Fish protein hydrolysates based on Atlantic salmon by-products

Enzyme cost-efficiency and characterization of sensory, surface-active and nutritional properties

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Scientific environment

The research activity presented in this thesis has been carried out at the Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima) under the supervision of Dr. Åge Oterhals. Prof. Nils-Kåre Birkeland at the University of Bergen, Department of Biology, was my co-supervisor. The experimental work on fish protein hydrolysate processing was performed at Nofima, Bergen. The chemical analyses were performed at Nofima BioLab (accredited according to ISO 17025). The sensory evaluations were performed at Nofima, Ås (accredited according to ISO 8586). ^{1}H-NMR analyses, used to determine critical micelle concentration, were performed at the University of Bergen, Department of Chemistry, in collaboration with Dr. Christian Totland.

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Abstract

The world fisheries and fish farming industries generate large amounts of by-products after the primary processing of fish to edible products. In Norway alone, this accounted for almost 900,000 tons in 2014. Based on present industrial practice, most of the by-products are either discarded or used in the manufacture of low-value commodity products such as fish silage, fishmeal and oil. By-product material from the primary filleting process, such as heads and backbones, contain high-quality food grade proteins with a great potential for value creation. The production of water-soluble protein hydrolysates using exogenous proteases may give an increased valorization of the by-products for human consumption and offers a mild and efficient processing approach without prejudicing the nutritional value.

Proteases act by cleaving proteins into smaller peptides and free amino acids that are more water-soluble and have altered sensory and surface-active properties compared to the intact protein. A major drawback in the production of commercial fish protein hydrolysates (FPHs) is the formation of bitter and unpalatable tastes due to exposure of hydrophobic amino acids and moieties during the hydrolysis process. Moreover, the cost of enzymes and high processing expenses may be a hindrance in a profitable production of FPHs for human consumption. This has led to a demand for new and improved knowledge of cost-efficiency of enzymes and the process conditions that influences the formation and reduction of bitter taste. Reduction of the bitter taste is of utmost importance in the production of FPHs, but also knowledge of the surface-active and nutritional properties of a hydrolysate may be important for its potential inclusion in food products.

The main objective of this study has been to produce FPHs based on Atlantic salmon (*Salmo salar*) head and backbone products with low bitter taste, good surface-active properties and high nutritional value. The hydrolytic and cost efficiency of five commercial endopeptidases (Alcalase 2.4L, Corolase 7089, Neutrase 0.8L, Promod 671L and Protex 7L) have been evaluated and compared in the hydrolysis of the salmon substrate, based on the pH-STAT method. The sensory properties of the hydrolysates

were assessed based on generic descriptive analysis by a trained sensory panel. The hydrolysate surface-active properties were evaluated based on critical micelle concentration (CMC) using ^{1}H NMR. Nutritional properties have been evaluated based on calculations of protein efficiency ratio (PER), amino acid score (AAS), digestible indispensable AAS (DIAAS) and protein digestibility corrected (PDCAAS) using FAO recommendations of indispensable amino acids for small children (six months to three years).

In Paper I, substrate specific numbers for nitrogen factor (f_N = 5.23 g protein/g nitrogen) and total number of peptide bonds (h_{tot} = 9.3 meqv/g protein) were developed to enable more accurate calculations of hydrolysis parameters, such as protein recovery (PR) and degree of hydrolysis (DH). Based on the experimental pH-STAT data, response surface regression models were established to evaluate the combined effects of hydrolysis time and enzyme activity-to-substrate ratio (U/S) on DH and yield of solubilized proteins. The models were combined with activity-specific enzyme cost to estimate the cost efficiency of the individual enzymes, important in upscale and industrial applications. The study demonstrated that all enzymes were equally efficient in hydrolyzing the substrate at low U/S, however, Alcalase 2.4L, Protex 7L and Promod 671L gave higher final DH at high enzyme addition, compared to Corolase 7089 and Neutrase 0.8L. All enzymes were equally efficient in solubilizing the substrate. This may be explained by the enzymes preferentially cleaving peptides already solubilized at high enzyme dose, rather than dissolving new proteins. A linear correlation of DH determined by the pH-STAT and OPA methods was established, which permits the use of the regression models in upscaling of processes where pH-STAT is not applicable.

The studies in Paper II confirmed that both molecular weight distribution and enzyme specificity were important for the formation of bitter taste and surface-active properties of the hydrolysates. High intensity of bitter, astringent and pungent attributes was associated with a high DH (≥25%) and peptides with molecular weight <2000 Da. Hydrolysates based on Alcalase 2.4L were significantly more bitter compared to Promod 671L and Protex 7L. Other relevant attributes tested (sweet, salt, umami, acidic, sea and fish) were separated based on the dilution gradient to reach an identical

test protein concentration. This suggests that these attributes are related to components inherent to the raw material rather than peptides and amino acids formed during the hydrolysis process. The determined CMCs revealed higher values for all hydrolysates compared to conventionally used food surfactants. The measured CMC was dependent on DH and molecular weight distribution, where low DH gave lowest CMC for all enzyme products. Superior properties (i.e. low bitter taste and low CMC) could be achieved without enzyme hydrolysis and only heat denaturation of the raw material, albeit, at a low yield of solubilized proteins.

In Paper III, the effects of exopeptidase activity and activated carbon (AC) adsorption in the debittering of moderately hydrolyzed FPH with DH ≤18% and broad molecular weight distribution were evaluated. Exopeptidase (Flavourzyme 1000L) treatment revealed high release of the hydrophobic amino acids leucine, isoleucine and valine compared to only endopeptidase activity. However, only minor and insignificant reduction of bitter taste was observed, possibly explained by high content of peptides >500 Da and the hydrolytic specificity of the used exopeptidase. Acidic ACs gave largest reduction of bitter taste when added at 1% on protein basis ($p = 0.09$). The reduction of bitter taste could not be explained by the adsorption of peptides and amino acids from the hydrolysates. Chemical analyses revealed a decrease in salt-free ash and an increase in crude protein on dry matter content in the hydrolysates after treatment with AC. This suggested that ACs adsorb non-protein constituents. The nutritional properties of the raw material and resulting hydrolysates revealed low levels of tryptophan, leucine, isoleucine and valine to meet dietary requirements of children under three years of age. Treatment with exopeptidase and AC did not influence the hydrolysates nutritional properties.

List of publications

I Aspevik, T., Egede-Nissen, H., Oterhals, Å.: "A systematic approach to comparison of the cost efficiency of endopeptidases to hydrolyze Atlantic salmon (*Salmo salar)* by-products". *Submitted.*

II Aspevik, T., Totland, C., Lea, P., Oterhals, Å.: "Sensory and surface-active properties of protein hydrolysates based on Atlantic salmon (*Salmo salar*) by-products". *Revised paper submitted Process Biochemistry*.

III Aspevik, T., Oterhals, Å.: Effect of exopeptidase and activated carbon treatment on sensory attributes and nutritional properties of moderately hydrolyzed Atlantic salmon (*Salmo salar*) by-product protein hydrolysates". *Submitted.*

Contents

Abbreviations

α	Degree of dissociation of α-NH_3 groups
AAS	Amino acid score
AC	Activated carbon
ANOVA	Analysis of variance
B	Base
CMC	Critical micelle concentration
CP	Crude protein
DH	Degree of hydrolysis
DIAAS	Digestible indispensable amino acid score
DM	Dry matter
f_N	Nitrogen factor
FAO	Food and Agriculture Organization of the United Nations
FPH	Fish protein hydrolysate
GRAS	Generally regarded as safe
h	Hydrolysis equivalents, defined as milliequivalents of peptide bonds cleaved per gram protein (meqv/g)
h_{tot}	Total number of peptide bonds in a protein, defined as milliequivalents per gram protein (meqv/g)
HPF	Hydrophobic peptide fraction
ISO	International Organization for Standardization
N_B	Normality of base
N	Nitrogen
NMR	Nuclear magnetic resonance
OPA	*ortho*-phthaldialdehyd
PCA	Principal component analysis
PDCAAS	Protein digestibility corrected amino acid score
PER	Protein efficiency ratio
PR	Protein recovery
QPS	Qualified presumption of safety

SDS	Sodium dodecyl sulfate
TR1	Taste receptor related to sweet and umami tastes
TR2	Taste receptor related to bitter taste
TRC	Taste receptor cell
TMA	Trimethylamine
TMAO	Trimethylamine oxide
U	Units of proteolytic activity, defined as release of tyrosine (μmol) per min
U/S	Units of proteolytic activity per gram substrate
WHO	World Health Organization

1. Introduction

The rapid growth, urbanization and increasing prosperity of the world population is demanding an improved utilization of existing protein sources along with the development of new and sustainable ones. By 2050, the global demand for protein is expected to double, not only due to the population pressure, but also by an increased recognition of the important role of proteins in a healthy diet in general and especially for the growing elderly population (Boland et al. 2013). Fish is an important source of proteins and provides nearly 17% of the global protein intake (FAO 2014). However, the availability of captured fish is partially limited by already overexploited resources, and the future increasing demand for fish proteins needs to come from aquaculture. At the same time, huge amounts of protein rich by-products from world fisheries and fish farming industries are either discarded or used in the manufacture of low-value feed products such as fish silage, fishmeal and oil (FAO 2014; Richardsen et al. 2015). Fish processed for human consumption gives food grade by-products of high quality with the possible utilization into a number of food products (Bechtel 2003). One of the high priority areas within the global seafood industry is a more sustainable utilization of fish by-products for human consumption (FAO 2014).

In Norway alone, the fish processing industry generated almost 900,000 tons of by-products in 2014 (Richardsen et al. 2015). Compared to other nations, Norway has a high exploitation level of these resources. Nonetheless, approximately 30% are un-exploited, mostly from the cod fisheries. This can mainly be ascribed to the use of large industrial fishing vessels with a lack of technology for processing the by-products, or small fishing boats without the capacity for bringing the by-product material ashore. In addition, the current perceived economics in bringing the by-products ashore are low (Olafsen et al. 2013). Fish by-products are parts of the fish that is removed before it reaches the final consumer market in order to improve their quality, reduce shipping weight, or increase the value of the products (Ramirez 2007). The by-products can be classified based on processing into heads, backbones, cut-offs, viscera, skin and blood. The head and backbone are the major by-product fractions and typically constitute around 70% of the by-products (Ramirez 2007).

Norway is the main producer of Atlantic salmon with a round-weight volume of 1.26 million metric tons in 2014 (Statistics Norway 2015). Based on current processing, around 55,000 tons of salmon head and backbone by-products were available in Norway in 2014 (Richardsen et al. 2015). By 2050, the production of Atlantic salmon is expected to have increased to up to 5.0 million metric tons (Olafsen et al. 2012). At the same time, the increased level and novel processing routes will leave more of the by-products inland at the processing plant. In the years to come there will be an increased exploitation of the fish raw material, including all by-products. To support building of this bio-economy, there is a need for new competence and increased knowledge within by-product processing and utilization.

A promising industrial exploitation route of marine by-products is the production of water-soluble fish protein hydrolysates (FPHs). Protein hydrolysates are defined as proteins that are chemically or enzymatically broken down to peptides and/or free amino acids (Adler-Nissen 1986). Protein hydrolysis by the use of endo- and/or exogenous enzymes offer the breakdown of proteins into water-soluble peptides with subsequent easy removal of residual bones, fat and particulate proteins by centrifugation. Enzymatic cleavage of proteins will decrease molecular weight, increase solubility and alter the hydrophobic/hydrophilic balance, due to the exposure of hydrophobic amino acids and moieties (Panyam and Kilara 1996). The molecular changes occurring during protein hydrolysis may result in altered sensory and surface-active properties of the hydrolysates compared to the intact protein. A main obstacle in the production of FPHs is the formation of bitter and unpalatable tastes generated during the hydrolysis process, mainly ascribed to small hydrophobic peptides. A prerequisite for the utilization of FPHs as a food ingredient is a neutral as possible sensory profile with negligible bitter taste.

A number of factors, including substrate composition, protease specificity and processing conditions, influences the hydrolysis process and the yield of solubilized proteins. There is a large number of commercial protease formulations available for the hydrolysis of fish substrates (Sustainable Chemistry Solutions 2015). Based on choice of protease, enzyme-to-substrate ratio, pH, temperature and processing time, different

products can be produced from the same substrate. Many scientific studies are published within the field of protein hydrolysis and comparison of protease performance. However, most studies have compared the proteases based on equal enzyme weight-to-substrate ratio and within the optimum pH-range of the respective proteases. The use of pH-adjustment and control is less attractive in industrial applications due to the addition of high amounts of acid or base that will increase the processing costs and the levels of salt in the final product. Increased salt will influence the sensory and nutritional properties of the resulting hydrolysates. There is a need for a more systematic comparison of different proteases and the properties of the resulting hydrolysates within the natural pH range of the substrate. This will enable a more industrially relevant comparison of different hydrolysate products. However, different proteases will have varying hydrolytic activity at the natural pH of the substrate and use of exogenous enzymes may introduce a high processing cost. This must be taken into consideration when comparing protease performance and hydrolysate properties.

1.1 Aims and objectives of the study

The main objective of the research activity was to develop knowledge supporting an improved utilization of Atlantic salmon by-products in the manufacture of food-grade FPH with low bitter taste, good surface-active properties and high nutritional quality.

Sub objectives:

- To develop a protocol for comparison of cost and hydrolytic efficiency of proteases.
- To assess the formation of bitter taste, and other sensory attributes, as a function of degree of hydrolysis, molecular weight distribution and enzyme specificity.
- To use critical micelle concentration to evaluate the surface-active properties of the FPHs.
- To assess the effects of debittering techniques to reduce the bitter taste of FPH.
- To evaluate the nutritional quality of the salmon substrate and FPHs produced based on short hydrolysis times and treated with debittering techniques.

2. Background

During the past 50 years, there has been an increasing interest in the utilization of fish by-products for food applications (Mackie 1982). The production of cheaper and more efficient food-grade proteases has opened up new possibilities within the processing of fish by-products. Challenges related to the formation of unpalatable bitter taste and high processing costs are the major limitations in the development of FPH for human consumption. The balance between enzyme performance, process time, yield of solubilized protein, and hydrolysate sensory, surface-active and nutritional properties are of utmost importance in the production of a competitive high-quality FPH for food applications. To obtain an increased profitability and food grade production of FPH, several criteria are required:

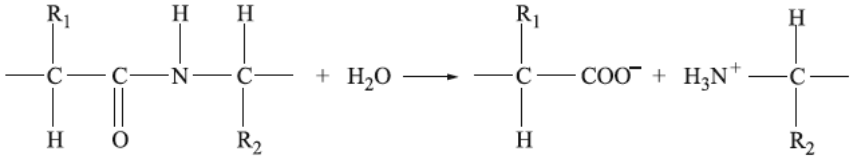
- The chosen protease must have high hydrolytic efficiency to effectively hydrolyze and solubilize the substrate at relatively short hydrolysis times.
- The FPH should taste as neutral as possible. Proper knowledge of the factors influencing the formation, and possible removal, of bitter taste, and other sensory attributes, is imperative for successful food utilization.
- Knowledge of the surface-active properties of the FPH to evaluate their potential applications as emulsifiers or foaming agents
- The FPH should have a high nutritional quality, with high content of all indispensable amino acids.

The work in this thesis will aim to meet these criteria through a systematic evaluation of protease performance and sensory, surface-active and nutritional properties of resulting hydrolysates. Hydrolysates based on different proteases were compared based on equal degree of hydrolysis (DH) and molecular weight distribution, rather than equal hydrolysis time, to enable a more accurate basis for comparison.

3. Production of fish protein hydrolysates

3.1 Protein hydrolysis

The main purpose of a hydrolysis process is increased protein recovery and yield of valuable components. Protein hydrolysis is the breakdown of proteins to smaller peptides and free amino acids, leaving the new molecules more water soluble than the intact protein. The word *hydrolysis* literally means reaction with water and protein hydrolysis requires the presence of H_2O molecules. The reaction releases H^+-ions at pH-levels > pH 6 and can be written as the following equation (Kunst 2003):



(1)

Hydrolytic breakdown of fish proteins can be obtained using chemical or enzymatic processes. Chemical processing includes the use of acid or alkali to cleave the peptide bonds. Acid hydrolysis is preferred over alkali reactions due to the adverse effects on the nutritive value with use of the latter. Thermal processing of proteins at alkaline pH partly destroys the amino acids threonine, cysteine, lysine, tyrosine and arginine, causes formation of toxic substance like lysinoalanine and leads to racemization of L-amino acids to undesired D-amino acids (Friedman 1978; Kristinsson and Rasco 2000a). Acid hydrolysis is frequently used for vegetable protein hydrolysis for the production of flavor enhancers used in bouillon. The substrate is treated with 4-6 M HCl at 100-130 °C followed by neutralization with NaOH (Aaslyng et al. 1998; Kristinsson and Rasco 2000a). There are several disadvantages with chemical processing: neither acid nor alkali hydrolysis are very specific and generate large amounts of salt in the neutralizing process. Alternatively, adjusting the pH to the isoelectric point of the protein (about pH 5.5) will cause minimum solubility and subsequent precipitation of the proteins; i.e. isoelectric precipitation. This method has

been suggested to allow for a high recovery of functional and nutritional proteins from sources that are difficult to process through conventional means (Gehring et al. 2011).

Enzymatic hydrolysis is carried out by proteolytic breakdown of the proteins to smaller peptides and free amino acids. It is regarded as a mild process resulting in products of high product yield without prejudicing the nutritional quality of the final products. Although enzymatic hydrolysis is preferred over thermal and chemical hydrolysis, it has some drawbacks; there is a need to inactivate the enzyme at the end of the reaction - either by chemicals, high temperatures or high or low pH. Both endogenous and exogenous enzymes may be used in the production of FPH. Endogenous enzymes are naturally occurring in the fish raw material, mainly digestive enzymes found in the viscera and digestive tract (Sovik and Rustad 2005). Fish processing using endogenous proteases is the production principle of silage. Formic acid is added to the raw material to obtain a pH less than 4 to avoid bacterial action. The liquefaction process usually lasts for 2-10 days and the resulting fish silage may be used as protein ingredients in animal feed (Tatterson and Windsor 2001). Endogenous enzyme hydrolysis is also a common process in Asia, where it is the method of producing fish sauce. Salt is added to conserve the fish by-products and the autolytic process usually lasts for 6-12 months (Gildberg et al. 2000). The use of endogenous enzymes is an inexpensive and mild process; however, it usually requires long hydrolysis times and gives an unspecific hydrolysis. The addition of commercially available proteases (exogenous enzymes), on the other hand, is considered the best choice for producing food-grade FPH (Guerard 2007). This process is regarded as highly specific and reproducible and may enable the tailoring of well-defined hydrolysate products. However, the use of exogenous enzymes will add to the processing costs.

Enzymatic hydrolysis of proteins is a complex process because of differences in the specific accessibility to enzymatic cleavage of different peptide bonds (Linder et al. 1995). Based on type of protease, enzyme-to-substrate ratio and process time, different levels of DH and solubilized protein can be achieved. At the start of the reaction, the majority of the substrate are insoluble and the enzymes adsorb to the solid particles and cleave peptide bonds that are exposed to the aqueous phase (Archer et al. 1973). This

will increase numbers of ionizable groups of the peptides, leaving the newly formed peptides more water-soluble than the intact protein (Panyam and Kilara 1996). A typical protein hydrolysis process is usually characterized by an initial rapid phase where many peptide bonds are available for cleavage. Dependent on enzyme activity, the rate of the initial phase will vary (Fig. 1). As the hydrolysis progresses there is a reduction of the reaction rate. This can be explained by a combination of reduced enzyme activity depending on a decrease in pH, fewer peptide bonds available for cleavage (O'Meara and Munro 1984), enzyme inactivation by hydrolysis products (Adler-Nissen 1986), substrate inhibition (Moreno and Cuadrado 1993) and the possible content of protease inhibitors present in the substrate (Hjelmeland 1983).

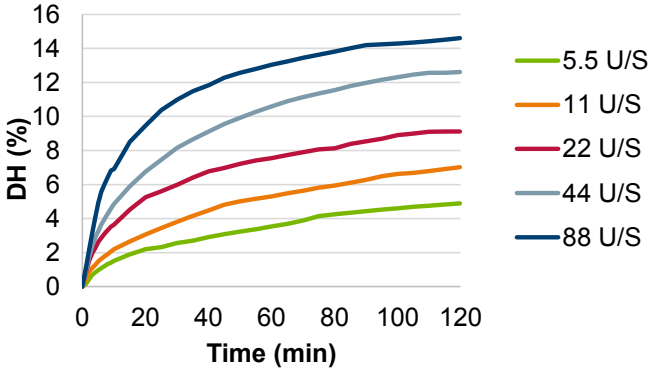


Figure 1. Protein hydrolysis of Atlantic salmon by-products (Paper I) by Alcalase 2.4L at five enzyme activity levels (5.5-88 U/S). The degree of hydrolysis (DH) increases as a function of hydrolysis time and enzyme activity. At first (0-20 min) there is a rapid increase in DH, due to many peptide bonds cleaved. As the hydrolysis progresses, fewer peptide bonds are available for cleavage and the reaction rate is reduced.

The factors that influences an enzymatic hydrolysis process are:

- Substrate (type and composition)
- Enzyme (hydrolytic specificity and efficiency)
- Enzyme activity-to-substrate ratio
- Hydrolysis conditions (pH, temperature, time of hydrolysis)

3.2 Substrate

The substrate is an important factor in a hydrolytic process. The amino acid composition and sequence of the protein not only determine the nutritional and physicochemical properties of the final hydrolysate, but also affects to what extent and how easily it can be hydrolyzed by a given protease (Kunst 2003). Food proteins include 21 amino acids (including hydroxyproline; Table 1), that provide 441 possible combinations of peptide bonds. Based on the amino acid sequences (primary structure), the peptide bonds align themselves in secondary structures (α-helixes, β-sheets and random coils) and tertiary structures (folding to a 3D geometric shape) (Damodaran 1997). Generally, there are two types of food protein tertiary structures: fibrous and globular proteins. Fibrous proteins are long, rod-like polypeptides and include structural proteins, such as collagen and muscle fibril protein (actin and myosin). Globular proteins are compact spherical molecules, e.g. myoglobin (Nelson and Cox 2008). Proteins with a large number of hydrophobic amino acids favor the globular tertiary structure. The hydrophobic amino acids orient toward the center of the protein molecule and interact with each other by hydrophobic interactions (Vaclavik and Christian 2008).

Both by-products from fish fileting lines and underutilized species (by-catch), suitable for human consumption, can be used as substrate in the manufacture of FPHs. Regardless, proper food-grade handling of the chosen substrate is imperative. Head and backbone by-products from fish filleting lines represent an excellent source in the production of FPHs with food-grade quality. This raw material has a high content of proteins (Ramirez 2007) and a low amount of endogenous enzymes (Sovik and Rustad 2005; Paper I). In contrast, the viscera fractions contain high levels of digestive enzymes that could influence the hydrolysis process (Sovik and Rustad 2005), are often regarded as less palatable and contain bile that gives an unpleasant taste (Dauksas et al. 2004). In addition to which parts of the fish are being hydrolyzed, the nature and quality of the raw material may have great impact on the hydrolysis and quality of the final products, e.g. FPH produced from fatty species may be exposed to off-flavor formation caused by lipid oxidation (Mackie 1982; Kristinsson 2007). Atlantic salmon

has a high lipid content (25-30%) and it is important to ensure proper removal of lipids in the downstream processing of the protein hydrolysates.

Table 1. Protein amino acids, their side chains characteristics, molecular weight and nutritional definition

Amino acid	Abbreviation	Side chain	MW (Da)	Definition
Arginine	Arg	Charged	174.2	Indispensable
Histidine	His	Charged, aromatic	155.2	Indispensable
Isoleucine	Iso	Nonpolar, branched	131.2	Indispensable
Leucine	Leu	Nonpolar, branched	131.2	Indispensable
Lysine	Lys	Charged	146.2	Indispensable
Methionine	Met	Nonpolar	149.2	Indispensable
Phenylalanine	Phe	Aromatic	165.2	Indispensable
Threonine	Thr	Polar neutral	119.1	Indispensable
Tryptophan	Trp	Aromatic	204.2	Indispensable
Valine	Val	Nonpolar, branched	117.1	Indispensable
Alanine	Ala	Nonpolar	89.1	Dispensable
Aspartic acid	Asp	Charged	131.1	Dispensable
Asparagine	Asn	Polar neutral	132.1	Dispensable
Cysteine	Cys	Polar neutral	121.2	Dispensable
Glutamic acid	Glu	Charged	147.1	Dispensable
Glutamine	Gln	Polar neutral	146.2	Dispensable
Glycine	Gly	Nonpolar	75.1	Dispensable
Hydroxyproline	Hyp	Polar neutral	131.1	Dispensable
Proline	Pro	Nonpolar	115.1	Dispensable
Serine	Ser	Polar neutral	105.1	Dispensable
Tyrosine	Tyr	Aromatic	181.2	Dispensable

Fish head and backbone by-products are complex substrates that contain numerous different proteins, including structural muscle proteins (e.g. myosin and actin), connective tissue protein (e.g. collagen) and sarcoplasmic proteins (e.g. globulin and myoalbumin) (FAO 2005), all of which have different compositions, tertiary structures

and denaturing temperatures. Most frequently, hydrolysis temperatures between 40 °C to 60 °C are used, as this is a valid temperature range for most commercially available proteolytic enzymes. Within this temperature range, both myosin and collagen proteins are expected to be denatured (Skipnes et al. 2008). Denatured proteins have a reduced efficiency of hydrolysis due to hydrophobic and sulfhydryl interactions between the peptides (Mutilangi et al. 1995). In addition, fish substrates may contain protease inhibitors that can influence the enzyme performance and overall hydrolysis process (Hjelmeland 1983).

The determination of protein content of foodstuffs is usually reported as crude protein (CP), and is based on analytical procedures for constituents of protein (Adler-Nissen 1986). Among these, the Kjeldahl Nitrogen method, first introduced by Johan Kjeldahl as early as 1883, is by far the most widely applied method (Kjeldahl 1883). CP is estimated by analysis of nitrogen (N) multiplied by a suitable nitrogen-to-protein conversion factor (f_N). Generally, a conversion number of 6.25 is applied, which assumes that a protein contains 16% N. In reality, food proteins can contain anywhere between 9% (polytyrosine) and 36% (polyarginine) nitrogen (Mosse 1990). Food proteins contain other nitrogenous organic compounds, such as non-protein amino acids and nucleotides that will influence the determination of true f_N. Ingredient-specific f_N of corn, soybean, poultry by-products and meat and bone meal have been calculated as 5.37, 5.39, 5.13 and 5.07, respectively (Sriperm et al. 2011). The determination of actual CP is particularly important for the dosage of enzyme as the dosage recommendations provided by the manufacturers are usually based on protein basis.

3.3 Proteases

The enzymes responsible for cleaving peptide bonds belong to the family of proteases (also called peptidases or proteinases). Several types of proteases are known (Table 2) and can be classified based on critical amino acid required for the catalytic function, pH-optimum for their activity, their site of cleavage or the requirement of a free thiol group (Rao et al. 1998). Proteases can be divided into endopeptidases and

exopeptidase, based on their preference for cleavage. Endopeptidases catalyze the peptide bonds in the interior of the peptide chain, leaving two new, smaller peptides. The exopeptidases, on the other hand, require the presence of an unsubstituted N- or C-terminus, only releasing free amino acids or small di- and tripeptides. Enzymes are systematically classified by a European Commission (EC) number according to rules of nomenclature defined by the Nomenclature Committee of International Union of Biochemistry and Molecular Biology (NC-IUBMB 1992). Proteolytic enzymes are defined as number 3 (hydrolases) 4 (proteases) and 11-19 (exopeptidases) or 21-99 (endopeptidases).

Table 2. Classification of proteases (Shankar 2010; Brenda 2016)

Protease	**EC Number**	**Peptidase type**	**Action**
Exopeptidases	3.4.11	Aminopeptidase	Releases N-terminals
	3.4.13	Dipeptidase	Acts only on dipeptides
	3.4.14	Dipeptidyl peptidase Tripeptidyl peptidase	Releases N-terminal di-peptides and tripeptides
	3.4.15	Peptidyl dipeptidase	Releases C-terminal dipeptides
	3.4.16	Carboxypeptidase (serine)	Releases C-terminals (serine at active site)
	3.4.17	Carboxypeptidase (metallo)	Releases C-terminals (metal requiring protease)
	3.4.18	Carboxypeptidase (cysteine)	Releases C-terminals (cysteine at active site)
	3.4.19	Omega peptidase	Releases modified residues from N- or C- termini
Endopeptidases	3.4.21	Serine endopeptidase	Serine at active site
	3.4.22	Cysteine endopeptidase	Cysteine at active site
	3.4.23	Aspartic endopeptidase	Aspartate at active site
	3.4.24	Metallo endopeptidase	Metal requiring protease
	3.4.25	Proteasome endopeptidase	Very broad specificity
	3.4.99	Endopeptidase of unknown catalytic mechanism	

The commonly used commercial proteases are of microbial, animal or plant origin (Rao et al. 1998). Microbial enzymes offer several advantages, including a wide variety of available hydrolytic specificity and great pH and temperature stabilities. The microorganisms used for production of food-grade enzymes need to be classified as "generally recognized as safe" (GRAS) (FDA 2015) and/or "qualified presumption of safety" (QPS) (EFSA 2013).

Choice of enzyme is determined by parameters such as required amounts of free amino acids or a demand for larger peptides. Depending on the enzyme hydrolytic specificity and efficiency, different peptide products can be produced from the same substrate. The hydrolytic *specificity* of a protease is an inherent property of the enzyme and determines where the protease cleaves the peptide bonds. The hydrolytic *activity,* on the other hand, determines how fast the enzymes cleave the peptide bonds and is often given in units (U). One U represents the quantity of liberated amino acids under standardized assay conditions (Cupp-Enyard 2008). The hydrolytic *efficiency* of a protease is a measure of how efficiently the protease cleave a certain substrate and is dependent on both specificity and activity. The use of highly specified proteases has an impact on the composition and chemical properties of the resulting peptide.

There are a large number of companies producing and offering different types of protease formulations suitable for the production of FPHs (Sustainable Chemistry Solutions 2015). Commercial protease formulations may have pure endopeptidase activity or combinations of endo- and exopeptidase activities and choice of appropriate enzyme is essential for the desired final product. Among the most common proteases that work well under neutral conditions are the subtilisins (3.4.21.62), the bacillolysins (3.4.24.28) and leucine aminopeptidases (3.4.11.1).

3.4 Hydrolysis conditions and downstream processing

The production of FPH includes the following steps:

1. Preparation of raw material (mincing and dilution with water)
2. Adjustment of reaction temperature and pH

3. Addition of enzyme
4. Hydrolysis reaction for a predefined hydrolysis time
5. Inactivation of enzyme
6. Separation of the water, oil and semi-solid phases

For most processes, the raw material is properly minced and diluted with water to allow good mixing and enzyme access (Kristinsson 2007). Proper dilution can prevent product inhibition and maximize product yield, but added water is also a factor influencing processing costs. In industrial operations, final products are often dried and a compromise between desired product yield and water that needs to be removed is imperative. Most commonly, the raw material is mixed with equal amounts of water.

The hydrolysis process and enzyme performance depends on reaction temperature and pH. These are often selected based on the optimum of the proteases (Kunst 2003). However, adjusting the pH requires acid or base that will introduce high levels of salt to the final hydrolysate after neutralization. This might reduce the nutritional value of the product and should be avoided if possible. Several commercial proteases working well under the substrate natural pH are available, avoiding the need for pH-adjustment. At the end of the reaction, the enzyme activity is terminated by irreversible denaturation of the enzyme by heating the slurry to temperatures above 85 °C for at least 10 minutes. Finally, the slurry is separated by centrifugation into three phases: an oil phase, a water phase and a sludge phase. The water phase constitutes the water-soluble protein hydrolysate, while the sludge phase contains insolubilized proteins and bones.

3.5 Quantification of a hydrolysis process

The main objective of a hydrolysis process is to obtain a high yield of solubilized proteins and peptides of desired molecular size for their intended application. Yield of solubilized protein can be defined as protein recovery (PR) that gives a measure of protein in the water phase compared to that in the original substrate:

$$\boldsymbol{PR} = \frac{\boldsymbol{Protein\ in\ the\ water\ phase\ (g)}}{\boldsymbol{Protein\ in\ the\ original\ sample\ (g)}} \times \mathbf{100}\ \% \qquad (2)$$

Without enzyme activity and only thermal coagulation of the raw material, only around 20% of the proteins will be solubilized (Papers I and II) and consist of mostly small water-soluble proteins, such as sarcoplasmic proteins, free amino acids and solubilized gelatin (Mohtar et al. 2010; Oterhals and Samuelsen 2015). Enzymatic hydrolysis will decrease the molecular weight of the peptides and increase the number of ionizable groups, leaving the new peptides smaller and more water-soluble than the intact proteins (Panyam and Kilara 1996). However, as the hydrolysis progresses, fewer proteins become solubilized, and the enzymes preferentially cleave the peptides already in solution. Consequently, the relationship between PR and the DH is not linear (Fig. 2). DH is the most frequently used parameter to describe the effectiveness of a hydrolysis process and is defined as the percentage of peptide bonds cleaved:

$$\boldsymbol{DH = \frac{h}{h_{tot}} \times 100\%} \tag{3}$$

h is peptide bonds hydrolyzed and h_{tot} is total number of peptide bonds per protein equivalents in the substrate (Nielsen et al. 2001).

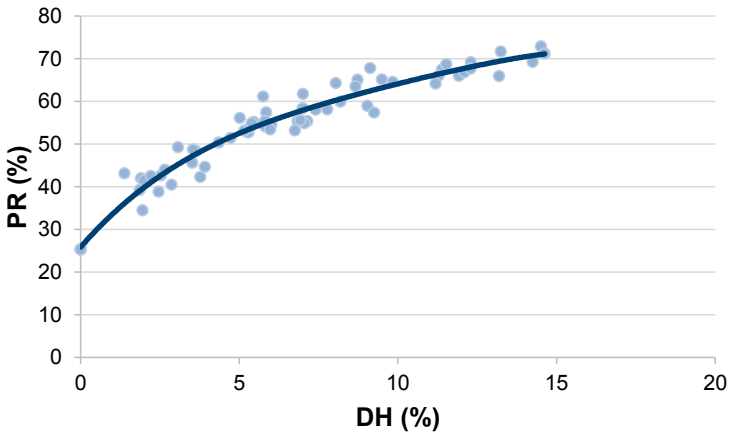


Figure 2: The relationship between protein recovery (PR) and degree of hydrolysis (DH). As the hydrolysis progresses, less protein is solubilized. The DH increases because the enzymes cleave the already solubilized peptides. Based on data from Paper I.

DH is an important parameter describing a hydrolysis process and provides the best basis for comparison between different hydrolysate products, rather than hydrolysis time. Several techniques are available for monitoring the DH in a hydrolytic process (Adler-Nissen 1986; Rutherfurd 2010). The methods for determining DH are based on three main principles: the determination of trichloroacetic acid soluble N (SN-TCA), the determination of free α-amino groups (TNBS, OPA and formol titration), and the titration of released protons (pH-STAT) (Rutherfurd 2010). However, it should be noted that DH values determined by different methods are often not directly comparable, due to different analytical principles. In this study, the pH-STAT (Paper I) and OPA (Papers I, II, and III) methods were used.

3.5.1 pH-STAT

The pH-STAT method was first described by Jacobsen et al. (1957) and is based on the liberation of protons when a peptide bond is cleaved. At neutral pH-levels, the amino groups of the polypeptides are protonated. During peptide bond hydrolysis free H^+ are released and will cause a decrease of pH in the reaction mixture. By continuous base titration, the increase in DH can be calculated from volume of base needed to maintain a constant pH. The pH-STAT is a straightforward and elegant technique to monitor the DH in a hydrolytic reaction and offers real-time monitoring of the process. However, the accuracy of the method depends on enzyme, pH and size of the released peptides and is generally regarded as imprecise at DH >20% (Adler-Nissen 1986). Free amino acids, di- and tripeptides are less protonated than polypeptides because they have higher pK-values. Accordingly, pH-STAT is not recommended when using enzyme preparations rich in exopeptidase activity (Spellman et al. 2003). High amounts of cations (e.g. Na^+ and K^+, depending on base used) are added in pH-STAT experiments, influencing sensory properties and nutritional quality of the resulting hydrolysates. Consequently, the pH-STAT method is not very applicable in industrial and upscale applications and is best suited for laboratory-scale experiments.

3.5.2 OPA method

In industrial and upscale applications, the final DH level is preferably quantified by determination of free α-amino groups. The OPA method is based on a direct assay of released amino groups in the hydrolytic reaction and has been found to be better suited than the TNBS reaction due to shorter incubation steps and less toxic reagents (Nielsen et al. 2001). The OPA method was first described by Church et al. (1983) and is based on the reaction between amino groups and *o*-phthaldialdehyde (OPA) in the presence of β-mercaptoethanol. This gives a colored compound that can be detected in a spectrophotometer at 340 nm. Nielsen et al. (2001) modified the method and used the more environmentally acceptable dithiothreitol instead of β-mercaptoethanol. The method offers a fast and easy method in the determination of DH. However, the method has some disadvantages: the OPA reagent does not react with proline and only poorly with cysteine (Rutherfurd 2010).

4. Sensory properties

The sensory properties of protein hydrolysates can be defined as the organoleptic properties that affect their taste and flavor. One of the main reasons for the slow development of commercial FPH is the formation of unpalatable and bitter tastes in the final products.

4.1 Taste and taste receptors

Within the field of sensory science, it is common to distinguish between taste and flavor. Taste is defined as the basic tastes that we are able to detect through the taste buds on our tongue. The flavors that we perceive in our foods, on the other hand, are a combination of senses, i.e. taste, smell and texture (Bakalar 2012).

Humans are able to perceive five basic tastes: sweet, salt, bitter, sour and umami via taste receptor cells (TRCs) on the tongue (Lindemann 2001; Meyerhof 2005; Chandrashekar et al. 2006). Sweet and umami tastes permits the identification of nutritious carbohydrates and amino acids, salt ensure a proper electrolyte balance, while sour and bitter tastes warn against unripen foods and poisonous chemicals, respectively. The TRCs are assembled into taste buds in structures that resemble garlic cloves. The taste buds are distributed in different papillae all over the tongue surface, illustrated in Fig. 3.

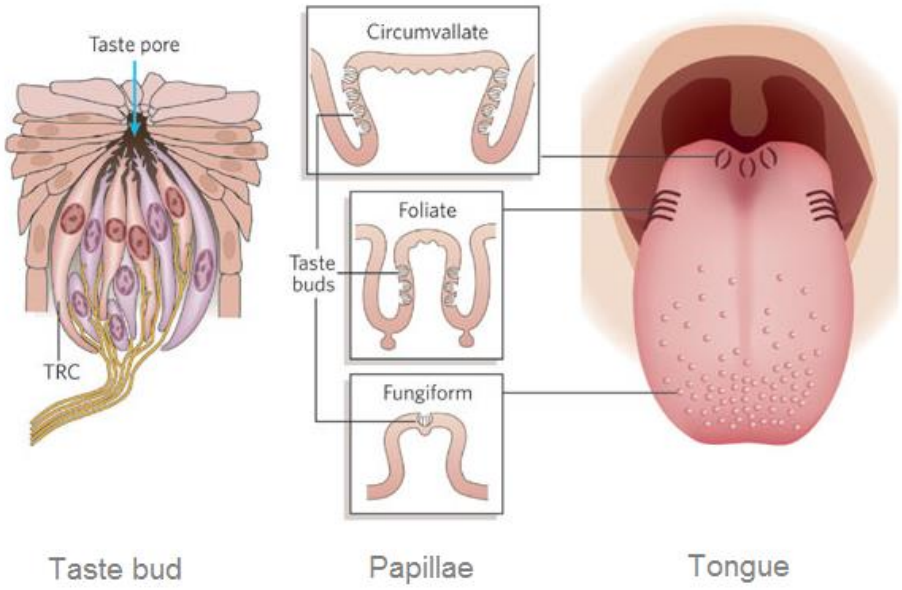


Figure 3. Taste buds are composed of taste receptor cells (TRCs) in structures that resemble garlic cloves. The taste buds are distributed across different types of papillae on the tongue. The circumvallate, foliate and fungiform papillae are found in the back, on the sides and in the front of the tongue, respectively. Reprinted with permission from Chandrashekar et al. (2006).

It is commonly accepted that the sour taste is elicited by acids, while the principal stimulus for salty taste is the cation Na^+. However, the transduction events and molecular details involved in the recognition of sour and salty tastes are only partially understood (Roper 2007; Roper 2015). The bitter, sweet and umami taste stimuli are transduced by different G-protein-coupled receptors (GPCRs), i.e. T1R and T2R receptors (Hoon et al. 1999; Lindemann 2001; Cygankiewicz et al. 2014). When the ligand binds to the TRC, an intercellular cascade of events take place and we experience a taste sensation. The T1R receptors are related to sweet and umami tastes, i.e. T1R2-T1R3 and T1R1-T1R3 heterodimers, respectively. The T2R receptors recognizes bitter taste. The T2R receptor family consists of at least 30 highly divergent GPRCs. This is consistent with mammals being sensitive to bitter taste and able to recognize a wide range of bitter substances (Adler et al. 2000).

4.2 Taste of protein hydrolysates

A protein hydrolysate is essentially an aqueous phase composed of peptides and free amino acids, along with small water-soluble components present in the substrate. Amino acids and peptides have been found to elicit all five basic tastes (Kirimura et al. 1969; Solms 1969; Temussi 2012). Sour and salty tastes are primarily due to charged terminals and side chains and do not reflect conformational features of a specific peptide. Sweet, umami and bitter tastes, on the other hand, are linked to different amino acids and peptides (Temussi 2012). The formation of the bitter taste constitutes the major limitation in the production of commercial protein hydrolysates for food applications. One of the earliest studies evaluating bitterness in protein hydrolysates was conducted by Murray and Baker (1952) who found that hydrolysates from casein and gelatin had a bitter and unpleasant taste. They suggested that the bitter taste was related to the presence of hydrophobic and aromatic amino acids released during the hydrolysis process. This may be explained by the following mechanism: in the intact globular protein, the hydrophobic moieties will be oriented towards the interior of the molecule. When the protein is hydrolyzed, the hydrophobic regions become exposed and can interact with the taste buds (Adler-Nissen and Olsen 1979).

The relationship between presence and position of several hydrophobic and aromatic amino acids, peptide chain length and the resulting taste have been extensively studied in more than 100 synthetic peptides by Japanese researchers (Kirimura et al. 1969; Otagiri et al. 1983; Asao et al. 1987; Ishibashi et al. 1987a; Ishibashi et al. 1987b; Ishibashi et al. 1988a; Ishibashi et al. 1988b; Ishibashi et al. 1988c; Tamura et al. 1990a). These studies suggested that peptide hydrophobicity, steric parameters, molecular size and primary amino acid sequence were of utmost importance for the perception of bitter taste. Both presence and position of hydrophobic amino acids are important for the formation of bitter taste. The bitter taste is most intense when both amino- and carboxyl groups of the hydrophobic amino acids are blocked by peptide bonds, and weakest when the hydrophobic amino acids are free (Matoba and Hata 1972). Kim and Li-Chan (2006) used partial least square regression analysis on available literature data on bitter peptides and found high correlations of bitterness with

total hydrophobicity, peptide length and amino acid position. They suggested that bulky amino acids at terminal ends were important for the bitterness of peptides. The formation of bitter taste is mainly ascribed to small peptides of less than 1000 Da, or up to 8-10 amino acids (Kukman et al. 1995; Kim and Li-Chan 2006; Tamura et al. 1990a). It is believed that large peptides have a higher molecular flexibility and may be able to mask the hydrophobic side chains, preventing them from interacting with the taste buds (Adler-Nissen and Olsen 1979). In that sense, low bitter taste can be achieved by restricting the hydrolysis to reach a low DH of 3-5% (Adler-Nissen 1984). However, a short hydrolysis process will also result in a low PR and process yield, leaving this option of limited interest. Alternatively, an extensive hydrolysis process to only free amino acids (DH = 100%) would significantly reduce the bitter taste because free hydrophobic amino acids have a weaker bitter taste compared to the corresponding peptides (Matoba and Hata 1972). However, it is neither desirable nor economically justifiable to run the hydrolysis process to a complete protein degradation. In realistic situations, the final DH will be anywhere between these extremes levels.

In addition to DH and peptide molecular weight, the amino acid sequence of the peptides is believed to be important for the formation of bitter taste (Kim and Li-Chan 2006; Spellman et al. 2009). Based on the specificity of the chosen protease it may be possible to produce products of different bitter potency from the same substrate. Many scientific studies have aimed to predict the formation of bitter taste based on amino acid sequence, hydrophobicity and spatial structure (Ney 1971; Shinoda and Okai 1985; Ishibashi et al. 1988a). However, neither the biological significance nor the relation to different T2Rs receptors have been established from these theoretical models. The detection of bitter tastes from peptides and amino acids is highly complex and involves at least six different T2Rs (Kohl et al. 2013).

Protein hydrolysates are complex products and do not solely contain peptides and amino acids. Small water-soluble components in the raw material will be present in the hydrolysate fraction and can influence the tastes and flavors of the final products. These components include NaCl and other mineral salts, nucleotides, non-protein amino acids and possibly small amounts of lipid oxidation products (Liaset et al. 2003; Sarower et

al. 2012; Shumilina et al. 2015). Many fish substrates contain the nitrogenous compound trimethylamine oxide (TMAO) (van Waarde 1988). TMAO can be converted to trimethylamine (TMA) during bacterial spoilage, responsible for the unpleasant "fishy" smell of stale fish products. Presence of TMA may be used as an indicator of low substrate quality.

4.3 Debittering techniques

Based on the current knowledge regarding the formation of bitter peptides in a hydrolysis process, several attempts have been made to prevent, remove or mask the bitterness of a protein hydrolysate. Available debittering techniques are based on the following mechanisms (Adler-Nissen 1986; Saha and Hayashi 2001):

- Plastein reaction
- Masking
- Application of exopeptidase
- Selective separation

The plastein reaction is the formation of a gel-like proteinaceous substance from a concentrated protein hydrolysate (Eriksen and Fagerson 1976; Gong et al. 2015). The hydrolysate is incubated with proteases, usually for long reaction times (24-72 hours), giving a protease-induced aggregate: the plastein. The plastein product essentially contains a cluster of peptides held together by hydrophobic and ionic bonds. The plastein reaction can be successfully applied to reduce the bitter taste of protein hydrolysates because the bitter tasting hydrophobic amino acids can be hidden in the plastein core, thereby limiting their interactions with TRC. However, the method is time consuming and not suitable in production of a water-soluble protein hydrolysate (Adler-Nissen 1986). Masking is performed by adding additives or molecules, e.g. cyclodextrin, to the hydrolysate to mask the bitter taste. Masking additives promotes conformational alterations of the peptides and introduction of sweet tastes that cover the bitterness (Linde et al. 2009). Tamura et al. (1990b) suggested that cyclodextrin wraps the hydrophobic functions of amino acids to decrease their bitterness. However,

a large excess of masking molecules may be needed (Tamura et al. 1990b) and the process is less suitable in the production of taste *neutral* FPH. In this study, the use exopeptidase and selective separation by activated carbons adsorption were used to reduce bitter taste of the FPH. The mechanisms of these techniques are elucidated in the following paragraphs.

4.3.1 Debittering by exopeptidase activity

Exopeptidases selectively release free amino acids from the peptides. Studies have demonstrated that free amino acids give less bitter taste than their corresponding peptides (Matoba and Hata 1972) and bitterness of peptides is high when branched or hydrophobic amino acids are at terminal ends (Kim and Li-Chan 2006). A selective removal of these may significantly reduce the bitter taste. Several exopeptidases are available and can generally be divided in two sub-groups: aminopeptidases and carboxypeptidases. These release free amino acids from N- and C-terminal ends of the peptide chain, respectively (Raksakulthai and Haard 2003). Exopeptidases are seldom effective on intact proteins and recommended to be used in combination with endopeptidases. Based on proper choice of endo- and exopeptidase activity it is possible to alter the peptide chain ends to reduce the bitter potency of the peptides. This approach offers an easy and straightforward method without prejudicing the amino acid composition of the hydrolysates. However, studies have suggested that a hydrolysis time of more than five hours and high DH (>50%) are required for significant reduction of bitter taste of protein hydrolysates by exopeptidase activity (Izawa et al. 1997; Nilsang et al. 2005; Cheung et al. 2015a).

4.3.2 Debittering by selective separation

Selective separation is based on removal of hydrophobic peptides and amino acids and thus reduction of bitter taste. The hydrophobic molecules can be removed by extraction with organic solvents, isoelectric precipitation and adsorbents (Saha and Hayashi 2001). Organic solvents, such as butanol have been found to be efficient in removing bitter peptides from hydrolysates (Lalasidis and Sjoberg 1978; Aubes-Dufau et al. 1995; Dauksas et al. 2004). The selective separation of hydrophobic amino acids is

based on the thermodynamic preference of hydrophobic amino acids and peptides to diffuse to the organic phase. After removal of the organic phase, the resulting hydrolysate tastes less bitter compared to the original hydrolysate. However, the method has some disadvantages: the organic solvent introduces a health risk and demands the use of explosion-proof equipment. Alternatively, the hydrophobic peptides may be removed by pH adjustment and isoelectric precipitation as the hydrophobic peptides have very low solubility around isoelectric point (Adler-Nissen 1984). Adsorbents include the use of activated carbons (ACs) (Murray and Baker 1952; Helbig et al. 1980; Cogan et al. 1981; Suh et al. 2000), polymeric resins (Doulia et al. 2001; Dauksas et al. 2004; Kamara et al. 2011) and siloxanes (Lin et al. 1997) to adsorb aromatic and hydrophobic peptides and amino acids from the solution to reduce bitter taste. The aromatic amino acids tyrosine, tryptophan and phenylalanine are particular susceptible for adsorption onto adsorption materials and this suggests that the main driving force for adsorption is through short-range van der Waal interactions (Doulia et al. 2001; Jensen et al. 2011).

ACs are unique and versatile adsorbents used extensively for the removal of undesirable odor, color, taste and impurities (Bansal and Goyal 2005), and were chosen as adsorbents in this study (Paper III). Essentially, all carbonous material can be converted into AC and the properties of the final product will vary depending on nature of the raw material used (Bansal and Goyal 2005). The carbons are processed and activated to make them extremely porous with a large surface area. The preparation of AC is based on two standard activation methods: chemical and gas (Yang 2003). Chemical activation is performed by direct carbonization of the raw material at temperatures between 500 °C and 900 °C in the presence of catalysts such as phosphoric acid, zinc chloride and potassium chloride. Gas activation is performed by the carbonization of the raw material at temperatures between 400 °C and 500 °C followed by partial gasification at 800-1000 °C using an oxidation gas such as CO_2 and steam. According to IUPAC classification, the pore sizes are divided into micropores, mesopores and macropores, with diameters of <2 nm, 2-50 nm and >50 nm,

respectively (Haber 1991). By judicious choice of precursor and by careful control of the activation method it is possible to tailor ACs with defined properties.

The adsorption onto AC is a spontaneous process that takes place if the free energy of adsorption, ΔG_{ads}, is negative (Moreno-Castilla 2004). The free energy of adsorption can be expressed as:

$$\mathbf{\Delta G_{ads} = \Delta G_{non\text{-}elect.} + \Delta G_{elect.}} \quad (4)$$

ΔG_{elect} represents the electrostatic contribution to the adsorption free energy such as coulombic and dipole interactions. $\Delta G_{non\text{-}elect.}$ is specific to the system and includes hydrophobic interactions and van der Waals forces. Factors that influences $\Delta G_{non\text{-}elect}$ include (Newcombe and Drikas 1997):

1) pore size distribution of the carbon and the relationship between the size of the pores and the size of the adsorbate
2) hydrophobic interaction between the carbon surface and hydrophobic parts of the adsorbing molecule
3) interaction between aromatic rings on the adsorbate and carbon
4) hydrogen bonding between functional groups on the surface and those on the adsorbate.

Debittering by ACs provide an efficient method for removal of hydrophobic bitter peptides. However, the selective separation of hydrophobic peptides and amino acids has some drawbacks. Most of the hydrophobic and aromatic amino acids available for adsorption onto ACs are indispensable for humans and need to be administered through the diet. In addition, adsorption materials are quite expensive and adds an additional step in the downstream processing. Moreover, the studies reporting a good debittering effect by ACs have used long hydrolysis times (6-24 hours of hydrolysis) and high dosage of AC (30-200% on protein basis) (Helbig et al. 1980; Cogan et al. 1981; Suh et al. 2000). There is a need for more knowledge of debittering properties of ACs on less hydrolyzed products, and at significantly reduced AC doses than those frequently reported in the literature.

4.4 Sensory analysis

Sensory evaluation is a science of measurement that applies principles of experimental design and statistical analysis to the use of human senses (Lawless and Heymann 2010). A vast array of sensory tests are available, where both trained judges and untrained consumers can be used. The three types commonly used sensory testing are listed in Table 3.

Table 3. Test methods in sensory evaluation (Lawless and Heymann 2010).

Question of interest	**Test**	**Panelists**
Are the products different?	Discrimination	Screened for sensory acuity, oriented to test method. Trained/untrained judges.
How do the products differ in sensory characteristics?	Descriptive	Screened for sensory acuity. Usually highly trained judges.
How well are the products liked?	Affective	Screened for product acceptance. Untrained judges.

The description of the tastes and flavors present in different food products are often assessed by descriptive analysis by an expert sensory panel. Descriptive sensory analyses allow the sensory scientist to obtain complete sensory descriptions of products and identify underlying ingredients, process variables and attributes that are important for the products (Lawless and Heymann 2010). Descriptive sensory analyses require a trained panel with high a degree of commitment and motivation. The training phase begins with the development of a common language that comprehensively and accurately describes the product attributes (Murray et al. 2001).

There are several different methods of descriptive analyses (reviewed in Murray et al. 2001), but most frequently generic descriptive analysis is used. This method combines different approaches from all methods of descriptive analyses. In a generic descriptive analysis, the samples are evaluated by a trained panel (8-12 judges) guided by a panel leader. The analysis is generally performed in the following stepwise procedure (Lawless and Heymann 2010):

1) **Attributes are chosen:** Attributes relevant for the products are chosen and discussed by the panelists.
2) **Panelists are calibrated:** Samples with variance in the sensory attributes tested are used to calibrate the judges of the potential variance in attribute intensity.
3) **The test is performed:** Samples are served in randomized order and the judges scale the chosen attributes on an unstructured line.
4) **The results are analyzed:** Statistical evaluations of the results are performed. Analysis of variance (ANOVA) and multivariate techniques are most commonly used to evaluate sensory data. The panelists' performance, repeatability and reproducibility are validated through statistical software, such as PanelCheck (Tomic et al. 2010).

5. Surface-active properties

It is desirable that the FPH possesses surface-active and physicochemical properties in order to be successfully utilized as a functional food ingredient, such as emulsifiers and foaming agents. The molecular properties of peptides relevant for both foaming and emulsifying are similar, i.e. amphilicity, surface activity, solubility, and segmental flexibility (Dickinson 1986; Panyam and Kilara 1996; Damodaran 2005). Proteins and peptides are amphiphilic molecules due to presence of both hydrophilic and hydrophobic amino acids. The hydrophobic moieties of such molecules will seek to minimize their contact with water and spontaneously adsorb at the interface between a polar (water) and non-polar (oil or air) interface, leading to a reduction of the interfacial tension between the phases. This phenomenon is thermodynamically favorable due to the simultaneous dehydration of the hydrophobic interface and hydrophobic portions of the peptide (Dickinson 1986). The emulsifying and foaming properties of FPH are closely related to how efficiently they can lower the interfacial tension between the polar and non-polar components in food.

Foams and emulsions are thermodynamically unstable dispersed systems that need to be stabilized by surface-active agents (Walstra 1993; Damodaran 2005; Patino et al. 2008) (Fig. 4). The principle of the formation of an emulsion is that, when energy is applied to the system, the phases will be dispersed. Without the presence of an emulsifying agent, the oil droplets will rapidly recoalescence to minimize their contact with water. Surface-active molecules will adsorb at the oil surface with their hydrophobic groups oriented towards the oil phase and hydrophilic groups in contact with water. This will facilitate breakup of the oil droplets and prevent recoalescence. Foam is essentially a colloidal system of tiny air bubbles dispersed in an aqueous continuous phase and may be formed after extensive whipping of the aqueous phase. Foaming agents will adsorb at the liquid interface and form a stabilizing film around the air bubbles to reduce disproportionation of the bubbles (Dickinson et al. 2002).

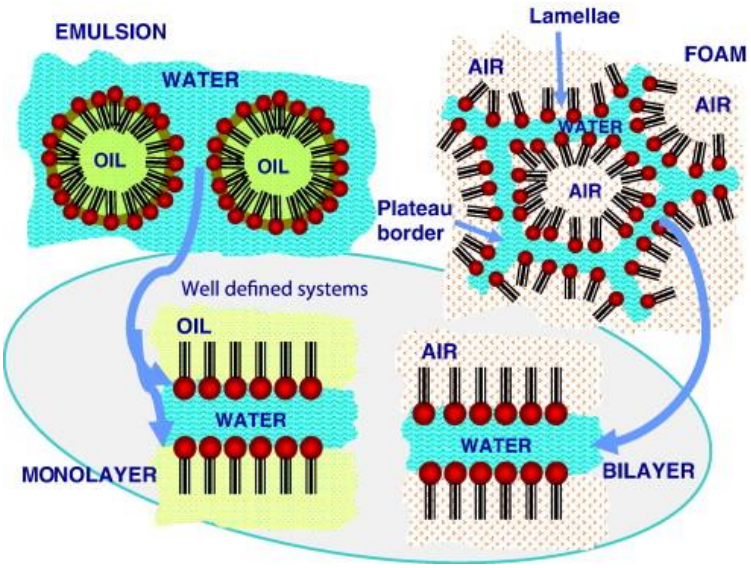


Figure 4. Surface-active agents form interfacial layer between polar (water) and non-polar (oil or air) phases in emulsions and foams. Emulsifiers form the interfacial film between oil and water phases in an emulsion and can be modelled as a monolayer. Bubbles in a foam are stabilized by a bilayer of surface-active molecules separated by the continuous phase. Reprinted with permission from Patino et al. (2008).

Hydrolysis of proteins will create smaller and more flexible peptides that can more easily be distributed and adsorbed at an interface compared to the intact protein (Panyam and Kilara 1996). However, extensive hydrolysis will usually give peptides with poor surface activities, presumably due to reduction of amphilicity and segmental flexibility. Molecular flexibility is of the utmost importance for the surface activity of proteins (Damodaran 2005) and the peptides should contain distinct clusters of hydrophobic and hydrophilic residues (Dexter and Middelberg 2008). It has been suggested that peptides should have a minimum molecular weight of 2-3000 Da to be an efficient emulsifier (Jeon et al. 2000; van der Ven et al. 2001). Enser et al. (1990) demonstrated that superior emulsifying and foaming abilities of synthetic amphiphilic peptides increased with chain length (>26 amino acids) and α-helix content. In general, the physicochemical properties of protein hydrolysates are negatively correlated to an increase in DH (Quaglia and Orban 1990; Kristinsson and Rasco 2000b; Gbogouri et

al. 2004; Ghribi et al. 2015; Imura et al. 2015). However, the enzyme specificity may also have an impact on the physicochemical properties of the resulting hydrolysate products because this will determine the final peptide sequences, i.e. amino acid composition, hydrophobic/hydrophilic balance and molecular flexibility. For example, the substitution of leucine with tryptophan produced a significant improvement in foaming properties in two otherwise identical peptides (Enser et al. 1990). Luo et al. (2014) demonstrated significant difference in emulsifying activity and stability of sodium caseinate hydrolysates based on three proteases at similar DH. However, it has also been suggested that protease specificity is inferior compared to molecular weight distribution on hydrolysate emulsifying properties (van der Ven et al. 2001).

Few standardized methods of determining the physicochemical and surface-active properties of protein hydrolysates exist. Frequently, maximum oil emulsified by a given protein dispersion is used to assess emulsifying capacity, while foaming properties are observed by measuring the foam volume after extensive of whipping the hydrolysate (Pearce and Kinsella 1978; Ferreira et al. 1995). However, different equipment and oils are used in literature studies, leaving most of the published data of limited use in the comparing of results. There is a need for a more standardized method to assess surface-active properties of protein hydrolysates. An important parameter for surface-active molecules and food surfactants is their critical micelle concentration (CMC; Fig. 5), defined as the concentration where the molecules start to self-assemble into micelles and aggregates (Bergström 2011). The driving force for the self-assembly of amphiphilic molecules in an aqueous solvent is the hydrophobic effect. When the interface between water and air phases is completely covered, excess concentration of amphiphilic molecules will start to self-assemble to micelles at the point of CMC. CMC is a key parameter for food surfactants, where efficient surfactants have a low CMC, i.e. less concentration of the molecules is required to decrease the surface tension. The CMC-value of food surfactants is a valuable tool for comparison of their suitability as functional food ingredient. However, the presence of salt (NaCl) and inorganic molecules present in the hydrolysates may influence its CMC and emulsifying and foaming properties, compared to pure surfactant solutions. In general, the presence of

salt will reduce the CMC (Yu et al. 2010) and increase emulsifying and foaming capacities (Ragab et al. 2004) due to reduced electrostatic repulsion of the molecules. Nevertheless, to my knowledge, little is known about the CMC of protein hydrolysates as a function of DH, molecular weight distribution and enzyme specificity. The determination of the CMC of FPH (Paper II) provides a novel technique for evaluating its potential use as a food surfactant.

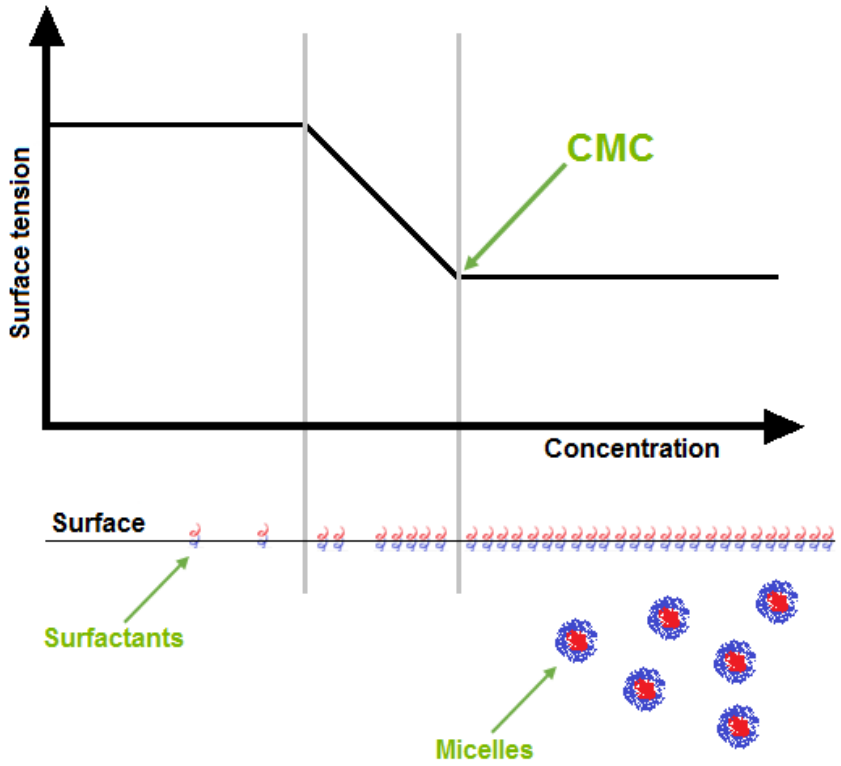


Figure 5. The principle of the determination of CMC. At concentrations below CMC, the surface-active molecules adsorb on the surface. As the concentration of molecules increases, more and more molecules adsorb to the surface with the polar groups directed towards the aqueous phase (blue) and non-polar groups directed towards the non-polar environment (red). Consequently, the surface tension decreases. At the CMC point, the surface is completely covered by molecules and remaining molecules form micelles and aggregates.

6. Nutritional properties

Proteins and amino acids are essential for growth and maintenance of the human body and sufficient intake through the diet is imperative. The amino acids are generally divided into two categories, based on their importance in protein synthesis: indispensable and dispensable (Table 2). The dispensable amino acids are produced by the human body. Others are not endogenously synthesized in adequate amounts and need to be obtained from the diet and defined as indispensable amino acids. For a protein hydrolysate to provide good nutritional value, it must contain well-balanced amino acid composition, containing all indispensable amino acids, and have a balanced peptide size distribution. The peptide chain length affects its adsorption from the human jejunum and is generally faster for small peptides (3-4 amino acids) than larger peptides (Grimble et al. 1987). It is neither necessary nor desirable that the protein hydrolysates should be made up of free amino acids alone, because these make the products hyperosmotic, and may be responsible for causing diarrhea (González-Tello et al. 1994).

Several methodologies and calculations are available for evaluation of food proteins nutritional quality (reviewed in Gilani 2012 and Friedman 1996). Common for all methods is the FAO recommendation of using the universally applied f_N of 6.25 to calculate CP (FAO/WHO/UNU 2013). Protein efficiency ratio (PER) is a widely used measure of a specific protein ability to promote growth. The PER method involves feeding of a test protein diet and casein control diet to weanling rats for a period of four weeks (Gilani 2012). In general, PER-values <1.5 are defined as a low-quality protein, PER-values of 1.5-2.0 an intermediate quality and PER-values >2.0 a high quality protein (Friedman 1996). Digestibility assays are both time consuming and expensive and thus Lee et al. (1978) attempted to predict PER with regression equations derived from the amino acid content of meat substrates:

$$\mathbf{PER = 0.06320\ (Thr + Val + Met + Ile + Leu + Phe + Lys + His + Arg + Trp) - 0.1539} \quad (5)$$

The equation can be used to estimate the nutritional quality of a food protein and offers a cheap, easy and rapid protocol of the assessment of food protein quality. However, the evaluation of PER has some disadvantages; it only provides a rough estimate of protein quality, it lacks proportionality and fails to credit protein used for maintenance purposes (Gilani 2012). Rats have significantly different dietary needs of sulfuric amino acids (cysteine and methionine), leaving the predictions of PER inaccurate (Deglaire and Moughan 2012). In addition, the accuracy of Eq. 5 for fish substrates is not known. Nevertheless, calculation of PER is a much used protocol for assessment of food protein quality and several studies have demonstrated high PER-values (>2.0) of fish substrates and corresponding hydrolysates, including cod (Shahidi et al. 1991; Slizyte et al. 2005), dogfish (Diniz and Martin 1997), black tilapia (Abdul-Hamid et al. 2002) and capelin (Shahidi et al. 1995). Less is known about PER-values of salmon proteins, but Opheim et al. (2015) demonstrated high PER-values of salmon viscera by-product protein hydrolysates.

The amino acid score (AAS) is a valuable tool in predicting how efficiently a protein will meet a person's amino acid need and indicates the level of each indispensable amino acid as a ratio to a reference value. Requirement values for preschool children (six months to three years) are used as reference value to calculate the AAS of food proteins:

$$\boldsymbol{AAS} = \frac{\boldsymbol{mg\ of\ amino\ acid\ in\ 1\ g\ test\ protein}}{\boldsymbol{mg\ of\ amino\ acid\ in\ 1\ g\ of\ reference\ protein}} \times \mathbf{100}\% \qquad (6)$$

The lowest score determines the most limiting amino acid and indicates a first approximation of its efficiency of utilization by children (Millward 2012). Accordingly, a dietary protein with AAS ≥100% contains a well-balanced amino acid composition. The AAS can be combined with fecal protein digestibility values to obtain the protein digestibility corrected amino acid score (PDCAAS) and ileal amino acid digestion to obtain digestible indispensable amino acid score (DIAAS). In calculating DIAAS, the ratio should be calculated for all indispensable amino acids, based on their individual ileal digestibility, and the lowest value designated as the DIAAS. According to the PDCAAS method, values that are higher than 100% are truncated to 100%,

arguing that digestible indispensable amino acids do not provide additional nutritional benefit (Schaafsma 2000). FAO/WHO/UNU (2013) recommends the use of DIAAS in the assessments of dietary protein quality as this provides the most accurate measurement of protein digestibility. Measures of digestibility based on fecal digestibility tend to overestimate the actual uptake of dietary amino acids due to the high metabolic activity of the hindgut microflora. However, ileal digestibility values for humans are inaccessible and only numbers from ileostomized adults are available (Rowan et al. 1994). Thus, ileal amino acid digestibility may be approximated using values from animals, such as rats and pigs, where the pig is the preferred model (Deglaire and Moughan 2012).

Besides high content of indispensable amino acids and high DIAAS, PDCAAS and PER-values, the FPH may also contain bioactive peptides with several health benefits. Bioactive peptides are believed to be small peptides of 2-25 amino acids with a positive impact on body functions and human health (Mine et al. 2010). It has been reported that marine bioactive peptides may possess anti-hypertensive activity, antioxidant activity and antimicrobial activity (Kim and Wijesekara 2010). In addition, recent studies have shown that daily supplementation of fish proteins gave beneficial effects on blood glucose levels, reduced percentage of body fat in overweight adults and reduced triacylglycerol and cholesterol levels in healthy adults (Vikøren et al. 2012; Aadland et al. 2015). Fish substrates also contain levels of water-soluble non-protein amino acids, including taurine, anserine and creatinine (Oterhals and Samuelsen 2015, Paper III) that may have several health benefits (Arihara and Ohata 2008). Taurine (2-aminoethanesulfonic acid) is one of the most abundant free amino acids in the human body. It is involved in several physiological processes and may have anti-obesity effects (Murakami 2015). Essential water-soluble B-vitamins and minerals, such as iodide and selenium will also be present in the FPH (Liaset and Espe 2008), adding to the nutritional quality. The salt (NaCl) content, however, may impair the nutritional value of the FPH. The recommended daily intake of NaCl for health adults is set at 5g/day (WHO 2012), approximate to the content of 125g dry salmon FPH (Paper III).

7. Potential applications

To my knowledge, only two commercial food-grade FPH products from Atlantic salmon by-products are currently available in Norway: *Salmigo* from Biomega, a low-molecule weight product for pet-food (Biomega 2016) and *ProGo* from Hofseth Biocare, a bioactive peptide product for medical food applications (Hofseth Biocare 2016). However, several protein hydrolysate products based on marine sources are available globally (Chalamaiah et al. 2012; Cheung et al. 2015b). Based on new technologies and knowledge of the factors influencing a hydrolysis process, combined with an altered consumer attitude towards fish proteins, there is reason to believe that the number of products will increase in the years to come. It is essential that the production of FPH is market-driven and is being sold at profit. In addition, the transformation of by-products to edible products must implement systems such as Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Point (HACCP) (Olsen, Toppe et al. 2014). Protein hydrolysates can be considered as a safe protein source if they are made from safe raw materials and proteases with GRAS/QPS classification (Schaafsma 2009). Proper control of peptide size distribution permits the use of protein hydrolysates within a wide range of products. Roughly, the use of protein hydrolysates for human nutrition can be divided into the following groups (Siemensma et al. 1993; Frøkjaer 1994; Clemente 2000; Neklyudov et al. 2000):

1) Functional food ingredients, such as emulsifiers, foaming agents or flavor enhancers
2) Formulas for infants with allergies towards intact food proteins or with inborn errors of metabolism.
3) Specialized adult nutritional formulas, e.g. diets for the elderly who need extra protein supplements to maintain their body weight. Protein hydrolysates may also be used in weight-control diets to ensure a controlled loss of body weight without malnutrition.
4) Nutritional supplements to provide amino acids in a form that is easily assimilated for restitution, e.g. sports nutrition.

5) Nutraceuticals, such as antihypertensive and cholesterol lowering dietary supplements

The remaining phases in a protein hydrolysis process, i.e. the oil and sludge phases, may also have a potential usage within higher value products. The lipid phase contains the essential fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), fat-soluble vitamins and cholesterol with potential within nutraceuticals (Rustad et al. 2011). The sludge phase contains non-solubilized proteins and bones. The fish bones are rich in minerals such as phosphorous, calcium and zinc, and collagen proteins (Toppe et al. 2007). The collagen proteins can be converted to fish gelatin with many potential uses within foods and nutraceuticals (Karim and Bhat 2009). The non-solubilized proteins contains high amount of indispensable amino acids (Liaset and Espe 2008) and have higher PER-values than the soluble hydrolysate (Slizyte et al. 2005).

8. Experimental and analytical approaches

8.1 Production of fish protein hydrolysates

The substrate used in the production of fish protein hydrolysates was Atlantic salmon (*Salmo salar*) heads and backbones, kindly provided by Sotra Fiskeindustri AS, Sotra, Norway. The raw material was collected less than four hours after filleting, to ensure a high food-grade quality, and were immediately ground using a Comitrol® Processor Model 1700 (Urchel Laboratories Inc., Valparaiso, IN) to pieces of approximately 1 cm^3. Every other head and backbone was added to the Comitrol Processor to ensure a balanced mix of the raw material. The substrate was vacuum packed in batches of approx. one kg and stored (-30 °C) before hydrolytic experiments. The proteases used in the hydrolytic experiments were chosen based on their reported food-grade quality and high hydrolytic activity at pH 6.5 and 50 °C, listed in Table 4.

Table 4: The proteases used in the respective papers.

Enzyme	Source	EC Number	Manufacturer	Paper
Alcalase 2.4L	*Bacillus licheniformis*	3.4.21.62	Novozymes (Bagsværd, Denmark)	I, II
Corolase 7089	*Bacillus subtilis*	3.4.24.28	ABEnzymes (Darmstadt, Germany)	I
Neutrase 0.8L	*Bacillus amyloliquefaciens*	3.4.24.28	Novozymes (Bagsværd, Denmark)	I
Promod 671L	*Bacillus licheniformis*	3.4.21.X/ 3.4.24.X	Biocatalysts (Cardiff, UK)	I, II
Protex 7L	*Bacillus amyloliquefaciens*	3.4.24.28 (3.4.21.62)	DuPont (Wilmington, DE)	I, II, III
Flavourzyme 1000L	*Aspergillus oryzae*	3.4.11.1 (3.4.2x.X)	Novozymes (Bagsværd, Denmark)	III

The protein hydrolysates in Paper I were produced in a 250 mL jacketed glass reactor connected to a heating circulator controlled by an external temperature sensor in direct contact with the fish slurry. The protein hydrolysates in Papers II and III were produced in a modified R10 Bear Varimixer (A/S Wodschow & Co. Brøndby,

Denmark) connected to a heating circulator monitored by an EBI 10-T240 temperature data logger.

During the hydrolysis process, the raw material was converted into a light pink-brown-colored mixture composed of water, lipids and solid particles. After the hydrolysis the enzyme activity was terminated by quickly heating the slurry to >90 °C, using a microwave oven, and holding at this temperature for 10 minutes. The hydrolyzed fish slurry was separated by centrifugation into three layers: the oil layer on top, the protein hydrolysate solution and a semisolid layer (sludge) at the bottom (Fig. 6).

Figure 6. After centrifugal separation the hydrolyzed fish slurry were divided into a semi-solid layer at the bottom, water-soluble protein hydrolysates in the middle and an oil-layer on top. Photo by Øyvind Ganesh Eknes.

After centrifugation, hydrolysates from Paper I were carefully collected by glass Pasteur pipettes and filtered through a 0.45 µm PES syringe filter (VWR Collection, Radnor, PA). The liquid phases (oil and water-soluble protein hydrolysate) in Papers II and III were successfully separated by a separation funnel (Fig. 7a) followed by filtering over a Büchner filter with a T-2600 depth (Mall Corporation, East Hills, NY) to remove any residual particles (Fig. 7b). All hydrolysates were kept frozen (-30 °C) before analyses.

Figure 7. a) Separation of the lipid phase and water-soluble hydrolysate through a separation funnel, b) Filtering of the water-soluble protein hydrolysate over a Büchner filter. Photo by Øyvind Ganesh Eknes.

8.2 Applied analytical methods

8.2.1 Chemical analyses

The following analytical methods have been applied to assess proximate composition and quality of substrate and protein hydrolysates in the respective papers:

Analytical method	**Reference**	**Paper**
Ammonia	Conway and Byrne (1933)	I
Kjeldahl Nitrogen	ISO 5983-2	I, II, III
Total ash	ISO 5984-2	I, III
Dry matter	ISO 6496-2	I, III
Free fatty acids	AOCS method Ca 5-40	I
Hydrolytic activity	Cupp-Enyard (2008)	I
Degree of hydrolysis (OPA)	Nielsen et al. (2001)	I, II, III
Total amino acid composition	Cohen and Michaud (1993)	I, III
Cysteine and cystine	Cohen and Michaud (1993)	I, III
Tryptophan	Miller (1967)	I, III
Fat content	Bligh and Dyer (1959)	I, III
Peptide size distribution	Wang-Andersen and Haugsgjerd (2011)	II, III
Free amino acids	Bidlingmeyer et al. (1987)	II, III
Salt (NaCl)	AOAC 937.09	III

8.2.2 Quantification of DH using pH-STAT

The pH-STAT experiments (Paper I) were performed using a Titrando 906 titrator (Methrom, Herisau, Switzerland) equipped with pH-meter and base pipette in direct contact with the hydrolysis slurry at all times. The liberation of H^+, released during cleaving of the peptide chains, was followed by the Tiamo v. 2.3 (Methrom, Herisau, Switzerland) software and added base was recorded every five sec. Base required to maintain a constant experimental pH (pH_{exp}) has a direct relationship with number of peptide bonds hydrolyzed, given by the following equation:

$$\boldsymbol{DH = \frac{B \times N_B}{\alpha \times M_P \times h_{tot}} \times 100\%} \quad (6)$$

B is base consumption (mL), N_B is the molarity of the base, α is the average degree of dissociation of the α-NH_2 groups, Mp is the mass of the protein being hydrolyzed (g) and h_{tot} is total number of peptide bonds in the substrate. The α-constant is dependent on pH and temperature and can be estimated by the following equation:

$$\boldsymbol{\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}}} \quad (7)$$

where pK can be calculated based on the Gibbs-Helmholtz equation (Steinhardt and Beychok 1964):

$$\boldsymbol{\Delta H = 2.303R\frac{T_1T_2}{T_1-T_2}(pK_{exp} - pK_a)} \quad (8)$$

ΔH is the ionization enthalpy for NH_3 (45 kJ/mol), R is the gas constant (8.315 J/K·mol), T_1 is temperature in Kelvin at 25 °C (298 K), T_2 is temperature at experimental conditions (K), pKa is pK-value of α-NH_3 at T = 298 K (7.8) and pK_{exp} is the pK-value at experimental conditions. By rearranging Eq. 8, the following relationship is established:

$$\boldsymbol{pK = 7.8 + \frac{298-T}{298T} \times 2400} \quad (9)$$

The parameters used in the calculation of DH by the pH-STAT method are listed in Table 5.

Table 5. The parameters used in the calculation of pH-STAT

Parameter	Value
pH_{exp}	6.5
T_2 (K)	323
pK_a	7.8
pK_{exp}	7.2
α	0.175
h_{tot} (meqv/g)	9.3
M_p (g)	N×5.23
N_b	0.5

In the pH-STAT experiment, samples were taken at various time intervals (10, 20, 40, 80 and 120 min) to evaluate PR and DH by the OPA method. Correction of base consumption (B) caused by sapling was accomplished by the following equation (Adler-Nissen 1986):

$$\boldsymbol{B = B_1 + \left[\frac{B_2 - B_1}{\frac{M}{M-m}}\right] + \left[\frac{B_3 - B_2}{\frac{M}{M-2m}}\right] + \ldots + \left[\frac{B_n - B_m}{\frac{M}{M-n \cdot m}}\right]} \quad (10)$$

B_1, B_2, etc. are the actual base consumption at the drawing of samples 1, 2, etc. M is reaction mass at start of the reaction and m is mass of sample drawn from the reaction.

8.2.3 Hydrophobic peptide fraction

The hydrophobic peptide fraction (HPF; Paper II) was determined based on 2-butanol partitioning (Adler-Nissen 1986). The solvent polarity of 2-butanol is 0.506 relative to water (Reichardt 2003), but is expected to be sufficiently apolar to attract small hydrophobic peptides and amino acids. Equal volumes of hydrolysate with 5% protein content and 2-butanol were mixed, manually shaken for two minutes and transferred to a separation funnel. After phase separation for one hour, the upper butanol-rich extract was recovered and analyzed for Kjeldahl N. The HPF was calculated from the concentration of protein in the 2-butanol phase to that in the original hydrolysate. The equilibrium final weight-distribution of the 2-butanol and hydrolysate phases were 53% and 47%, respectively.

8.2.4 Sensory analysis

Generic descriptive analysis was used to evaluate the sensory properties of the FPHs. Eight to ten trained judges, without any knowledge of product specificities to avoid bias, evaluated the products. The sensory assessments were performed in a sensory laboratory in accordance with ISO 8586. The judges were chosen based on their sensory acuity, ability to discriminate between similar samples and ability to rate products for intensity. All judges had their own booth to avoid influence by external distractions such as noises and smell. Prior to the sensory assessments, multiple FPHs were evaluated to find common attributes relevant for all products, listed in Table 6. A preliminary test using hydrolysates based on Promod 671L with high (30%) and low (17%) DH was performed to find the most optimal test concentration level for all products and attributes. Protein concentrations of 0.5%, 0.75%, 1.0%, 1.25% and 1.5% were used and products tested for all relevant attributes (Table 6).

Table 6. Sensory attributes and their description used in the respective studies.

Attribute	Description	Paper
Sweet taste	Related to the basic taste sweet	II, III
Salt taste	Related to the basic taste salt	II, III
Sour taste	Related to the basic taste sour	II
Bitter taste	Related to the basic taste bitter	II, III
Umami taste	Related to the basic taste umami	II, III
Acidic flavor	Related to a fresh, balanced flavor due to organic acids	II, III
Metallic flavor	Related to flavor of ferro sulfate	II
Shellfish flavor	Related to the flavor of shellfish, a sweet taste of shrimp, crab and crayfish	II
Sea flavor	Related to the flavor of fresh, salty sea	II, III
Fish flavor	Related to the flavor of boiled white fish	II, III
Pungent flavor	Related to the flavors from ammonia, chemicals and medicine	II, III
Rancid flavor	Related to all rancid flavors including grass, hay, stearic and paint	II
Astringent flavor	Related to a complex feeling of contraction and dryness of the mouth	II
Aftertaste	Related to the flavors left in the mouth after 30 sec.	II, III

The experimental sensory evaluations were performed by presenting the FPHs at equal protein concentration, coded by three-digit codes and served in randomized order. The FPH was evaluated separately, with expectorating and rinsing between each sample. The different sensory attributes were scaled on an unstructured line, ranging from 1 (no intensity) to 9 (high intensity).

8.2.5 Critical micelle concentration

CMC was determined by nuclear magnetic resonance (NMR). The principles of the NMR method are as follows: Atomic nuclei that contain an odd numbers of protons and neutrons possess a spin. When such nuclei are placed in an external magnetic field, they arrange in two energy states (spin quantum number = ½). The magnetic field around the nuclei has a spin with a certain frequency, depending on the nuclei and magnetic field, e.g. ^{1}H in 11.75 Tesla magnetic field has a spin of 500 MHz (Gonnella 2012). If an external magnetic pulse with frequency of 500 MHz is applied, an energy transfer of the nuclei is possible. A nucleus with spin at higher energy generates a magnetic field in the opposite direction to the external magnetic field. A nucleus with spin at lower energy generates a magnetic field in the same direction of the external magnetic field. A nucleus with spin at the lower energy state can absorb a photon and end up in the upper energy state. It is then said to be in resonance. A detector can measure the frequency of this absorption and plot it on a spectrum. Based on the chemical environment of the hydrogen (e.g. CH_3 and COOH), there is a need for slightly different external magnetic fields to bring them into resonance at a particular frequency. The *chemical shift* is the resonance frequency of the nucleus relative to the standard in a magnetic field. Several calibration standards are available, but 2-dimethyl-2-silapentane-5-sulfonic acid is commonly used for protein NMR (IUPAC recommendation) (Marion 2013).

Detailed descriptions on NMR can be obtained from Hornak (1999), Winter (2014) and Gonnella (2012).

At CMC, the peptides undergo a substantial change in their environment and water is forced out of the micelle. This can be reflected by changes in chemical shift of the

nucleus of interest. The CMC of FPH (Paper II) was estimated by observing ^{1}H chemical shifts of methyl resonance of lactate, clearly visible at about 1.3 ppm for all samples. The CMC was determined by plotting the chemical shift of lactate as a function of log protein concentration (Fig. 8). CMC was defined as the intersection between the linear trend lines above and below the increase in chemical shift (Lesemann et al. 1998) (Fig. 8).

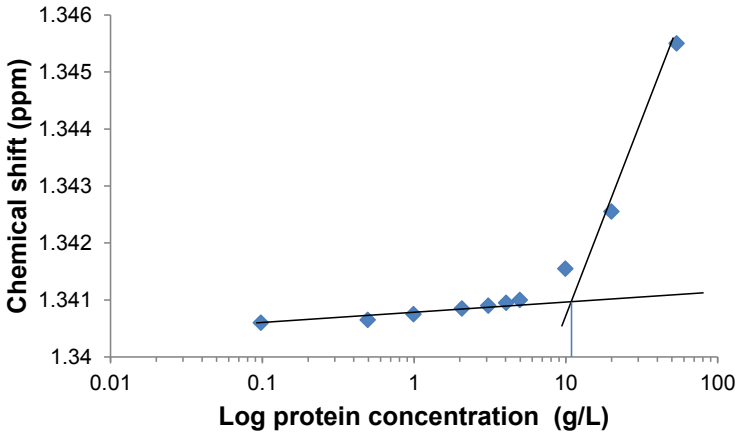


Figure 8: Observed ^{1}H chemical shifts of methyl resonance of lactate. A change in chemical environment causes changes in chemical shift. The critical micelle concentration (CMC) is obtained from the intersection of the two lines. The plot is based on data from Paper II (Pm25%).

8.2.6 Active carbon adsorption

Several adsorbents are available for the selective separation of hydrophobic and bitter peptides. AC was chosen as adsorbent based on its wide range of industrial applications, food-grade quality and easy removal from the solution. Food approved ACs were obtained from Cabot Norit (Amersfoort, The Netherlands): Norit CASPF, a phosphoric acid activated AC (CASPF); Norit CGP SUPER, a phosphoric acid activated, partially neutralized AC (SUPER) and Norit SX-PLUS, a steam activated, acid washed AC (SX-PLUS). The ACs were chosen based on their varying mode of activation and chemical and physical properties (Table 7).

Table 7. General properties of the ACs CASPF, SUPER and SX-PLUS used in the AC adsorption trials. Analyses provided by Norit (Amersfoort, The Netherlands).

Parameter	**Unit**	**CASPF[a]**	**SUPER[b]**	**SX-PLUS[c]**
pH (water extract)	-	2.7	4.2	7
Specific surface area (BET)	m^2/g	1584	1840	970
Methylene blue adsorption	g/100 g	32.9	38.1	20.3
Iodine number	-	975	1135	960
Phenol adsorption	mg/g	0.5	0.6	4.6
Total pore volume (DFT)	cm^3/g	1.01	1.18	0.37
Pore volume distribution (DFT)				
Micropores (<2 nm)	%	36	41	70
Mesopores (2-50 nm)	%	63	58	29
Macropores (>50 nm)	%	1	1	1
Water-soluble matter	Mass-%	3.62	0.47	0.18
Moisture content	Mass-%	7.8	7.4	5.9
Particle size				
D10	µm	4.2	3.5	3.7
D50	µm	14.8	12.9	18.5
D90	µm	32.6	28.4	120.6
Price[d]	EUR/kg	3.94	7.04	10.16

[a] Sample #150338
[b] Sample #150339
[c] Sample #150337
[d] Provided from the manufacturer June 2015

The adsorption process was performed at 50 °C for one hour under continuous stirring (50 rpm) (Paper III). The AC were removed by centrifugation (11400×*g*, 20 min) followed by filtration through a K-900 depth filter (Mall Corporation, East Hills, NY). Two concentrations of AC were used: 0.1% and 1% based on protein content. The concentration levels were chosen based on preliminary results suggesting that 1% AC would be efficient in reducing bitter taste.

8.3 Statistical analyses

Protein hydrolysis is a multivariate system where both enzyme concentration and hydrolysis time have an effect of DH and PR. The sensory and surface-active properties of the resulting hydrolysates are influenced by enzyme specificity, DH and peptide size distribution. In this thesis, response surface regression was used to model the combined effects on hydrolysis time and enzyme concentration on DH and PR (Paper I). Principal component analysis (PCA) was used to evaluate the effect of enzyme, DH and peptide size distributions on sensory and surface-active properties (Paper II). In addition, ANOVA was employed on the data to find chemical and sensory attributes that statistically varied between products.

8.3.1 Response surface regression

Response surface methodology is a collection of mathematical and statistical techniques useful for modeling and the analysis of problems (Meyers and Montgomery 2002). The objective is to measure a response as a function of independent variables. The first step in RSM is to find a suitable approximation to the relationship between the response and the variables. The simplest form is a first-order model that assumes a linear relationship between the response and variables:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \varepsilon \quad (11)$$

where y is the estimated response, x_1 and x_2 the predictor variables, β_0 the intercept, β_1, β_2 and β_{12} the regression coefficients of each factor and the interaction term between them, respectively. ε is the residual error.

If there is a curvature in the response surface, then a higher degree polynomial should be used. The approximation with two variables is called a second-order model:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \varepsilon \quad (12)$$

The response surface models enable regression equations for the prediction of the response at a particular point on the process variable space.

8.3.2 Principal component analysis

Principal component analysis (PCA) is a statistical method that uses latent variables, called principal components (PC), to describe the systematic pattern of variation between the samples (Martens and Martens 2001; Esbensen 2002). PCA decomposes the original data matrix, X, into two smaller matrices, T and P plus residual error (E):

$$\mathbf{X = TP^T + E = \sum t_a p_a^T + E_a} \quad (13)$$

The columns in T, t_a, are called score vectors and the rows in P, p_a, are called loading vectors. $\sum t_a p_a^T$ is the sum of all possible PCs. PC1 ($t_1 p_1^T + E_1$) are located in the direction that explains most of the variance between the samples. The second PC (PC2; $t_2 p_2^T + E_2$) are orthogonal (do not co-vary) to PC1 and explains as much of the remaining variance as possible. This can be repeated until the number of PCs are equal to the number of objects or variables (whichever comes first). The explanatory importance of the PCs is measured through the ratio of the explained variance to the variance obtained in the original variables. Because most of the variance in the data matrix is explained by the first two PCs, it is most common to plot the score and loading vectors of PC1 against the corresponding vectors of PC2. This gives a score plot and loading plot, respectively. The score plot gives a visualization of the relationship between the original objects and the loading plot the relationship between original variables. The PCA plots are helpful for studying correlation, similarities and grouping of variables and objects.

8.3.3 Analysis of variance

ANOVA is a collection of statistical models used to analyse the differences among group means (Lane 2013). One-way ANOVA is used to compare means of more than three samples. The hypothesis can be written as:

H_0: $\mu_1 = \mu_2 = \ldots . \mu_i$

H_1: Not all mean values are the same

Two-way factorial ANOVA was used to evaluate significant variance of sensory attributes between the products because the sensory analysis is dependent on two factors: the product and the assessor. The model used can be written as:

$$\mathbf{Y_{ij} = P_i + A_j + PA_{ij} + \varepsilon_{ij}} \quad (14)$$

P is the product effect, A is the assessor effect and PA is the product × assessor interaction effect. Product was taken to be a fixed effect and the assessor and product × assessor effects were taken to be random effects, giving a mixed model ANOVA (Næs and Langsrud 1998).

The ANOVA test will only determine whether there are significant difference between samples, but not which of the sample(s) are deviant. There is a need for a *post hoc* ("after this") test. There are a great number of different post hoc tests available, but most frequently, Tukey HSD test is used on sensory data. The test is based on the calculation of a difference score between the samples means. The difference score is compared to a critical value to see if the means are significantly different. The critical Honestly Significance Difference (HSD) is computed by the following equation:

$$\boldsymbol{HSD = q\sqrt{\frac{MS_{within}}{n}}} \quad (15)$$

q is a studentized range statistics and n is number of values. If the difference between the samples mean is larger than HSD they are significantly different.

9. Summary and discussion of experimental work

9.1 Characterization of the raw material (Paper I)

The proximate composition of the raw material revealed a protein content of 126 g/kg, including all indispensable amino acids and taurine (2.9 g/kg DM; Aspevik 2016, unpublished results). The DM content was 429 g/kg and included 267 g/kg lipids and 39 g/kg ash. Based on the amino acid composition, substrate specific numbers for h_{tot} and f_N were calculated as 9.3 meqv/g protein and 5.23 g N/g protein, respectively. The numbers deviate from those commonly used for fish substrate, i.e. h_{tot} = 8.6 and f_N = 6.25 (Adler-Nissen 1986), mostly ascribed to the high content of connective tissue amino acids, mainly glycine.

The presence of endogenous lipase activity was negligible as the formation of free fatty acids only increased from 11 ± 1.5 g/kg to 12 ± 1 g/kg after two hours of hydrolysis. In addition, the presence of endogenous protease activity was determined as insignificant at 50 °C, with only a minor increase in DH (<0.5%) observed without addition of enzyme during a two-hour pH-STAT run.

9.2 Hydrolytic and cost efficiency of commercial endopeptidases (Paper I)

There is a vast array of food-grade proteases available for protein hydrolysis. In this study, five commercial endopeptidases were chosen based on four criteria (Table 5): 1) food-grade quality and GRAS/QPS classification of the production strain, 2) reported primarily endopeptidase activity, 3) hydrolytic activity at pH 6.5 and 50 °C and 4) diversity in manufacturers. The specific hydrolytic activity of the chosen proteases was quantified at the experimental conditions (pH 6.5, 50 °C), based on a casein assay, which would enable dosing of the proteases at equal activity levels. Alcalase 2.4L had highest hydrolytic activity (1.4 U/mg), followed by Corolase 7089 (1.0 U/mg), Protex 7L (0.8 U/mg), Neutrase 0.8L (0.6 U/mg) and Promod 671L (0.3 U/mg).

The pH-STAT experiments were performed at pH 6.5 to keep the pH as close as possible to the natural pH of the substrate, but still have a degree of protonated α-amino groups. A pH of 6.5 is in the lower range applicable for pH-STAT experiments, with α-value of only 0.175. However, preliminary hydrolysis tests without addition of base confirmed poor buffering capacity of the substrate in this pH-range. Five activity levels of protease were used (5.5 U/S, 11 U/S, 22 U/S, 44 U/S and 88 U/S) and the hydrolysis processes were run for 120 min. At the lowest activity levels (5.5 and 11 U/S), there were only negligible difference in final DH between enzyme products. At highest U/S levels (22-88 U/S), Protex 7L, Alcalase 2.4L and Promod 671L gave higher final DH compared to Corolase 7089 and Neutrase 0.8L after two hours of hydrolysis. Protex 7L, Neutrase 0.8L and Corolase 7089 are reported to have identical EC numbers (Table 4) and a similar performance of these enzymes were expected. However, based on possible differences in the gene expression of the applied production strains and protease purification steps, the formulations may contain traces of other proteases. The manufacturer of Protex 7L confirmed that this enzyme also contained a lesser amount of endopeptidase 3.4.21.62, which may explain the better performance of this protease formulation. Even though the enzymes gave different final DH values at the highest U/S, all enzymes were equally able to solubilize the protein. On average the PR was increased to 54 ± 1%, 58 ± 2%, 64 ± 3%, 67 ± 1% and 71 ± 2% after two hours of hydrolysis at 5.5, 11, 22, 44 and 88 U/S, respectively. Thus, two-fold increases in enzyme activity only gave a small effect on the increase in solubilized protein. The results are in agreement with Bhumiratana et al. (1977), who demonstrated that a five-fold increase in enzyme activity only gave a minor increase in yield of solubilized proteins. Excess amount of enzyme will preferentially cleave already solubilized peptides giving an increased DH, but only contribute to further solubilization of insoluble protein to a minor extent.

For each of the tested enzymes, response surface regression models with R^2 of 0.942 to 0.960 ($p < 0.001$) were established for the combined effects of hydrolysis time and enzyme activity. Only insignificant differences were observed between the enzymes ability to solubilize the substrate and a common PR model was established ($R^2 = 0.924$,

$p < 0.01$). The main effects of hydrolysis time and enzyme activity were positive, reflecting that an increase in either would give increased PR and DH. The models may be used to predict hydrolysis time and enzyme activity needed to reach a predefined DH- or PR-value. Combined with activity-specific enzyme cost, this enables the calculation of cost of enzyme needed to reach a target DH or PR. In this system, Protex 7L was found to be the most cost-efficient enzyme, due to low price in EUR/U and high hydrolytic efficiency, followed by Alcalase 2.4L, Corolase 7089, Neutrase 0.8L and Promod 671L.

A linear correlation ($R^2 = 0.84$, $p < 0.001$) between DH determined by the pH-STAT and OPA methods was observed ($DH_{OPA} = 10.5 + 1.53 \times DH_{pH\text{-}STAT}$). The intercept of 10.5 can be explained by the reaction of OPA reagent with water-soluble proteins and free amino acids in the raw material. The slope of 1.53 indicate a deviance between the two methods, and similar results have been observed between the pH-STAT and TNBS methods in cod substrate (Himonides et al. 2011). In general, the TNBS and OPA methods are believed to be most accurate due to direct assessment of newly formed α-amino groups. The underestimation of DH by the pH-STAT method may be explained by the higher pKa-value of small peptides generated in the hydrolysis process. Small peptides generated in the hydrolysis process may have pKa-values half a unit higher than polypeptides (Adler-Nissen 1986). The observed linearity between the methods make it possible to transfer the response surface models to an unbuffered system and reach equivalent DH_{OPA} by different proteases.

9.3 Impact of DH, molecular weight distribution and enzyme specificity on sensory and surface-active properties (Paper II)

9.3.1 DH and molecualar weight distribution

The aim of this study was to evaluate the effects of enzyme specificity, PR, DH and molecular weight distribution on the sensory and surface-active properties of FPH. The commercial proteases Alcalase 2.4L, Promod 671L and Protex 7L were chosen based

on their good ability to hydrolyze the Atlantic salmon substrate and their different EC numbers (Table 5), i.e. different hydrolytic specificity. Based on the regression models developed in Paper I, upscale hydrolysis processes were performed to reach equal DH-levels of 17%, 25% and 30%. A reference sample, made by direct thermal coagulation of the raw material (>90 °C, 10 minutes), was included to assess the sensory and surface-active properties of water-soluble peptides and amino acids inherent to the substrate. Only 19.1% of the total protein was solubilized in the reference sample and consisted of mainly (61%) large polypeptides (<20,000 Da), confirming that the raw material contained low amounts of endogenous protease activity. Even a short hydrolysis process of 15-18 min of hydrolysis to reach DH of 17% significantly increased PR to more than 34%, and decreased the molecular weight distribution to peptides of less than 10,000 Da. At the highest DH of 30%, a PR of about 65% was achieved, with more than 68% of the peptides less than 1000 Da. An increase of molecules with molecular weight <200 Da was observed, reflecting mostly free amino acids and small dipeptides. The quantification of free amino acids demonstrated an increase from 0.8% in the reference sample to 6.4%, 5.1% and 4.5% in products hydrolyzed by Alcalase 2.4L, Promod 671L and Protex 7L, respectively, at DH of 30%. This confirms that the protease formulations contain some undeclared exopeptidase activity.

9.3.2 Sensory properties

The influence of enzyme specificity, DH and molecular weight distribution on the sensory attributes of the FPH were evaluated based on generic descriptive analysis. Prior to the sensory evaluations, a concentration test was performed using hydrolysates with DH of 17% and 30% to find the most optimal test protein concentration for all products. It was imperative that the chosen concentration level would be high enough so that all sensory attributes could be detected. At the same time, the concentration level should not pass a level where some of the strongest attributes dominated and possibly overshadowed the remaining. Based on the PCA bi-plot from the concentration test (Fig. 9), a protein concentration level of 1.0% was chosen as the best compromise for the tested samples and attributes and used in the sensory analysis.

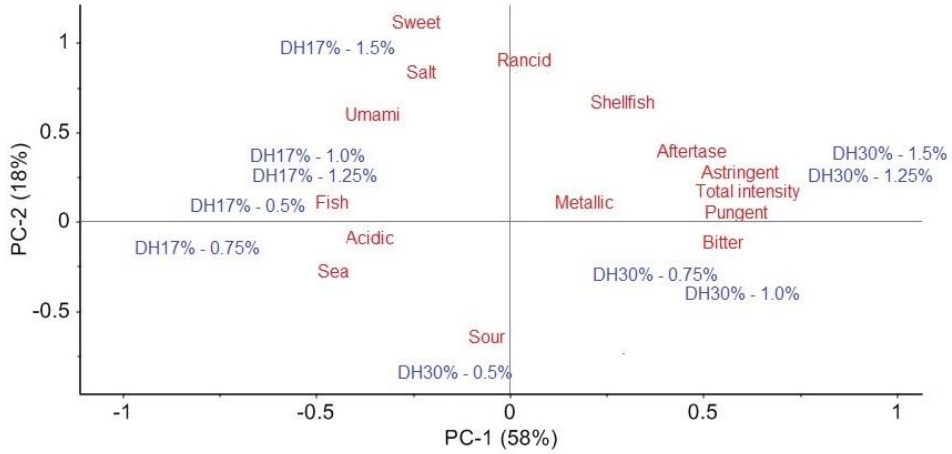


Figure 9: PCA bi-plot from concentration test using hydrolysates based on Promod 671L with DH of 17% and 30%. Protein concentration levels of 0.5%, 0.75%, 1.0%, 1.25% and 1.5% were used (Aspevik 2016, unpublished results).

The PCA of the sensory analysis explained less than 50% of the variance of aftertaste, sour, shellfish and metallic attributes and these tastes and flavors were regarded as not relevant for the interpretation of the sensory variance between the FPHs. The intensity of bitter, astringent and pungent attributes increased significantly when DH was elevated from 17% to 25% and 30% for all enzyme products. No significant differences in bitter taste intensity were observed between the two latter DH-levels. A positive correlation between bitter taste, high numbers of HPF and PR, and small peptides of 500-2000 Da was observed. Bitter taste showed no correlation with peptides of 2000-4000 Da, whereas a negative correlation was observed for peptides >4000 Da. The weakest bitter taste intensity was detected in the reference sample, reflecting a low content of water-soluble bitter peptides inherent to the substrate compared to the hydrolyzed proteins.

The formation of bitter taste was also dependent on enzyme specificity, where hydrolysates based on Alcalase 2.4L were the most bitter and astringent products at all DH-values. No significant variance was observed between hydrolysates based on Protex 7L and Promod 671L, however, Protex 7L gave least bitter taste intensity at the tested DH. This reflects a higher bitter potency of the subtilisin activity of Alcalase

2.4L compared to the bacillolysin activity of Protex 7L. Alcalase 2.4L is a commonly used endopeptidase preparation in the production of FPHs, due to its high hydrolytic efficiency, low price and well-established brand. In addition, literature studies have reported less bitter taste of FPHs based on Alcalase 2.4L compared to other commercial protease formulations (Gildberg et al. 2002; Hou and Li 2011). However, these studies have compared the hydrolysates based on equal hydrolysis time, rather than equal DH and similar molecular weight distributions. This gives and an imprecise basis of the comparison of bitter peptides based on different proteases. Spellman et al. (2009) demonstrated higher bitter taste of whey hydrolysates based on Alcalase 2.4L compared to other endopeptidases (Corolase 7089 and Prolyve 1000) at equal DH. They attributed the findings to the presence of glutamyl endopeptidase activity in the Alcalase 2.4L formulation.

The remaining tested attributes (sweet, salt, umami, acidic, sea and fish tastes and flavors) showed high intensity levels in the reference sample and hydrolysates with DH 17%, and a positive correlation to high molecular weight peptides (>4000 Da) and low PR. The negative correlation of these attributes and PR indicate that they are related to the dilution gradient to reach an identical test concentration of 1% protein. This suggest that these tastes and flavors are more related to water-soluble components in the substrate, rather than specific amino acids and peptides formed during the hydrolysis process.

9.3.3 Hydrophobic and surface-active properties

The effects of enzyme specificity, DH and molecular weight distribution on the hydrophobic and surface-active properties of the FPH were assessed based on HPF and CMC, respectively. The HPF revealed that only small amounts (≤6.4%) of the peptides were small and apolar enough to partition to the organic 2-butanol phase. A dependence of DH was observed ($R^2 = 0.927$, $p < 0.001$), with a positive correlation of HPF to small molecular weight peptides (<2000 Da). However, the reference sample gave a HPF of 4.1%, indicating a hydrophobic nature of some of the free amino acids and soluble polypeptides present in fish solubles. Hydrolysates based on Alcalase 2.4L

gave slightly higher HPF-values compared to hydrolysates based on Promod 671L and Protex 7L, at all DH-levels, reflecting a higher content of small hydrophobic peptides in the Alcalase 2.4L hydrolysates.

Low CMC, i.e. good surface-active properties was observed at low DH and positively correlated to peptides of more than 4000 Da. The reference sample gave the lowest CMC-value (6.0 g/L), followed by hydrolysate based on Alcalase 2.4L with DH 17% (8.5 g/L). The CMC-values increased dependent on DH for all enzymes, indicating a reduction of amphilicity and segmental flexibility of the peptides as the DH increases. Small, but insignificant, differences were observed between hydrolysates based on Alcalase 2.4L and Protex 7L. Hydrolysates based on Promod 671L gave highest CMC at all tested DH-levels, with significantly higher numbers compared to Alcalase 2.4L at DH 17% and 25%. This indicates poor surface-active properties of peptides based on Promod 671L and also confirms that enzyme specificity is important for the surface-active and physicochemical properties of FPH. Regardless, the observed CMC-levels were much higher than conventionally used food surfactants such as sodium dodecyl sulfate (SDS) with CMC of 2.39 g/L (Cifuentes et al. 1997). Therefore, FPH requires higher inclusion levels to function as a functional food ingredient compared to SDS. However, in contrast to SDS, FPH also acts as a nutritional component and may contribute to reduce the need for synthetic emulsifiers in food formulations.

9.4 Debittering of the FPH (Paper III)

The objective of this study was to further reduce the bitter taste of FPH based on Protex 7L endopeptidase by treatment with exopeptidase (Flavourzyme 1000L) activity and AC adsorption. The hydrolysates were produced based on 10 and 60 min of hydrolysis, with DH of 12% and 18%, respectively, to reveal the effects of debittering treatments on hydrolysates with low DH.

9.4.1 Effect of exopeptidase treatment

The enzymatic hydrolysis using endo- and exopeptidase activity was evaluated based on combined (COMB) and sequential (SEQ) hydrolysis steps. In the COMB

experiments, the endo- and exopeptidases were added at the same time. In the SEQ experiments, the hydrolysis was first run with endopeptidase activity, followed by inactivation of the enzyme (>90 °C, 10 min), before exopeptidase was added in a second hydrolysis step. Higher levels of free amino acids and higher DH were observed in hydrolysates with exopeptidase activity compared to only endopeptidase activity. The COMB experiments were slightly more efficient in the release of free amino acids compared to the SEQ experiments. This might be explained by the heat inactivation of enzyme activity leading to a thermal denaturing of the substrate before addition of exopeptidase in the sequential hydrolysis processes. This will cause a reduced efficiency of the hydrolysis process due to hydrophobic and sulfhydryl interactions between the peptides (Mutilangi et al. 1995).

In particular, levels of the hydrophobic amino acids leucine, valine and isoleucine increased in hydrolysates with exopeptidase activity, reflecting the hydrolytic specificity of Flavourzyme 1000L. However, an increase in reaction time from 10 to 60 min of hydrolysis also caused an increased release of free amino acids, confirming that the Protex 7L protease formulation contains some exopeptidase activity. Even though DH was directly correlated to release of free amino acids ($R^2 = 0.919$, $p = 0.003$), PR was not influenced by exopeptidase activity in the hydrolysis process. This indicates a primary action of exopeptidase on already solubilized peptides and a negligible action on the insoluble protein.

The bitter taste was less intense compared to hydrolysates from Paper II, and a protein concentration of 2% was used in the sensory analysis. The results, however, did not reveal any significant reduction of bitter taste after treatment with exopeptidase activity. Only a significant effect of hydrolysis time was observed where products hydrolyzed for 60 min were significantly more bitter than products hydrolyzed for 10 min ($p < 0.05$). This confirms the dependence of DH and molecular weight distribution on bitter taste as demonstrated in Paper II. Only a minor insignificant reduction of mean bitter intensity was observed in products with exopeptidase activity ($p \geq 0.577$). The intensity of the remaining attributes tested (salt, sweet, umami, acidic, fish, sea, pungent tastes and flavors) decreased depending on hydrolysis time, possibly reflecting

the dilution effect to reach identical test concentration of 2% protein, as discussed in section 9.3.2. No difference in aftertaste was observed between the products.

The observed non-significant reduction of bitter taste by inclusion of exopeptidase activity may possibly be explained by the following factors: 1) the broad molecular weight distribution consisting mostly of peptides >500 Da might be less susceptible for the debittering effect. In general, the significant debittering effect by exopeptidase is ascribed to proteins hydrolyzed for more than five hours and with a high DH (>50%), i.e. high content of small peptides less than 500 Da (Izawa et al. 1997; Nilsang et al. 2005; Cheung et al. 2015a). Peptides of 200-500 Da accounted for less than 11% in the tested hydrolysates and removal of terminal amino acids from larger peptides (>500 Da) is likely of less effect in debittering applications; 2) the choice of exopeptidase activity might be a limiting factor in the debittering of FPHs. In this study, a leucine aminopeptidase was used. However, the use of leucine carboxypeptidases has been reported as more efficient in debittering applications (Cheung et al. 2015a). Moreover, it has been shown that the release of leucine from C-terminus decreased the bitter taste ten-folds compared to release from the N-terminus (Ishibashi et al. 1987a).

9.4.2 Effect of activated carbon treatment

The effect of AC adsorption on reduction of bitter taste was evaluated on hydrolysate with DH 18% using three types of AC at two concentration levels (0.1% and 1% on protein basis). The ACs were selected based on their different activation methods and chemical properties (Table 7). A sensory test protein concentration of 3.5% was used to ensure that small reductions in bitter taste would be detected.

The largest reduction of bitter taste was observed in samples treated with 1% CASPF, followed by 1% SUPER where a reduction of mean bitter taste intensity from 6.4 in the untreated hydrolysate to 5.3 and 5.4, respectively, was observed. However, the reductions of bitter taste were not significant ($p \geq 0.09$). The intensity of the remaining attributes tested (salt, sweet, umami, acidic, fish, sea, pungent and aftertaste) did not vary between products and suggests that the ACs do not adsorb components influencing these tastes and flavors.

The minor reduction of bitter taste could not, however, be explained by a reduction of hydrolysate protein content. On the contrary, levels of total N increased on DM basis. The ACs did not release soluble N to the products and the only possible explanation would be that the ACs adsorbs non-protein DM from the hydrolysates. Analysis of salt and ash revealed a reduction in salt-free-ash of 21-45% in hydrolysates treated with 1% AC compared to the untreated product. No reduction of salt (NaCl) content was observed, with about 40 g/kg DM in all products. The relative content of individual amino acids demonstrated a minor reduction of serine, alanine and cysteine ($p < 0.05$) and tyrosine ($p = 0.09$) in AC treated samples compared to the untreated hydrolysate. The content of arginine and threonine increased ($p < 0.05$) in AC-treated hydrolysates, possibly reflecting a minor accumulation of polar amino acids after treatment with AC. The aromatic amino acids tyrosine, tryptophan and phenylalanine are reported to be particularly susceptible for adsorption onto AC, and other adsorption materials (Murray and Baker 1952; Cogan et al. 1981; Doulia et al. 2001). These amino acids only constituted about 2.6%, 0.5% and 2.8% of the total amino acid content, respectively and less than 0.8% as free amino acids combined. The low levels make any quantification of potential reduction effect difficult.

The observed small reduction of bitter taste and negligible removal of hydrophobic and aromatic amino acids may have several potential explanations: 1) the employed AC concentrations were significantly less than those reported in literature studies (30-200% on protein basis) and possibly too low for proper adsorption of bitter peptides and amino acids; 2) the hydrolysate was only moderately hydrolyzed for one hour with a DH of 18%, whereas published studies have used prolonged hydrolysis times (6-18 hours of hydrolysis) (Cogan et al. 1981; Suh et al. 2000) and consequently high levels of low-molecular weight peptides available for adsorption; 3) the applied method for statistical analysis of sensory data will influence the interpretation of sensory results. Sensory data should be calculated by a mixed model ANOVA, with assessor effect as a random effect (Næs and Langsrud 1998). This gives a *p*-value of 0.09 for the analysis of effects of AC adsorption on bitter taste. However, a one-way ANOVA model would give a *p*-value of 0.14, while a fixed two-way model would give a *p*-value of 0.04. In

general, the studies reporting debittering by AC do not explain the statistical approach used and this make comparison of results difficult.

9.5 Nutritional properties (Paper III)

The calculations of AAS revealed that the tryptophan was first-limiting amino acid in the raw material, followed by leucine, valine and isoleucine to meet the dietary protein criteria for small children (six months – three years) (FAO/WHO/UNU 2013). The low levels of tryptophan may be explained by the high content of bones and connective tissue that are deficient in this amino acid (Toppe et al. 2007). Leucine was first-limiting amino acid in hydrolysates based on 10 min of hydrolysis, followed valine, tryptophan, isoleucine, threonine and aromatic amino acids (tyrosine and phenylalanine), indicating a slow release of the two former amino acids from the substrate. Hydrolysate based on 60 min of hydrolysis reflected the amino composition of the raw material, being most deficient in tryptophan followed by leucine, valine, isoleucine and, to a minor degree, aromatic amino acids. The calculations of PDCAAS and DIAAS were only 70.6% for the raw material and less than 60.1% for all hydrolysates, ascribed to the low AAS-values. In comparison, Opheim et al. (2015) demonstrated significantly higher PDCAAS and DIAAS-values for highly hydrolyzed (DH >30%) Atlantic salmon viscera hydrolysates, with leucine being the first-limiting amino acid. This might reflect a more well balanced amino acid composition in viscera compared to the head and backbone. Moreover, the presence of endogenous enzymes gave higher release of indispensable amino acids compared to only exogenous enzyme activity (Opheim et al. 2015). The calculations of PER revealed high numbers of >1.9 for both raw material and hydrolysates and reflects high nutritional properties of the hydrolysates In the PER-equation (Eq. 5), all indispensable amino acids are equally weighted, explaining the better performance by calculation of PER compared to AAS. Nevertheless, it should be pointed out that the calculations of nutritional properties are based on protein basis of f_N = 6.25, according to FAO recommendations (FAO/WHO/UNU 2013), that will be deviant from actual protein content. By using the substrate specific f_N of 5.23, higher AAS-values are obtained for the raw material

(91.7%) and resulting hydrolysates (≥62.7%). Moreover, by using $f_N = 5.23$ gives PER-values of >2.3 for all hydrolysates.

It is generally accepted that a major drawback with AC adsorption is removal of indispensable amino acids. However, no negative effects on nutritional value were observed in this study after treatment with AC. In addition, the effect of exopeptidase activity did not reveal any effect on nutritional properties and confirmed that the exopeptidase activity primarily acts on solubilized peptides and does not cause further solubilization of non-polar indispensable amino acids.

10. Conclusions

The work presented in this thesis has provided new insight and knowledge into the influence of enzyme specificity and peptide molecular weight distribution on the sensory properties of FPH. The experimental work has improved the understanding of factors influencing the formation of bitter taste and challenges met in the production of a taste neutral product. In addition, important surface-active and nutritional properties of the hydrolysates have been assessed. The main conclusions are summarized in the following:

- Direct thermal coagulation of the salmon by-products give a soluble protein (stickwater) recovery of about 20%. The obtained soluble proteins consists primarily of large peptides (>20,000 Da), reflecting low endogenous enzyme activity, with low level of and free amino acids.
- PR shows a non-linear relationship with DH and increased enzyme dose gives minor effects after two hours of hydrolysis.
- The combined effects of hydrolysis time and enzyme activity on DH and PR can be modelled based on a second-order equation. The models can be combined with activity-specific enzyme cost to evaluate the cost-efficiency of the different proteases to reach a target DH or PR.
- High bitter taste is related to high yield of solubilized proteins, high DH and peptides of low molecular weight (500-2000 Da). At high DH (≥25%), the effect of enzyme specificity is of relevance. Hydrolysates based on Alcalase 2.4L are significantly more bitter than products based on Protex 7L and Promod 671L.
- The hydrolysis process directly influences the formation of bitter, pungent and astringent attributes, with an increasing intensity related to increasing DH. Other attributes present in the FPH (sweet, salt, sour, umami, acidic, metallic, shellfish, sea, fish tastes and flavors) are correlated to the degree of dilution required to reach the protein concentration used during sensory assessment, reflecting a correlation to water-soluble components inherent to the substrate.

- HPF is directly correlated to DH and small molecular-weight peptides. Hydrolysates based on Alcalase 2.4L give slightly higher HPF-values than hydrolysates based on Protex 7L and Promod 671L.
- ^{1}H-NMR can be used to measure CMC of protein hydrolysates. Hydrolysates with high DH and large number of low molecular weight peptides give high CMC, reflecting a reduction of surface-active properties. Hydrolysates based on Alcalase 2.4L and Protex 7L give no significant difference in CMC-values, while Promod 671L produces highest CMC at all tested DH.
- The CMC-values of the FPHs are higher than conventionally used food surfactants and reflect a need for higher inclusion level to function as emulsifiers or foaming agents.
- Superior properties, with respect to low bitter taste and low CMC, may be achieved by direct thermal coagulation of the raw material (stickwater), albeit, at low yield of solubilized protein.
- Leucine aminopeptidase treatment has negligible effect on bitter taste reduction in FPH with DH ≤18%
- AC adsorption (1% on protein basis) gave only a minor reduction of bitter taste in FPH with DH 18%. Acid activated carbons are more effective for the removal of bitter taste compared to neutral carbons.
- The raw material and resulting hydrolysates were most deficient in tryptophan and leucine to meet dietary protein requirements for small children.
- Salmon head and backbone by-products contain high levels of connective tissue protein. This gives a substrate specific nitrogen factor (f_N = 5.23 g N/g P) and total number of peptide bonds (h_{tot} = 9.3 meqv/g) deviating from commonly used values.

11. Future outlooks

Fish by-products are a valuable resource with great potential for human consumption. The production of food-grade FPH is a promising alternative for improved and economical utilization of these resources. Such applications will also support and improve the sustainability of the global aquaculture and fishing industry. Further research should focus on improved understanding of the molecular composition and physicochemical, nutritional and sensory properties of FPH.

The main limitation of FPH in food applications is the development of bitter taste during the hydrolysis process. Significant reduction of bitter taste is possible with proper control of DH and enzyme specificity. However, a complete removal of bitter taste was not achieved in this study and further research is needed on evaluation of proteases with other specificities. In addition, other post-hydrolysis debittering techniques could be worth investigating in the production of a low-bitter FPH.

Further studies are needed to develop a protocol for the sensory analysis of FPH. The development of a standardized scale and relevant bitter standards would enable comparison of hydrolysates produced by different conditions, times and laboratories.

The performance of FPH as a food ingredient or emulsifier should be more thoroughly assessed. The actual food pH may deviate from the natural pH-level of the FPH, usually in the acidic range. This may have huge impact on the actual performance of FPH as a functional food ingredient.

The potential bioactive properties of FPH should be evaluated by pre-clinical trials to assess possible health benefits.

Only the head and backbone by-products from Atlantic salmon have been used in this study. The production of FPH based on by-products from other marine species and by-catch should be conducted. Moreover, the remaining fractions after the production of a water-soluble FPH, i.e. lipid and solid phases, are rich in essential lipids, bones and insolubilized proteins, respectively. These may also have potential within higher-value

products and future research should have more focus on these fractions as well to ensure a sustainable and economical feasible utilization of the marine resources.

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