Journal of Aquatic Food Product Technology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/wafp20

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To cite this article: Jannicke Remme, Guro Møen Tveit, Bendik Toldnes, Rasa Slizyte & Ana Karina Carvajal (2022) Production of Protein Hydrolysates from Cod (*Gadus morhua)* Heads: Lab and Pilot Scale Studies, Journal of Aquatic Food Product Technology, 31:2, 114-127, DOI: 10.1080/10498850.2021.2021341

To link to this article: https://doi.org/10.1080/10498850.2021.2021341

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Published online: 03 Jan 2022.

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JOURNAL OF AQUATIC FOOD PRODUCT TECHNOLOGY
2022, VOL. 31, NO. 2, 114–127
https://doi.org/10.1080/10498850.2021.2021341

Production of Protein Hydrolysates from Cod (*Gadus morhua)* Heads: Lab and Pilot Scale Studies

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ABSTRACT
Cod heads were hydrolysed using different enzymes (Alcalase, Flavourzyme, Papain, Bromelain, and Protamex) at concentration of 0.1% for 1 hour and different water/raw material ratios. The most promising processing conditions were further verified in large-scale trials in a pilot plant. The results showed that the water/raw material ratios had little effect on hydrolysis yield, protein content, or the molecular weight distribution in hydrolysate. However, different enzymes led to differences in chemical composition and molecular weight distribution. Protein recovery was lower in the pilot trial, primarily because pilot and industrial processing equipment must be adapted and optimized to the raw material and process. Upscaling proved that high-quality protein powder can be produced from cod heads. The product has a neutral smell, light color, contains more than 80% protein, and could serve as an excellent source of protein for human consumption.

KEYWORDS
Enzymatic hydrolysis; Protein Hydrolysate; cod heads; rest raw material; whitefish

Introduction

Cod heads could be a valuable rest raw material from the Norwegian whitefish industry. In 2019, about 400,000 tonnes of cod (*Gadus morhua*) were caught by the combined Norwegian coastal and oceanic fishing fleets. The coastal fisheries land fresh cod at processing plants three to 12 hours after catching. Traditionally, cod heads have been air-dried outdoors before being packed and exported to markets in Africa. For drying, 15–20 heads are manually threaded on a rope and hung on wooden racks. Up to 60 tons (about 25 000 heads) must be hung on the same day they are landed. In recent years, both prices and export volumes have been severely reduced due to market instability. Cod heads represent about 20% of whole-fish weight and constitute a major proportion of the 43% rest raw materials (Hjellnes et al. 2020; Slizyte et al. 2005; Tveit et al. 2020) from whitefish. They contain high levels of protein (14–15%), low lipid contents (4%), and about 6% ash (Tveit et al. 2020), and their chemical composition makes them a highly suitable source for the manufacture of high-quality protein products. The need for innovative processing approaches is required to convert cod heads into more profitable and marketable products, while still retaining the nutritional value of the raw material. Enzymatic hydrolysis is one of the most effective technologies for recovering valuable proteins from marine rest raw materials, including cod (*Gadus morhua*) heads.

The aim of this study was to identify process parameters for an efficient and affordable production of high-quality cod head protein hydrolysate and verify the process on an industrially relevant scale. The most promising results from lab-scale tests were used in pilot-scale trials. Though there are many advantages of enzymatic processes, there are several challenges that should be solved when upscaling the process (He et al. 2013). There are many scientific works describing enzymatic hydrolyses (Dauksas et al. 2005; Gildberg et al. 2002; Slizyte et al. 2005; Šližytė et al. 2009) using different marine

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raw materials (Bougatef et al. 2012; Gildberg 1993; Quaglia and Orban 1990). Previous studies have often focused on functionalities (Gao et al. 2021; Gbogouri et al. 2004; Klompong et al. 2007; Kristinsson and Rasco 2000; Spinelli et al. 1972) and bioactivity (Batista et al. 2010; Chabeaud et al. 2009; Guérard and Sumaya-Martinez 2003; Klompong et al. 2007) of protein hydrolysates, rather than industrial process development.

In the laboratory, the type and amount of enzyme, water/raw material ratio, and hydrolysis time has little effect on energy consumption linked to heating and drying. In industrial settings, water addition drives energy costs up, because the water must be heated and subsequently dried off. Some published laboratory studies have used water/raw material ratios such as 1:1 (Batista et al. 2009; Cancre et al. 1999; Dauksas et al. 2005; Gbogouri et al. 2004; He et al. 2013; Opheim et al. 2015), 2:1 (Awuor et al. 2017; Jafarpour et al. 2020), and 4:1 (Rodriguez-Diaz et al. 2011), while others utilize a variety of buffers to adjust reaction pH (Ovissipour et al. 2010). In 2000, Alcalase was suggested as the best enzyme for use with marine materials (Kristinsson and Rasco 2000). Since then, several new commercial enzymes have become available, and enzymes are now tailored to be substrate specific (Klompong et al. 2007). In industrial settings, it is important to identify enzymes that give a high yield of white hydrolysate with a high protein content and neutral taste and odor. The amount of enzymes added is also of great interest, and published studies include concentrations such as 3.5–6.5% (Gbogouri et al. 2004), 2.0% (Awuor et al. 2017), 1.5% (He et al. 2013; Jamnik et al. 2017; Tan et al. 2018), and 0.5% (Batista et al. 2009; Cancre et al. 1999; He et al. 2013). However, for industrial applications, the use of concentrations higher than 0.1% generally incurs higher production costs (Dauksas et al. 2005; Opheim et al. 2015). Hydrolysate production efficiency and profitability demand the shortest possible hydrolysis reaction times, and a number of studies have tested times such as 6 hours (Awuor et al. 2017; Jamnik et al. 2017), 4 hours (Rodriguez-Diaz et al. 2011), and 2 hours (Cancre et al. 1999; Gbogouri et al. 2004; Kristinsson and Liang 2006), when 60 minutes (Dauksas et al. 2005) or even less may be sufficient to produce acceptable product quality and yield (Bougatef et al. 2012; Gildberg 1993; Quaglia and Orban 1990). Thus, the processing parameters must be specifically tailored to the individual application to achieve optimal profitability and verified at the relevant scale. Enzymatic hydrolysis is known to enhance the functional properties of dietary proteins without affecting their nutritional value by converting them into easily digestible peptides with the desired size, charge, and surface properties (Moure et al. 2006; Shahidi et al. 1995; Slizyte et al. 2005). Products can thus be marketed in food products for human consumption, rather than simply as animal feed or fertilizer (Benjakul and Morrissey 1997; Bougatef et al. 2012).

Norway has an advanced industry for enzymatic hydrolysis of salmon rest raw material, but it is unclear whether this technology is applicable to enzymatic hydrolysis of cod heads. Therefore, a mobile unit for production of protein hydrolysates in pilot scale was used for verification of processing and production of protein hydrolysates. The mobile unit was transported to a fish processing plant, as it was important to show that by using fresh cod heads directly, it is technologically possible to obtain high-quality marine protein hydrolysates.

Material and methods

Raw material

Cod (*G. morhua*), caught in January (for laboratory studies), February and March (for pilot studies) 2017, was landed and deheaded at a processing plant in Rolvsøy, Norway. Cod heads for laboratory studies were collected in 20 kg batches, frozen (−20°C) and transported to SINTEF Ocean in Trondheim. Prior to lab hydrolysis, cod heads were thawed in a cold room at 4–6°C for 16–20 hours before being minced using an AE200 Hobart mincer with 10 mm diameter holes. Prior to pilot trials, fresh heads were collected in 400 kg batches and hydrolyzed in Mobile Sealab 3–12 hours after catch and 1–2 hours after deheading.

Chemicals and enzymes

The enzymes employed were Alcalase (2.4 U/g), Flavourzyme (1000 LAPU/g), and Protamex (1.5 AU-N/g), all manufactured by the company Novozymes based in Bagsvaerd, Denmark. Protamex has been shown to be an effective proteolytic enzyme in the hydrolysis of cod backbones (Gildberg et al. 2002) and is commonly used in industrial applications. Papain (Performase ®GSM80) and Bromelain (2400 GDU/g) were obtained from Enzybel International S.A., based in Villers-le-Bouillet, Belgium. All the enzymes used comply with the recommended purity specifications for food-grade enzymes issued by the joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC). The methanol, chloroform, hexane, and formaldehyde used during chemical analysis were obtained from Merck, based in Darmstad, Germany. Cytochrome C, aprotinin, insulin A, leucine enkephaline, Val-Tyr-Val and Gly-Tyr (all of which were obtained from the Sigma Chemical Co. in St. Louis, MO, USA) were used as standards for molecular weight distribution. All the chemicals used were of reagent grade.

Enzymatic hydrolysis

At laboratory scale, hydrolysis was performed in closed, 4-liter glass reactors with an electrical impeller. Minced cod heads (1 kg) were mixed with preheated water at different ratios (Table 1). Enzymatic hydrolysis was started by adding 0.1% enzyme (by wet weight of raw material) when the mixture reached 50°C. Samples were taken from the reactor after 0, 30, and 60 minutes. After 60 minutes, enzymes were inactivated by heating the hydrolysis mixture to 90°C for 10 minutes, before centrifugation at 2250 x g for 15 minutes. The two fractions, water-soluble proteins, and insoluble matter were separated by manual decanting. The water-soluble fraction was then frozen and freeze-dried prior to further analytical work.

In pilot scale, nine hydrolysis trails were performed (Table 2). The transportable unit Mobile Sealab had a production capacity of 1000 liters of material (cod heads and water) per batch. The cod heads were run through a processing line containing a coarse grinder (Monster, Stette AS, Skodje, Norway), a fine grinder (Ultra 2 MEW 623, Mado GmbH, Schwarzwald, Germany) (hole diameter 10 mm), a scraped surface heat-exchanger (Votator 2, Waukesha Cherry-Burrell, Delavan, WI, USA) (heated to 55°C), a 1000 L stirred hydrolysis reactor and a continuous tricanter centrifuge (Z23-3, Flottweg, Vilsbiburg, Germany). Water was added directly into the stirred reactor, and the temperature was adjusted by the heating jacket. The enzymatic hydrolysis was begun when the solution was 50°C, by adding 0.1% (of wet weight of raw material) of either Protamex (P) or a mixture of papain and bromelain 1:1 (PB). After 60 minutes, the hydrolysis mixture was heated to 90°C by the heating jacket and held for 10 min to ensure inactivation of the enzymes. The heated material was pumped through the tricanter, where protein water and

Table 1. Experimental design for laboratory scale trials.

Sample name	Raw material [kg]	Water [kg]	Enzyme [%]	Enzyme type,ratio and abbrevation	Hydrolysis temperature [°C]	Samples [min]
E 1	1.0	1.0	0.1	Endogenous	50	0, 30, 60, H
P 1	1.0	1.0	0.1	Protamex (P)	50	0, 30, 60, H
PB 1	1.0	1.0	0.1	Papain & Bromelain (1:1) (PB)	50	0, 30, 60, H
AF 1	1.0	1.0	0.1	Alcalase & Flavorzym (2:1) (AF)	50	0, 30, 60, H
E 0.75	1.0	0.75	0.1	Endogenous	50	0, 30, 60, H
P 0.75	1.0	0.75	0.1	Protamex	50	0, 30, 60, H
PB 0.75	1.0	0.75	0.1	Papain & Bromelain (1:1)	50	0, 30, 60, H
AF 0.75	1.0	0.75	0.1	Alcalase & Flavorzym (2:1)	50	0, 30, 60, H
E 0.5	1.0	0.5	0.1	Endogenous	50	0, 30, 60, H
P 0.5	1.0	0.5	0.1	Protamex	50	0, 30, 60, H
PB 0.5	1.0	0.5	0.1	Papain & Bromelain (1:1)	50	0, 30, 60, H
AF 0.5	1.0	0.5	0.1	Alcalase & Flavorzym (2:1)	50	0, 30, 60, H

H – inactivated, centrifugated and freeze-dried hydrolysate (final product).

Table 2. Experimental design for pilot scale trials at the processing plant, Tufjordbruket (T). Trials were performed with papain and bromelain (PB) or Protamex (P).

Sample name	Month	Water/raw material ratio	Enzyme concentration (% of kg raw material)	Enzyme
T1	Feb	1:1	0.1	PB[c]
T2	Feb	3:4	0.1	PB
T3	Feb	3:4	0.05	P[d]
T4	Mar	3:4	0.1	PB
T5	Mar	1:2	0.1	PB
T6	Mar	1:4	0.1	PB
T7	Mar	1:2	0.1	P
T8 [b]	Mar	3:4	0.1	P
T9 [a]	Mar	1:2	0.1	PB

[a]200 liters of stickwater from the marine oil factory at the processing facility was added instead of tap water.
[b]The cod heads were pre-minced with hole diameter 13 mm, before mincing in Mobile Sealab.
[c]PB = Papain and bromelain (1:1)
[d]P = Protamex

sediments were separated. The cod bones were left in the reactor and had to be removed manually. Samples of hydrolysate were frozen and transported to SINTEF Ocean, where they were subject to chemical characterization.

Chemical characterization

Moisture in the hydrolysate powders was determined gravimetrically after drying at 105°C for 24 hours. Ash content was estimated using the AOAC Official Method 942.05. Total nitrogen (N) was determined using a CHN-S/N elemental analyzer (1106, Carlo Erba Instruments S.Pa., Milan, Italy), and crude protein was estimated by multiplying total N by a factor of 6.25 (Mariotti et al. 2008). Lipids were extracted from the samples using the method described by Bligh and Dyer (1959). The degree of hydrolysis was evaluated as the proportion (%) of α-amino nitrogen with respect to the total N in the sample (Taylor 1957). All measurements were performed in triplicate.

Molecular weight distribution

Dry hydrolysate powders were dissolved in water to a concentration of 1 mg/ml. Samples were analyzed using a Hitachi high-performance liquid chromatographer (HPLC) with a UV detector set at 220 nm, using a Superdex peptide 10/300 column (GE Healthcare, product code: 17517601). The run was isocratic with 30% acetonitrile and 0.1% TFA in water at 0.4 ml/min, with a sample volume of 30 µl. All tests were conducted at room temperature. The standards used were cytochrome C (12327 Da), aprotinin (6512 Da), insulin A (2531 Da), leucine enkephalin (555.6 Da), Val-Tyr-Val (379.5 Da), and Gly-Tyr (238.2 Da). The linear regression obtained for the standards gave a correlation coefficient of $r^2 = 0.994$. Molecular weight results were divided into the following intervals: 200 Da or lower, 200–500 Da, 500–1,000 Da, 1,000–2,000 Da, 2,000–4,000 Da, 4,000–6,000 Da, 6,000–8,000 Da, 8,000–10,000 Da, 10,000–15,000 Da, 15,000–20,000 Da, and 20,000 Da or higher. All samples were analyzed in triplicate.

Statistical analysis

All experiments and measurements were performed in triplicate. Hydrolysis trials on laboratory scale were conducted in duplicate. Statistical analysis was performed using Microsoft Excel 2010 (Redmond, WA, USA). Significance values were calculated using a t-test, where a probability of $p < .05$ was required for statistical significance. All results are reported as a mean ± standard deviation.

Results and discussion

Raw material

The chemical composition of the cod heads was analyzed and revealed a stable chemical composition with a protein content of 15.4 ± 1.0%, lipid 0.9 ± 0.1%, water content of 77.9 ± 1.3%, and ash content of 6.7 ± 1.0%. For comparison, a previous study (Tveit et al. 2020) revealed values for minced cod head compositions of 11.3 ± 1.9% protein (n = 20), 3.6 ± 0.4% lipids (n = 10), 78.7 ± 1.3% water (n = 15), and 6.7 ± 1.1% ash (n = 15). The comparison suggests that the amount of dry material and ash is stable, while lipids and protein vary. Slizyte et al. (2005) characterized viscera and backbones from cod, and all fractions contain similar amounts of protein: 15.4 ± 1.0% (heads), 14.9 ± 2.3% (viscera) and 16.1 ± 0.4% (viscera and backbones),; but lipid content exhibits greater variation with very low values in heads (0.9 ± 0.1%) compared to 21.0 ± 0.5% in viscera. Ash content also differs among these fractions, with the highest in cod heads at 6.7 ± 1.0%, compared to 4.4 ± 0.3% in viscera.

Hydrolysate yield

Hydrolysis trials, in lab scale, produced two fractions: (1) fish protein hydrolysates (FPHs) and (2) sediments. In pilot scale, cod bones had to be removed manually from the reactor, adding an extra fraction. No oil or emulsion phases were detected in lab or pilot-scale studies. The amount of lipids in the cod heads (0.9 g of lipid per 100 g of wet weight) was insufficient to form a separate lipidic fraction after hydrolysis (Spinelli et al. 1972), indicating that any lipids present were distributed between the hydrolysate and sediment fraction. In trials using a raw material consisting of a mixture of cod viscera and backbones, the release of oil did not occur when the lipid content of raw material was below 6% (Slizyte et al. 2004).

The FPHs produced in laboratory scale had 92–96% water and 4–8% dry matter (Table 3). Dry matter in the FPHs increased with hydrolysis time using commercial enzymes, and the results are consistent with those from previous studies involving cod rest raw materials (Slizyte et al. 2005) and cod heads (Tveit et al. 2020). Hydrolysis based on endogenous enzymes gave less rise in dry matter with increased hydrolysis time compared to commercial enzymes. When the cod heads were hydrolyzed using a combination of the enzymes PB, using a 1:2 water/raw material ratio, the amount of dry matter in the FPHs increased from 5.7% to 8.0% during 60 minutes of hydrolysis. This demonstrates that PB form an effective enzyme combination. Hydrolysis progressed rapidly during the first 30 minutes, and then appeared to stabilize for the next 30 minutes. This result is similar to typical hydrolysis curves reported for other fish raw materials such as Atlantic salmon (Kristinsson and Rasco 2000), herring (Liceaga-Gesualdo and Li-Chan 1999) and sardine (Barkia et al. 2010; Bougatef et al.

Table 3. Dry matter (%) in lab scale FPHs after 0, 30, and 60 minutes of hydrolysis of cod heads using four different enzymes (E-Endogenous, P-Protamex, PB-Papain and Bromelain, AF-Alcalase and Flavorzyme) and three different water concentrations. Values are given as a mean ± STD.

Sample	Water:raw material ratio	0 min	30 min	60 min
E 1	1:1	4.30 ± 0.01	4.34 ± 0.01	4.61 ± 0.01
P 1	1:1	4.11 ± 0.02	5.27 ± 0.03	5.52 ± 0.02
PB 1	1:1	4.21 ± 0.02	5.42 ± 0.02	5.90 ± 0.01
AF 1	1:1	4.03 ± 0.01	4.83 ± 0.02	5.36 ± 0.03
E 0.75	3:4	4.93 ± 0.01	4.98 ± 0.03	5.06 ± 0.03
P 0.75	3:4	4.72 ± 0.07	6.00 ± 0.01	6.33 ± 0.02
PB 0.75	3:4	4.92 ± 0.01	6.23 ± 0.02	6.55 ± 0.02
AF 0.75	3:4	4.83 ± 0.01	5.80 ± 0.00	6.18 ± 0.01
E 0.5	1:2	5.72 ± 0.02	5.87 ± 0.00	6.08 ± 0.02
P 0.5	1:2	5.37 ± 0.02	7.21 ± 0.01	7.52 ± 0.01
PB 0.5	1:2	5.70 ± 0.01	7.21 ± 0.53	7.98 ± 0.02
AF 0.5	1:2	5.52 ± 0.00	7.08 ± 0.07	7.52 ± 0.00

Table 4. Mass balance, dry matter (%), and theoretical protein recovery for the nine different pilot scale trials at Tufjordbruket AS. Values are given as a mean ± STD.

Sample	Hydrolysate [kg]	Insoluble matter [kg]	Bone and residue [kg]	Dry matter [%]	Protein recovery [%]
T1	695	12	93	5.4	53.4
T2	557	27	82	6.6	53.6
T3[a]	537	35	84	6.4	50.4
T4	557	30	77	6.7	56.1
T5	435	20	83	8.3	58.4
T6	308	15	68	10.8	59.0
T7[a]	399	20	72	8.1	55.9
T8[a]	567	30	76	6.3	53.4
T9	541	25	54	8.5	59.0

[a]*Hydrolysis trials where Protamex were used. All other trials are done with PB.*

2010; Souissi et al. 2007). The dry matter in FPHs from pilot trials, which varied from 5.4% to 10.8%, correlated ($r^2 = 0.95$) to the amount of added water (Table 4). Reducing the addition of water did not affect viscosity of FPHs, and all mixtures produced were easily transferable and homogenous.

Freeze-dried FPHs

The proximate chemical composition of FPHs depends on several factors, such as raw material composition, the specificity of the enzyme, and the conditions under which hydrolysis takes place. The results from the lab-scale studies are shown in Table 5.

Lipid oxidation is of great concern to the food industry and its consumers because it results in unpalatable flavors, unpleasant odors, dark coloring, and potentially toxic reaction products (Kristinsson and Rasco 2000; Lin and Liang 2002; Spinelli et al. 1972). The Food and Agriculture Organization of the United Nations has issued a standard stipulating that the lipid content in FPHs used for human consumption should not exceed 0.5% (w/w) (He et al. 2013). The lab tests show that commercial enzymes have a positive effect on the amount of lipids in dried hydrolysates, resulting in 0.2–0.6% lipids, compared to 2.1–3.2% lipids in hydrolysates based on endogenous enzymes.

In pilot scale, the lipid content in freeze-dried hydrolysates was 4.7–11.2%. Stick water from the local marine oil factory was tested as a sustainable water source, not successfully, in trial T9, resulting in 17% lipid in FPH. Lipid content is high in pilot trial hydrolysates compared to hydrolysates from laboratory tests, probably due to centrifugation efficiency. Lab scale centrifugation effectively reduced the lipid content in the hydrolysate, transferring the lipids to the sediments, indicating that the

Table 5. Chemical composition (protein, lipids, ash, and water) of freeze-dried FPHs (lab scale) using four different enzymes (E-Endogenous, P-Protamex, PB-Papain and Bromelain, AF-Alcalase and Flavorzyme) and three different water concentrations (1–1:1 water/heads, 0.75–3:4 water/heads, 0.5–1:2 water/heads). Values are given as a mean ± STD.

Sample	Water [%]	Ash [%]	Protein [%]	Lipid [%]
E 1	1.4 ± 0.7	18.5 ± 0.6	80.2 ± 0.4	3.2 ± 0.2
P 1	2.7 ± 0.7	16.2 ± 0.1	82.5 ± 1.0	0.3 ± 0.0
PB 1	4.3 ± 0.3	13.1 ± 0.4	85.1 ± 0.1	0.2 ± 0.1
AF 1	7.9 ± 0.1	13.8 ± 0.5	81.3 ± 0.4	0.2 ± 0.1
E 0.75	1.0 ± 0.6	19.1 ± 0.7	81.7 ± 1.5	2.1 ± 0.6
P 0.75	4.5 ± 1.9	16.3 ± 0.1	83.2 ± 0.1	0.2 ± 0.0
PB 0.75	4.1 ± 0.3	15.3 ± 0.1	83.6 ± 0.2	0.0 ± 0.1
AF 0.75	9.4 ± 0.6	15.7 ± 0.1	78.9 ± 0.3	0.3 ± 0.1
E 0.5	3.1 ± 1.8	17.2 ± 1.1	85.1 ± 0.3	2.1 ± 0.5
P 0.5	5.8 ± 0.0	15.7 ± 0.6	83.2 ± 0.2	0.0 ± 0.1
PB 0.5	4.1 ± 0.6	9.6 ± 0.0	86.1 ± 0.1	0.6 ± 0.0
AF 0.5	8.7 ± 0.0	13.7 ± 0.1	82.0 ± 0.6	0.3 ± 0.0

Table 6. Ash (%) and protein (%) content in freeze-dried FPHs after 0, 30, and 60 minutes of hydrolysis using four different enzyme combinations (E-Endogenous, P-Protamex, PB-Papain and Bromelain, AF-Alcalase and Flavorzyme) and three different water concentrations (1–1:1 water/heads, 0.75–3:4 water/heads, 0.5–1:2 water/heads). Values are given as a mean ± STD.

	Ash			Protein		
Sample	0 min	30 min	60 min	0 min	30 min	60 min
E 1	19.6 ± 0.0	19.2 ± 0.3	18.5 ± 0.6	80.2 ± 0.5	80.3 ± 0.6	80.2 ± 0.4
P 1	20.0 ± 1.6	16.8 ± 0.4	16.2 ± 0.1	79.6 ± 0.8	81.1 ± 0.6	82.5 ± 1.0
PB 1	17.9 ± 0.3	14.2 ± 0.3	13.1 ± 0.4	83.5 ± 0.2	86.1 ± 0.2	85.1 ± 0.1
AF 1	18.2 ± 1.2	15.6 ± 0.5	13.8 ± 0.5	83.4 ± 0.4	83.9 ± 0.4	81.3 ± 0.4
E 0.75	19.0 ± 1.0	19.3 ± 0.4	19.1 ± 0.7	81.5 ± 0.5	82.0 ± 0.7	81.7 ± 1.5
P 0.75	19.8 ± 1.3	17.3 ± 0.2	16.3 ± 0.1	81.5 ± 0.5	82.7 ± 0.7	83.2 ± 0.1
PB 0.75	19.6 ± 0.2	16.2 ± 0.1	15.3 ± 0.1	80.1 ± 0.6	83.1 ± 0.2	83.6 ± 0.2
AF 0.75	19.9 ± 0.5	17.1 ± 0.1	15.7 ± 0.1	80.8 ± 0.5	82.8 ± 0.2	78.9 ± 0.3
E 0.5	18.0 ± 0.0	18.0 ± 0.7	17.2 ± 1.1	85.2 ± 0.3	85.3 ± 0.3	85.1 ± 0.3
P 0.5	20.1 ± 0.7	16.4 ± 0.6	15.7 ± 0.6	80.6 ± 0.8	83.0 ± 0.2	83.2 ± 0.2
PB 0.5	17.9 ± 0.5	13.8 ± 0.2	9.6 ± 0.0	84.0 ± 0.7	83.6 ± 0.6	86.1 ± 0.1
AF 0.5	16.7 ± 0.7	14.3 ± 0.3	13.7 ± 0.0	83.0 ± 0.7	84.3 ± 0.6	82.0 ± 0.6

tricanter performance could be enhanced or that a polishing step will increase the quality of the protein hydrolysate. Newer and larger tricanters can utilize higher G-force and longer retention time and should be able to separate more lipids.

The high concentration of ash may be related to high bone substance in cod heads. In laboratory trials, ash content varied between 16.7% and 20.1% (Table 5). In pilot scale, the ash content varied between 8.4% and 10.5% (Table 7). Ash content depends on the enzyme used and hydrolysis time (Table 6). PB produced hydrolysates with significantly lower ash contents ($P < .05$) compared to Protamex in all trials. Ash content at 0 min varied from 16.7% to 20.1%. After 30 minutes of hydrolysis, it was significantly lower ($P < .05$) at 13.8% – 19.3%. At 60 minutes, it was further reduced to 9.6% – 19.1%. This is significantly lower than at 0 minutes ($P < .05$), although the difference in ash content between 30 and 60 minutes is not statistically significant ($P = .11$). Reduction in ash concentration during hydrolysis may be related to the separation in two phases, where salt and some minerals enters the water phase, but bone fragments enter the sediments.

Protein content in dried FPHs is above 80% for most hydrolysates, as shown in Table 5 for lab-scale trials and Table 7 for pilot-scale trials. In lab scale, the AF combination had a very efficient protein recovery during the first 30 minutes, showing approximately the same protein recoveries as all the PB combination, but subsequently slowed in its effect, resulting in lower values of protein recovery compared with PB after 60 minutes of hydrolysis. Similarly, P was highly effective for the first 30 minutes of hydrolysis, but also resulted in lower protein recoveries compared to PB. The highest protein recovery was obtained using the enzyme combination of PB (Figure 1). Hydrolysis with endogenous enzymes resulted in the lowest protein recovery. In pilot scale trials, the protein recovery was significantly lower ($p < .05$) (54.2 ± 5.1%) compared to laboratory scale (62.2 ± 2.2%), when hydrolyzed with PB. Using P, the protein recovery in pilot scale was also lower (53.2 ± 2.8), although not statistically

Table 7. Chemical composition (protein, lipids, ash, and water) and degree of hydrolysis (DH) (%) of freeze-dried FPHs from nine pilot scale trials. Stickwater was used instead of water in trial T9. Values are given as a mean ± STD.

Sample	Water [%]	Ash [%]	Lipid [%]	Protein [%]	DH [%]
T1	3.4 ± 0.0	8.4 ± 0.1	8.1 ± 0.1	81.6 ± 0.5	12.8 ± 0.6
T2	3.3 ± 0.1	9.5 ± 0.3	6.9 ± 0.3	81.4 ± 1.8	13.5 ± 0.5
T3	3.0 ± 0.0	9.8 ± 0.0	8.8 ± 0.1	80.2 ± 0.9	14.3 ± 0.3
T4	1.4 ± 0.4	9.0 ± 0.2	7.7 ± 0.1	84.8 ± 0.6	13.0 ± 0.0
T5	2.0 ± 0.1	8.8 ± 0.0	8.0 ± 0.4	83.5 ± 0.4	14.4 ± 0.3
T6	1.7 ± 0.0	8.4 ± 0.1	7.9 ± 0.2	83.9 ± 0.1	15.8 ± 0.3
T7	1.3 ± 0.1	9.8 ± 0.1	11.2 ± 2.3	83.5 ± 0.7	15.0 ± 0.5
T8	2.0 ± 0.2	10.5 ± 0.1	4.7 ± 0.0	85.6 ± 0.3	16.2 ± 0.9
T9	1.5 ± 0.1	7.1 ± 0.1	17.0 ± 0.2	70.9 ± 0.2	16.2 ± 0.1
Stick water	6.3 ± 0.2	12.9 ± 0.1	8.0 ± 2.2	30.5 ± 0.5	

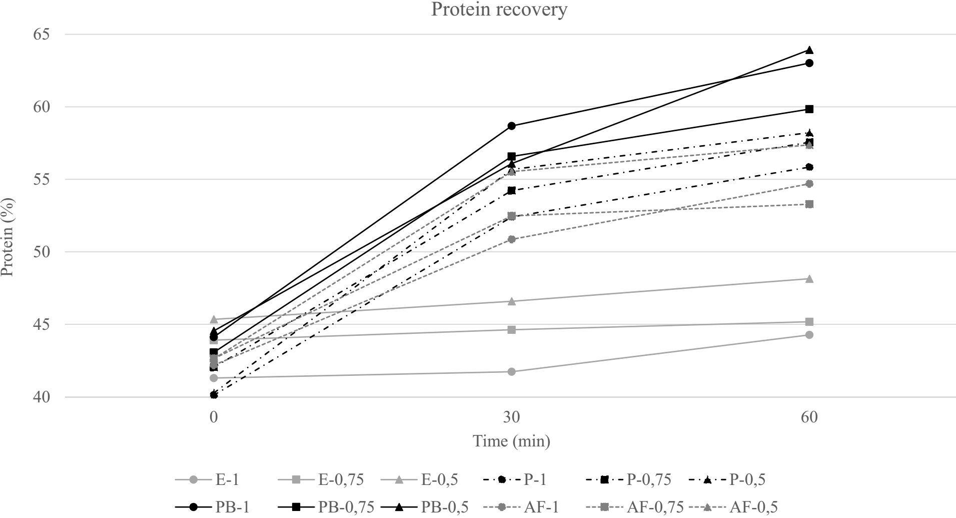

Figure 1. Recovery of accessible protein from cod heads after 0, 30, and 60 minutes of hydrolysis using four different enzyme combinations (E-Endogenous, P-Protamex, PB-Papain and Bromelain, AF-Alcalase and Flavorzyme) and three different water concentrations (1–1:1 water/heads, 0.75–3:4 water/heads, 0.5–1:2 water/heads).

significant, compared to laboratory scale (57.1 ± 1.2). The raw material had the same surface area, so this can partly be explained by the difficulties during the trials. The bone mass is heavy and tends to sediment and capture fish meat between layers of bones. Some of the fish meat is consequently difficult for the enzymes to reach. It is likely that a better reactor would contribute to a higher protein recovery.

Degree of hydrolysis

Since size of the peptide molecule is very important for the surface activity and bioactivities of FPHs, the degree of hydrolysis (DH) is also key (Jeon et al. 1999; Kristinsson and Rasco 2000). The degree of hydrolysis is defined as the proportion of cleaved peptide bonds in a protein hydrolysate. An important factor that impacts FPH usefulness as a food ingredient is solubility. The solubility of FPHs increases with increasing DH (Gbogouri et al. 2004; Jamdar et al. 2010). A downside of high DH, however, is the production of bitter peptides, which affect palatability. Also, very high DH can have negative effect on the functional properties (Kristinsson and Rasco 2000).

DH varied from 8% to 18% in laboratory trials (Figure 2) and was dependent on the enzymes used, as previously reported (He et al. 2013). Hydrolysates from endogenous enzymes had low DH values, ranging from 7.8 ± 0.4% to 8.0 ± 1.3%. In comparison, the enzyme combination of AF yielded the highest degree of hydrolysis, with 17.4 ± 0.4%, using a water/raw material ratio of 3:4. This was higher than P (16.6 ± 0.4%) with a water/raw material ratio of 1:1, and PB, at 13.9 ± 0.2%, 14.6 ± 0.3%, and 15.1 ± 0.7%, using water/raw material ratios of 1:2, 3:4, and 1:1, respectively. Pilot scale trials confirm that DH in hydrolysates using P is higher, compared to the DH in hydrolysates from PB. It has been reported that higher DH leads to higher protein recovery, because more cleaved peptide bonds result in protein hydrolysates with smaller molecular weights, which are more soluble in water (He et al. 2013). Here, the protein recovery was significantly higher in laboratory trials, compared to pilot trials, without significant difference in DH.

Molecular weight distribution

Since enzymes have specific cleavage positions on polypeptide chains, the resulting FPH products will consist of peptide molecules of different lengths (Barkia et al. 2010). In this study, fish protein hydrolysates were separated using gel chromatography to analyze peptide size composition. The

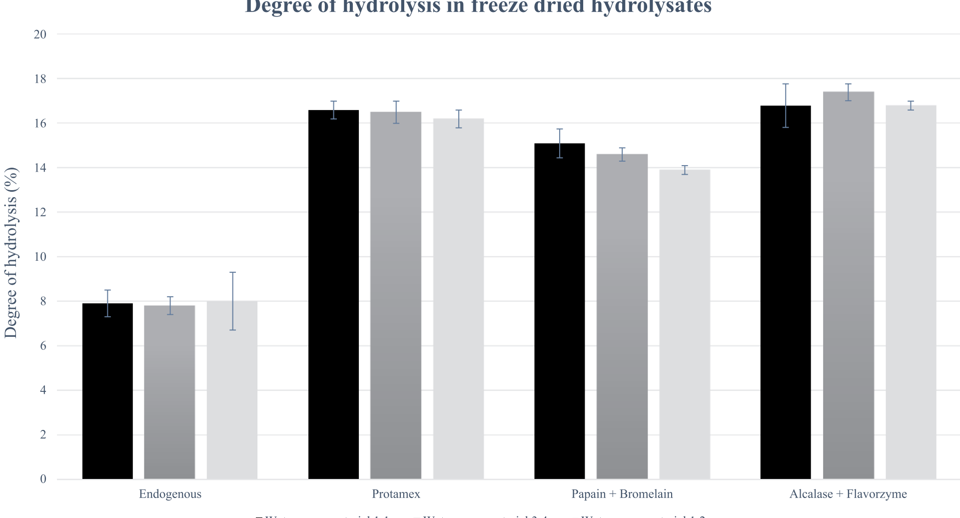

Figure 2. Degree of hydrolysis in freeze-dried hydrolysates produced in lab scale. Values are given as a mean ± STD.

chain length of peptides, which depends on the DH, is of special interest in relation to organoleptic FPH characteristics such as bitterness, as well as functional properties, such as emulsion capacity and solubility (Gbogouri et al. 2004). Extensive previous research has suggested that there is an optimum molecular size or chain length for peptides that ensures good foaming and emulsifying properties, and that extensive hydrolysis that produces small peptide molecules reduces these properties (Adler-Nissen and Olsen 1979; Jeon et al. 1999; Klompong et al. 2007; Kristinsson and Rasco 2000; Lee et al. 2014; Quaglia and Orban 1990; Šližytė et al. 2009).

Analysis of peptide size distribution in lab-scale hydrolysates (Table 8) shows that different commercial enzymes generated different molecular weight (M_w) distributions. Hydrolysates derived using the commercial enzyme P and the combination AF were made up of peptides in the M_w range, from approx. 15,000 Da to 200 Da. The proportion of peptides measuring below 5,000 Da was 65.0–66.8% for P-based hydrolysates and 61.2–64.8% for the AF hydrolysates. Thus, it appears that the M_w distribution for peptides of this size does not vary significantly following hydrolysis using these enzyme combinations. The hydrolysates from PB consisted of peptides in the M_w range, from approx. 10,000 Da to 200 Da. Here, the peptide content lower than 5,000 Da was 77.2–79.0%, which is not significantly different from the M_w distributions of peptides produced following hydrolysis using P. Hydrolysates obtained using only endogenous enzymes exhibited lower levels of breakdown and consisted mainly of peptides larger than 10,000 Da. The results suggest that size and charge of peptides may be different for hydrolysates produced by different enzymes, as previously reported (Klompong et al. 2007). In pilot trials, peptide content lower than 5,000 Da was 70.6%–89.6% in hydrolysates from PB and 79.7%–85.2% in hydrolysates from Protamex (excluding trial T3 where only 0.05% enzyme was added).

Results show a shift in M_w distribution between laboratory and industrial scale (Table 8, Table 9) for hydrolysis using P, with a higher degree of peptides below 5,000 Da hydrolysates in pilot scale. The results for PB are within the same range in laboratory and industrial scale. The results also reveal that the amount of added water had little effect on the M_w distribution, and no statistically significant differences in yield were recorded for different water concentrations. This means that it may be possible to achieve good results using smaller water volumes, although this study does not indicate how small such volumes could be.

Many studies have focused on bioactivities, which is associated with molecular weights (He et al. 2013). Levels and compositions of free amino acids and peptides have been reported to determine antioxidant activities for protein hydrolysates (Wu and Bechtel 2012). Other studies on cod protein

Table 8. Molecular weight distribution for lab scale hydrolysates from four different enzyme combinations (E-Endogenous, P-Protamex, PB-Papain and Bromelain, AF-Alcalase and Flavorzyme) and three different water concentrations (1–1:1 water/heads, 0.75–3:4 water/heads, 0.5–1:2 water/heads). Values are given as a mean ± STD.

Size (Da)	E 1	E 0.75	E 0.5	P 1	P 0.75	P 0.5	PB 1	PB 0.75	PB 0.5	AF 1	AF 0.75	AF 0.5
>20 000	56.0 ± 6.9	60.8 ± 0.7	59.8 ± 3.5	0.6 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.4 ± 0.1
15000–20000	11.2 ± 0.6	10.9 ± 0.8	11.6 ± 0.1	1.8 ± 0.1	1.3 ± 0.1	1.6 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	1.8 ± 0.3	1.2 ± 0.1	1.3 ± 0.1
10000–15000	9.8 ± 0.6	9.3 ± 0.3	9.4 ± 0.1	9.2 ± 0.0	8.3 ± 0.1	9.0 ± 0.0	2.2 ± 0.2	1.9 ± 0.4	1.6 ± 0.3	11.0 ± 1.2	8.5 ± 0.0	9.6 ± 1.1
5000–10000	5.0 ± 1.3	3.9 ± 0.8	3.7 ± 0.6	23.2 ± 0.5	23.4 ± 0.9	24.1 ± 0.4	20.5 ± 0.4	19.5 ± 1.1	19.3 ± 1.3	25.5 ± 1.2	25.2 ± 0.1	25.5 ± 0.4
2000–5000	1.9 ± 1.8	0.9 ± 0.1	1.1 ± 0.1	24.4 ± 0.1	25.6 ± 0.1	24.8 ± 0.1	38.5 ± 0.9	39.0 ± 0.1	39.2 ± 0.3	25.7 ± 0.8	27.4 ± 0.1	27.6 ± 0.6
1000–2000	0.2 ± 0.0	0.2 ± 0.2	0.7 ± 0.4	14.1 ± 0.2	14.7 ± 0.4	14.4 ± 0.1	19.1 ± 0.4	19.7 ± 0.5	20.0 ± 1.1	13.3 ± 0.6	14.1 ± 0.4	13.5 ± 0.4
500–1000	0.8 ± 0.9	1.0 ± 0.5	0.9 ± 0.2	10.1 ± 0.1	10.2 ± 0.1	9.6 ± 0.0	8.0 ± 0.0	8.4 ± 0.3	8.6 ± 0.4	6.9 ± 0.5	7.4 ± 0.1	6.6 ± 0.2
200–500	6.1 ± 1.3	5.5 ± 1.3	5.1 ± 0.8	8.5 ± 0.1	8.0 ± 0.4	8.2 ± 0.4	5.7 ± 0.3	6.2 ± 0.6	6.1 ± 0.4	7.2 ± 0.5	7.7 ± 0.4	7.5 ± 0.1
<200	9.1 ± 3.0	7.8 ± 0.9	8.1 ± 2.1	8.4 ± 0.2	8.4 ± 0.2	8.0 ± 0.1	6.0 ± 0.1	5.3 ± 0.4	5.2 ± 0.0	8.3 ± 0.4	8.4 ± 0.2	8.3 ± 0.4
SUM <5000	18.05	15.25	15.65	65.25	66.8	64.95	77.2	78.45	78.95	61.2	64.75	63.35

Table 9. Molecular weight distribution for pilot scale hydrolysates. Values are given as a mean ± STD.

Size (Da)	T1	T2	T3	T4	T5	T6	T7	T8	T9
>20 000	0.0 ± 0.0	0,0 ± 0.0	0,7 ± 0.0	0,10 ± 0,0	0,00 ± 0,0	0 ± 0,0	0,00 ± 0	0,00 ± 0,0	0,40 ± 0,0
15000–20000	0.1 ± 0.0	0,1 ± 0.0	1,4 ± 0.1	0,00 ± 0,0	0,00 ± 0,0	0 ± 0,0	0,00 ± 0,0	0,00 ± 0,0	1,00 ± 0,1
10000–15000	0.5 ± 0.0	1,4 ± 0.1	6,9 ± 0,3	0,20 ± 0,0	0,70 ± 0,0	0,7 ± 0,0	0,50 ± 0,0	1,20 ± 0,1	4,10 ± 0,2
5000–10000	10,8 ± 0.5	22,5 ± 1.1	32,3 ± 1,6	9,90 ± 0,5	20,00 ± 1,0	18,6 ± 0,9	14,40 ± 0,7	19,00 ± 1,0	23,80 ± 1,2
2000–5000	37,5 ± 1.9	42,5 ± 2.1	26,7 ± 1,3	40,30 ± 2,0	43,60 ± 2,2	43,3 ± 2,2	42,60 ± 2,1	32,80 ± 1,6	29,10 ± 1,5
1000–2000	22,0 ± 1.1	16,9 ± 0.8	12,1 ± 0,6	25,80 ± 1,3	19,10 ± 1,0	19,9 ± 1,0	22,40 ± 1,1	18,60 ± 0,9	16,00 ± 0,8
500–1000	13,0 ± 0.7	7,0 ± 0.4	6,4 ± 0,3	11,70 ± 0,6	7,90 ± 0,4	8 ± 0,4	9,50 ± 0,5	13,00 ± 0,7	9,90 ± 0,5
200–500	7,8 ± 0.4	4,0 ± 0.2	6 ± 0,3	6,70 ± 0,3	4,30 ± 0,2	4,5 ± 0,2	5,20 ± 0,3	7,70 ± 0,4	7,50 ± 0,5
<200	8,4 ± 0.4	5,5 ± 0.3	7,5 ± 0,4	5,10 ± 0,3	4,40 ± 0,2	4,9 ± 0,2	5,50 ± 0,3	7,60 ± 0,4	8,10 ± 0,4
SUM <5000	88.7	75.9	58.7	89.6	79.3	80.6	85.2	79.7	70.6

Table 10. The operational time, measured in minutes, for different steps during the hydrolysis process.

Hydrolysis trial	T1	T2	T3	T4	T5	T6	T7	T8	T9	Mean	Stdev
Feed	130	94	161	166	110	79	205	59	247	139	61,4
Hydrolysis	60	60	60	60	60	60	60	60	60	60	0,0
Inactivation	36	36	40	38	37	45	29	47	37	38	5,3
Separation	53	43	37	47	28	22	25	42	39	37	10,4
Clean up	40	47	49	29	32	55	49	49	60	46	10,2
Total	319	280	347	340	267	261	368	257	443	320	61,7

hydrolysates have shown that peptides below 10 kDa had the strongest antioxidative activity (Jeon et al. 1999). Antihypertensive activity in cod protein hydrolysates increased from 60% with M_w 10,000–30,000 Da to 88% with M_w below 1,000 Da (Je et al. 2004). Another study revealed that M_w of 100–500 Da and 1,000–3,500 Da were the two ranges with the most bioactive peptides (Vandanjon et al. 2009).

Pilot trials and operational challenges

Although the results above demonstrate the ability to produce the same high-quality protein in pilot scale as in laboratory scale, there were several struggles along the way. Cod bones were especially challenging in the pilot trials as they were (1) difficult to mince, (2) heavy and jam-packed at the bottom of the reactor, and (3) not very pumpable. This resulted in varying processing times, as shown in Table 10. Using a mincer originally designed for meat without bones is not ideal for cod heads as it often was interrupted by stuck bones. This, however, lead to convenient feeding speed. Feeding the reactor too fast led to an unmovable mass at the bottom of the reactor. This study demonstrated that it is possible to produce high-quality proteins from cod heads, but to make it industrial and profitable, proper and suitable technological equipment should be introduced.

Conclusions

Our study verified that high yield and good quality fish protein hydrolysates can be produced not only at lab, but also at industrial scale. Taking production sustainability and costs into consideration, the best possible FPH yields and qualities should be obtained using minimal amounts of added enzyme and water and a minimal hydrolysis reaction time. In our study using cod heads as the raw material, a high yield of high-quality protein FPHs was obtained using the combination of 0.1% PB and a reaction time of 60 minutes, in both laboratory and pilot trials. The study has shown that the amount of added water has little effect on the chemical composition and molecular weight distribution of the resulting FPHs and has also suggested that the amount of added water can be further reduced; for cod heads, additional water possibly could be unnecessary. For further industrialization, process equipment able to handle both cod bones and a very low oil content must be adapted and optimized. Compared to the traditional

hanging and drying of cod heads, hydrolysis processing has larger investment costs, but products are likely to suit markets that are more stable and willing to pay. The protein fraction derived from cod heads can be used as ingredients in dietary supplements or as food additives for human consumption.

Acknowledgments

We are also grateful to the company Fjordlaks AS for supplying the cod heads and facilitating the industrial-scale trials where we were able to use Mobile Sealab. We also extend our thanks to Arnstein Johannesen, Erlend Indergaard, Merethe Selnes and Marte Schei at SINTEF Ocean for their assistance during the experiments and analytical work.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research study was funded by the Norwegian Seafood Research Fund [grant no. 901308] and the Research Council of Norway [grant no. 294539 and 313945] for which we are most grateful.

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