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Review

Formation of Oxidative Compounds during Enzymatic Hydrolysis of Byproducts of the Seafood Industry

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Abstract: There is a significant potential to increase the sustainability of the fishing and aquaculture industries through the maximization of the processing of byproducts. Enzymatic hydrolysis provides an opportunity to valorize downstream fish industry byproducts for the production of protein hydrolysates (FPH) as a source of bioactive peptides (BAP) with health benefits. Deteriorative oxidative reactions may occur during the enzymatic hydrolysis of byproducts, influencing the safety or bioactivities of the end product. Lipid oxidation, autolysis mediated by endogenous enzymes in viscera, protein degradation, and formation of low-molecular-weight metabolites are the main reactions that are expected to occur during hydrolysis and need to be controlled. These depend on the freshness, proper handling, and the type of byproducts used. Viscera, frames, trimmings, and heads are the byproducts most available for enzymatic hydrolysis. They differ in their composition, and, thus, require standardization of both the hydrolysis procedures and the testing methods for each source. Hydrolysis conditions (e.g., enzyme type and concentration, temperature, and time) also have a significant role in producing FPH with specific structures, stability, and bioactivity. Protein hydrolysates with good safety and quality should have many applications in foods, nutraceuticals, and pharmaceuticals. This review discusses the oxidative reactions during the enzymatic hydrolysis of byproducts from different fish industry sectors and possible ways to reduce oxidation.

Keywords: fish processing byproducts; storage; enzymatic hydrolysis; oxidation; fish protein hydrolysates

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1. Introduction

In recent years, sustainability of the fishing and aquaculture industries has become more important on a global scale in response to recognition of the limits of marine sources and the crisis of food security. Consequently, maximum valorization of food waste and processing byproducts has been adopted as a component of the future strategy to optimize these resources [1]. According to the Food and Agriculture Organization of the United Nations (FAO) global statistics [2], of the 179 million tons of global fish production in 2018, 22 million tons (or 12%) were used for non-food purposes. Byproducts obtained after industrial processing of most commercially farmed or captured fish, shellfish, and invertebrates constitute a significant part of non-edible seafood [2].

Enzymatic hydrolysis was used to process byproducts to obtain digestible proteins containing BAP that are known to show various physiological functions [3]. This process involves the breakdown of proteins by cleaving peptide bonds in polypeptide chains using a protease to produce protein hydrolysates containing peptides of varying size and free amino acids. Therefore, the degree of hydrolysis (DH) represents the potency of the proteases used to cleave peptide bonds [4]. Hydrolysis conditions affect the DH and

formation of specific peptides. The type of enzymes used for producing the hydrolysates will determine the composition and properties of the end product. In addition, enzyme concentration, temperature, pH, and time of hydrolysis are other variables that influence enzymatic hydrolysis [5]. The type of byproducts (e.g., frames, trimmings, heads, and viscera) and their freshness also will determine the composition of BAP, while the hydrolysis determines which peptides are formed and their biological activities [6].

In recent years, the utilization of fish and other marine species byproducts as a source of BAP has been explored [3–6]. Enzymatic hydrolysis has been used for the conversion of byproducts of fish processing into smaller protein fragments with 2–20 amino acid residues that, then, result in higher solubility, digestibility, and absorption [3,5]. Fish protein hydrolysates are the basic source of BAP whose structure is dependent on the native protein matrix, the degree of hydrolysis, enzyme specificity, and other hydrolysis conditions, such as time and temperature [7,8]. The specific biological activities of peptides are mainly determined by their amino acid sequence and size [3–5]. BAP from seafood byproducts have shown antioxidant [9,10], antihypertensive [11], anti-cancer [12], anti-allergic [13], anti-diabetic [14,15], immunomodulatory [16], and anti-hyperlipidemic activities [17,18], and appetite-suppressing properties, among others [19]. Therefore, some BAP may be used as functional ingredients in health-promoting foods, nutraceuticals, and pharmaceuticals [4,20].

During enzymatic hydrolysis, heating the solution to achieve the optimal activity for proteases, changes of pH, the presence of pro-oxidants (i.e., hemoglobin and metal ions) and unsaturated lipids in addition to initial quality of the starting material can cause oxidation, influencing the nutritional and organoleptic properties of the FPH [21,22]. Therefore, it is essential that the hydrolysis conditions are controlled to reduce the rate of oxidation while obtaining the desired functionality in the FPH for each particular source of seafood byproduct. The aims of the present review were to address the undesirable biochemical reactions that occur during the enzymatic hydrolysis of byproducts, the formation of oxidation compounds, and the possible ways to control oxidation.

2. Fish Industry Byproducts: Sources and Properties

In the fish industry the edible portion is the "principal product", the remaining fractions have a much lower value, and those that can be recycled after treatment are considered "side stream" or "byproducts" [23]. These may include blood, viscera, heads, frames, skin, fins, and fillet trimmings. Byproducts may represent up to 70% of the processed fish depending on the species and the type of processing. The byproducts of fish processing are highly perishable raw materials, thus, by ensuring high quality and hygiene of the byproducts (unfortunately often not the case in many fish processing plants) during principal product processing, value added components will generally have better nutritional and economic profitability [24]. Of the 22 million tons of fish that were not used for human consumption, 80% (~18 million tons) were used for aquaculture feed, mainly in Latin America, followed by Asia and Europe [2]. Production of FPH is seen as a potentially more profitable process to utilize byproducts compared to fish meal, especially in developed economies. Byproducts of different species such as fish, shellfish, and invertebrate vary in their composition and that may affect the extent of their oxidative reactions during enzymatic hydrolysis and, thus, the functional properties of the end product.

2.1. Fish Byproducts

Fish byproducts are generally composed of heads (9–12% of total weight), viscera (12–18%), skins (1–3%), bones (9–15%), and scales (~5%) [2]. Frames and trimmings can be regarded as clean fractions of the most contaminating component, when compared to viscera [25]. The presence of abdominal fats, blood, non-digested feed in the stomach, digesta and fecal matter in the intestines, and endogenous proteases and bile are major concerns with viscera, which may make any enzymatic hydrolysis non-repeatable as well as lead to the formation of off-flavor in FPH [3]. Heads, frames, and trimmings of several

industrial fish species such as Atlantic salmon, rainbow trout, tuna, seabass, seabream, and Atlantic codfish have been used as sources for FPH [26–31]. One of the main concerns with heads would be the presence of gills as a source of large amounts of blood and hemoglobin, which is often an oxidant and a nutritional source of iron. Therefore, where possible, this pro-oxidant rich fraction might be separated [25] or, if the heads are large enough, gills can be removed before enzymatic hydrolysis to decrease the rate of oxidation.

2.2. Shellfish Byproducts

Shrimp byproducts are composed of the cephalothorax, internal organs, ovaries, and the exoskeleton, which accounts for 50–60% of the weight after processing [32]. They contain several valuable compounds such as proteins, lipids, chitin, cholesterol, and the red pigment astaxanthin. Endogenous proteases and polyphenoloxidases in shrimp hepatopancrease are the enzymes associated with spoilage and the economically important black spot defect [33]. Heating at 80 °C for 30 s inhibited enzymatic activities in Pacific white shrimp (*Litopenaeus vannamei*) [34] during storage. However, due to pre-cooking, the decreased trypsin activity in shrimp hepatopancreases may lower the rate of the peptides bond cleavage mediated by endogenous proteases [3,35].

Lipids are present in complexes with proteins and carotenoids forming carotenolipoprotein complexes. Therefore, unlike fish byproducts, lipids are not extracted during hydrolysis, although lipid oxidation still takes place [36]. This facilitates the separation of FPH or the red paste caroteno-protein and peptides at a centrifugation step and removes the need for a decanter to separate the oil. Shrimp byproducts are, thus, a cleaner source of protein compared to fish byproducts (such as rainbow trout viscera).

2.3. Marine Invertebrate Byproducts

Cephalopod catches reached 3.6 million tons in 2017 and 2018. Among marine invertebrate, jumbo flying squid (*Dosidicus gigas*), Argentine shortfin squid (*Illex argentinus*) and Japanese flying squid (*Todarodes pacificus*) are the three main squid species [2]. Squid byproducts constitute about 52% of the total weight after processing and are composed of heads and tentacles (25% of total weight), fins (15%), viscera (8%), skins (3%), and pens (1%) [37,38]. The main edible portion is the cone shaped trunk of the body (mantle), which accounts for 48% of the total body weight. Squid protein rich byproducts have been used to produce FPH using commercial proteases [39,40] and are high in attractant amino acids such as alanine, glycine, and taurine that are stimulants (attractants) for use in aquaculture feeds as well as growth promoting factors [38,41]. The pen that could be used for producing β-chitosan and chitooligosaccharides (COS) with antioxidant and antimicrobial activities [42] may be removed from other byproduct fractions to avoid a dark color in the hydrolysis solution.

Aquaculture production of shelled mollusca species including bivalve mussels, oysters, and cockles reached 17.3 million tons in 2018, representing 56.3% of marine and coastal aquaculture production [2]. Among them, cupped oysters (*Crassostrea* spp.), Japanese carpet shells (*Ruditapes philippinarum*), and scallops (Pectinidae) were the major species [2]. The byproducts of bivalves consist of shells, byssus threads, extracellular fluids (containing hemolymph and extrapallial fluid (EP)) and rejected individuals [39]. Proteins, low molecular weight enzymes, pigments, polyunsaturated fatty acids (PUFA), collagen, antimicrobial peptides (AMP), such as myticins, and calcite are potential functional food ingredients that can be derived from bivalve byproducts [43].

3. Enzymatic Hydrolysis

Enzymatic hydrolysis is a promising technique as it produces FPH and peptides with varying interesting functionalities that may include nutrition and health-promoting effects [6]. Hydrolysis is referred to as a process of decomposition that involves the splitting of a bond and addition of the hydrogen cation and hydroxide anion of water [3]. There are several major processing steps in enzymatic hydrolysis of food or byproduct proteins,

including raw material grinding, hydrolysis, thermal inactivation, separation, concentration or drying, packaging, and storage [44] (Figure 1).

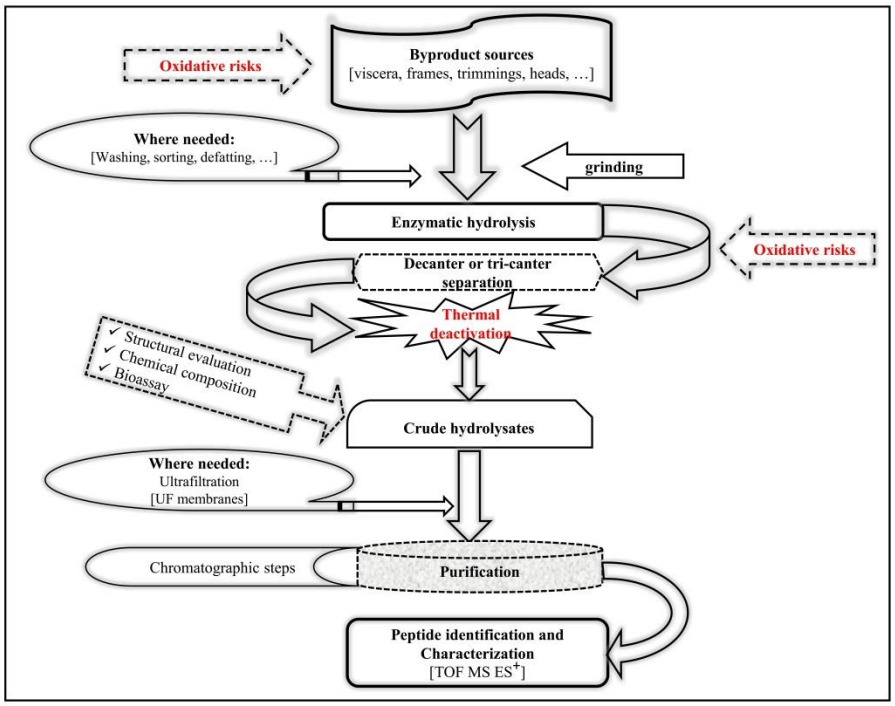

Figure 1. Schematic figure for enzymatic hydrolysis of byproducts of the seafood industry.

Enzymatic hydrolysis is done with the aid of proteolytic enzymes such as endopeptidases that cleave the peptide bonds within polypeptide chains, and exopeptidases that break peptide bonds from the end of the protein chain. Each protease has a unique specificity for peptide bonds adjacent to certain amino acid residues [6]. Proteases of microbial (Alcalase, Flavourzyme, and Neutrase), plant (papain, bromelain, and ficin), or fish (pepsin, trypsin, and chymotrypsin) origin have been used for the enzymatic hydrolysis of byproducts. Microbial proteases have been used for producing FPH from various seafood byproducts due to their higher efficiency in hydrolyzing protein substrates at higher pH with more temperature stability [26,27,30]. The use of commercial enzymes is generally required to obtain peptides for food applications because of the greater control of the process compared to autolytic hydrolysis, which may be used when the final aim is to prepare feed ingredients with somewhat less stringent requirements [36,45].

Several operational parameters, such as the type of enzymes and enzyme concentrations, pH shift, substrate pretreatments (including washing, defatting, and protein isolate preparation), water content, time, temperature, and composition and freshness of byproducts, are factors affecting FPH structure and properties [6]. Those factors also have a significant role in oxidation during enzymatic hydrolysis, influencing the stability and organoleptic properties of FPH [22,28]. Table 1 shows the enzymatic hydrolysis of byproducts and FPH characteristics from different aquatic species.

Table 1. Enzymatic hydrolysis and chemical properties of FPH from seafood byproducts.

Species	Byproducts	Hydrolysis Conditions	Chemical Properties of FPH	References
Squid *	Heads	Flavourzyme (51 °C, pH 7.0) was used at 1% for 210 min, solid to liquid (S:L) ratio = 1:2	FPH with protein content of 23.95 mg/mL was obtained after 210 min; total soluble solid (TSS) increased from 16 to 21 °Brix at 0–210 min of hydrolysis; salt content was increased from 0.81 to 1.13%; the highest overall liking (6.64) obtained after 3 h of hydrolysis while further increasing hydrolysis time to 210 min increased bitterness of FPH solution, solubility > 97%; yield and protein content of freeze-died FPH powder were 14.42% and 76.42% while those of foam-mat dried sample was lower (10.37% and 70.31%, respectively); freeze-died FPH powder had umami taste and light brown color	[40]
Pearl oyster (*Pinctada fucata*)	Shell	Proteases Nucleicin (50 °C, pH 7.0) and Orientase 22 BF (60 °C, pH 9.0) from *Bacillus subtilis* were used at 4% or 0.8%, respectively for up to 9 h to hydrolyze dried shell powder (having protein content of 2.5%)	Highest DH (26%) was obtained with Orientase 22 BF after 6 h peptide yields in solutions of water (0% ethanol (EtOH), 10% EtOH, 20% EtOH, and 30% EtOH were 71.4, 51.3, 40.1, and 33.9%, respectively; peptide Gly-Val-Gly-Ser-Pro-Tyr (MW: 578.7 Da) was isolated as the active peptide possible functional food or pharmaceutical ingredient	[46]
Cuttlefish (*Sepia officinalis*)	Viscera (containing digestive gland, esophagi, stomach, digestive ducts, pyloric caeca, pancreatic diverticula, gonads, and accessory nidamental glands), ink gland was removed, the content of protein, lipids, ash and moisture were 17.45, 4.78, 1.95, and 74.99%, respectively	Hydrolysis was done for 24 h at 50 °C and pH 8.0 using Protamex (1.5%), Alcalase (0.1%), Flavourzyme, S:L = 1:1 (*w*/*v*)	Total amounts protein recovered in soluble phase after hydrolysis were 57.2, 64.3, and 60.3 when Protamex, Alcalase, and Flavourzyme were used; DH was 3.2, 7, and 6.8 when Protamex, Alcalase, and Flavourzyme were used; most peptides in UF membrane fractionation had MW less than 1000 Da, mainly oligopeptides and free amino acids.	[47]
Rainbow trout (*Oncorhynchus mykiss*)	Heads and viscera	Alcalase was used at 3% (*v*/*w*) protein basis; 50 °C, pH 8; 3 h, solid to liquid (S:L) = 1:1	Less TBARS and protein carbonyls were formed during enzymatic hydrolysis when byproducts were washed before hydrolysis and oxidation was reduced; 68–80% peptides had MW < 1 kDa; free amino acids represented 32–39% of FPH followed by 0.18–0.5 kDa (26–27%) peptides	[48]

Table 1. *Cont.*

Species	Byproducts	Hydrolysis Conditions	Chemical Properties of FPH	References
Monkfish (*Lophius piscatorius*)	Heads and viscera	Optimal proteolytic digestion: 57.4 °C, pH 8.31, Alcalase = 0.05% (*v*/*w*), 3 h, and S:L = 1:1 ratio (*w*/*v*), Stirring rate: 200 rpm	Peptides with MW < 1 kDa were 73.5 and 54.5% in FPH from viscera and heads, respectively; yield of dry powder was 6% (60 g dry FPH per kilogram of wet head) for heads and 5% for viscera; total essential amino acids (TEAA)/total amino acids (TAA) were 42.1 and 40.3% for head and viscera FPH; glutamic acid, glycine, aspartic acid, and alanine were the dominant amino acids in both FPH; the content of protein, moisture, ash, and lipids were 69.80, 9.25, 18.47, and 2.39% for head FPH and 67.41, 5.19, 19.74, and 4.82% for viscera FPH, respectively	[49]
Turbot (*Scophthalmus maximus*)	Heads (Tu-H), trimmings and frames (Tu-TF), viscera (Tu-V)	Alcalase was used at the optimal conditions of 60.3 °C, pH 8.82, Alcalase = 0.2% (*v*/*w*), 3 h, S:L = 1:1 ratio (*w*/*v*) and 200 rpm	TEAA/TAA amino acids for Tu-H, Tu-TF, and Tu-V was 28.04, 30.54, and 30.41%, respectively; peptides in the range of 1–3 kDa were the dominant peptide fraction, accounting for 46, 40.3, and 52.9% in Tu-H, Tu-TF, and Tu-V, respectively; Tu-V showed the highest percentage of peptides >1 kDa (65%), compared to Tu-H (54%) and Tu-TF (45%); higher than 92% digestibility for all sample; DH = 30–37%, soluble protein > 62 g/L, and high yield of digestion (>83%)	[50]
Seabream (*Sparus aurata*)	Heads (He), Viscera (Vis), and frames and trimmings (FT) representing 18.7, 8, and 20.4% of fish weight, respectively.	Alcalase (0.2%), temperature 57.13 °C, pH 8.17, S:L = 1:1	Glutamic acid was the dominant amino acid followed by aspartic acid and glycine, alanine, leucine, and lysine; TEAA/TAA amino acids for He, Vis, FT were 41.94, 44.73, and 41.84%, respectively; peptides with MW of 1–3 kDa (28.3–51.2%) were the main peptides followed by those with MW of 0.2–1 kDa (24.6–33.9%) and 0–2 kDa (18.5–28.5%), respectively; DH was 18.3, 19.27, and 21.5% for He, Vis, FT, respectively; digestibility > 86% in all samples	[51]
Seabass *Dicentrarchus labrax*)	Heads (He), Viscera (Vis), and frames and trimmings (FT) representing 16.7, 9.1, and 26.9% of fish weight, respectively	Alcalase (0.2%), temperature 58.43 °C, pH 8.46, S:L = 1:1	TEAA/TAA amino acids for He, Vis, FT were 40.32, 44.61, and 42.42%, respectively; the highest proportion of peptides had MW in the range of 1–3 kDa; peptide with MW < 0.2 kDa represented 24.7, 17.6, and 26.3% in He, Vis, FT, while those having MW of 0.2–1 kDa represented 17.9, 11.3, and 38.8% of all peptide fractions, respectively; DH ranged from 12.96 to 21.7, being lowest in protein hydrolysates from Vis; digestibility > 86% in all samples	[51]

Table 1. *Cont.*

Species	Byproducts	Hydrolysis Conditions	Chemical Properties of FPH	References
Rainbow trout	Heads	Minced heads with different pretreatments (H_2O_2 and Fe^{2+}, butylated hydroxyltoluene (BHT)) hydrolyzed using Alcalase, bromelain, or papain at 50 °C for 1 h, S:L = 1:1, 150 rpm, E/S 0.05% (*w*/*w*)	Crude protein ranged from 79.7 to 88.5%; yield ranged from 6.2 to 6.8%; DH ranged from 17.4% in FPH produced from heads (FPH-CON) and heads plus pro-oxidants (FPH-OX) to 18.2% in FPH-OX in the presence of antioxidant (FPH-OXAX); no significant differences in peptides MW distribution between FPH-OX and FPH-OXAX; peptides with MW of 2000–5000 Da dominated the peptidic fractions, followed by 1000–2000 Da fractions; the content of 200–500 Da (mainly di-peptides and tri-peptides) were low; EAA was between 36.34 and 39.04%	[52]
Atlantic codfish (*Gadus morhua*)	Frames	Alcalase was used at the optimal conditions: temperature 56.8 °C, pH 8.35, enzyme concentration 0.25% (*v*/*w*), 3 h. Stirring rate (200 rpm) and S:L = 1:1	A total of 49% of peptides had MW < 1 kDa and 41% in the range of 1–3 kDa; DH = 37%; in vitro digestibility 92%; the content of organic matter, moisture, ash, and lipids were 84, 1, 15, and 2%, respectively; small brown powder with an intense fishy odor yield was 12% (dry powder in relation to the initial frames wet weight); TEAA/TAA = 36.67%	[27]
Yellowfin tuna (*Thunnus albacares*)	Heads	Different enzymes including Alcalase (1% E/S, 60.5 °C, pH 8.65), papain (0.025% E/S, 45 °C, pH 6.5), Protamex (1% E/S, 45 °C, pH 6.5) and esperase (1.6% E/S, 60.5 °C, pH 8.65) at S:L = 1:2, 200 rpm, for 3 h hydrolysis of upper and lower portions of heads	Weight average MW for FPH from Alcalase, esperase, papain, and Protamex were 1839, 1417, 2020, and 2175 Da, respectively; EAA was >40% for all samples; asparagine, glutamine, glycine, alanine, lysine, and leucine were the dominant amino acids; the yield of dried FPH produced from 1 kg upper head hydrolyzed using Alcalase was 150 g	[26]
Atlantic salmon (*Salmo salar*)	VHF (50% viscera and 25% minced heads and 25% minced frames) or V (100% viscera), crude protein content of viscera, heads, frames, and the mixed byproducts were 8, 13, 15, and 11%, respectively	Protamex (01%), and bromelain and papain (0.05% each as wet-weight basis of byproducts) were used for hydrolysis at 52 °C for 2 h, in viscera, endogenous enzymes in some samples were inactivated at 70 °C for 5 min	The content of protein in VHF was >82% while FPH from Vis had lower value (maximum 77.4%); at industrial level, protein content of VHF and Vis was lower (78.8 and 71.9%, respectively); FPH from enzymatic Vis had slightly lower protein content than that produced using endogenous enzymes (73%); peptides with MW < 500 Da were the main fractions, being highest (80.5%) in FPH from viscera hydrolyzed using Protamex and endogenous enzymes (V ePr) while V PaBr samples produced using papain and bromelain showed the lowest content of small peptides (44%); VHF showed 62.6–69.9% di-peptides and tri-peptides depending on the type of protease; at industrial scale, percentage of <500 Da peptides decreased (44–80.7%) compared to lab scale (54.6–71%)	[30]

Table 1. *Cont.*

Species	Byproducts	Hydrolysis Conditions	Chemical Properties of FPH	References
Bighead Carp (*Hypophthalmichthys nobilis*)	Heads	Alcalase and alkaline protease (40–60% ammonium sulfate fraction) from rainbow trout viscera, temperature 58 °C and PH 8.5 for Alcalase and 60 °C and PH 7.0 for viscera alkaline protease, enzyme level 1.5 (*w*/*w*), 150 min hydrolysis time, 600 rpm, hydrolysates were fractionated using ultrafiltration membranes (UF) to >30, 30–10, 10–3, and <3 kDa MW peptide size	Protein hydrolysates and UF fractions showed the highest solubility at pH of 9 and 3, also fraction with the lowest MW had the highest solubility compared to peptides with bigger size; TEAA/TAA ranged from 42.03 to 44.98% in all samples; aspartic acid, glutamic acid, leucine, isoleucine, lysine, and proline were the dominant amino acids	[53]

* Scientific name was not mentioned.

4. Formation of Oxidative Compounds in Fish Protein Hydrolysates

During enzymatic hydrolysis, heating the solution to achieve the optimal activity for proteases, changes of pH, the presence of pro-oxidants (i.e., hemoglobin and metal ions) and unsaturated lipids in addition to initial quality of byproducts can cause oxidation, which should be studied at both substrative and hydrolysis levels (Figure 2).

4.1. Lipid Oxidation Products

Oxygen is vital for oxidative reactions. The solubility of oxygen is higher in oils than in water [54]. Lipids in byproducts gradually release into the hydrolysis solution, causing oxidation. In Sind sardine, hydrolysis of pretreated fish by 5% Alcalase in the presence of nitrogen gas (N_2) significantly decreased lipid oxidation along with having higher DH and antioxidant activity for the FPH [22]. Decomposition of formed peroxides during enzymatic hydrolysis [36,48] by pro-oxidative metal ions is a driving factor for lipid oxidation. Metal-catalyzed hydroperoxides degradation generally takes place via cleavage of an oxygen–oxygen bond in the peroxide (LOOH), producing some highly reactive alkoxyl lipid radicals ($LO^{\bullet}$) and hydroxyl ions (OH^{-}). $LO^{\bullet}$ further degrades rapidly by carbon-carbon cleavage on either side of the radical to form volatile decomposition products or off-odors [54]. In addition, the initial oxidative status of lipids in byproducts is very important for the extent of lipid oxidation during hydrolysis as well as the sensory quality and oxidative stability of FPH. The TBARS, PV, and undesirable fishy odor in FPH produced from fresh Nile tilapia were significantly lower than that produced from non-fresh fish [55].

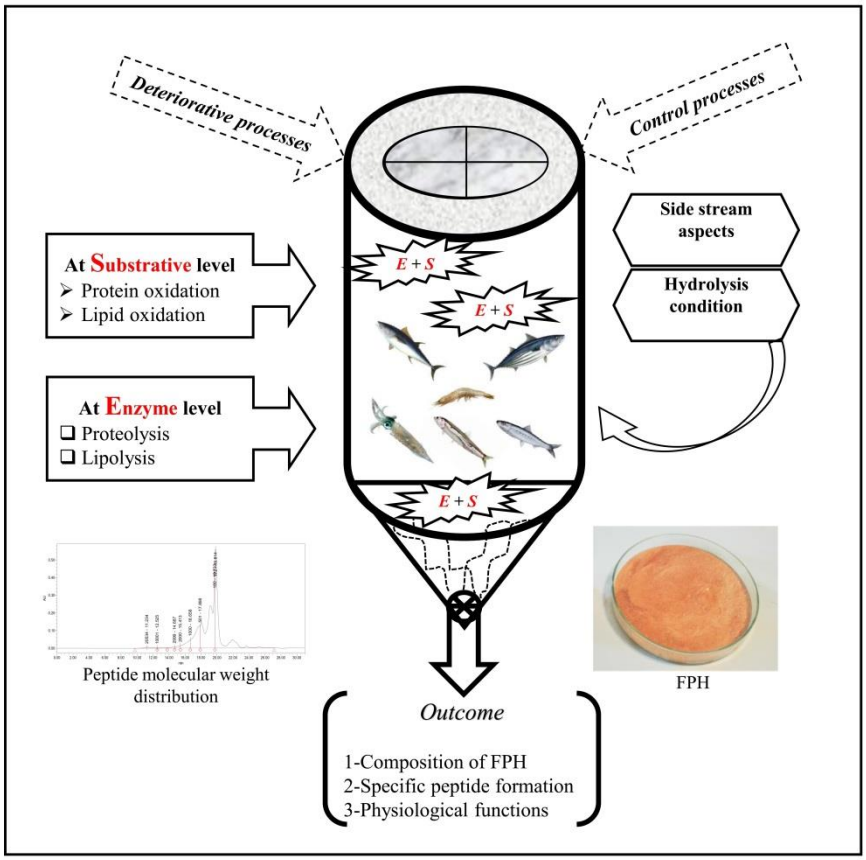

Figure 2. Main oxidative processes, control measures, and properties of FPH from byproducts of the seafood industry.

4.2. Blood Components and Degradation

Fish byproducts contain considerable amounts of blood containing hemoglobin (Hb) and iron as pro-oxidants. These components can react with each other during enzymatic reactions [56]. The brown color development during the enzymatic hydrolysis of byproducts may be explained by the auto-oxidation of Hb and changes of Fe^{2+} to Fe^{3+} to form metHb, which has increased pro-oxidant activity compared to its reduced form. Dissociation of the iron-protoporphyin IX moiety (or heme) from metHb is relevant in the context of lipid oxidation. Iron-protoporphyin IX released from metHb intercalates within the phospholipid bilayer of cellular membranes facilitating the breakdown of any lipid hydroperoxides (LOOH) formed to alkoxyl and peroxyl radicals that propagate lipid oxidation [56]. Hemoglobin (5 and 20 μM/kg) and iron (100 or 200 μM) in the presence of fish oil (5%, *v*/*v*) induced oxidation during hydrolysis of cod using a commercial protease at 3% *w*/*v* and 36 °C. In frames, trimmings, and heads with remaining fish meat, Hb might contribute to both lipid and protein oxidation during hydrolysis, the same as can happen with fillets, which could have impacts on the structural properties and oxidation of peptides [21]. Larsson and Undeland [57] investigated Hb-mediated lipid and protein oxidation in washed cod mince and found an increased peroxide value (PV), rancid odor, protein carbonylation, and insolubilisation in the presence of 20 μmol/L Hb. The higher Hb and iron in herring heads compared to other fractions of byproducts, including frames, belly flap, and viscera, led to greater lipid oxidation during ice storage [25]. DeoxyHb could easily react with O_2 to rapidly form metHb and superoxide radicals that can dismutate to hydrogen peroxide (H_2O_2) and facilitate lipid oxidation [56]. Therefore, reducing oxygen in reactors may decrease undesirable oxidative reactions during enzymatic hydrolysis. With tuna byproducts, hydrolysis in the presence of nitrogen gas decreased TBA, off-odor, brown discoloration, and bitterness in the FPH [58].

4.3. Protein Carbonyls, Amino Acid Degradation, and Solubility

The gastrointestinal tract and internal organs could be exposed to the cytotoxic and mutagenic potential of harmful free radicals formed in oxidized proteins and peptides such as alkyl, peroxyl, and alkoxyl radicals and carbonyls [59]. Oxidation products may interact with other components, influencing FPH quality, and this can influence the oxidative deteriorations during enzymatic hydrolysis where all components of byproducts are present at their original concentrations, unless subjected to substrate pretreatment [7]. Rainbow trout byproducts subjected to washing using distilled water (BPW-FPI) or distilled water prior to calcium chloride and citric acid washing (W-CaCi-BPFPI) had a lower content of protein carbonyls in the dried FPH [45]. Secondary protein carbonyls can be introduced into proteins using a covalent linkage of lipid carbonyls (i.e., protein-bound malondialdehyde (MDA)) [48]. Both lipid and protein oxidation can occur during the enzymatic hydrolysis of byproducts that makes the process more biochemically complicated. Oxidized peptides may lose their nutritional value due to the destruction of essential amino acids and impaired digestibility [60].

The solubility of peptides influences their functional properties (including emulsifying and foaming properties, and water holding capacity) for food applications [53]. During enzymatic hydrolysis, after unfolding the protein structure, polar (but generally uncharged) and non-polar amino acid groups inside the protein come to the protein surface, and then polar residues interact with water molecules to form hydrogen bonds and electrostatic interactions, thereby affecting solubility [7,45]. The reduced solubility (62.6%) of FPH produced from oxidized rainbow trout heads (FPH-OX) was associated with higher levels of protein carbonyls (5.14 nmol/mg protein) showing that protein oxidation and aggregation were occurring during enzymatic hydrolysis [52].

4.4. Autolysis Mediated by Endogenous Enzymes

Visceral and muscle proteases are the main groups of endogenous enzymes in byproducts, contributing to the deterioration (i.e., autolysis) of byproducts with inappropriate storage, which then may increase oxidative deterioration during enzymatic hydrolysis. In frames, heads, and trimmings, muscle proteases, including lysosomal cathepsins, alkaline proteases, and neutral proteases, are present and show high capacity for hydrolyzing myofibrillar proteins at neutral or slightly alkaline pH, especially with improper handling and storage (i.e., high storage temperatures and longer times). In viscera, four main groups of proteases are available in the stomach, pyloric caeca, and intestine. These include acidic/aspartyl proteases (pepsins), serine proteases (such as trypsin and chymotrypsin), cysteine (thiol) proteases, and metalloprotease. These enzymes could be activated during enzymatic hydrolysis with optimal conditions for their activity (e.g., pH and temperature) accelerating the reactions unless deactivated by heating to avoid autolytic hydrolysis [3]. Endogenous enzymes along with bacterial enzymes are responsible for the process of autolysis in byproducts after a fish's death. Consequently, the degradation of proteins and the development of off odors and off flavors takes place that lowers the quality of byproducts for enzymatic hydrolysis. Action of lipases leads to the formation of degradation products such as free fatty acids that are responsible for oxidation during hydrolysis and oxidative instability of FPH [61].

5. Control of Oxidative Deteriorations during Enzymatic Hydrolysis

5.1. Cold Storage of Byproducts

The most important biochemical changes that can occur during storage and that affect the quality of byproducts for enzymatic hydrolysis include: lipid oxidation, autolysis, reactions with lipid oxidation products, and changes in pH, which can lead to protein oxidation (i.e., aggregation, denaturation, cross-linking, and breakdown of polypeptide chains). Such changes in protein structure can reduce the nutritional value (e.g., reduced digestibility), decrease the yield, change the organoleptic properties (e.g., color and odor), and alter protein solubility and other functional properties of the FPH [23]. Storage and

transportation of byproducts at low temperature could preserve quality and safety for a few days before enzymatic hydrolysis [3]. Short-time storage of salmon byproducts at two different temperatures (4 and 10 °C) showed the formation of various metabolites that were affected by the storage temperature and mincing of byproducts [62]. Nuclear magnetic resonance (NMR) spectra of TCA extracts of heads, backbones, and viscera showed the formation of trimethylamine (TMA), acetic and succinic acids, ethanol and 1,3-propanediol, biogenic amines (including tyramine, histamine, putrescine and cadaverine), and proteolysis of the byproducts. The formation of TMA and proteolysis were the most important temperature dependent processes that occurred during storage. Byproducts stored at 4 °C retained their quality up to 7 days without significant changes in the concentration of harmful compounds. However, those stored at 10 °C could not be kept with acceptable quality for >3 days. Mincing of heads because of greater formation of TMA reduced storage time to 2–3 days and was not suggested. TMA could have a major role in the development of unpleasant fishy odor and unacceptable taste in fish products because of its low odor threshold. Salmon byproducts rejection based on TMA concentration were as follows: minced heads stored at 10 °C after two days, minced heads at 4 °C, viscera at 10 °C, whole heads at 10 °C after the fourth day of storage, and the other fractions could be stored for >7 days. Therefore, elevated temperatures for byproducts storage should be avoided to decrease the formation of harmful compounds in byproducts that can follow the aqueous hydrolysate phase during enzymatic hydrolysis that negatively influence the overall quality, safety, and marketability of the FPH [3]. In cuttlefish byproducts, a greater activity of proteolytic enzymes was found after storage and during handling, indicating zymogen vesicles lysosomal breaking that decreased byproducts quality for further hydrolysis [63].

5.2. Antioxidants

Antioxidants are substances that delay, control, or inhibit oxidative processes leading to food quality deteriorations when used at low concentrations. These additives exert their inhibitory effect though different mechanisms including hydrogen atom transfer (HAT), single electron transfer (ET), metal chelation, and reducing power [64]. An antioxidant strategy has been suggested to lower oxidation during enzymatic hydrolysis. However, the efficacy of antioxidants in complex food systems depends on many factors, including their structure, concentration, temperature, the oxidation substance and physical state of the system, and the presence of pro-oxidants [54,64]. Therefore, these factors should be considered when selecting a suitable antioxidant to control oxidation processes during enzymatic hydrolysis of fish byproducts containing susceptible oxidation substrates (such as n-3 polyunsaturated fatty acids) and pro-oxidants (blood and heme iron). Halldorsdottir et al. [65] reported that a brown algae (*Fucus vesiculosus*) extract (0.16 g extract/1 L of 3.7% protein solution) reduced oxidation during the enzymatic hydrolysis of cod backbones, as evidenced by lower formation of peroxides and TBARS. Sarteshnizi et al. [22] showed that pistachio green hull (PGH) extract (260 µg/mL) controlled oxidation of Sind sardine hydrolyzed using Alcalase. The resulting FPH showed higher DPPH radical scavenging and metal chelating activity.

5.3. Sorting of Byproducts

Some studies indicated a higher content of pro-oxidants (such as Hb and metal ions) in some factions of byproducts than other parts. The blood-rich and large gills in tuna and sturgeon heads may increase oxidation during enzymatic hydrolysis. Their removal prior to hydrolysis of heads could lower Hb-mediated oxidation [3]. In herring (*Clupea harengus*), the higher Hb levels (100 µmol/kg) and iron (>60 mg/kg) in minced heads, associated with the presence of gills, compared to other byproducts factions such as frames, tail, mixed viscera, and belly flap, increased lipid oxidation during 4 days of ice storage, confirming the role of the composition of fileting byproducts on the rate of oxidation [25]. In lean fish species such as cod, lipids are mainly stored in the liver. Thus, storage of liver together with other fractions increases the susceptibility of byproducts to lipid oxidation during

storage. Thus, where possible, liver should be separated from the more stable fractions such as heads or frames [66]. On the other hand, due to high concentrations of endogenous enzymes in fish viscera, byproducts that contain or are stored with viscera are even more susceptible to oxidation and spoilage [23]. A recent survey of fish industry byproducts handling practices in some Nordic countries indicated the sorting of byproducts fractions was done in 63% of the processing plants, while the remainder do not handle their side streams effectively [67]. Therefore, sorting of byproducts would be beneficial to lower oxidation during enzymatic hydrolysis.

5.4. Hydrolysis Parameters

5.4.1. Enzyme Type and Concentration

Since different proteases have different cleavage sites on polypeptide chains, protein hydrolysates with different properties and bioactivities are expected. The type of enzymes determines which peptides are formed. Structural properties of FPH such as amino acid composition, molecular weight distribution, and surface hydrophobicity are influenced by enzyme types, which then affect their antioxidant or other functional properties [5,6]. In hydrolyzing salmon frames, Alcalase led to higher DH than papain while, the latter increased the metal chelating and reduced capacity of protein hydrolysates [68]. Salmon skin hydrolyzed using Alcalase, Neutrase, Flavourzyme, and Protamex showed differences in DH, molecular weight distribution, secondary and tertiary structure. Among all samples, FPH from Alcalase had higher DH (20%) and the lowest MW distribution (<2000 Da), antioxidant activity, and surface hydrophobicity due to the exposure of buried hydrophobic peptides resulting from the highly specific cleavage of Alcalase. However, Neutrase influenced protein quality as evidenced by the lower content of α-helix (17.8%) and higher β-turn (32.8%) with lower stability against temperature, pH, and sodium chloride [69]. The amount of water during enzymatic hydrolysis is another factor that influences the hydrolytic efficiency of proteases. Higher water content is expensive because of the higher amount of energy needed for water evaporation during vacuum concentration or the final drying step [3]. In hydrolyzing salmon frames, water percentages of 75% and 100% byproducts weight were suggested to reduce the drying cost while ensuring efficient hydrolysis in terms of DH, nitrogen recovery, and peptide size [70].

5.4.2. Time and Temperature

The suitable time and temperature necessary for enzymatic hydrolysis is determined by the optimal activity of the selected proteases. At the optimal conations, enzymes basically possess higher proteolytic capacity towards cleavage of peptidic bonds in byproducts [6,7]. Therefore, extreme times and temperatures are not necessary and must be avoided as they decrease FPH quality and bioactivities while increasing energy consumption. A higher hydrolysis temperature together with longer hydrolysis time increased the rate of lipid and protein oxidation during hydrolysis of rainbow trout [54] and Pacific white shrimp [32] byproducts, decreasing antioxidant activity and changing the amino acid composition. Protein hydrolysates rich in <500 Da molecular weight peptides derived from salmon and rainbow trout byproducts using Alcalase showed differences in DH, solubility, and digestibility that was affected by the temperature (30–80 °C) and pH (6–10) of hydrolysis [30].

5.4.3. Presence of Oxygen and Stirring

The effect of stirring that is coupled to industrial reactors on the rate of oxidation was investigated. With tuna byproducts (heads and viscera), shaking of the hydrolysis solution was found to influence organoleptic and chemical properties of protein hydrolysates. FPH with shaking had more brown color, bitterness, fewer free amino acids, and higher TBARS, indicating more vigorous lipid oxidation with shaking rate [58]. Therefore, harsh stirring or shanking should be avoided and be adjusted according to chemical properties of FPH.

At the same time, a reduction in oxygen would be beneficial to lower oxidation rates by replacing it with inert gas such as nitrogen [22].

5.4.4. Role of the Equipment Used for Processing

Despite the general processing steps during enzymatic hydrolysis (Figure 1) that influence FPH quality, recently some novel equipment has been studied for its efficacy in the improvement of byproducts hydrolysis. To avoid extended hydrolysis times and nonselective hydrolysis, tilapia byproducts were subjected to protein isolation followed by pressure (38–462 MPa) before a 6–35 min hydrolysis [71]. The trichloroacetic acid solubility index, protein content, solubility, and antioxidant activity of protein hydrolysates were improved by pressure, time, and adjusting process variables. The process also influenced oxidation. Peroxides of peptides using ambient conditions (8–12 meq/kg) were higher than those of pressure-assisted hydrolysis (4–10 meq/kg), indicating both pressure and reaction time had significant effects on PV. Ultrasound pretreatment of cod heads at 150–600 W reduced hydrogen- and alkane-containing odor components in the hydrolysate solution compared to conventional enzymatic hydrolysis and reduced the bitterness and astringency of peptides. Ultrasound pretreatment increased the degree of hydrolysis (DH), soluble peptides, and the percentage of <3 kDa peptides, while increasing umami and sweet tasting amino acids, salty amino acids, nucleotides, and succinic acid. FPH obtained after a 450 W ultrasound pretreatment smelled and tasted better and had a higher formation of pleasant volatile compounds than the non-treated sample [72]. Therefore, better proteolysis technology to utilize cod heads was suggested.

6. Conclusions

Several parameters of enzymatic hydrolysis influence the properties and constituents of the protein hydrolysates from byproducts such as the composition of and variation in the starting raw materials, freshness of byproducts, the specificity, activity, and concentration of the enzymes used, time, pH, and temperature. Deteriorative reactions can be studied at the substance or enzyme levels and the control measures should be applied on byproducts and hydrolysis conditions. With farmed species, fasting prior to harvest will eliminate the intestinal content to a large extent, thus, a consistent quality of byproducts can be ensured for enzymatic hydrolysis of viscera. Furthermore, removal of gills from heads can reduce the content of blood, which may decrease oxidation. The use of fresh byproducts directly after such operations as shrimp processing or fish filleting can result in the desired quality of the initial raw material for further enzymatic hydrolysis with controlled conditions. Controlling oxidation using an antioxidant during hydrolysis along with other controls can have a significant role on FPH organoleptic, nutritional, and biological properties.

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