Paper I chicken was included to provide more product variation in the study aiming to elucidate metabolite-attribute associations. This was also the only study using fillets to reduce variation within sample groups of the same raw material. In the other studies different fish residual raw material fractions were used. All the species used in the presented studies are relevant in regard to residual raw material utilization.

Table 1. Overview of hydrolysis parameters in the different studies

Paper	***Specie***	***Fraction***	***Enzyme***	***Temp. °C***	***Time (min)***
I	Cod (*Gadus mohua*), salmon (*Salmo salar*), chicken (*Gallus gallus*)	Fillets	FoodPro PNL, Bromelain	50	10, 50
II	Cod (*Gadus morhua*), salmon (*Salmo salar*)	Backbones	FoodPro PNL, Bromelain	50	5, 10, 30, 60
III	Cod (*Gadus morhua*), salmon (*Salmo salar*)	Heads, Backbones (mixted)	FoodPro PNL, Bromelain	50	50
IV	Salmon (*Salmo salar*), mackerel (*Scomber scombrus*)	Heads, Backbones, Viscera (separate)	FoodPro PNL	55	50

The raw materials were milled in a kitchen grinder (aperture 4 mm, Electrolux AKM 3110 W, Stockholm Sweden; Paper I) or in a Comitrol 1700 Processor (Urchel laboratories Inc., Valparaiso, IN; Paper II, III and IV). The hydrolysis reaction in Paper I was conducted in a modified R10 Bear Varimixer (A/S Wodchow & Co., Brøndby) and in Paper II a Distec Model 2500 Dissolution System (Distek Inc., North Brunswick, NJ) was used. For Papers III and IV, a 200 l jacketed stirred tank reactor was used. In all experiments a water-substrate ratio of 1:1 was applied. The mixture was heated to a predetermined reaction temperature (Table 1) under continuous stirring before adding the enzyme. The reaction was terminated by heating the slurry to > 90°C and holding that temperature for 10 min. In the lab scale experiments, centrifugation was applied for phase separation (Sorvall, LYNX 6000, Thermo scientific, Waltham, MA). This method provided a relatively clear water phase and a solid pellet. In the pilot scale experiments a 3-phase decanter centrifuge was used (Flottweg Tricanter Z23-3, Vilsbiburg, Germany), after coarse sieving to remove bone fragments. The apparatus gave a good separation of the lipid phase, but the water phase appeared muddier than in the lab scaled separation. The difference is likely a result of higher g-force and batchwise centrifugation in the laboratory compared to the continuous flow in the decanter. The higher levels of solids in the pilot scaled hydrolysate possibly affected the downstream membrane filtration by increasing membrane fouling.

6.1.1 Memebrane filtration

All products were filtered to remove suspended solids and residual lipids. For Paper I a vivaflow 200 crossflow cassette (Sartorius, Goettingen, Germany) with a MWCO of 100 kDa was used for hydrolysate purification. In Paper II a Centramate 500S Tangential Flow Filtration System (Pall, Port Washington, NY) was used, which had higher capacity compared to the system used in Paper I. In addition, a membrane of 0.1 µm was applied, likely reducing the rejection of peptides by the membrane. In the pilot scaled experiments a 0.1µm ceramic filter was applied in a filtration unit supplied by Membranteknikk (Flekkefjord, Norway). Common for all the filtration steps was a reduction in flow throughout the process due to fouling, thus no specific flow rate could be maintained.

6.2 Applied analytical methods

6.2.1 Chemical analyses

The methods presented in Table 2 were used to determine raw material and hydrolysate composition.

Table 2. Analytical methods used to determine the chemical properties of raw materials and products in the studies.

Paper	*Analytical method*	*Reference*
I, II, III, IV	Nitrogen (Kjeldahl)	ISO 5983-2
I, III, IV	Fat	Bligh and Dyer (1959)
I, III, IV	Ash	ISO 5984-2
I, III, IV	Dry matter	ISO 6496-2
I, II, III, IV	MWD	Wang-Andersen & Haugsgjerd (2011)
I, II, IV	Amino acid composition	Cohen & Michaud (1993)
I	Cystein (cystine)	Cohen & Michaud (1993)
I, II	Ammonia	Conway & Byrne (1933)
I	Tryptophan	Miller (1967)
III	Sodium	ISO 11885
IV	Free amino acids	Bidlingmeyer *et al.* (1987)
IV	TMA/TMAO	Conway & Byrne (1933)
IV	Biogenic amines	Mietz & Karmas (1978)

6.2.2 Sensory analysis

Sensory assessments were performed in the studies for Papers I, III, and IV. The hydrolysates were presented at 1% based either on protein contents (Paper I) or dry matter (Papers III and IV). The latter will give a dilution effect in regard to protein concentration, affecting peptide-associated sensory attributes. However, as most sensory attributes appeared to be mostly correlated to non-peptide metabolites (Paper I), dilution on dry matter basis may be a more representative method, unless bitterness is the focus of the study.

A highly trained panel of 8-10 assessors performed the descriptive analysis in accordance with Lawless and Heymann (2010a) and the ISO standard 13299 (2016) under the supervision of a panel leader. The assessors are regularly tested and trained in accordance with ISO standard 8586 (2012), and are experienced in profiling complex protein hydrolysates which provided the basis for attribute inclusion in the studies

(Table 3). The variation in the attributes used for the three studies reflect upon both product differences and increased panel experience. The assessors were calibrated on the attribute intensity scale in pre-test with two samples deemed high and low in generic intensity. For the descriptive analysis, 20 ml samples were served in plastic glasses at room temperature with a three-digit number in a full balances design. The attributes were evaluated on a 15 cm unstructured line at individual speed and directly recorded in a computer system (EyeQuestion, Software Logic8 BV, Utrecht, the Netherlands).

Table 3. Sensory attributes used in the studies and their descriptions.

Paper	***Attribute***	***Description***
I, III, IV	Total flavour intensity	Strength of all flavours in the sample
I, III, IV	Sweet taste	Basic sweet taste (sucrose)
I, III, IV	Salt taste	Basic salt taste (NaCl)
I, III, IV	Acidic (sour) taste	Basic sour taste
I, III, IV	Bitter taste	Basic bitter taste
I, III, IV	Umami taste	Basic umami taste
I	Acidic flavour	Related to fresh, balanced flavour from organic acids
I, III	Metallic flavour	Related to flavour of metal (ferrous metal)
I	Chicken flavour	Related to flavour of chicken meat
I	Swine flavour	Related to flavour of swine/pork meat
I, III	Sea/Marine flavour	Related to flavour of fresh, salty sea
I, III, IV	Fish flavour	Flavour of boiled white fish
I, IV	Cloying flavour	Non-fresh, nauseating flavour
I, III, IV	Rancid flavour	All rancid flavours (grass, hay, stearin, paint)
I, III	Fullness	Textural properties related to flow resistance
I, III, IV	Astringent	Related to complex feeling of contraction and dryness
I, III, IV	Fatness	Surface textural property related to perception of fat
III, IV	TMA flavour	The flavour of trimethylamine
III, IV	Flavourless	Describes a product which has no flavour
IV	Shellfish flavour	Flavour of shellfish and shrimp
IV	Burned flavour	Processed and burned flavour

6.2.3 Metabolite composition by NMR

The NMR spectroscopy for Papers I and III was conducted on samples of the same dilution as presented to the sensory panel. Any variation in metabolite dilution between samples would be the same for the NMR spectra as for the sensory panel. In Paper III, where the aim was to evaluate the effect of nanofiltration, the relative composition of all components was of interest. As the resonance frequency is pH dependent to varying

extent, the samples were diluted a sodium phosphate buffer to a final concentration of 100 mM and pH of 6.8. The pH was chosen based on its proximity to the value of most of the hydrolysates. A final concentration of 10% D_2O was used, which is common in NMR metabolomics. The spectra were acquired at 300 K using a Bruker AVANCE NEO ultrashielded 600 MHz spectrometer with a cryoprobe (Karlsruhe, Germany). In Paper I, both 1D ^{1}H NOESY, ^{1}H CMPG and ^{1}H-^{13}C HSQC data were acquired. The latter shows direct coupling between carbon and hydrogen and was used primarily for peak identification. 1D NOESY with pre-saturation are used for water suppression and was used for further elucidation on attribute prediction capability of the methodology and determination of metabolite-attribute associations. CPMG minimizes the signal of larger molecules, but the difference between these spectra and the 1D NOESY spectra were minimal, thus the latter was chosen for further data handling. Only 1D NOESY spectra were acquired for Paper III. Details on specific acquisition parameters and spectra processing methods can be found in the respective papers.

The spectra were processed in Topspin (v.4.0, Bruker Biospin, Karlsruhe, Germany). For Paper I data processing, the spectra were exported and pareto scaled in Microsoft Excel (v. 2013) prior to multivariate analyses in Unscrambler (v. 10.4.1, Camo, Oslo, Norway). Principal component analysis (PCA) showed associations between the hydrolysates in the study, and partial least squares regression (PLSR) were used to create prediction models for sensory attribute intensity based on the NMR data. The covariance ($p \leq 0.05$) of NMR spectra and sensory attributes were evaluated using the predicted attribute scores (MATLAB R2018b, The Mathworks, Inc. Natick, MA).

The assigned peaks in Paper III were quantified with the following formula using dimethyl sulfone ($DMSO_2$) as an external reference standard:

$$Metabolite\ concentration\ \left(\frac{g}{l}\right) = \frac{\frac{A_{peak}}{A_{ref}}}{\#H_{met}} \times M_{ref} \times \frac{g}{mol_{met}} \quad (2)$$

Where met is the metabolite in question, A is the total peak area, ref is the reference standard and #H is the number of protons eliciting the peak. The peak areas were

determined by deconvolution in MestReNova (v. 14.1.2-25024, Santiago de Compostela, Spain).

6.2.4 Critical micelle concentration

CMC was determined by NMR spectroscopy. The use of this method for protein hydrolysates was first presented by Aspevik *et al.* (2016b). As the peptides start to form micelles, there is a change in the chemical environment causing a shift in resonance frequencies relative to the internal standard (2,2-dimethyl-2-silapentane-5-sulfonate; DSS). In this study, CMC was estimated by observing the shift in CH_3 signal of lactate, visible around 1.3 ppm for all the products included in the study (Paper II).

Dilution series were produced for all hydrolysates with a final concentration of 100 mM sodium phosphate buffer (pH 6.5) to eliminate any potential effect of pH on the ^1H shift of lactate. A pre-study was performed to determine suitable dilution area to achieve a good basis for linear trend lines. The spectra were acquired using a water suppression pulse program (zgesgppe) on a Bruker AVANCE NEO ultrashielded 600 MHz spectrometer (Bruker Biospin, Karlsruhe, Germany). The chemical shift of lactate was plotted against log protein concentration as illustrated in Figure 10. Trend lines were fitted for the lag- and exponential phase, and intercept between these indicate the critical peptide micelle concentration.

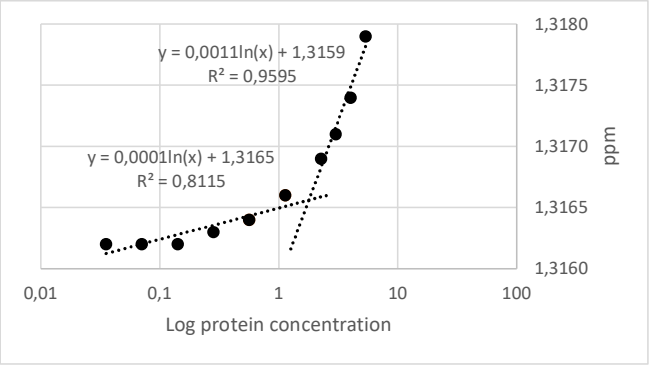

Figure 10. Illustration of the graphical determination of critical micelle concentration of protein hydrolysates by the intercept between trend lines.

6.2.5 Emulsion activity index

EAI was determined based on the method by Pearce & Kinsella (1978). The hydrolysates were diluted to 1% based on protein concentration. Below this concentration the homogenization method may have a higher effect on the results. The emulsions were made in triplicate with a 1:4 ratio of rapeseed oil and sample. Tween 20 was included as a control in each round to verify repeatability. The emulsified samples were collected immediately after homogenization and diluted 200-fold in 0.1% sodium dodecyl sulphate (SDS) for stabilizing purposes. Additional samples were collected after 10 and 30 min to evaluate the ESI. Casein was included in the study to compare the emulsifier capabilities of the hydrolysates with proteins known to have excellent physicochemical properties. A detailed description of the assay can be found in Paper II.

6.3 Statistical analysis

Several statistical methods were used for the presented studies. Analysis of variance (ANOVA) was used in all papers and a form of multivariate data analysis was applied in Papers I-III.

6.3.1 Analysis of Variance

The basic principle of ANOVA is to evaluate if the means of two or more data sets are equal. There are several statistical methods available for analysis of differences between sample groups, the simplest being one-way ANOVA (Lea *et al.* 1997). This is a test to check the probability of whether two or more means are equal or not in samples sets of usually only one variable and one response. In a two-way ANOVA the effect of two independent variables, and any interactions thereof, on a dependent variable (response) is determined. In Papers I, III, and IV a mixed effect model was used for the sensory data. It has been proposed that, while the products are fixed variables, the assessors should be seen as random representatives of the population, so that the conclusion can be extrapolated to the whole population (Naes & Langsrud 1998). The mixed model allows for a random independent variable to be set. The model can be written as:

$$\mathbf{Y_{ij} = P_i + A_j + PA_{ij} + \varepsilon_{ij}} \quad (3)$$

Where P is the effect of the product, A is the assessor effect, PA is the interaction between the fixed and random independent variables and ε is the error term. For Paper II, a general linear model was applied to assess the effect of hydrolysis parameters on emulsion properties, as all variables could be considered fixed.

The ANOVA determines if there are significant differences between the analysed samples but does not provide information on the source of the difference. Pairwise comparisons can be calculated by *post hoc* tests. For sensory data, Tukey's comparison is commonly used, as it is considered a conservative method for determining whether group means are significantly different.

In Paper I, a 50-50 multivariate ANOVA (MANOVA) was used to elucidate the influence of hydrolysis parameters on variation in metabolite composition. Such methods are useful when the number of responses far exceeds that of the observations. In spectroscopic data sets there are generally a vast number of responses (Langsrud 2002). In NMR spectra, small regions of chemical shifts make one response, adding to thousands of responses, depending on size of bins (summary of areas over defined spectral chemical shifts), if any. The method is based on PCA and handles multiple collinear responses and calculates overall sum-of-squares and p-values for each experimental factor. Familywise adjusted single response p-values were computed by rotation testing, being the more conservative choice compared to false discovery rate as a method for significance testing.

6.3.2 Principal Component Analysis

PCA is a multivariate method that reduces a dataset by removing "noise", whilst maintaining the informative variation. This is accomplished by decomposing the original data matrix with *n* objects (the observations) and *p* variables (the measurements) into two new matrixes of scores and loadings:

$$\mathbf{X = TP^T + E} \quad (4)$$

Where X is the original matrix, usually centred, T are the scores, P^T the loadings and E a residual matrix with all the variation excluded from the principal components (PCs), or the noise (Esbensen & Swarbrick 2018). The PCs are thus the product of TP^T. The highest level of object variation will be explained by PC1, which is the best linear fit for all data points, but much information still resides in E_1. PC2 is extracted from E_1, and PC3 from E_2 and so forth to the *n*-1 possible PCs. This results in PCs of continuously lower level of variance explanation. To get an overview of the data, it is beneficial to plot the score values in a coordinate system based on the PCs containing most information. The scores, T, gives the coordinate values for each sample for the respective PC; t_1, t_2, etc. These score plots show the correlation between the samples, that is, similarities, dissimilarities, and groupings. The loadings, P, can be plotted in a similar fashion and provides information on the correlations between the variables, *p*. The loading values, illustrated by the distance from the centre in a two-dimensional plot, indicate the importance of the variable in explaining object variation. The two plots can sometimes be combined in a bi-plot that gives a full overview of scores and loading. For spectral data, however, it is more common to visualize the loadings by plotting each loading vector against the variables, such as each chemical shift in a NMR spectra, creating a linear graph-like plot (Isaksson & Næs 1996).

6.3.3 Partial Least Squares Regression

PLS can be seen as an extended form of combining two PCAs, but instead of focusing on object variance, it aims to explain the relationship between two sets of variables, X and Y(Grung 1996). The method tries to find the information in X that coincides with the variation in Y by using a linear multivariate method and is a good tool for analysis of data with a high number of X-variables with collinearity and noise (Wold *et al.* 2001). The X-variables are used as predictors of the response Y, and can be written as:

$$\mathbf{X} = \mathbf{TP^T} + \mathbf{E} \quad (5)$$

$$\mathbf{Y} = \mathbf{UC^T} + \mathbf{F} \quad (6)$$

Where X is the matrix of predictor variables, T is the score matrix, P the loading matrix and E the matrix of X-residuals. For Y, the matrix of response variables, U is the score matrix, and C the Y-weight matrix. F is the matrix of Y-residuals (Wold *et al.* 2001). In general, it has been found that the use of one Y-variable at the time (PLS1) will

provide the best prediction models. This was the chosen method for Paper I, but it is possible to include multiple Y-variables (PLS2) simultaneously (Esbensen & Swarbrick 2018). Calibration fit (root mean squared error; RMSEC) and predictive ability (root mean squared error of prediction/cross validation; RMSEP/RMSECV) are measurements for the PLSR uncertainty. The best validation principle is to use a test set to evaluate the predictive ability, but due to a relatively small sample set in Paper I, cross-validation (leave one out) and RMSECV was applied as an alternative.

7. Summary and discussion of the main results

The papers included in this thesis deals with elements important for the inclusion of enzymatic protein hydrolysates in food products. The aim was to address factors that should be considered and understood prior to hydrolysate inclusion in foods. Papers I and III address sensory properties and how they are influenced by metabolite composition. Paper I focus on the effect of hydrolysis parameters on sensory properties and metabolite-attribute associations, while Paper III concentrates on effects of industrially relevant membrane filtration on the metabolite composition, and thus sensory properties. Paper II deals with surface-activity of protein hydrolysates, primarily emulsion properties. Paper IV compares sensory and quality properties as an effect of different side stream material fractions.

7.1 Association of hydrolysate metabolites and sensory profiles (Paper I)

Sensory attributes of high intensity are considered limiting for the inclusion of enzymatic protein hydrolysates in foods. In the interest of producing taste neutral hydrolysates, it is beneficial to understand how the various attributes develop as an effect of process factors. The objective of this study was to assess the formation of sensory attributes as an effect of hydrolysis factors and associate the attribute intensity with metabolite composition as determined by NMR spectroscopy. The results were thought to aid in the evaluation of suitability of applying NMR metabolomics in predicting sensory profiles.

Muscle tissue from chicken (*Gallus gallus*), salmon (*Salmo salar*), and cod (*Gadus morhua*) were used as raw materials. Pure muscle tissue was applied to minimize unknown variables, and the raw material variation to provide a substantial spread in data. This facilitates elucidation of metabolite-attribute associations. In addition, all the species are important protein sources and generate substantial amounts of residual raw materials. The two endo-proteases applied in the study, Bromelain and FoodPro PNL, have different specificity, provoking sample variations in MWD within hydrolysate groups of the same raw material and hydrolysis time.

In total, 12 hydrolysates were produced. The spider plot in Figure 11 illustrates that all hydrolysates produced in this study, had relatively high total flavour intensity, and significant product differences were found for most sensory attributes. The variation could mostly be attributed to raw material variation, but for some, hydrolysis time and/or enzyme specificity were the critical factors. The strong influence of raw material may have dwarfed effects of the other factors, but for the purpose of elucidating metabolite effects this does not necessarily pose a problem.

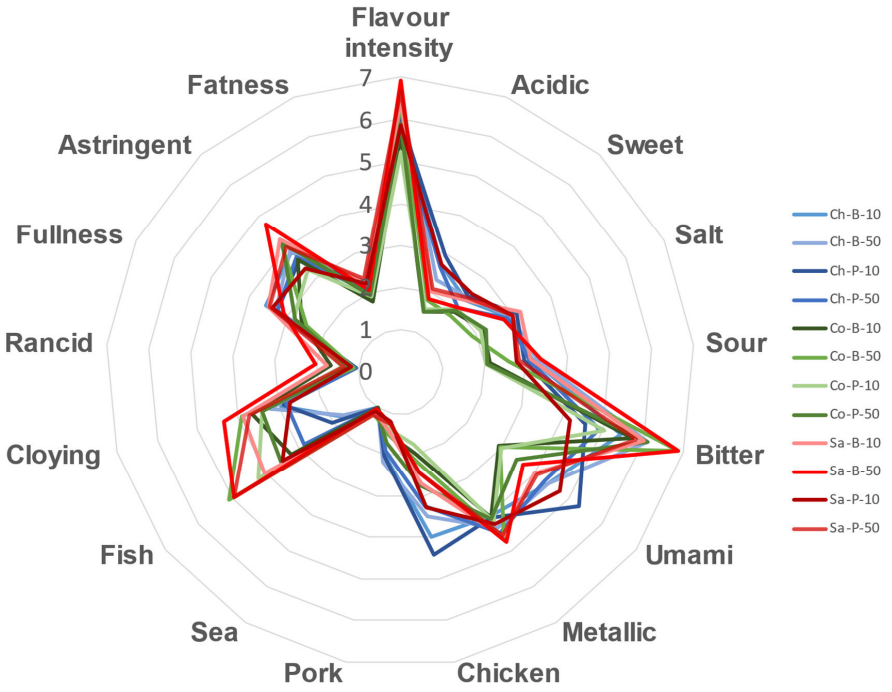

Figure 11. Spider plot showing sensory profiles of the enzymatic protein hydrolysates produced for Paper I. Muscle tissue of chicken (Ch), cod (Co) and salmon (Sa) were hydrolyzed with Bromelain (B) and FoodPro PNL (P) for 10 and 50 minutes.

The NMR spectra showed that the products were mainly composed of the same metabolites, but at varying amounts. MANOVA on the metabolite data revealed that 51% of variation was due to raw material, while 17 and 13% were dependent on enzyme and hydrolysis time.

PLSR showed that the variation in metabolites coincided with variation in sensory attribute intensity. Eleven of the 17 evaluated attributes could be modelled with the

metabolomic data, giving acceptable prediction ability based on cross-validation (Q^2 = 0.55-0.89). The calibrated PLS values were used to compute spectral areas significantly correlated ($p < 0.05$) to changes in attribute intensity. We found several metabolite associations for all modelled attributes. Some attributes were, however, found to associate with the same combination of metabolites. This is likely affected by the strong positive correlation of the attributes in question, causing similar PLSR loadings and thus corresponding to variations in the same spectral areas. Undetected or unassigned components could be the differentiating factor.

Bitterness was, as expected, positively associated with some hydrophobic amino acids. A decrease in lactate and inosine compounds caused an increase in bitter taste sensation. Fish flavour, which can be limiting for the application of FPH, could not be modelled. Neither TMA nor DMA explained attribute intensity variations. Albeit, other volatile components, such as carbonyls and alcohols, are known to contribute to fish flavour, but hydroxyl protons were difficult to observe in these overlapping NMR spectra.

7.2 Modification of emulsion and surface-active properties by protein hydrolysis (Paper II)

There are a considerable number of studies addressing physicochemical properties of protein hydrolysates. There is no consensus as to whether hydrolysis leads to improved protein functionality or not. Some studies have found that restricted hydrolysis improve functionality, and others indicate that any hydrolysis disrupt protein properties necessary for emulsification. However, the consensus is that a high degree of hydrolysis is detriment to the amphiphilic nature of peptides necessary for high surface-activity.

The aim of this study was to evaluate and compare functional properties (EAI, ESI, and CMC) of direct thermal extracts (stickwater) of cod and salmon backbones with hydrolysates of increasing hydrolysis time, and to evaluate the association between MWD, process yield and the functional properties. Two different enzymes (FoodPro PNL and Bromelain) and a time series of 5, 10, 30 and 60 minutes were used on both

raw materials to elucidate effects of hydrolysis parameters. In addition, the emulsion properties were compared to those of casein, as a reference to commercial emulsifiers.

The results showed that the properties of direct extracts and hydrolysates varied considerably, making the former outliers, or close to, in the PCA. To get an overview of the effect of hydrolysis parameters, the direct extracts were excluded from the model. The hydrolysate variation was mostly based on hydrolysis time, as apparent by the separation along PC1 (Figure 12), followed by raw material with the variation shown along PC2. The product separation was based on all CMC, PR and MWD, while emulsion properties have little contribution. High CMC, indicating poor surface-activity, were strongly correlated with high PR, and negatively associated with big peptides as MWD are displaced toward smaller MW with increasing hydrolysis time. No correlations were found between CMC and emulsion properties.

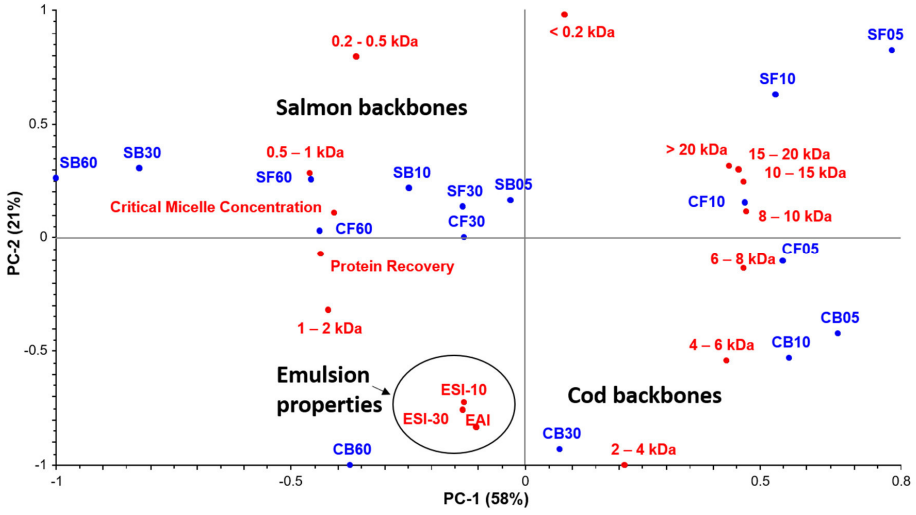

Figure 12. PCA bi-plot of hydrolysates based on cod (C) and salmon (S) backbones, the proteases FoodPro PNL (F) and Bromelain (B) and hydrolysis times of 5, 10, 30 and 60 mins. The plot showed the product association to molecular weight distribution, critical micelle concentration and emulsion properties.

EAI was significantly higher for the direct extracts but showed little variation between hydrolysates. The difference can likely be attributed to the higher levels of peptides > 10 kDa in the direct extracts, but it also indicates that even a small degree of hydrolysis

is detriment to the flexibility and amphiphilicity needed to create emulsions. The EAI values of the direct extract were, however, only slightly lower (12-13 m^2/g protein) compared to casein (16 m^2/g protein), but the low PR of the extracts (6.2 and 7.0% for cod and salmon) does not entail sufficient raw material utilization. The ESI values were more varied than the EAI, and lowest for the direct extracts, which may be due to a combination of electrostatic attraction and slow diffusion rates. Hydrolysis time of 60 min gave best stability for both raw materials, but the values were considerably lower than for casein with ESI values of 22-27% for the hydrolysates compared to 54 for casein after 10 min of standing. The ANOVA to elucidate the effect of the process parameters showed that choice of enzyme was the only parameter affecting the EAI. Bromelain gave the highest values, which was also the case for ESI. The results showed varying effects of hydrolysis time on ESI. The stability reduced some from 5 to 10 min hydrolysis, after which it increased. The exact physiochemical processes responsible for the effect on the stability is not understood, but small peptides appear to be important for emulsion stabilization.

7.3 Influence of membrane filtration on protein hydrolysates (Paper III)

In Paper I it was shown that small water-soluble metabolites influence sensory attributes of enzymatic protein hydrolysates. In this study the objective was to elucidate the effect of nanofiltration (NF) on hydrolysate metabolite composition and sensory profile. The effect of NF on products hydrolysed with enzymes of different specificity. and variations in raw material inherent metabolite composition, was evaluated. In addition, the protein recovery throughout the whole process was monitored.

Protein hydrolysates were produced in pilot scale (approx. 80 kg raw material) with head and backbones from cod and salmon. Both raw materials were hydrolysed with Bromelain and FoodPro PNL for 50 min. The hydrolysates were microfiltered (0.1 μm) to remove residual lipids and suspended solids. The high lipid contents of the salmon raw material (21.5%) gave slightly smaller volumes compared to the cod-based hydrolysates, as the latter raw material contained more water. Consequently, there was a difference in degree of concentration between products in the first round of NF

resulting in a slight product variation in dry matter concentration. The MF process resulted in a PR of 46-59% relative to the MF feed, illustrating the need for optimization of the process prior to a commercialized product. The PR of the NF process (60% of the MF permeate) mostly reflected the level of free amino acids and small peptides. The recovery may be improved by a membrane of smaller MWCO, but the permeation of flavour-contributing metabolites would have to be verified.

All the microfiltered hydrolysates were subjected to NF. The process resulted in a considerable change in MWD. Analysis of the NF permeates showed that peptides > 1 kDa were generally rejected by the membrane. Components < 0.2 kDa, which is mostly free amino acids, showed high degree of permeation (> 60%). This was confirmed by the ^{1}H NMR spectra of NF retentates. The region 0.8-1.5 ppm (Figure 13) clearly illustrates how the signals of free leucine (triplet, 0.94 ppm), valine (duplets, 0.98 and 1.03 ppm) and isoleucine (duplet, 1.00 ppm) decreased through NF and diafiltration. As a result, the concentration of peptides containing said amino acids increased, likely contributing to the observed enhancement of bitterness in the NF retentates.

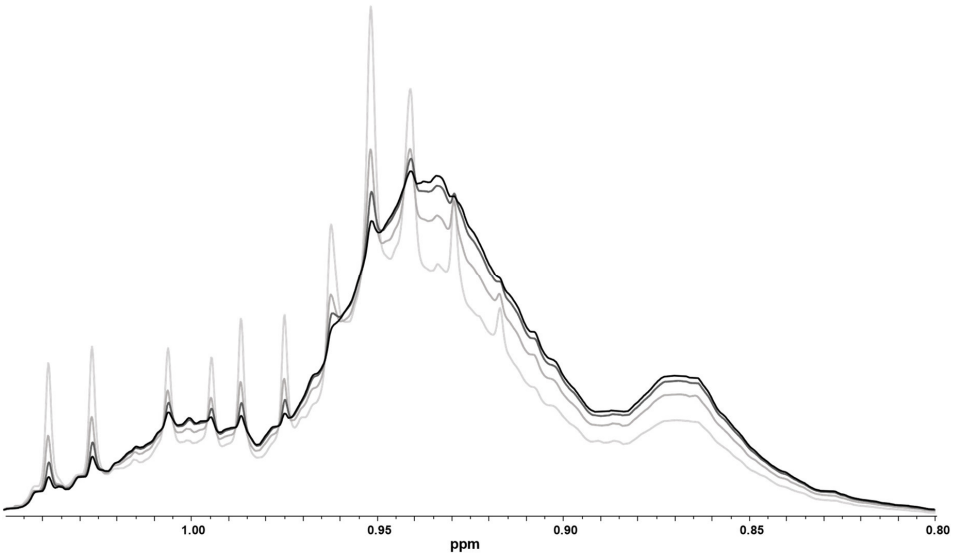

Figure 13. 600MHz ^{1}H NMR 0.8 to 1-5 spectra region of salmon hydrolysed with FoodPro PNL, illustrating decrease in free amino acids and increase in peptides through a nanofiltration and diafiltration cascade with the black line being the final product.

The other sensory attributes tested generally decreased as an effect of nanofiltration and the reduction of flavour-contributing metabolites. Most of the assigned metabolite-peaks in the NMR spectra were considerably reduced after the first round of NF, and even more so after the two batchwise diafiltration steps (Figure 14), some of which could explain the change in sensory attribute intensity. The decrease in lactate probably influences both the increase in bitterness and decrease of other attributes, as was described by the metabolite-attribute associations found in Paper I.

The contents of TMAO and its degradation products, DMA and TMA were also reduced by NF (Figure 14). Although these components are not singlehandedly responsible for generating fish flavour, their reduction contributes to the explanation in attribute intensity drop. Anserine has been found positively correlated to a variety of palatable attributes. However, the change in concentration of the metabolite did not provide a significant reduction for all associated attributes.

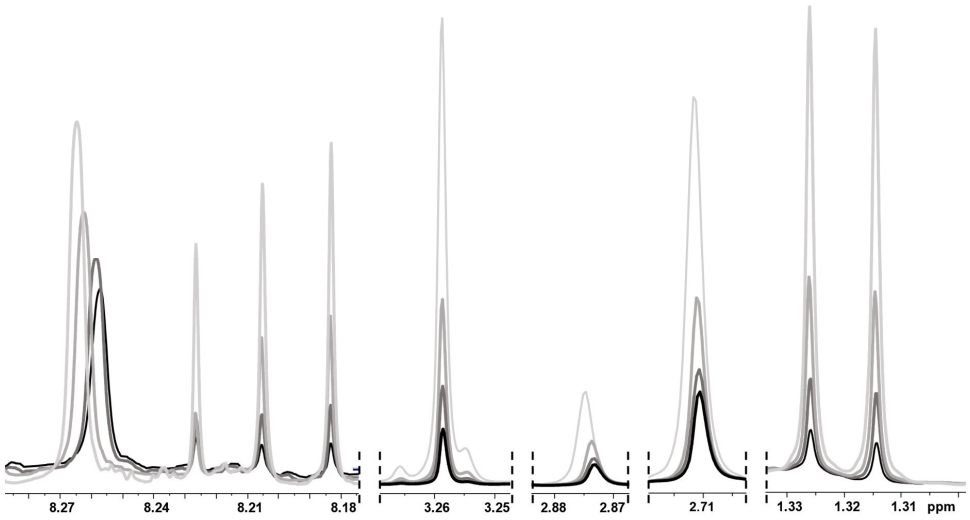

Figure 14. 600MHz ^{1}H NMR spectral areas (scaled differently) of MF permeate (the peaks of highest intensity), NF retentate, and diafiltered NF retentates (the peaks of lowest intensity). 1.32ppm: lactate, 1.71ppm: DMA, 1.87ppm: TMA, 3.26: TMAO, 8.18 and 8.19: Hypoxanthine, 8.23: Inosine, 8.26: Anserine.

Sodium and ash were considerably reduced by NF in all products. The level of ash in the NF retentates was similar, ranging from 32 to 44 g/kg dry matter. The variation may be due to volume differences in the MF permeates, resulting in varying degrees of increased concentration in dry matter. Diafiltration led to additional reduction in ash and sodium levels, albeit, not to the same extent. The levels were reflected by the sensory assessment, where the MF permeates had significantly higher salty taste compared to the corresponding nanofiltered products, and the attribute intensity decreased with diafiltration, although not significantly. This indicates that the variation in salt and ash content between the three NF products is too small to be detected by a sensory panel.

The effects of raw material and enzyme specificity were small compared to those of NF, but there were some significant effects. Salmon hydrolysates gave hydrolysates of higher attribute intensity for bitter, acidic, umami, and total intensity, while cod had highest TMA flavour. The MWD variation in the MF permeates, where salmon products had higher levels of 0.2-1 kDa components, is a likely explanation for the observation. This caused some differences in NF effect on MWD depending on the hydrolysate raw material source, giving higher concentration of bitter peptides in nanofiltered salmon products. Enzyme specificity had significant effect on acidic taste and metallic flavour, with bromelain giving the highest intensity.

7.4 Variaration in sensory profiles and chemical properties as an effect of residual raw material fraction (Paper IV)

The objective of this study was to assess how different fractions of residual raw material influence sensory properties, chemical composition, and nutritional quality. Enzymatic protein hydrolysates were produced from heads, backbones, and viscera of salmon and mackerel with FoodPro PNL for 50 min in pilot scale. The products were purified by MF, concentrated on a 4-stage falling film evaporator and spray dried.

Contents of essential amino acids, MWD, and biogenic amines influence the nutritional value of the protein hydrolysates. The biogenic amines histamine, putrescine, and cadaverine are metabolites found in seafood, but high levels indicate microbial

proliferation in the raw material. The two latter are considered potentiators of the former, of which a toxicity threshold limit of 90 mg/dose is reported (FAO/WHO 2012). TMA is also formed from the osmolyte TMAO by bacterial activity in the raw material and will influence the sensory profile of hydrolysates.

All raw materials applied in the study contained high levels of essential amino acids and the hydrolysates had comparable amino acid levels. All the produced hydrolysates consisted mainly of peptides < 2 kDa, and the viscera products had almost 40% compounds < 0.2 kDa, i.e. mostly free amino acids. The difference can be ascribed to endogenous enzyme activity in the visceral raw material. A considerable product difference was found in the ash content. The mackerel hydrolysates had higher ash content compared to the corresponding salmon hydrolysates. The most extreme level was observed in the mackerel head product with 38%, compared to 12.6% in the salmon head product. This was reflected by the sensory assessment, where the former hydrolysate had the highest intensity of salty taste. Significant differences were found for all sensory attributes except sweet, acidic, shellfish, and rancid. The strongest fish flavour intensity was found in the mackerel head hydrolysate, which did not coincide with the highest TMA concentration found in the salmon viscera hydrolysate. However, the latter product was the most bitter, in addition to high intensity of many other attributes, potentially obscuring some of the fish flavour intensity.

Two-way mixed model ANOVA was used to evaluate effect of raw material fraction and fish species. Viscera products had significantly higher bitter taste, and TMA, burned, cloying, and astringent flavours, adding up to the highest total flavour intensity. In addition to the level of small peptides, viscera contain bile that may add to the bitter taste sensation. The high intensity of unpalatable attributes indicates low suitability of visceral hydrolysates in food formulations without subsequent processing for improvement of sensory properties. Salty taste was the only attribute of which heads gave the highest intensity, influence by the exceptionally high salt levels in mackerel heads, while backbones was perceived as most flavourless. Total flavour intensity, and the attributes salt, umami, fish, TMA, and fatness were significantly influenced by fish species, with mackerel having the highest intensities. In general, NF should be included

in the downstream processing when using mackerel heads as raw materials to reduce the salt content.

8. Conclusions

The work presented in this thesis has expanded the knowledge on enzymatic protein hydrolysate properties important for their inclusion in foods. The experimental work increased the understanding of how raw material, enzyme specificity and extent of hydrolysis affected molecular weight distribution and metabolite composition in relation to sensory properties. The potential of nanofiltration as a method for reduction in sensory attribute intensity was evaluated. In addition, emulsion properties and critical micelle concentration were assessed. The main conclusions of the study are summarised below.

- Salmon, cod, and chicken muscle tissue have similar nitrogen-to-protein conversion factors (5.2, 5.3, and 5.3, respectively), but they do not coincide with the commonly used factor of 6.25, which is important to consider when aiming for production of comparable protein hydrolysates.
- Enzyme specificity determines bitter taste development to a large extent, but NF in the downstream processing reduces the influence of enzyme on the attribute while the effect of raw material increases.
- Water-soluble raw material compounds are the most important factor influencing sensory attributes other than bitterness. Fish flavour is a complex attribute and cannot be determined based solely on TMA and DMA contents in the hydrolysates.
- ^{1}H NMR spectroscopy is a valuable tool in evaluation of hydrolysate properties. It has potential for prediction of sensory properties based on metabolite composition and can be used to determine specific attribute-metabolite associations.
- Microfiltration is efficient for purification of protein hydrolysates, removing suspended solids and residual lipids, keeping rancidity levels of the products low. A downside is a considerable loss of protein in the process, resulting in approx. 40% protein recovery without the use of diafiltration.

- Hydrolysis was detrimental to the EAI of fish proteins but is positive for the ESI. Fish proteins were inferior to emulsion properties of casein by both measurements.
- There were no clear positive associations between the emulsion properties and peptide sizes, nor with the critical micelle concentration.
- Low critical micelle concentration, indicating good surface-activity, was associated with relatively large peptides (> 6 kDa), and increased along with degree of hydrolysis.
- Nanofiltration reduces the concentration of water-soluble flavour-contributing metabolites in hydrolysates, causing intensity reduction for several attributes. However, the increased concentration of peptides > 1 kDa results in higher bitter taste sensation. Diafiltration with approx. 1.5-fold addition of water to the nanofiltration retentate gave additional reduction in metabolites, but generally not enough to give significantly different sensory attributes.
- Microfiltration up to 0.1 µm cause a substantial peptide loss, particularly when applied to direct thermal protein extracts, but also in protein hydrolysates.
- The fractions of residual raw materials used for hydrolysis influences the chemical composition of the product. Ash is relatively high in hydrolysates based on mackerel, while viscera is generally high in TMA and biogenic amines, and more taste-intense compared to head- and backbone-products.

9. Future outlooks

The inclusion of enzymatic protein hydrolysates in food formulations provides both a potential for increased valorization of residual raw materials and sustainable use of resources. The main limitation of any food inclusion is unpalatable tastes. In this thesis, we have shown that most of the sensory attributes can be ascribed to water-soluble metabolites inherent to the raw material. Through membrane filtration technology it is possible to reduce the intensity of many of the attributes. However, optimization studies should be performed to increase protein recovery throughout the process. This may include testing different molecular weight cut-offs and types of membranes.

In addition to the hydrolysate, the other process fractions should be used for some application to ensure optimal raw material utilization. The solids, including the bone fractions may have potential as food ingredients, but is frequently used for feed applications. The bone fraction contains minerals and proteins with high nutritional value, as does the membrane filtration fractions (the microfiltration retentate in the case of this study) that is not considered the main product of the process. In the case of high-fat species, the oil fraction may also have high potential for human nutrition given further refining. A potential method to increase hydrolysis yield while simultaneously decreasing amounts of other fractions, is a cascade hydrolysis reaction. This could facilitate solubilization of more protein while restricting the degree of hydrolysis.

This thesis did not demonstrate excellent functional properties of the tested hydrolysates. However, this does not mean that it is impossible to produce hydrolysates with good surface-activity. Other process parameters or raw materials may prove better, or it could be possible to use them in combination with other surfactants. In any case, more detailed rheological understanding of the emulsion systems is needed, and it would be beneficial if they were tested in conjunction with a formulated food product.

Researchers have started to test food formulations containing hydrolysates. This is imperative to really start the process of commercializing such products, which have been so extensively studied the past decades. As these are complex systems, crowded

with various components, it is necessary to continue testing actual food formulations to see the effect of hydrolysate variables in the potential food products of the future.

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I

Paper I

Steinsholm, S., Oterhals, Å., Underhaug, J., Måge, I., Malmendal, A. & Aspevik, T.
Sensory assessment of fish and chicken protein hydrolysates. Evaluation of NMR metabolomics profiling as a new prediction tool.

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Paper II

Steinsholm, S., Oterhals, Å., Underhaug, J. & Aspevik, T.

Emulsion and surface-active properties of fish solubles based on direct extraction and after hydrolysis of Atlantic cod and Atlantic salmon backbones.

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II

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Article

Emulsion and Surface-Active Properties of Fish Solubles Based on Direct Extraction and after Hydrolysis of Atlantic Cod and Atlantic Salmon Backbones

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Abstract: The focus on natural foods and "clean" labeled products is increasing and encourages development of new biobased ingredients. Fish solubles derived from downstream processing of side stream materials in the fish filleting industries have potential as emulsifiers based on their surface-active and emulsion stabilizing properties. The aim of this study was to evaluate and compare emulsion properties and critical micelle concentration (CMC) of direct protein extracts and protein hydrolysates based on fish backbones, and to identify associations between molecular weight distribution and process yield with the studied physicochemical properties. Protein extracts and enzymatic protein hydrolysates were produced based on two raw materials (cod and salmon backbones), two enzymes with different proteolytic specificity, and varying hydrolysis time. Emulsion activity index (EAI), emulsion stability index (ESI) and CMC were measured and compared with casein as a reference to protein-based emulsifiers. Protein hydrolysis was found to have negative impact on EAI and CMC, likely due to generation of small peptides disrupting the amphiphilic balance. The direct protein extracts had comparable EAI with casein, but the latter had superior ESI values. Protein hydrolysates with acceptable EAI could only be obtained at the expense of product yield. The study emphasizes the complexity of physicochemical properties of protein hydrolysates and discusses the challenges of achieving both good surface-active properties and high product yield.

Keywords: enzymatic protein hydrolysates; emulsion activity; critical micelle concentration; fish by-products; emulsion stability

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1. Introduction

Emulsifiers are important ingredients in a variety of formulated food products containing two immiscible phases, such as mayonnaise, spreads, and salad dressings [1]. Their surface-activity reduces the interfacial tension between the phases and promotes the formation of stable emulsions. Present consumer attention and increasing preference for natural products and "clean" labeled food products is encouraging the development of new natural surface-active biobased ingredients [2,3]. Food-approved emulsifiers include proteins, polysaccharides, phospholipids, and synthetic surfactants [1,4]. Fish-based peptides may be a coming alternative. Given adequate surface-activity, this type of emulsifier will also add to the nutritional value while exerting a function in food formulations.

Emulsions in foods are often in the form of oil-in-water (O/W), where small droplets of lipids are distributed in a continuous aqueous phase, or water-in-oil (W/O), where oil is the continuous phase [1]. The formed emulsions are thermodynamically unstable and require the presence of an emulsifier for stabilization through reduction of surface-tension and prevention of droplet aggregation and coalescence. The amphiphilic nature of proteins facilitates adsorption at the interphase between polar and non-polar environments, after

which they will reorient in a manner maximizing the contact between hydrophilic areas with the oil, while repelling other emulsion droplets [5,6]. The adsorption rate of native proteins to an interphase varies depending on the protein, but often the net charge of the proteins will not provide repulsion between droplets, causing aggregation [1]. Partial hydrolysis may improve the physicochemical properties due to exposure of hydrophobic moieties, improving the electrostatic balance, and increase the solubility and flexibility of the peptides compared to the intact protein [7,8].

Several studies have addressed the physicochemical properties of protein hydrolysates[9–21], where influences of both hydrolysis time and choice of enzyme have been assessed. The degree of hydrolysis determines the reduction in peptide molecular weight, while enzyme specificity influences the balance between hydrophilic and hydrophobic regions, both imperative for peptide surface-activity. Enzymatic protein hydrolysates of side stream products from the fish filleting industry, such as heads, backbones, and trimmings, have been proposed as a source of emulsifiers for food formulations [8]. This represents a possibility for valorization of side streams and increasing the yield of water-soluble protein, while simultaneously improving the functionality of the native proteins [22]. Hydrolysis of herring protein has been shown to improve emulsion activity and stability [23], and sardine protein hydrolysates showed better emulsion properties compared with sodium caseinate [24]. However, hydrolysis of the common emulsifiers casein and whey have not always been found to improve physicochemical properties (emulsion capacity and stability), having emulsifying abilities either inferior or comparable to that of the native proteins [24]. In general, large peptides (>2–4 kDa) are essential for proper functionality, and it has been suggested that peptides should contain more than 20 amino acids to exhibit good emulsifying capability [16]. In addition, peptide–peptide interactions are particularly important [5,10,16].

Several approaches can be used in the determination of surface-activity and emulsion properties of proteins and peptides. Emulsion activity (emulsion activity index; EAI) determines the obtainable interfacial area between oil and water per unit weight of protein or product, and emulsion stabilizing ability (emulsion stability index; ESI) indicates the emulsifying effect over time [25]. Critical micelle concentration (CMC) indicates the minimum concentration of a product needed for maximum reduction of the surface-tension and can be assessed by different methods based on fluorescence, conductivity, surface-tension, or ^{1}H nuclear magnetic resonance (NMR) [26,27]. High CMC values imply poor surface-activity, i.e., a high concentration of the given surfactant is needed to reduce the surface-tension [10,27]. Measurements of properties related to emulsion capabilities of protein hydrolysates are challenging to standardize. Pearce and Kinsella [25] showed that EAI results are dependent on assay variations, such as homogenization factors and protein concentration, and the latter was confirmed by Nalinanon et al. [17]. This makes interstudy comparisons difficult and may add to the contradictory results from previous emulsion studies of protein hydrolysates.

There is a lack of knowledge on the effect of raw material and process variables on properties related to surface-activity of fish solubles. The aims of this study were (1) to evaluate and compare physicochemical properties (i.e., emulsion activity (EAI), stability index (ESI) and CMC) based on direct extraction and enzymatic hydrolysis of salmon and cod backbones, and (2) to assess the association between peptide molecular weight distribution, physicochemical properties, and process yields.

2. Materials and Methods

2.1. Materials

Atlantic salmon (*Salmo salar*) and cod (*Gadus morhua*) backbones were kindly provided by Sotra Fiskeindustri AS (Glesvær, Norway) and Halstensen Granit AS (Bekkjarvik Norway), respectively. The raw materials were milled on a Comitrol 1700 (Urschel laboratories, Chesterton, IN, USA), vacuum packed, and stored at −20 °C until use. The applied enzymes were Bromelain BR1200 (EC 3.4.22.32, Enzybel, Waterloo, Belgium) and FoodPro

PNL (EC 3.4.24.28, DuPont, Wilmington, DE, USA). Refined rapeseed oil was purchased at a local supermarket (Rema 1000 store brand, Kjerreidviken, Norway). Peptide standards were purchased from Sigma-Aldrich (Oslo, Norway) except lysozyme (Fluka biochemicals, Buchs, Switzerland) and Alberta standards (Alberta Peptide Institute, Department of Biochemistry, University of Alberta, Edmonton, AB, Canada). Technical grade Tween 20 (VWR, Oslo, Norway) and bovine casein (Sigma, Oslo, Norway) were applied in the emulsion assay. All other chemicals were analytical or food grade.

2.2. Chemical Analyses

Analysis of nitrogen (N) was performed by the Kjeldahl method [28] and the crude protein level determined based on substrate specific N-to-protein conversion factor [29]. Amino acid composition was quantified by High performance liquid chromatography (HPLC) using fluorescence detection with excitation/emission at 250/395 nm. Proteins were hydrolyzed to free amino acids with 6N HCl and amino acids derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate before passing through the HPLC column (Waters Accq Tag 3.9 × 150 mm, Milford, MA, USA) and detector [30]. Asparagine and glutamine were estimated based on the release of ammonia in the HCl digest compared to a neutral control sample [31]. Released ammonia was quantified by the method of Conway and Byrne [32]. Analysis of molecular weight distribution (MWD) was performed by HPLC (1260 series HPLC, Agilent Technologies, Santa Clara, CA, USA) using size exclusion chromatography [33], as described by Oterhals and Samulesen [34]. All chemical analyses were performed in duplicate with predetermined allowances for replicate variation.

2.3. Enzymatic Protein Hydrolysis

Raw materials were thawed overnight at 4 °C. The raw material was mixed with purified water (1:1) and transferred to a Distek Model 2500 Dissolution System (Distek Inc., North Brunswick, NJ, USA). The slurry was heated to 50 °C at continuous stirring (70 rpm) before adding 10 U enzyme per gram protein [35]. The proteolytic reactions were terminated after 5, 10, 30 or 60 min by heating to >90 °C in a microwave oven (Menumaster commercial, Cedar Rapids, IA, USA) for a minimum 10 min. The slurry was cooled to <40 °C in a water bath before phase separation by centrifugation at 15,000× *g* for 20 min (Sorvall, LYNX 6000, Thermo Scientific, Waltham, MA, USA). Direct protein extracts by thermal coagulation were produced with the same method, with the exception of enzyme addition, of both raw materials. The water phase was filtered through a Seitz-T2600 filter (Mall Corporation, East Hills, NY, USA) to remove larger particles and thereafter subjected to 0.1 µm cross flow membrane filtration (Centramate 500S Tangential Flow Filtration System, Pall, Port Washington, NY, USA) to remove remnant fine particles and fat. The final hydrolysates were stored at −20°C until further use.

Protein recovery (PR) was determined based on protein content in the filtered hydrolysate compared to that in the raw material:

$$\mathrm{PR} = \frac{\text{Protein in the hydrolysate} \times \text{g hydrolysate}}{\text{Protein in the raw material} \times \text{g raw material}} \times 100\% \quad (1)$$

volume of collected hydrolysate after membrane filtration was corrected for the remaining dead volume in the Centramate filter apparatus.

2.4. Determination of Critical Micelle Concentration by NMR Spectroscopy

All samples were diluted 3:4 with 400 mM sodium phosphate buffer (pH 6.5) containing 15% D_2O and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The pH was adjusted to 6.5 with 0.1 M HCl. Dilution series of 10 samples were prepared from all hydrolysates with the final concentration of 100 mM sodium phosphate buffer and 7.5% D_2O. A volume of 600 µL was transferred to 5 mm NMR tubes. ^{1}H spectra were acquired at 300 K with a Bruker AVANCE NEO ultrashielded 600 MHz spectrometer with cryoprobe using Bruker pulse program zgesgppe (Karlsruhe, Germany). Acquisition parameters were set to four

dummy scans, 32 real scans, 1 s relaxation delay, 32k time-domain points, and spectral width of 11.9 ppm. The NMR spectra were processed using TopSpin (v. 4.0.4, Bruker BioSpin, Karlsruhe, Germany). Exponential line broadening of 0.5 Hz was applied prior to Fourier transformation, and the chemical shifts were referenced to DSS.

The CMC was estimated by plotting the ^{1}H shift of the lactate methyl group as a function of log protein concentration [10]. The methyl resonance of lactate was visible around 1.3 ppm for all samples. Separate linear trend lines were drawn for the lag phase and the exponential phase in the plot. The data points used for the lag phase tangent were adjusted to obtain best fit by removing points in the transition between lag and exponential phase. The intercept of these lines indicated the start of protein aggregation and thus the critical micelle concentration. Propagation of uncertainty assuming independent variables indicated standard measurement error < $\pm$ 0.2 based on the fitted regression lines and standard error for the coefficient provided by the LINEST function in Microsoft Excel (v. 2013). Aspevik et al. [10] estimated the standard deviation for the used protocol to be 0.5 g/L.

2.5. Emulsion Properties

Emulsion activity index (EAI) and emulsion stability index (ESI) were determined based on the method by Pearce and Kinsella [25] and modified by Liceaga-Gesualdo and Li-Chan [23]. The hydrolysates were diluted to 1.0% protein in purified water and pH adjusted to 6.5 with 0.1 M HCl. Tween 20 was used as control to ensure method repeatability, and casein (1% solubilized in 50 mM potassium phosphate) as a reference to commercial emulsifiers. Emulsions were made by adding 2 mL of rapeseed oil to 6 mL of standardized hydrolysate and homogenized (T 10 basic Ultra-Turrax, Ika, Staufen, Germany) at 16,000 rpm for 1 min in a glass container. Emulsions were made in triplicate. Immediately after homogenization, 25 µl was collected from the bottom of the mixture, added to 5 mL 0.1% sodium dodecyl sulfate (SDS) and the mixture inverted two times before measurement of EAI. Sample collection was repeated after 10 and 30 min for determination of ESI. Absorbance was measured at 500 nm and EAI was calculated based on the following equation:

$$\mathrm{EAI}\left(\mathrm{m}^2(\mathrm{g\ protein})^{-1}\right) = \frac{2 \times \frac{2.303\mathrm{A}}{\mathrm{l}} \times \mathrm{df}}{\varphi \times \mathrm{c}} \quad (2)$$

where A = absorbance measured at 0, 10 or 30 min; l = path length of cuvette in m; df = dilution factor (200); φ = oil phase volume of total mixture volume; c = weight of protein per unit volume of aqueous sample before emulsion. ESI was calculated based on the percent EAI remaining after a defined stagnant standing period:

$$\mathrm{ESI}(\%) = 100 - \left(\frac{\mathrm{EAI} - \mathrm{EAI_t}}{\mathrm{EAI}} \times 100\right) \quad (3)$$

where $\mathrm{EAI_t}$ = EAI value of samples collected after 10 or 30 min.

2.6. Statistics

Analysis of variance (ANOVA) was performed using Minitab (v. 19.2, Pennsylvania State University, PA, USA). One-way ANOVA was used to determine significant product differences of EAI and ESI results. Two-way ANOVA determined significant parameters. Hydrolysates were used to model effect of raw material, enzyme, and hydrolysis time. Tukey's pairwise comparison was used when significance ($p < 0.05$) was found.

Principal component analysis (PCA) was used to evaluate relationships between EAI, ESI, and MWD using Unscrambler (v10.4.1, Camo, Oslo, Norway). All data were unit variance scaled and centered before analysis.

3. Results and Discussion

3.1. Raw Material and Hydrolysate Composition

Salmon and cod backbones showed similar amino acid compositions (Table 1), with slightly higher levels in the cod raw material, reflecting higher protein concentration in the latter substrate. Both substrates contained high levels of glycine, proline, and hydroxyproline, ascribed to the high proportion of bones and connective tissue protein. N-to-protein conversion factors (f_N) for cod and salmon backbones were calculated to 5.5 and 5.2, respectively, in agreement with previous findings for pure muscle proteins from the two species [35], and illustrated the deviance of fish raw material from the commonly used factor of 6.25. The use of substrate specific conversion factors facilitated a more accurate quantification of protein content, and thus enzyme addition on protein basis and standardization of hydrolysis conditions in studies applying different protein sources [30].

Table 1. Amino acid composition in the raw material (g kg^{-1}; n = 2) and substrate specific nitrogen-to-protein conversion factor data of salmon (*Salmo salar*) and Cod (*Gadus morhua*) backbones.

AA (g/kg) **	Cod Backbone	Salmon Backbone
Alanine	11.0	9.9
Arginine	11.0	9.0
Asparagine *	7.1	6.3
Aspartate	8.9	7.7
Glutamate	16.1	12.0
Glutamine *	7.9	7.0
Glycine	17.0	14.0
Histidine	3.3	3.6
Isoleucine	6.4	5.7
Leucine	12.0	9.6
Lysine	13.0	11.0
Methionine	5.4	4.6
Phenylalanine	5.9	5.2
Proline	8.8	7.8
Serine	8.6	6.5
Threonine	6.9	6.2
Tyrosine	6.9	4.0
Valine	7.4	6.7
Hydroxyproline	3.8	3.5
Nitrogen	27	25
NH3 (acid digest)	1.8	1.6
Total AA	167.4	140.3
Total n	27.0	25.0
f_N ***	5.5	5.2

* Calculated based on released NH_3, and assuming 1:1 ratio of released NH_3 between Asp and Glu [29]. ** Amino acid. *** Nitrogen-to-protein conversion factor.

The enzymes were added based on similar activity-to-protein ratio [31], and the resulting peptide molecular weight distribution (MWD; Table 2) showed an increase in smaller peptides as the hydrolysis progressed. Furthermore, levels of molecules <0.2 kDa were higher in products based on FoodPro PNL compared to the equivalent Bromelain hydrolysate, indicating some exopeptidase activity in the former enzyme [31]. In general, hydrolysates based on cod backbone contained a larger proportion of peptides, >1 kDa, compared with salmon. For both raw materials, the PR increased with prolonged hydrolysis time, with Bromelain giving slightly higher levels compared with FoodPro PNL (Table 3). This is possibly explained by the broad specificity of Bromelain and efficiency on connective tissue proteins [36]. As expected, the direct protein extracts contained mostly large peptides, >10 kDa, and small molecules, <0.2 kDa, characteristic for a product based on direct thermal coagulation and separation [10,34].

Table 2. Apparent molecular weight distribution (MWD; kDa) and average amino acid units in the molecular size of direct protein extracts (-ext) and hydrolysates made from cod (C) and salmon (S) backbones with FoodPro PNL (F) and Bromelain (B) for 5, 10, 30 and 60 min.

MWD (%)	C-ext	CF05	CF10	CF30	CF60	CB05	CB10	CB30	CB60	S-ext	SF05	SF10	SF30	SF60	SB05	SB10	SB30	SB60	AA *
>20	8.9	1.3	1.2	0.4	0.2	1.3	1.4	0.8	0.3	4.9	2.6	1.5	0.4	0.1	1.1	0.8	0.1	<0.1	>198
15–20	3.9	1.1	0.9	0.3	0.1	1.0	0.9	0.5	0.2	2.2	1.7	1.1	0.4	0.1	0.6	0.4	0.1	<0.1	138
10–15	5.7	2.9	2.7	1.1	0.5	3.0	2.6	1.3	0.5	3.1	4.1	3.1	1.3	0.4	1.2	0.8	0.2	<0.1	99
8–10	3.4	3.0	2.9	1.5	0.8	3.5	3.2	1.8	0.8	1.7	4.0	3.4	1.9	0.9	1.0	0.7	0.2	0.1	71
6–8	4.0	6.0	6.1	3.9	2.5	7.8	7.4	5.1	2.8	2.2	7.0	6.5	4.5	2.7	2.5	2.0	0.6	0.3	55
4–6	3.8	12.0	12.6	9.7	7.2	15.9	16.0	13.7	10.0	2.4	11.6	11.2	8.9	6.7	7.6	6.6	2.9	1.7	32
2–4	3.7	21.4	23.0	22.8	21.2	26.4	27.9	30.0	29.3	2.5	18.6	20.0	20.0	18.2	23.3	23.0	16.0	12.0	20
1–2	1.9	16.1	17.2	20.2	21.4	14.6	16.2	20.7	25.0	1.3	12.3	14.0	16.9	18.4	22.2	23.7	25.7	24.5	12
0.5–1	1.2	10.0	11.0	14.9	17.5	6.2	6.9	9.7	13.3	0.8	7.7	9.2	13.0	16.0	12.8	14.3	21.1	24.2	5.9
0.2–0.5	3.3	6.6	7.1	10.3	13.0	3.3	3.4	4.4	6.0	18.4	10.3	11.0	13.6	16.4	10.7	11.5	16.5	20.0	2.8
<0.2	60.3	19.7	15.4	14.8	15.7	17.0	14.1	12.1	11.9	60.7	20.2	19.1	19.1	20.1	16.9	16.4	16.4	17.2	1.0

* Estimated average number of amino acid units in the molecular size group based on weighted average MW [30].

Table 3. Measured emulsion activity index (EAI *) and stability index after 10 and 30 min (ESI-10/30 *), critical micelle concentration (CMC **) and protein recovery (PR) of hydrolysates and direct protein extracts (-ext) based on cod (C) and salmon (S) backbones with FoodPro PNL (F) and Bromelain (B) for 5, 10, 30 and 60 min of hydrolysis.

	EAI (m^2/g)	ESI-10	ESI-30	CMC (g/l)	PR (%)
C-ext	13 ± 0.9^{a}	21 ± 1.9^{cde}	16 ± 3.0^{de}	1.6	6.2
CF05	11 ± 1.2^{bcd}	33 ± 2.7^{a}	25 ± 0.6^{ab}	3.7	18.1
CF10	9.0 ± 0.2^{cd}	18 ± 1.9^{def}	9 ± 0.6^{efg}	4.8	23.4
CF30	11 ± 0.7^{bcd}	14 ± 2.1^{ef}	7 ± 1.3^{fgh}	6.0	31.4
CF60	9.0 ± 0.2^{d}	27 ± 0.5^{abcd}	22 ± 2.9^{abc}	6.2	35.6
CB05	11 ± 0.5^{bcd}	31 ± 1.7^{abc}	22 ± 2.9^{bcd}	5.1	24.0
CB10	11 ± 0.7^{bcd}	25 ± 1.6^{abc}	17 ± 1.5^{cde}	5.4	27.0
CB30	11 ± 0.4^{bcd}	33 ± 1.4^{abc}	26 ± 3.1^{ab}	6.0	31.5
CB60	11 ± 0.1^{abc}	31 ± 2.2^{a}	27 ± 1.6^{a}	6.6	36.6
S-ext	12 ± 1.2^{ab}	10 ± 1.0^{f}	6 ± 1.8^{gh}	1.8	7.0
SF05	10 ± 1.2^{bcd}	11 ± 2.4^{f}	6 ± 1.0^{gh}	5.8	26.5
SF10	10 ± 0.3^{bcd}	12 ± 0.0^{f}	6 ± 1.2^{gh}	5.3	25.8
SF30	10 ± 0.4^{bcd}	30 ± 2.2^{ab}	23 ± 1.6^{abc}	6.7	31.1
SF60	10 ± 0.3^{bcd}	32 ± 3.6^{a}	25 ± 3.7^{ab}	6.8	33.7
SB05	11 ± 0.3^{bcd}	11 ± 3.5^{f}	4 ± 0.7^{h}	5.3	26.1
SB10	10 ± 0.5^{bcd}	13 ± 0.6^{ef}	5 ± 1.0^{h}	6.2	30.0
SB30	10 ± 0.5^{bcd}	22 ± 1.7^{bcd}	13 ± 0.4^{ef}	7.6	40.0
SB60	11 ± 0.5^{bcd}	31 ± 3.3^{ab}	22 ± 1.4^{abc}	7.6	40.8

* Different letters indicate statistically different values ($p \leq 0.05$) by one-way ANOVA and Tukey's pairwise comparison. ** Measured based on single samples. Standard deviation was estimated to 0.5 g/L by Aspevik et al. [10].

All products were purified by microfiltration to eliminate suspended solids and residual lipids with possible bias effects in the surface-activity assays. The protein recoveries (Table 3) were lower than observed in earlier studies without a microfiltration step [10], particularly for the direct extracts (PR = 6–7% compared to expected approximate 20%). This confirms a partial retention of large proteins and peptide fragments by microfiltration, as earlier reported [37]. The shift in MWD toward smaller peptides probably influenced the surface-active properties of the respective products; however, effects of membrane filtration were outside the scope of this study.

3.2. Associations between MWD and Physicochemical Properties

Principal component analysis (PCA; Figure 1) was used to evaluate associations between EAI, ESI, CMC, PR, and MWD of the hydrolysates. Data from the direct protein

extracts were excluded due to the deviant MWD and PR compared with the hydrolysates (Tables 2 and 3), adding too much leverage to the model and dominating the variation of the two variables. Based on the score plot, hydrolysates with similar or different properties could be identified. Two principal components (PCs) were found to be relevant for the interpretation of results. The first and second PCs explained 58% and 21%, respectively.

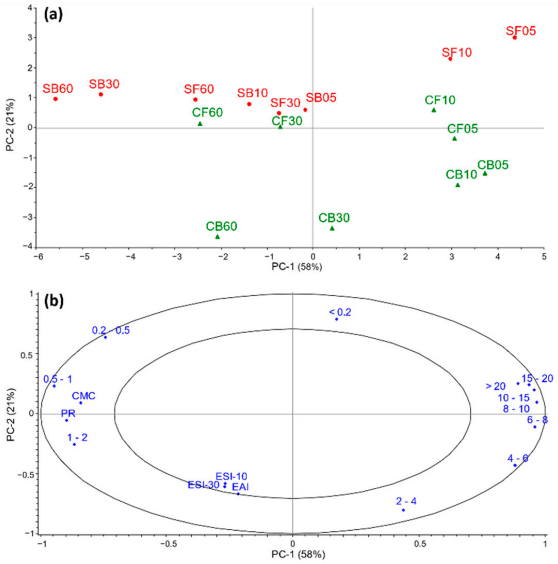

Figure 1. Principal component analysis score plot (**a**) shows similarities and differences between salmon (S) and cod (C) backbone hydrolysates made with FoodPro PNL (F) and Bromelain (B) for 5, 10, 30 and 60 min. The correlation loading plot (**b**) illustrates associations between molecular weight distribution (kDa), protein recovery (PR), critical micelle concentration (CMC), emulsion stability after 10 min (ESI-10), emulsion stability after 30 min (ESI-30), and emulsion activity index (EAI). The two ellipses represent 50% and 100% of explained variance.

In the score plot (Figure 1a), PC-1 mainly explains the effect of hydrolysis time, while the raw material variation is explained by PC-2. The correlation loading plot (Figure 1b) mostly shows a product separation based on CMC, PR, and MWD. High values for the two former variables and small molecules of 0.5–2 kDa were associated and negatively correlated with molecules of 4–>20 kDa, in agreement with Aspevik et al. [10]. The emulsion responses were less than 50% explained by the model, thus interpretation should be done with care. The loading plot shows no positive correlations between emulsion properties and specific MW groups. This was also the case in studies on salmon muscle protein hydrolysates [15], and whey and casein protein hydrolysates [24]. The results indicate that extended hydrolysis is detrimental to surface-activity, expressed by CMC. The MWD (Table 2) and considerably lower CMC values (Table 3) for the direct protein extracts supported this conclusion.

3.3. Effect of Process Parameters on Emulsion Properties

The EAI of the products (Table 3) showed small differences between the enzymatic hydrolysates, but the direct protein extracts gave the highest values for both cod and salmon. This may be attributed to the higher levels of large peptides (>10 kDa) being sufficiently flexible for effective interfacial surface coverage. Furthermore, a decrease in surface hydrophobicity of the hydrolysates may also add to this observation, as discussed for whey hydrolysates [20]. Negligible differences between the EAI (Table 3) of the hydrolysates

were observed, with no clear pattern of differences influenced by raw material, enzyme, or hydrolysis time (Figure 2).

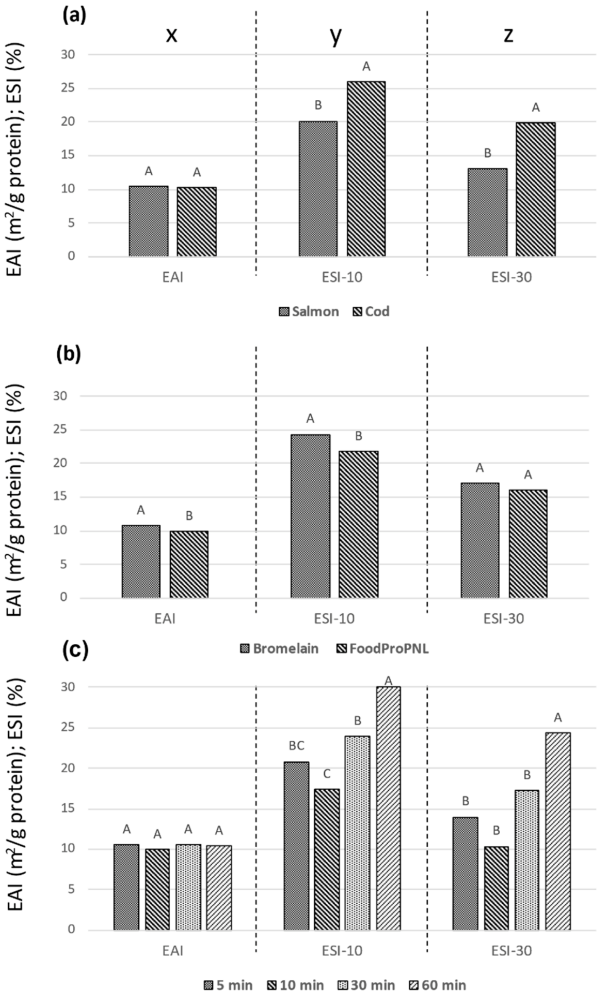

Figure 2. Mean emulsion activity index (EAI) and emulsion stability index (ESI) after 10 and 30 min for the raw materials salmon and cod (**a**), the enzymes FoodPro PNL and Bromelain (**b**), and hydrolysis times (**c**). Different letters indicate statistical effect of the hydrolysis parameter on the emulsion variable. EAI (x), EAI-10 (y) and EAI-30 (z) are separate statistical entities, indicated by the dotted lines.

Casein is an excellent emulsifier in milk-based products [6], and the EAI of casein was measured at 16 ± 1 m^2/g protein, only slightly higher than the direct protein extracts in this study (EAI = 12–13, Table 3). We have found few studies comparing the EAI of fish-based protein hydrolysates with direct protein extraction of the raw material. Contrary to this study, Liceaga-Gesualdo and Li-Chan [23] showed enhanced EAI of herring hydrolysates compared to this type of reference sample. More common is to use a commercial protein emulsifier as reference. Tan et al. [21] found that restricted hydrolysis on catfish gave EAI and ESI comparable to those of soy protein isolate. However, Alves et al. [9] found the EAI

of soy proteins to be superior to that of chicken blood hydrolysates and associated this to large interfacial areas of the soy proteins. The interfacial properties of proteins could possibly explain the relatively good results for the casein protein and the direct thermal extracts compared to the hydrolysates in this study. The mentioned studies [6,9,21,23] had assay variations comparable to the current work, such as sample dilution media, protein concentration, and pH, likely influencing the results.

The ESI showed more variation between the samples compared to the EAI (Table 3), with lowest values obtained by the direct protein extracts. The low stability of the two latter emulsions may be due to a combination of electrostatic attraction between unfolded protein and interactions with other small molecules present in the samples [1]. An increase in ESI with prolonged hydrolysis time for the salmon hydrolysates was observed, suggesting that a higher release of small molecules is required for stabilization, compared with shorter hydrolysis time (Figure 2). Other studies have reported a decrease in emulsion stability in hydrolysates based on salmon heads [13], muscle proteins [15], and chicken blood [9] when the degree of hydrolysis is increased. They attributed the reduction in ESI to a reduction in interfacial tension and increased hydrolysis, indicating a loss of emulsion stabilizing properties when the salmon peptides are substantially hydrolyzed.

The ESI values for the cod hydrolysates, on the other hand, were more ambiguous, with low levels at intermediate hydrolysis times. The highest observed levels were, however, similar for both salmon and cod, and the values decreased after 30 min holding time. The ESI values of casein were about two times higher compared with the hydrolysates, with values for ESI-10 = 54 $\pm$ 5 and ESI-30 = 40 $\pm$ 4, likely due to an appropriate balance of hydrophobic regions [1] and high film viscosity.

ANOVA of the individual enzymatic hydrolysis parameters demonstrated that there were no significant effects of species or hydrolysis time on EAI (Figure 2(ax,cx)), whereas Bromelain gave significantly higher values compared with FoodPro PNL (Figure 2(bx)). The proteases Bromelain and FoodPro PNL (formerly named Protex 7L) were chosen based on studies suggesting good emulsion properties and CMC values in hydrolysates based on these enzymes [10,12]. The observed difference, although small, was in agreement with a study on tilapia hydrolysates [12], where Bromelain gave superior emulsion properties of the four proteases tested.

All hydrolysis parameters significantly influenced the ESI-10 levels (Figure 2(ay–cy)). where cod, Bromelain, and 60 min hydrolysis were superior to salmon, FoodPro PNL, and shorter hydrolysis time, respectively. This suggests that smaller peptides may be important for emulsion stability, in agreement with other studies on fish-based substrates [17,18]. On the other hand, there have been studies suggesting a general decrease in emulsion properties of fish-based substrates as the hydrolysis progressed [14,15], but very high protease concentration [14] and different emulsion assays [15] were applied. The PCA-plot (Figure 1) indicates a negative association between MW < 0.2 kD and ESI. Characteristic for the two direct protein extracts is a very high content (60%) of this MW fraction (Table 2) and a low ESI. Combined, this indicates a negative effect of free amino acids on EAI and a positive effect of higher molecular weight peptides in general. No correlations were found between specific peptide fractions and ESI; however, the effect might reflect a low contribution to film viscosity of free amino acids compared to peptides.

Interpretation and comparison between separate studies should be made with caution. The protein concentration applied in EAI assays strongly influences the results [17], along with pH [18] and the equipment used [25]. These factors have been taken into consideration when assessing similarities with other studies, but the challenges emphasize the need for more standardized assay methodology. The process of emulsion formation and stabilization is complex with many influencing factors [5], especially peptide–peptide interactions [16]. Furthermore, the relatively low ESI of the direct extracts (Table 3) shows that the formation and stability of emulsions cannot be seen as co-dependent responses, as they appear to depend on different peptide properties, in agreement with previous studies [24,38].

3.4. Critical Micelle Concentration of Fish Protein Hydrolysates

The use of ^{1}H NMR is a well-established method for determination of CMC [10,27]. In this study, the chemical shift of lactate was used to measure changes in the chemical environment due to micelle formation (Figure 3). A full overview of the chemical shifts with the corresponding protein concentration can be found in Table S1. A low CMC is favorable and implies that less of the surfactant is needed to obtain maximum reduction of the surface-tension. The lowest value of CMC was observed for the direct extracts (Table S1) and indicated that the undigested proteins present were flexible enough to exert a better reduction of surface-tension than their peptide moieties. The NMR technology measures a change in the chemical environment; however, it cannot discern if the micelles or aggregates are homogenously distributed, which indicates electrostatic repulsion necessary for emulsifying capabilities. This may add to an explanation of the lack in correlation between CMC and ESI (Figure 1).

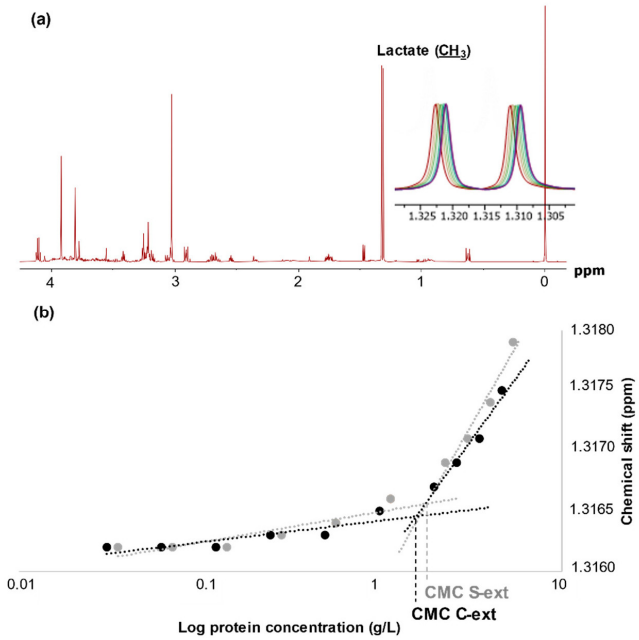

Figure 3. (**a**) ^{1}H NMR spectra showing the methyl resonance of lactate around 1.3 ppm, with an insert illustrating the change in chemical shift of lactate with decreasing protein concentration of the salmon protein extract dilution series. (**b**) Determination of the critical micelle concentration (CMC) of the direct protein extracts based on cod (C-ext) and salmon (S-ext).

An increase in hydrolysis time, and thus reduction in peptide size, gave higher CMC values (Table 3). This was in agreement with a previous observation [10] and likely due to a decrease in the amphiphilic nature of the peptides by extended hydrolysis [6]. The CMC of cod hydrolysates was slightly lower compared with the corresponding salmon hydrolysates and may be explained by the generally higher content of larger peptides in cod-based hydrolysates (Table 2). Furthermore, the CMC values of hydrolysates based on Bromelain were higher than the corresponding FoodPro PNL hydrolysates, probably due to the broad specificity of the former enzyme, leading to more disruption of the hydrophobic areas in the peptides [36]. The obtained CMC values were lower than those reported by Aspevik et al. [10] for salmon heads and backbones, which ranged from 6 g/L in the control sample (without proteolysis) to 11.5 g/L after prolonged hydrolysis with FoodPro

PNL. The respective values of the current study are 1.8 and 6.8 g/L. A major difference between the two studies was the use of a 100 kDa [10] and 0.1 µm (this study) membrane filter to remove residual lipids and fine particles in the hydrolysate before measurement of physicochemical properties. A microfiltration step is needed to remove interfering compounds; however, it will also partly remove higher molecular weight peptides with negative impact on CMC. The variation in CMC between the two studies shows that a 100 kDa filter removes more of the high MW molecules required for low CMC values.

The negative correlation between PR and low CMC (Figure 1) suggests that a compromise must be met between high surface-activity and product yield. Although by restricting hydrolysis to a degree where peptide surface-activity is retained, the lower PR may be compensated by introduction of a cascade approach where peptide fractions with different physicochemical properties are obtained after successive hydrolysis steps to improve the overall process yield. Additional studies on droplet size distribution and reduction in interfacial tension may be included to further elucidate the physicochemical properties.

4. Conclusions

No associations between CMC and emulsion properties (EAI and ESI) of protein hydrolysates based on cod and salmon backbones were observed. Low CMC, implying good surface-activity, was correlated with peptides > 4kDa and hydrolysates of restricted proteolysis. The lowest CMC and highest EAI values were obtained for products based on direct protein extraction without hydrolysis and reflected a negative effect of hydrolysis on CMC and EAI. The ESI values of the hydrolysates were both increased and reduced compared with direct protein extraction. The process combination of cod, Bromelain, and 60 min of hydrolysis was superior to salmon, Food Pro, and shorter hydrolysis times with respect to this property. The EAI values of direct protein extracts were slightly lower than casein; however, ESI values were less competitive. Hydrolysates showed both lower EAI and ESI values compared to casein. Direct protein extraction gives superior physicochemical properties measured as CMC and EAI; however, it also results in lower ESI and product yield compared with hydrolysis. This is further reinforced by microfiltration to remove residual lipids and fine particles. A cascade approach is suggested as a potential method both to improve product yield and optimize emulsifier properties.

Supplementary Materials: The following are available online at https://www.mdpi.com/2304-8158/10/1/38/s1, Table S1: Chemical shifts of lactate methyl resonance.

Author Contributions: Conceptualization, S.S., Å.O. and T.A.; Formal analysis, S.S.; Investigation, S.S.; Methodology, S.S., Å.O., J.U. and T.A.; Project administration, T.A.; Supervision, Å.O., J.U. and T.A.; Visualization, S.S.; Writing—original draft, S.S.; Writing—review and editing, S.S., Å.O., J.U. and T.A. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available in the article and the supplementary material.

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Paper III

Steinsholm, S., Oterhals, Å., Thoresen, L., Underhaug, J.,
Kousoulaki, K. & Aspevik, T.

Reduction in flavor-intense components in fish protein hydrolysates by membrane filtration.

Submitted to *Journal of Food Science*.

III

Paper IV

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IV

Sensory and chemical properties of protein hydrolysates based on mackerel (*Scomber scombrus*) and salmon (*Salmo salar*) side stream materials

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Abstract

The comparative sensory, nutritional, and chemical quality properties of protein hydrolysates produced from mackerel and salmon backbones, heads and viscera were evaluated. All hydrolysates had high essential amino acid and low biogenic amine levels, implying good raw material quality. The mackerel head-based hydrolysate was rich in ash, influencing the salty taste of the product. Hydrolysates based on viscera were significantly more taste intense and bitter compared with hydrolysates based on backbones and heads. There were only small differences in sensory intensity scores of hydrolysates based on either salmon or mackerel, with no significant differences in bitter taste.

Key words: side stream materials, enzymatic hydrolysis, salmon, mackerel, sensory properties

Introduction

In 2018, the Norwegian aquaculture and fisheries industries generated almost 1.000.000 tons of side stream materials, such as heads, backbones and viscera (Richardsen et al. 2018). More than 80 % of this were utilized; however, mostly as low-cost feed ingredients, and there is a great potential for upgrading this material for human consumption (Stevens et al. 2018). All side streams from the fish filleting industry are food grade after the primary processing and represent raw material with a high protein content, including all essential amino acids, vitamins and minerals (Stevens et al. 2018; Liaset and Espe 2008).

A promising approach for utilizing marine side stream materials is the production of enzymatic protein hydrolysates using commercial enzymes. Enzymatic protein hydrolysis is a mild processing technology that decreases the molecular weight and increases the water-solubility of the peptides, thus facilitating their recovery as a protein hydrolysate (Panyam and Kilara 1996). A major challenge in the production of enzymatic protein hydrolysates is the formation of bitter taste and unpalatable flavors. Bitter taste is related to the liberation of small hydrophobic peptides in the hydrolysis process (Fu et al. 2019; Kim and Li-Chan 2006). Bitterness may be reduced by proper choice of enzyme and processing conditions (Aspevik et al. 2016; Steinsholm et al. 2020). In addition to bitter taste, a protein hydrolysate will contain several other tastes and flavors, which are more dependent on the raw material. Water-soluble molecules and metabolites present in the raw material will follow the aqueous hydrolysate phase and may influence the overall product sensory properties (Steinsholm et al. 2020).

Fish is highly perishable, and the formation of various metabolites associated with spoilage starts right after harvesting (Prabhakar et al. 2020). Thus, proper handling and processing of as-fresh-as-possible raw materials is imperative in products destined for human consumption. Many fish contain significant amounts of trimethylamine-oxide (TMAO) which can be bacterially reduced to trimethylamine (TMA), which has a distinctive stale and unpleasant

"fishy" flavor (Wu and Bechtel 2008). Moreover, several fish species may develop biogenic amines during storage and processing that can cause food-poisoning if present in high amounts. Biogenic amines are formed by bacterial decarboxylation of amino acids, and especially scombroid fish, such as mackerel, are highly susceptible to the formation of histamine during storage (Sone et al. 2019; Biji et al. 2016). These amines are water-soluble and thus may follow the hydrolysate fraction. Today, mackerel is mostly sold as round frozen in Norway, but the share that is filleted is increasing, generating large amounts of side stream materials available for utilization in novel food applications. Side stream materials from farmed salmon, on the other hand, are highly utilized today; however, the visceral fraction is mostly used for low-value silage production (Richardsen et al. 2018). The visceral fraction is highly perishable and susceptible to the formation of TMA and biogenic amines during storage, due to high proteolytic and bacterial activity in the digestive tract.

Several studies have addressed flavor development during protein hydrolysis of marine substrates (Aspevik et al. 2016, Dauksas et al. 2004, Steinsholm et al. 2020). However, to our knowledge, no studies have compared the sensory attributes and chemical properties of hydrolysates based on different side stream fractions from mackerel and salmon, at similar hydrolysis conditions. Such studies may improve the understanding of sensory attribute development depending on raw material and hydrolysis parameters. Furthermore, understanding of the chemical composition and quality of the final products is of utmost importance for their use towards human consumption. The objectives of this study were to produce enzymatic protein hydrolysates based on salmon and mackerel heads, backbones and viscera and compare their chemical composition, nutritional quality and sensory properties.

Materials and Methods

Materials

Salmon (*Salmo salar*) heads, backbones and viscera were collected fresh from the filleting factory at Sotra Seafood (Sotra, Norway), stored cold (4 °C) and processed within 48 hours. Mackerel (*Scomber scombrus*) heads, backbones and viscera were collected directly from the filleting line at Pelagia (Selje, Norway) in October 2019 and stored frozen (- 22 °C) until processing (one month). On the day of hydrolysis, the raw materials were milled on a Comitrol 1700 processor (Urchel laboratories Inc., Valparaiso, IN) using a 3K030120U cutting head (3 mm horizontal and 5 mm vertical gaps). The protease used was FoodPro PNL (EC 3.4.24.28, DuPont, Wilmington, DE). All other chemicals used were of analytical grade.

Methods

Chemical analyses

Nitrogen was analyzed by the Kjeldahl method (ISO 5983-2, 2009) and crude protein estimated based on N×6.25. Ash was determined by combustion of raw material at 550 °C (ISO 5984-2, 2002). Dry matter was determined by drying at 103 °C (ISO, 6469-2, 1999). Fat content was determined by chloroform-methanol extraction (Bligh and Dyer 1959). Peptide size distribution was measured by HPLC size exclusion chromatography (SEC) (1260 series HPLC Agilent Technologies) using a Superdex Peptide 10/300GL column (GE Healthcare, Uppsala, Sweden), acetonitrile with TFA as eluent and UV detection at 190-600 nm. Total amino acid composition was quantified by fluorescence detection with excitation/emission at 250/395 nm. Proteins were hydrolyzed to free amino acids with 6 N HCl and derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate before HPLC (column: Waters Accq Tag 3.9 x 150 mm (Cohen and Michaud 1993). Free amino acids were measured by HPLC using Waters Pico-Tag method and UV-detection at 254 nm (Bidlingmeyer et al 1987). TMAO and

94 TMA were determined based on the micro-diffusion technique described by Conway and
95 Byrne (1933). The biogenic amines cadaverine, histamine and putrescine were measured by
96 HPLC according to Mietz and Karmas (1978).

97 *Enzymatic protein hydrolysis*

98 All raw materials, except mackerel viscera, were combined with tap water 1:1 by weight in a
99 200 L jacketed reactor equipped with overhead stirring. The mixtures were stirred vigorously
100 while raising the temperature to 55 °C before adding the enzyme. The enzyme was added at
101 concentrations between 0.17 and 0.22 wt % (Table 1) based on initial tests to reach similar
102 enzyme activity of 10 U/g protein, and the hydrolysis was run for 50 min at 55 °C. The
103 temperature was then raised to 90 °C, over approx. 15 minutes, and held at that temperature
104 for 10 minutes to inactivate the enzyme. The temperature was then lowered to 60 °C. The
105 coarse bone particles were removed (where applicable) by filtration through a 2 mm sieve
106 before pumping the remaining hydrolysate into a 3-phase separating centrifuge (Flottweg
107 Tricanter Z-23-3). Due to small raw material volume, the mackerel viscera were hydrolyzed
108 on laboratory scale. in a modified R10Bear Varimixer (A/S Wodschow &Co. Brøndby,
109 Denmark). The reaction was stirred (20 rpm) for 60 min at 55 °C, and enzyme activity was
110 terminated by heating to 90 °C in a microwave oven. The hydrolysis slurry was then
111 centrifuged (20 000 × *g*, 10 min; Sorvall LYNX 6000, Thermo scientific, Waltham, MA). The
112 liquid phase was decanted from the solid phase, and the oil and aqueous phases were
113 separated in a separatory funnel. The remaining downstream processing was the same for all
114 test raw materials.

115 The aqueous phase was further purified by crossflow filtration using a ceramic filter (0.1 µM
116 ceramic ZrO_2/TiO_2 "Kleansep" membranes; Orelis Environment, SAS, Salindres, France) to
117 remove small particles and residual lipids. The filtrate was concentrated on a 4-stage falling
118 film evaporator before spray drying (inlet temperature 220 to 225 °C, outlet temperature 94 to

100 °C). The purified hydrolysates were isolated as hygroscopic, white to off-white, fluffy powders.

Hydrolysis yield was calculated from the mass of dry powder divided by the mass of raw material:

$$\text{Hydrolysis yield} = \frac{Dried\ hydrolsate\ (kg)}{Raw\ material\ used\ in\ the\ hydrolysis\ process\ (kg)} \times 100\ \%$$

Sensory evaluation

Spray-dried hydrolysates were dissolved in tap water at 1.0 % dry matter concentration for sensory evaluation. A highly trained panel of 10 assessors (10 women; aged, 37–64 years) performed a sensory descriptive analysis according to the "Generic Descriptive Analysis" as described by Lawless and Heymann (2010) and the ISO standard 13299 (2016). The assessors are regularly tested and trained according to ISO standard 8586 (2012), and the sensory laboratory follow the practice of ISO standard 8589 (2007). The assessors agreed upon 15 attributes describing the hydrolysate samples (Table 2). Samples were served in glasses of plastic (20 ml) with a lid at a room temperature of 18°C± 2°C. All attributes were evaluated on an unstructured 15 cm line scale with labelled end points going from "no intensity" (1) to "high intensity" (9). Each assessor evaluated all samples at individual speed on a computer system for direct recording of data (EyeQuestion, Software Logic8 BV, Utrecht, the Netherlands).

In a pretest session before the main test, the assessors were calibrated on samples that were considered the most different on the selected attributes typical for the hydrolysate samples to be tested. All samples were served to the panel coded with a three-digit number in a full balanced design. Tap water and unsalted crackers was available for palate cleansing and red light was used in the sensory laboratory to masque differences in appearance between samples.

Statistical analysis

Analysis of variance (ANOVA) of the sensory profiling data was performed using Minitab (v19.2, Pennsylvania State University, PA). First, a two-way mixed effects ANOVA model was conducted to assess differences between products for all sensory attributes. Product was set as a fixed variable whereas assessor and interaction effects were set as random variables (Næs and Langsrud 1998). Mixed effects ANOVA was used to evaluate the individual fixed effects of specie and fraction on sensory attributes, still treating assessor as a random variable. Tukey's pairwise comparison was applied where significant ($p<0.05$) differences were found.

Results and Discussion

Chemical composition of raw material

The raw materials contained high levels of protein and fat (Table 3). The mackerel heads contained a significant level of ash, indicating high proportion of bones in this raw material. Both salmon and mackerel contain high levels of polyunsaturated fats, so proper handling and processing is imperative to avoid lipid oxidation (Shumilina et al. 2016, Prabhakar et al. 2020). In this study, the raw materials were collected directly from the processing lines and either frozen immediately (mackerel) or stored cold (4 °C; salmon) until processing (within 48 hours) to avoid quality degradation before enzymatic hydrolysis. The raw materials contained high levels of essential amino acids and especially mackerel backbones were rich in lysine and leucine (Table 4). Furthermore, the heads and backbones from both mackerel and salmon contained notable levels of the connective tissue amino acids glycine, proline and hydroxyproline. The discrepancy between protein levels (Table 3) and sum of amino acids (Table 4) is mainly ascribed to the commonly used N-to protein factor of 6.25, which is inaccurate for fish substrates (about 5.2) (Steinsholm et al. 2020).

167

168 ***Chemical composition of protein hydrolysates***

169 Protein hydrolysates were produced based on the different side stream fractions from salmon
170 and mackerel. Products based on mackerel contained higher levels of ash compared with
171 products based on salmon (Table 5). The hydrolysate based on mackerel heads had ash levels
172 above 38% and indicates that further processing, such as nanofiltration, should be performed
173 on these products to reduce the salt content. The amino acid profiles of the hydrolysates
174 (Table 6) were comparable, with high levels of essential amino acids. The viscera products
175 contained the highest levels of essential amino acids, followed by hydrolysates based on
176 backbones and heads for both species. Furthermore, the products based on heads contained
177 the highest levels of glycine, proline and hydroxyproline, indicating higher release of
178 connective tissue proteins from this raw material.

179 All products consisted mainly of peptides smaller than 2000 Da (Table 7). The products based
180 on viscera contained significantly higher levels of peptides < 200 Da, i.e. mostly free amino
181 acids (Table 8), accounting for almost 40 % of the total protein content in the viscera
182 products. Peptides <200 Da were less than 10% of the total protein content in head and
183 backbone hydrolysates (Table 6). This indicates significant endogenous enzyme activity in the
184 viscera, augmenting the exogenous enzyme hydrolysis process, causing higher levels of free
185 amino acids. Furthermore, all products contained more than 2.4 %, on protein basis, non-
186 protein amino acids, which may add to the nutritional properties of the products (Wu, 2020).

187 The estimation of biogenic amines and TMA in protein hydrolysates is of great importance,
188 not only from a toxicological point of view, but also as indications of degree of freshness in
189 the raw material used. Histamine, putrescine and cadaverine are three common biogenic
190 amines found in seafood, formed by decarboxylation of histidine, ornithine and lysine,

191 respectively (Biji et al. 2016). TMA is formed by bacterial reduction of TMAO (Wu and
192 Bechtel 2008). For both salmon and mackerel, the backbone-hydrolysates had highest levels
193 of TMAO, but rather low levels of TMA, indicating fresh raw-material (Table 9). On the
194 other hand, no TMAO was detected in the viscera hydrolysates and suggests that all TMAO
195 had been converted to TMA.

196 Due to high contents of free histidine (Table 8), mackerel is highly susceptible to the
197 formation and accumulation of histamine (Lehane and Olley 2000). However, there were only
198 negligible differences in histamine levels between the hydrolysates based on salmon and
199 mackerel, and all were below the acceptable toxicity threshold limit of 90 mg/ dose
200 (FAO/WHO 2012). Furthermore, the average content of biogenic amines was slightly lower
201 in the mackerel hydrolysates compared with the salmon hydrolysates. The mackerel raw
202 materials were only partially thawed before grinding and hydrolysis, whereas the salmon raw
203 materials were stored cold (4 °C) up to 48 hours before hydrolysis, possibly explaining this
204 observation. The visceral fractions were not heat treated prior to the hydrolysis process and
205 endogenous bacteria, mainly from the digestive tracts, were active in the raw materials during
206 the hydrolysis process. This could explain why the SV hydrolysate contained the highest
207 levels of biogenic amines of all the hydrolysates (Table 9) and was the only product with
208 detectable cadaverine levels (Table 9). High cadaverine levels indicate substantial microbial
209 decarboxylation of free lysine and formation of cadaverine is one of the main spoilage
210 processes during salmon visceral storage (Shumilina et al. 2016). Cadaverine and putrescine
211 are considered to be histamine potentiators, however minimum levels that potentiate toxicity
212 is unknown (FAO/WHO 2012).

213

214 ***Sensory evaluation***

215 Generic descriptive analysis was performed on the hydrolysates to compare the effect of fish
216 species and side stream fraction on sensory attributes. Except for sweet and acidic tastes, and
217 shellfish and rancid flavors, all tested attributes varied significantly between the products
218 (Table 10). Salt taste demonstrated the largest variance in intensity between products, with
219 MH giving the highest value. This may be attributed to the extreme levels of ash in this
220 product (Table 5). Furthermore, total flavor intensity and bitter taste demonstrated large
221 variance between products, with SV and SH giving highest and lowest intensity values,
222 respectively for both attributes. The intensity of rancid flavor was very low, indicating a
223 negligible level of lipid oxidation in the raw material and hydrolysis process.

224 The hydrolysate based on mackerel heads was perceived as the most intense in TMA and fish
225 flavor. This does not coincide with the chemical analysis (Table 9), where levels of TMA in
226 the MH-product were low and similar to other product, except SV. The SV product had high
227 levels of TMA (Table 9) and did also show high intensity score for TMA flavor, whereas the
228 intensity score for fish flavor was average (Table 10). This hydrolysate was also perceived as
229 the most bitter, astringent and taste intense product, possibly overshadowing the fishy flavors
230 thus demonstrating that the formation of fish flavor is complex and not fully understood, also
231 discussed by Steinsholm et al. (2020).

232 The effects of fish species and side stream fraction on the sensory attributes were evaluated
233 using mixed model ANOVA (Figure 1). Products based on viscera were significantly more
234 flavor intense and displayed significantly stronger bitter, burned and astringent attributes
235 compared with products based on heads and backbones (Figure 1a). The strong bitter taste of
236 viscera-based products may be explained by the additional endogenous proteolytic activity, as
237 previously mentioned, adding to the formation of small, bitter peptides. Furthermore, the
238 viscera contained bile that may add to the bitter taste sensation (Dauksas et al. 2004). The
239 high contents of free amino acids in the viscera hydrolysates (Table 8) may also influence the

sensory profiles by contributing to bitter, umami, sweet and acidic tastes (Kirimura et al. 1969). The visceral fractions were also associated with the highest intensity of the non-pleasant flavor cloying and astringent mouthfeel (Figure 1a). The products based on heads were significantly saltier compared with backbones and viscera hydrolysates, which can be explained by the high ash contents of the head hydrolysates as discussed above (Table 5).

Hydrolysates based on mackerel were in general perceived as more flavor intense, salt, umami and fish tasting, and with a stronger mouthfeel of fatness, compared with hydrolysates based on salmon (Figure 1b), indicating substrate-inherent metabolites influencing the overall taste sensation. On the other hand, there were no significant differences between salmon and mackerel with regards to bitter taste, reflecting similar hydrolysis conditions and substrate-independent release of bitter-peptides, in accordance with previous findings by Steinsholm et al. (2020).

Conclusion

For both salmon and mackerel, hydrolysates based on viscera were the most taste intense and more bitter compared with hydrolysates based on heads and backbones. Furthermore, hydrolysates based on viscera were rich in small peptides and free amino acids, as well as biogenic amines and TMA. Still, the levels of biogenic amines were low for all products, suggesting good quality of the raw materials and appropriateness of the processing methods used towards novel food applications. Hydrolysates based on mackerel were slightly more taste intense and had higher scores for umami, salty and fish taste as compared to those based on salmon side streams. This may partly be explained by the high levels of ash in the mackerel hydrolysates, especially in the product based on heads, and indicate the need for salt removal for human consumption purposes.

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347 oxide from raw and processed fish by-products. J Aquat Food Prod Technol. 17(1):

348 27-38.

349 Table 1 Overview of the hydrolysates and enzyme dose used.

Raw material	**Enzyme added (wt %)**	**Product coding**
Mackerel backbones	0.22	MB
Mackerel heads	0.17	MH
Mackerel viscera	0.18	MV
Salmon backbones	0.19	SB
Salmon heads	0.17	SH
Salmon viscera	0.13	SV

350

351

352 Table 2 Sensory attributes determined in the hydrolysates and their respective descriptions

Attribute	**Description of tastes/flavors related to the attributes**
Total flavor intensity	Strength of all flavors in the sample
Sweet taste	Basic sweet taste (sucrose)
Salty taste	Basic salt taste (sodium chloride)
Acidic taste	Basic sour taste (citric acid)
Bitter taste	Basic bitter taste (caffeine)
Umami taste	Basic umami taste (mono sodium glutamate)
Fish flavor	Flavor of boiled white fish
Shellfish flavor	Flavor of shellfish and shrimp
TMA flavor	Flavor of trimethylamine
Burned flavor	Processed and burned flavors
Rancid flavor	All rancid flavors (grass, hay, stearin, paint)
Flavorless flavor	Related to the flavor of water from boiled potato
Cloying flavor	Related to an un-fresh and nauseating flavor
Astringent (mouthfeel)	Related to complex feeling of contractions and dryness in the mouth
Fatness (mouthfeel)	Surface textural property related to perception of fat in a product

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355 |

356 Table 3 Chemical composition (g/100 g) of mackerel and salmon backbones, heads and viscera

	Mackerel			Salmon		
	Backbones	Heads	Viscera	Backbones	Heads	Viscera
Protein (N×6.25)	17.5	12.3	13.5	14.9	14.2	9.9
Dry matter	35.1	41.1	32.5	44.0	38.1	48.8
Ash	3.5	7.2	2.2	3.9	3.3	0.9
Fat	14.5	21.0	15.9	25.4	26.9	34.5

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358

359 Table 4 Amino acid composition (g/100 g sample) of backbones, heads and viscera from
360 mackerel and salmon

	Mackerel			Salmon		
	Backbones	Heads	Viscera	Backbones	Heads	Viscera
EAA[1]						
Arginine	1.0	0.8	0.8	0.9	0.9	0.6
Histidine	0.8	0.3	0.4	0.3	0.4	0.3
Isoleucine	0.7	0.4	0.5	0.5	0.5	0.4
Leucine	1.3	0.7	0.9	0.8	0.9	0.7
Lysine	1.5	0.7	0.9	0.9	1.1	0.7
Methionine	0.5	0.3	0.3	0.4	0.4	0.3
Phenylalanine	0.6	0.4	0.5	0.4	0.5	0.4
Threonine	0.7	0.5	0.5	0.5	0.6	0.5
Valine	0.8	0.5	0.6	0.5	0.6	0.5
Sum EAA	7.9	4.7	5.3	5.4	5.8	4.1
NEAA[2]						
Alanine	1.0	0.8	0.7	0.9	1.0	0.6
Aspartic acid	1.5	1.0	1.1	1.1	1.2	0.9
Glutamic acid	2.2	1.4	1.5	1.8	1.9	1.3
Glycine	1.1	1.4	0.9	1.8	1.6	0.8
Hydroxyproline	0.2	0.6	0.2	0.5	0.4	0.2
Proline	0.6	0.7	0.5	0.9	0.8	0.4
Serine	0.7	0.6	0.6	0.6	0.6	0.5
Tyrosine	0.6	0.3	0.3	0.3	0.4	0.3
Sum NEAA	7.9	6.7	5.9	8.0	7.9	4.9
Sum protein AA	15.8	11.4	11.2	13.3	13.7	9.0

361 [1]EAA = Essential amino acids
362 [2]NEAA = Nonessential amino acids

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Table 5 Chemical composition (g/100 g) of protein hydrolysates based on mackerel (M) and salmon (S) backbones (B), heads (H) and viscera (V)

	MB	**MH**	**MV**	**SB**	**SH**	**SV**
Protein (N × 6.25)	82.4	59.3	75.5	89.8	89.3	82.8
Total dry matter	96.3	96.4	94.9	96.3	98.0	97.3
Ash	15.7	38.3	11.9	9.3	12.6	8.5
Yield (%)*	5.0	1.5	5.0	4.0	3.3	1.8

*Calculated by dividing the mass of spray-dried hydrolysate by the mass of raw material. No effort was made to optimize these yields as the focus of this study is the chemical and sensory properties of the products.

368 Table 6 Amino acid composition (g/100 g crude protein) of protein hydrolysates based on
369 mackerel (M) and salmon (S) backbones (B), heads (H) and viscera (V).

Amino acids	**MB**	**MH**	**MV**	**SB**	**SH**	**SV**
EAA[1]						
Arginine	5.5	6.2	6.1	5.9	6.3	6.6
Histidine	6.1	3.0	2.5	2.0	1.9	1.8
Isoleucine	2.9	2.5	4.1	2.9	2.4	3.6
Leucine	6.1	5.4	7.0	5.6	4.8	6.3
Lysine	8.4	7.3	7.7	7.2	6.2	6.6
Methionine	2.3	2.4	2.8	2.6	2.7	2.5
Phenylalanine	2.3	2.5	3.4	2.6	2.6	3.3
Threonine	3.5	3.4	4.4	3.6	3.2	3.9
Valine	3.8	3.4	5.0	3.6	3.1	4.6
Sum EAA	40.8	36.1	43.0	35.9	33.1	39.3
NEAA[2]						
Alanine	5.6	7.1	5.4	6.2	6.7	5.6
Aspartic acid	7.9	7.4	8.9	7.8	7.6	7.7
Glutamic acid	13.0	12.5	12.8	12.1	12.1	11.7
Glycine	6.4	10.5	6.9	9.8	13.5	7.4
Hydroxyproline	1.7	3.2	2.1	3.3	4.6	2.2
Proline	3.6	5.2	4.2	5.0	6.8	4.6
Serine	3.8	4.2	4.8	3.8	4.3	4.2
Tyrosine	2.1	1.9	2.0	1.9	1.6	3.1

Sum NEAA	44.1	51.9	47.2	50.0	57.2	46.5
Sum protein AA	84.8	88.0	90.2	85.9	90.4	85.7

370 [1]EAA = Essential amino acids

371 [2]NEAA = Nonessential amino acids

372 Table 7 Molecular weight distribution (wt%) of hydrolysates based on mackerel (M) and
373 salmon (S) backbones (B), heads (H) and viscera (V)

MW (Da)	**MB**	**MH**	**MV**	**SB**	**SH**	**SV**
> 20000	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
20000-15000	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
15000-10000	0.1	0.1	<0.1	0.1	0.1	<0.1
10000-8000	0.1	0.3	<0.1	0.2	0.2	<0.1
8000-6000	0.5	1.0	0.1	0.9	1.0	0.1
6000-4000	1.9	3.7	0.2	3.3	3.6	0.5
4000-2000	8.2	13.9	0.8	12.9	15.9	2.3
2000-1000	14.5	17.7	1.9	18.4	22.2	5.6
1000-500	17.7	16.6	5.5	18.9	19.0	10.1
500-200	19.7	16.9	16.8	20.3	17.3	16.6
200-	37.3	29.7	74.5	24.9	20.7	64.7

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375 Table 8 Levels of free amino acids (g/100 g protein) in hydrolysates based on mackerel (M)

376 and salmon (S) backbones (B), heads (H) and viscera (V)

	MB	**MH**	**MV**	**SB**	**SH**	**SV**
Aspartic acid	0.07	0.12	1.85	0.10	0.11	1.21
Glutamic acid	0.47	0.46	2.12	0.40	0.36	1.81
Hydroxyproline	0.01	0.02	0.07	0.02	0.03	0.06
Serine	0.11	0.20	1.46	0.13	0.18	1.45
Asparagine	0.02	0.02	0.19	0.02	0.02	0.40
Glycine	0.18	0.35	0.94	0.17	0.24	1.01
Glutamine	0.25	0.73	3.18	0.48	0.50	4.59
Histidine	3.88	1.37	1.22	0.24	0.22	0.76
Threonine	0.12	0.17	1.46	0.16	0.16	1.33
Alanine	0.35	0.46	2.25	0.49	0.45	2.78
Arginine	0.30	0.57	3.84	0.21	0.24	3.99
Proline	0.07	0.15	0.99	0.11	0.08	0.72
Tyrosine	0.22	0.44	1.13	0.20	0.16	2.17
Valine	0.12	0.24	2.25	0.26	0.21	2.29
Methionine	0.35	0.61	3.18	0.52	0.41	2.78
Cysteine	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Isoleucine	0.10	0.22	2.12	0.27	0.21	1.93
Leucine	0.51	1.32	4.50	0.92	0.81	4.23
Phenylalanine	0.36	0.73	2.38	0.53	0.75	2.05
Tryptophan	0.08	0.12	0.86	0.13	0.11	0.70
Lysine	0.68	0.66	3.84	0.45	0.34	3.38

Sum protein amino acids	8.29	8.92	39.83	5.82	5.59	39.65
Non-protein amino acids						
Creatinine	1.14	0.25	<0.01	0.45	0.38	<0.01
β-alanine	<0.01	<0.01	0.03	0.16	0.24	0.16
Taurine	1.03	2.36	2.38	0.51	1.46	3.26
4-aminobutanoic acid	<0.01	<0.01	<0.01	<0.01	0.01	0.01
Citrulline	<0.01	<0.01	0.20	<0.01	0.01	0.07
Carnosine	0.07	0.05	0.25	0.09	0.03	0.17
Anserine	0.13	0.08	0.15	2.56	0.72	0.13
L-Ornithine	0.02	0.03	0.08	0.02	0.02	0.18
Sum non-protein amino acids	2.40	2.78	3.09	3.79	2.87	3.99

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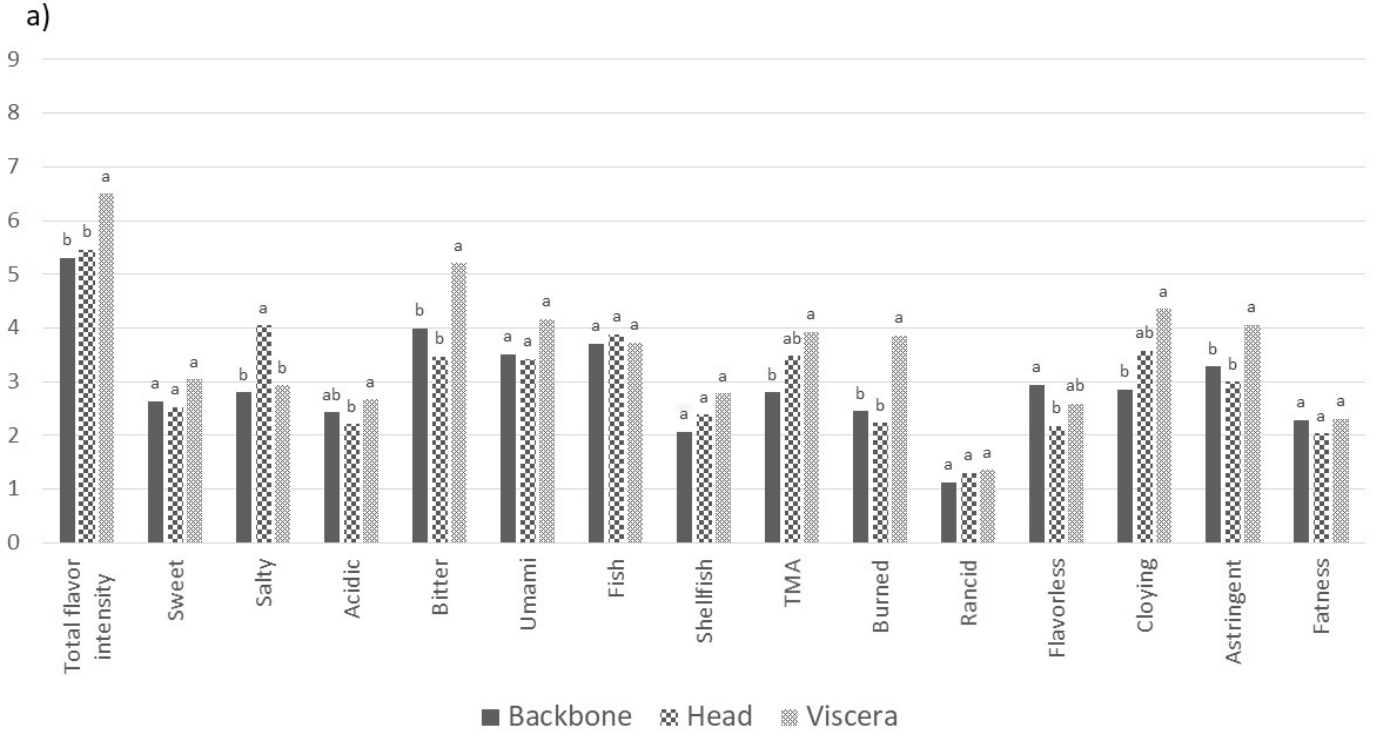
380 Table 9 Levels of trimethylamine (TMA), trimethylamine-oxide (TMAO) (mg N/100 g) and
381 biogenic amines (mg/kg) in spray-dried protein hydrolysates based on mackerel (M) and salmon
382 (S) backbones (B), heads (H) and viscera (V).

	MB	**MH**	**MV**	**SB**	**SH**	**SV**
TMA	17	15	23	14	<1	250
TMAO	162	53	<1	125	<1	<1
Putrescine	63	44	78	110	180	410
Cadaverine	<20	<20	<20	<20	<20	380
Histamine	74	38	<20	67	68	22

383

Table 10 Mean sensory of protein hydrolysates based on mackerel (M) and salmon (S) backbones (B), heads (H) and viscera (V). Different letters indicate statistical difference ($p<0.05$) among the hydrolysates by two-way ANOVA and Tukey's multiple comparison test.

	Total flavor intensity	Sweet	Salty	Acidic	Bitter	Umami	Fish	Shellfish	TMA	Burned	Rancid	Flavorless	Cloying	Astringent	Fatness
MB	5.7^{bc}	2.8	3.3^{b}	2.3	3.8^{cd}	4.2^{ab}	4.3^{ab}	2.1	3.1^{ab}	2.1^{b}	1.2	3.2^{a}	3.1^{abc}	3.3^{abc}	2.3^{ab}
MH	6.7^{a}	2.6	5.8^{a}	2.4	3.8^{cd}	3.9^{abc}	4.9^{a}	2.8	4.6^{a}	2.4^{b}	1.5	1.7^{b}	4.7^{a}	3.5^{abc}	2.6^{a}
MV	6.1^{ab}	3.1	3.0^{bc}	2.6	4.7^{ab}	4.6^{a}	3.7^{abc}	2.9	3.7^{ab}	3.2^{b}	1.4	2.8^{ab}	4.1^{abc}	3.8^{ab}	2.4^{ab}
SB	4.9^{cd}	2.4	2.3^{c}	2.6	4.2^{bc}	2.8^{c}	3.2^{bc}	2.1	2.5^{b}	2.8^{b}	1.1	2.7^{ab}	2.6^{bc}	3.2^{bc}	1.7^{c}
SH	4.2^{d}	2.5	2.3^{c}	2.1	3.1^{d}	3.0^{bc}	2.8^{c}	2.0	2.3^{b}	2.1^{b}	1.1	2.7^{ab}	2.4^{c}	2.6^{c}	2.0^{bc}
SV	6.9^{a}	3.0	2.8^{bc}	2.8	5.7^{a}	3.7^{abc}	3.8^{abc}	2.6	4.2^{a}	4.5^{a}	1.3	2.4^{ab}	4.6^{ab}	4.3^{a}	2.2^{ab}
p-value	<0.001	0.099	<0.001	0.052	<0.001	0.001	<0.001	0.079	<0.001	<0.001	0.133	0.014	0.003	<0.001	<0.001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Total flavor intensity
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Salty
Acidic
Bitter
Umami
Fish
Shellfish
TMA
Burned
Rancid
Flavorless
Cloying
Astringent
Fatness
Backbone
Head
Viscera

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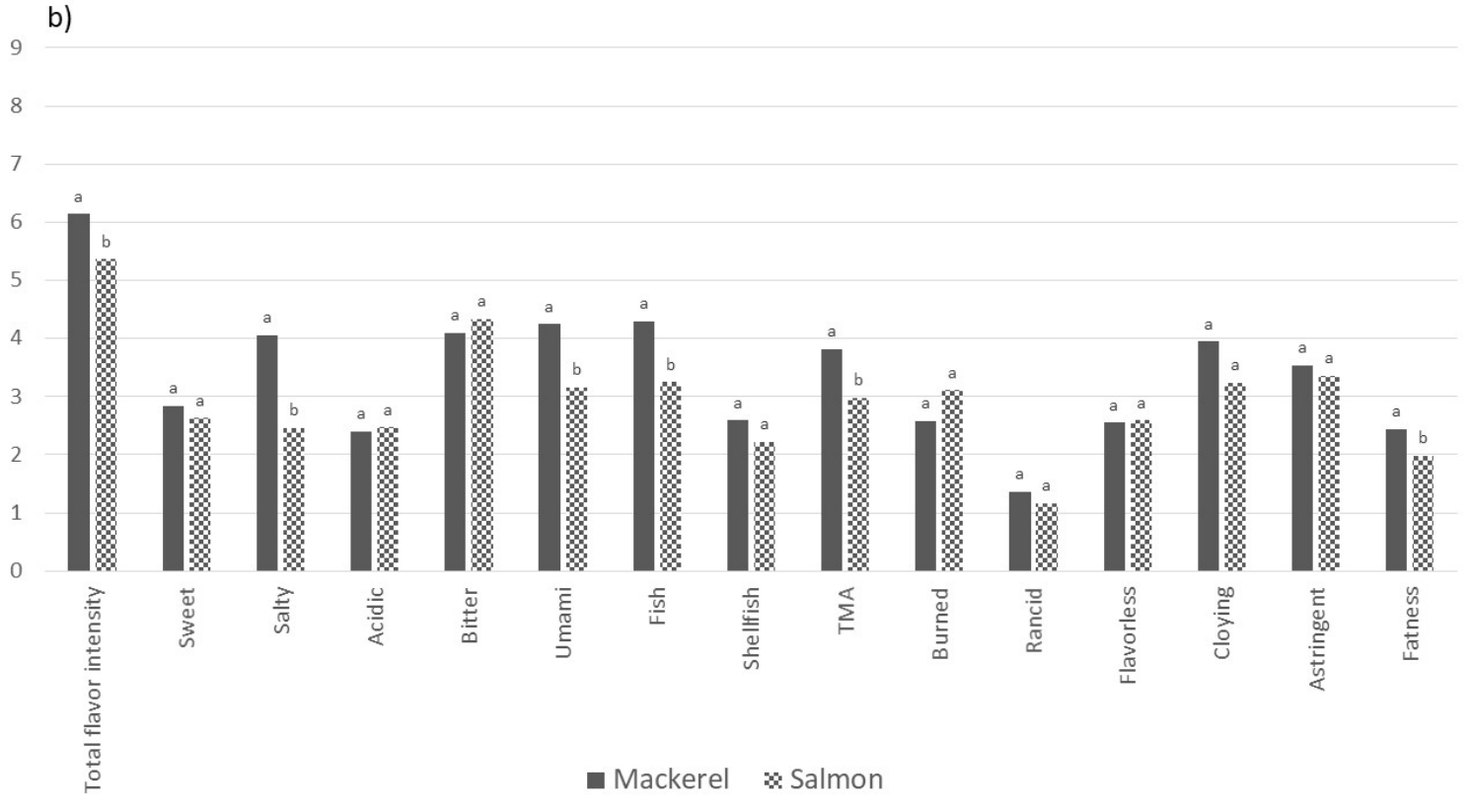
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Total flavor intensity
Sweet
Salty
Acidic
Bitter
Umami
Fish
Shellfish
TMA
Burned
Rancid
Flavorless
Cloying
Astringent
Fatness
Mackerel
Salmon

393

394 Figure captions

395

396 Figure 1 Mean intensity of sensory attributes based on (a) backbones, heads and viscera, and

397 (b) mackerel and salmon. Different letters indicate statistical difference ($p<0.05$) based on

398 mixed model ANOVA and Tukey's multiple comparison test

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