Turk. J. Fish.& Aquat. Sci. 24(4), TRJFAS25347
https://doi.org/10.4194/TRJFAS25347

PROOF
REVIEW

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Functional Ingredients from Seafood Processing Wastes: Protein Hydrolysate and Biocalcium

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How to Cite

Patil, U., Nilsuwan, K., Benjakul, S. (2024). Functional Ingredients from Seafood Processing Wastes: Protein Hydrolysate and Biocalcium. *Turkish Journal of Fisheries and Aquatic Sciences*, *24(4), TRJFAS25347.* https://doi.org/10.4194/TRJFAS25347

Article History
Received 29 September 2023
Accepted 03 January 2024
First Online 12 January 2024

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Keywords
Fish protein hydrolysate
Fish biocalcium
Production
Bioactivity and bioavailability
Applications

Abstract

The fish processing industry generally generates large amounts of byproducts, including skin, bone, scale, or meat residues, etc. These byproducts contain a wide range of nutritional components such as protein, lipid, mineral, etc. These leftovers can be converted to functional ingredients or nutraceuticals. To produce high value-added functional ingredients, the remaining proteins in fish processing leftovers are hydrolyzed, in which hydrolysate containing different peptides and amino acids can be generated. Frame or backbone as well as scale can be converted to fish biocalcium, which serves as a potential source of calcium and minerals. Nevertheless, potential processing or technology must be adopted to obtain desired products with target bioactivitities. This review covers production, characteristics, and bioavailability of fish protein hydrolysate (FPH) and fish biocalcium (BC). This review also focuses on the recent applications of the FPH and BC in drink and foods. Overall, the review points out the better exploitation of fish processing leftovers and simultaneously lower pollution caused by the improper discard or disposal of those wastes to environment. Also, consumers have more choices to consume functional ingredients or nutraceutical from marine resources.

Introduction

In Asia, the total fisheries and aquaculture production reached a record of 214 million tons in 2020, comprising 178 million tons of aquatic animals and 36 million tons of algae as highlighted in the State of World Fisheries and Aquaculture 2022 report. The increasing global fish production has given rise to a large quantity of raw material utilized for processing. Consequently, significant quantities of fish processing leftover, comprising inedible tissues such as bones/frame, skin/scales, gills, swim bladders, fins, intestines, blood, roes, liver, etc. are generated (Kumar et al., 2018). These remnants are often disposed into the river, waterways, and oceans, resulting in severe environmental contamination. Over the past few decades, efforts have been made to exploit these leftovers via transforming them into fish meal, fertilizer, or animal feed with a low market value (Patil et al., 2022b). Nonetheless, it is crucial to recognize that these leftovers are rich sources of valuable components such as protein, bioactive peptides, enzymes, minerals, lipid, vitamins, flavorant, pigments, etc. (Kumar et al., 2018). Therefore, it is imperative to convert these wastes into value-added products in order to increase their market value and minimize environmental pollution.

Fish frame or trimming still has some meat retained. These leftovers can be utilized as raw material for protein hydrolysate production. The common method for production of fish protein hydrolysate (FPH) is enzymatic process, which is be easily controlled to end up with the designed degree of hydrolysis (DH) (Kristinsson & Rasco, 2000). Also, such a mild condition does not lead to side effects such as the development of toxic compounds, etc. FPH has several functional properties such as emulsification, foaming, oil binding, and water retention, and various bioactive properties including antioxidant, antimicrobial, antihypertensive, etc. (Misir, 2022). To produce high quality FPH, undesirable attributes, especially fishy odor, must be minimized. Also, bitterness is another drawback of FPH, in which debittering could be adopted to increase consumer acceptability (Idowu & Benjakul, 2019). Simultaneously, the bioactivity or nutraceutical properties must be maximized, mainly contributing to the active peptides in FPH. Sequence of amino acid in peptides are also known to determine bioactivity. Due to the hygroscopic in nature of FPH, the handling and storage must be done properly to avoid absorption of moisture from environment (Chiodza & Goosen, 2023). FPH with bioactivities has been widely employed in various processed foods, in order to enhance their intrinsic value and give them "functional food" feature. Moreover, FPH also has been used in pharmaceutical and nutraceutical industries (Rana et al. 2023).

Calcium (Ca) is known to be an important element required for many physiological functions in the human body, including cofactor in enzymatic reactions, muscle contraction, blood clotting, nerve function, and strengthening teeth and bones (Anderson & Garner, 1996). Dairy products including cheese, milk, calcium fortified products, and dietary supplements are generally the essential sources of calcium (Tunick, 1987). However, milk or dairy products are rarely consumed in some populations, particularly Asian people, owing to lactose intolerance and indigestion (Gaskin & Ilich, 2009). Consequently, calcium-fortified foods or calcium salts can be alternative calcium supplements. Fish calcium showed a comparable bioavailability to calcium from skim milk in both rats (Larsen et al., 2000) and humans (Hansen et al., 1998). Frame or backbone and scale of fish, which are byproducts from fish processing industry, can serve as a potential source of minerals and calcium. Minerals including calcium, phosphorus and hydroxyapatite have been found in fish bones at approximately 60-70% (Kim & Mendis, 2006b). The calcium/phosphorus (Ca/P) mole ratio of hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$) and tricalcium phosphate (TCP: $Ca_3(PO_4)_2$) were 1.67 and 1.50, respectively (Piccirillo et al., 2013). HA crystals are localized between gap zones of collagen fibrils. Collagen fibrils align together with HA in bones to provide strengthening effect (Benjakul et al., 2019) (Figure 1). In addition, clinical trial and biological study revealed that fish bone calcium is an excellent source of bioavailable calcium, suggesting that fish bone can serve as the promising source of dietary calcium (Hansen et al., 1998; Idowu et al., 2019a).

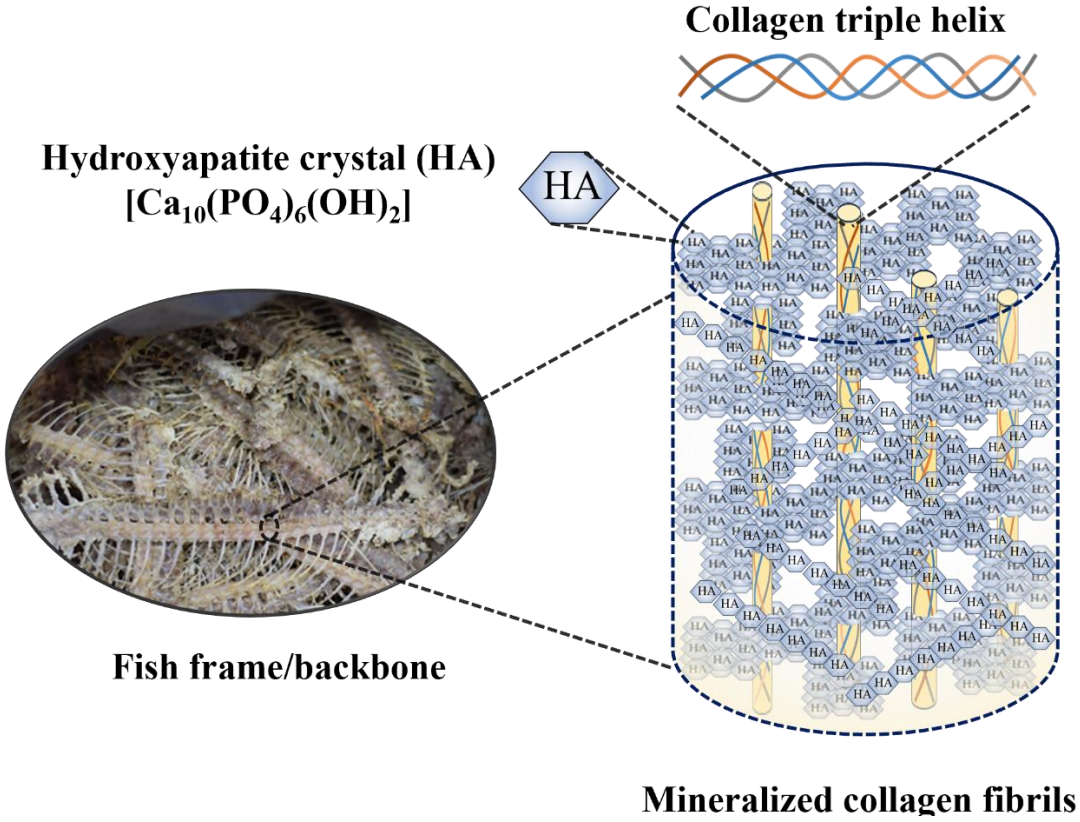

Figure 1. The structure of a hydroxyapatite-collagen composite in fish frame/backbone tissues.

Protein Hydrolysates from Fish Processing Leftover

Seafood processing by-products can contain up to 60% of proteins (on dry weight basis) (Sasidharan & Venugopal, 2020). This has urged the production of fish protein hydrolysates (FPH) through hydrolysis process to produce bioactive peptides and essential nutrients from these by-products, thereby increasing their value. The schematic presentation of general approach to produce FPH and their applications is shown in Figure 2. The desire for the sustainable utilization of fish processing by-products has spurred the advancement of protein hydrolysis processes and recovery, in which their functionalities can be improved, and application can be achieved (Nirmal et al., 2022).

Production of Fish Protein Hydrolysate (FPH)

Hydrolyzing proteins into smaller peptides has been demonstrated to enhance their nutritional and functional properties (Kudo et al., 2009). Protein hydrolysis has been used to transform by-products into more profitable forms (Patil et al., 2023). Head, bone/frame, skin, viscera, and other by-products have potential to be utilized in the production of FPH. These FPH, composed of relatively short peptides (2–20 amino acids), can be generated by breaking down proteins into peptides either chemical or enzymatic hydrolysis (He et al., 2013). Chemical hydrolysis involves the use of acid or base to facilitate protein hydrolysis. This method is straightforward and advantageous for its cost-effectiveness. However, chemical hydrolysis presents challenges in controlling the molecular weight (MW) of the resulting peptides, and it may yield a significant quantity of salt along with adverse side reactions including decomposition of tryptophan and racemization (Gao et al., 2021).

Alternatively, the enzymatic hydrolysis process has emerged as the preferred method due to fast, safe, and easily controlled methods with mild reaction conditions (Patil et al., 2019). This process has been proven highly effective in producing novel peptides from byproducts, with superior bioactivities and functionality (Kristinsson & Rasco, 2000). Enzymatic hydrolysis employs proteolytic enzymes, both endopeptidases, which cleave the peptide bonds within proteins and exopeptidases, which hydrolyze peptide bonds from either N or C terminal (Clemente, 2000; Raksakulthai & Haard, 2003). Commercially available proteases used for FPH production come from different sources: (a) microbial sources, e.g. flavourzyme, alcalase, neutrase,

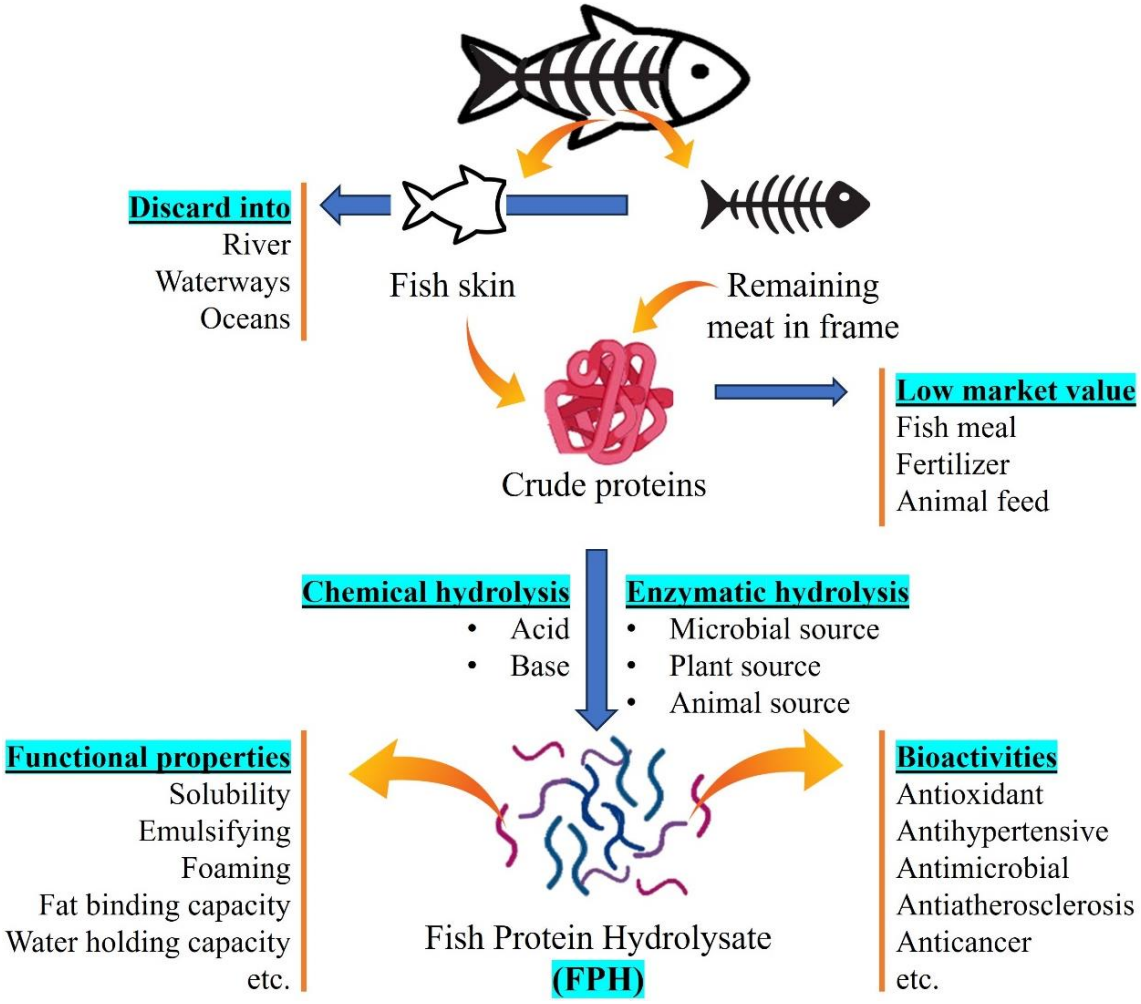

Figure 2. The schematic presentation of FPH production and its potential applications.

umamizyme, protamex; (b) plant sources, e.g. bromelain, ficin, papain; and (c) animal sources, e.g. chymotrypsin, trypsin, pepsin (Gao et al., 2021). FPH were produced with the help of commercial microbial enzyme namely Alcalase from *Bacillus licheniformis*, plant enzyme namely papain from the latex of *Carica papaya* (Idowu et al., 2019b) and animal gastrointestinal enzymes including pepsin and trypsin from porcine and bovine sources (Ketnawa et al., 2017). In addition to commercial enzymes, crude proteases from fish viscera were also used to obtain FPH (Nalinanon et al., 2011). Partially purified proteases such as pepsin and trypsin from fish viscera are also utilized for production of FPH (Patil et al., 2022a).

Nevertheless, each protease exhibits a distinct specificity for peptide bonds adjacent to specific amino acid residues, allowing for selective protein hydrolysis to achieve desired properties (Wu et al., 2003). Factors like raw material composition, enzyme type, and hydrolyzing condition including, time, temperature, pH, enzyme-to-substrate ratio, solid-liquid ratio, amount of enzyme, and production conditions (e.g. ultrasonic treatment) directly affect the physicochemical, functional properties and bioactivities of FPH (Misir & Koral, 2019; Patil et al., 2022c). Controlled hydrolysis has been known as a potential means to improve the functional and bioactive properties of proteins (Karami & Akbari-Adergani, 2019).

Properties of FPH

Protein hydrolysis results in the generation of hydrolysates or peptides possessing altered or improved bioactivities and functional properties when compared to their native proteins (Kitts & Weiler, 2003). FPH possess desirable functional properties (e.g., solubility, emulsifying, foaming, oil, water/oil binding capacities, etc.) (Kristinsson & Rasco, 2000) and several beneficial bio-activities (anti-oxidative, anti-hypertensive, anti-microbial, anti-tumor activities, etc.) with potential applications in foods, nutraceuticals and pharmaceutical products (He et al., 2013; Nirmal et al., 2022).

Functional Properties

The hydrolysis process plays a vital role in influencing the molecular size, hydrophilicity, and hydrophobicity of FPH (Kristinsson & Rasco, 2000), consequently impacting their functional properties (Karami & Akbari-Adergani, 2019). Solubility is considered as a crucial functional property of FPH. Other functional properties like foaming and emulsifying, are directly linked to solubility (Benjakul et al., 2014). Protein hydrolysis contributes significantly to the enhancement of solubility. This can be ascribed to the breakdown of proteins into smaller soluble peptides, resulting in a higher exposure of charged carboxyl and amino groups (Yin et al., 2008). The high solubility of FPH implies their potential applicability in formulated food products (Foh et al., 2010; Pires & Batista, 2013). Strong correlation between solubility and degree of hydrolysis (DH) was established (Dinakarkumar et al., 2022). However, an extensive hydrolysis process may negatively impact other functional properties (Benjakul et al., 2014). For example, salmon heads FPH produced by hydrolysis with Alcalase with higher DH exhibited superior solubility. Nonetheless, better properties such as fat absorption capacity, emulsifying capacity, and emulsion stability were obtained when DH was lower (Kristinsson & Rasco, 2000).

Emulsifying properties of FPH are strongly affected by their solubility (Benjakul et al., 2014). Peptides with low molecular weight (LMW) owing for high solubility, which immediately disperse and attach to the interface. Nevertheless, these smaller peptides are less capable of reducing the interfacial tension, compared to larger peptides, primarily because of unfolding and reorganization at the interface (Patil & Benjakul, 2019). Consequently, this causes speedy flocculation and subsequently affects the stability protein layer. The molecular weight (MW) distributions of FPH play an important role in emulsion stabilization. Peptides generated through limited hydrolysis exhibited superior emulsifying and foaming properties compared to those subjected to extensive hydrolysis (Mazorra-Manzano et al., 2012).

FPH with LMW peptide fractions enhanced water holding capacity, since they were highly hydrophilic in nature (Halim et al., 2016). Fat binding capacity can be influenced by the enzyme-substrate specificity, DH and bulk density of proteins (Villamil et al., 2017). Two FPH from *Rastrineobola argentea* had the decreased fat binding ability when the peptide chain length upsurged (Mbatia et al., 2014).

Moreover, functional properties of protein hydrolysate were further modified by formation of different complexes, such as protein-polysaccharide (Xie et al., 2023), protein-polyphenols (Patil et al., 2023), etc. The influences of chitosan and rice protein hydrolysate (CH/RH) ratio and oil-water ratio on the emulsification efficiency, as well as the impact of RH on the formation of CH/RH complex coacervates was investigated by Xie et al. 2023. CH/RH at a 1:1 ratio is more effective in enhancing the interfacial adsorption and emulsification stability than CH or RH alone, especially at 50% oil volume fraction.

Bioactivities

The bioactivities of FPH or peptides are linked to their structural properties, amino acid composition, and sequences. FPH derived from the enzymatic hydrolysis of fish processing by-products have been demonstrated to be a rich source of bioactive peptides, known for their beneficial effects on consumer health (Bougatef et al., 2010; Ren et al., 2008). Nevertheless, the choice of protease in the enzymatic hydrolysis process plays a crucial role. It can lead to significantly different

characteristics in the resulting peptides, particularly in terms of their bioactivity. The use of multi-step hydrolysis is an effective method to enhance the bioactivities of peptides (Phanturat et al., 2010). The proteases sequence in multi-step hydrolysis also influences the bioactivities of the resultant peptides (Klompong et al., 2007).

Bioactive peptides exhibit a diverse range of biological functions, such as antioxidant, antihypertensive, antimicrobial activities, etc. Among these, the most extensively documented bioactivities associated with FPH are antioxidant and antihypertensive properties (Zamora-Sillero et al., 2018). Some peptides exhibited multiple bioactivities, both antioxidant and antihypertensive activities such as a single peptide from FPH derived from *Merluccius productus* (Samaranayaka et al., 2010). Additionally, peptide fractions obtained from tuna gelatin hydrolysate prepared by Alcalase had both antimicrobial and antioxidant properties (Guillén et al., 2010).

Antioxidant peptides derived from fish processing by-products tend to have a LMW and are predominantly produced through enzymatic hydrolysis. Type of protease is a crucial factor affecting the antioxidant activity. Papain is the most frequently used enzyme for FPH production (Cunha & Pintado, 2022). Studies were primarily focused on the ability of these peptides from FPH to scavenge hydroxyl, peroxyl, DPPH and ABTS radicals, with relatively fewer evaluating their protective effects on DNA and Oxygen Radical Absorbance Capacity (ORAC) activity (Cunha & Pintado, 2022). Some researchers have noted that hydrophobic amino acids may enhance the efficiency of antioxidant peptides (Zamora-Sillero et al., 2018). Additionally, different marine sources yield antioxidant peptides with varying capacities to scavenge free radicals. These peptides often contains amino acids like lysine, tyrosine, proline, and histidine (Wang et al., 2008). Antioxidant activities of selected FPHs from several fish species are tabulated in Table 1.

The inhibition of Angiotensin-Converting Enzyme (ACE) has emerged as a primary target in hypertension treatment. Several peptides have ACE-inhibiting activity, acting as natural hypotensive ACE inhibitors due to their high competitive affinity with the ACE active site (Lee et al., 2010). ACE-inhibiting peptides have been isolated from fish processing by-products such as skipjack roe (Intarasirisawat et al., 2013), salmon pectoral fin (Ahn et al., 2012), Atlantic salmon skin (Gu et al., 2011), tuna frame (Lee et al., 2010), sardinelle by-products (Bougatef et al., 2008), yellowfin sole frame (Jung et al., 2006b), Alaska pollock skin (Byun & Kim, 2001) and Tub gurnard skin (Bougatef et al., 2023).

Antimicrobial peptides are also prevalent in FPH, typically containing amino acids less than 50 with MW below 10 kDa (Sinaga et al., 2023). These peptides play a crucial role in natural defenses and not only have the ability to kill microorganisms but also modulate inflammatory responses (Yeaman & Yount, 2003). Nearly all fish antimicrobial peptides functions against both Gram-positive and Gram-negative strains (Najafian & Babji, 2012). Additionally, Ennaas et al. (2015) demonstrated that FPH from mackerel by-products exhibited antibacterial activity against Gram-negative (*E. coli*) and Gram-positive (*L. innocua*) strains. Hasani et al. (2022) investigated the antioxidant, anti-cancer and antimicrobial properties of hydrolyzed Indian mackerel protein (*Rastrelliger kanagurta*) using two enzymes, alcalase and flavourzyme. It was found that highest antioxidant, anticancer and antimicrobial properties were observed in FPH produced by alcalasefor 30 min.

These antimicrobial peptides derived from fish and fish processing byproducts hold great promise for new antibiotic advancement in both pharmaceuticals and food industries (Kim & Wijesekara, 2010).

Furthermore, FPH has a wide range of bioactive properties including antiatherosclerosis, anticancer, anticoagulant, antidiabetic, calcium binding, and anti-inflammatory activities (Cunha & Pintado, 2022; Gao et al., 2021; Zamora-Sillero et al., 2018). The bioactivities of selected FPHs from different fish species are given in Table 2. FPH derived from *Liza abu* has anti-cancer properties against colon cancer cells and Hela cancer cells (Shahosseini et al., 2022). Taroncher et al. (2023) reported that natural antioxidants (vitamins C and E, quercetin, and resveratrol) improved the cell proliferation of FPH in Caco-2 cells. FPH derived from *Trachinotus ovatus* showed significant antidiabetic effects, which alleviated body weight loss, polyphagia, blood glucose elevation and insulin secretion decline in diabetic mice (Wan et al., 2023).

Bitterness and Debittering

Bitterness is a significant challenge that impacts the overall sensory acceptability of FPH. Bitterness in FPH is attributed to the presence of hydrophobic amino acids, including Leucine (Leu), Valine (Val), Tyrosine (Tyr), Isoleucine (Ile), Phenylalanine (Phe), and Tryptophan (Trp). Some factors such as enzyme types, DH, and MW also determine bitterness of FPH (Zhou et al., 2023). The hydrolysis process often exposes the concealed hydrophobic amino acids, contributing to the bitterness (Idowu & Benjakul, 2019). Peptides with bulky hydrophobic groups towards their C-terminal have been identified as the primary contributor to bitterness (Sinthusamran et al., 2020b). The average free energy (Q values) necessary for the transition of amino acid chains from ethanol to water has been used as a bitterness index. Bitter peptides have Q value greater than 1,400 kcal/mole with MW below 6 kDa, while non-bitter peptides exhibit Q value lesser than 1,300 kcal/mole. This practical relationship between average hydrophobicity and bitterness is termed as Q rule. The rule related with average hydrophobicity is implemented for bitterness prediction (Idowu & Benjakul, 2019).

Table 1. Antioxidant activities of selected FPHs from different fish species and their isolated peptides.

Fish species and body part used for FPH	Enzymes used for FPH production	Antioxidant activities exhibited	Hydrolysis condition	References
Muscle from Mahseer (*Tor tambroides*)	Papain and bromelain	Antioxidant activities and antibacterial activities	Papain and bromelain: pH 7.0 and Temperature 50 °C	Sinaga et al. (2023)
Bighead Carp (*Hypophthalmichthys nobilis*)	Flavourzyme	Antioxidant activities	pH 6.0, Temperature 50 °C and Time 60 min	Alahmad et al. (2023)
Salmon (*Salmo salar*) and mackerel (*Scomber scombrus*) heads and backbones	FoodPro PNL	Antioxidant activities	pH 7.0, Temperature 50 °C and Time 60 min	De la Fuente et al. (2023)
Featherback (*Chitala ornata*) skin	Alcalase	Antioxidant activities	pH 7.5, Temperature 55 °C and Time 5 h	Vo et al. (2023)
Liza abu muscle	Alcalase and flavourzyme	DPPH free radical scavenging activity, ferric reducing power, Fe^{2+} chelating activity, and ABTS free radical scavenging activity	Alcalase and flavourzyme: pH 7 and pH 8.5 Temperature 58 °C	Shahosseini et al. (2022)
Skipjack tuna (*Katsuwonus pelamis*) roe	Flavourzyme	Antioxidant activities	pH 7.5, Temperature 45 °C and Time 8 h	Wang et al. (2022)
Klunzinger's mullet (*Liza klunzingeri*)	Alcalase	Antioxidant activities	pH 8.0, Temperature 50 °C and Time 3 h	Rabiei et al. (2022)
Atlantic codfish (*Gadus morhua*) frames	Alcalase	Antioxidant and antihypertensive activities	Alcalase: pH 8.35 Temperature 56.8 °C Time 3 h	Rodrigues et al. (2021)
Pacific thread herring (*Opisthonema libertate*)	Alcalase® 2.4 L and subtilisin A	ABTS, FRAP, and DPPH radical scavenging activity	Alcalase and subtilisin A pH 9.0 and Temperature 55 °C Time 1h	Martínez-Montaño et al. (2021)
Snakehead fish (*Channa striata*) viscera	Papain	DPPH and ABTS radical scavenging activity	Papain: pH 7.0 and Temperature 55 °C	Agustin et al. (2021)
Frame of salmon (*Salmon salar*)	Alcalase and papain	DPPH radical scavenging ability, ABTS, ferrous reducing antioxidant power (FRAP), metal chelating activity and oxygen radical antioxidant capacity (ORAC)	Alcalase: pH 8.0 Temperature 60°C Time 15 min Papain: pH 7.0, Temperature 40°C Time 15 min	Idowu et al. (2019)
Muscle of *Nemipterus hexodon*	Pepsin from skipjack tuna	DPPH radical scavenging activity, ABTS and FRAP	Pepsin extract: pH 2 Temperature 50°C Time 15 min	Nalinanon et al. (2011)
Scale gelatin from *Oreochromis niloticus*	Alcalase, Pronase E, trypsin and pepsin	DPPH radical scavenging activity, hydroxyl radical scavenging activity and superoxide radical anion scavenging activity	Alcalase, Pronase E & trypsin: pH 8.0 Pepsin: pH 2 Temperature 60°C Time 4 h	Ngo et al. (2010)
Viscera from *Parastromateus niger*	Pepsin, trypsin and ά-chymotrypsin	DPPH radical scavenging activity, FRAP and metal chelating activity	Pepsin: pH 2.5 trypsin & α-chymotrypsin: pH 6.5 Temperature 37°C Time 2 h	Jai Ganesh et al. (2011)
Meat of *Misgurnus anguillicaudatus*	Papain	DPPH radical scavenging activity, linoleic acid autoxidation inhibition activity, hydroxyl radical scavenging activity, and CU^{2+} ion chelating activity	Papain: pH 7 Temperature 55°C Time 4 h	You et al. (2010)
Muscle from *Decapterus maruadsi*	Flavourzyme	DPPH radical scavenging activity, FRAP and metal chelating activity	Flavourzyme: pH 8 Temperature 50°C Time 1 h	Thiansilakul et al. (2007)
Body meat of *Selaroides leptolepis*	Alcalase and flavourzyme	DPPH radical scavenging activity, FRAP, and reducing power activity	Alcalase: pH 8.5 Temperature 60°C Flavourzyme: pH 7 Temperature 50°C DHs: 5, 15 & 25%	Klompong et al. (2007)
Backbones of Tuna	Alcalase, ά-chymotrypsin, Neutrase, papain, pepsin and trypsin	DPPH radical scavenging activity, lipid peroxidation inhibition activity, superoxide radical scavenging activity and hydroxyl radical scavenging activity	Alcalase: pH 7, Temperature 50°C Trypsin, α-Chymotrypsin & Neutrase: pH 8.0, Temperature 37°C Pepsin: pH 2.0, Temperature 37°C Papain: pH 6.0, Temperature 37°C	Je et al. (2007)
Protamine derived from Salmon (*Salmon salar*)	Pancreatin	DPPH radical scavenging activity, hydroxyl radical scavenging activity and superoxide anion radical scavenging activity	Pancreatin: pH 8.5 Temperature 37°C Time 5h	Wang et al. (2008)

Table 2. Bioactivities of selected FPHs from different fish species.

Fish species and body part used for FPH	Bioactivities	Reference
Silverfish *(Trachinotus ovatus)* muscle protein	Antidiabetic	Wan et al. (2023)
Tub Gurnard *(Chelidonichthys lucerna)* Skin	Antihypertensive	Bougatef et al. (2023)
Yellowfin tuna frame (*T. albacares*)	Myeloperoxidase inhibitory activity	Cai et al. (2022)
Liza abu muscle protein	Anti-cancer	Shahosseini et al. (2022)
Farm Rainbow Trout	Antihypertensive	Pérez-Escalante, et al. (2022)
Pacific thread herring (*Opisthonema libertate*) Stickwater	Antihypertensive	Martínez-Montaño et al. (2021)
Meagre (*Argirosomus Regius*) bones, gills and skin	Antiproliferative	Kandyliari et al. (2020)
Pacific hake (*Merluccius productus*)	Neuroprotective effect	(Lee et al., 2019)
Blue whiting (*Micromesistius poutassou*) muscle	Antidiabetic	Harnedy et al. (2018)
Rainbow trout viscera	Antibacterial	Wald et al. (2016)
Lanternfish (*Benthosema pterotum*)	Neuroprotective effect	Chai et al. (2016)
Rohu (*Labeo rohita*) roe	Antiproliferative	Chalamaiah et al. (2015)
Salmon pectoral fin	Anti-inflammatory	Ahn et al. (2015)
Half-fin anchovy (*Setipinna taty*)	Antiproliferative	Song et al. (2014)
Norwegian salmon	Anti-atherosclerotic	Parolini et al. (2014)
Goby fish muscle protein	Anticoagulant	Nasri et al. (2012)
Tilapia (*Oreochromis mossambicus*)	Antitumor	Chang et al. (2011)
Tuna frame protein	Antihypertensive	Lee et al. (2010)
Tuna skin	Antimicrobial	Guillén et al. (2010)
Sardinelle heads and viscera	Antihypertensive	Bougatef et al. (2008)
Hoki (*Johnius belengerii*) frame	Calcium binding	Jung and Kim (2007)
Loach *(Misgurnus anguillicaudatus)*	Antimicrobial	Dong et al. (2002)

Various methods including liposomal encapsulation, plastein reaction, enzymatic hydrolysis with exopeptidase, chromatographic separation, and extraction with cyclodextrin, activated carbon treatment, alcohol and Maillard reaction have been discovered to reduce or remove the bitterness of FPH (Idowu & Benjakul, 2019; Sharma et al., 2023a; Sharma et al., 2023b; Singh et al., 2020; Sinthusamran et al., 2020b). These approaches effectively reduce bitterness and enhance the overall taste profile. However, it is important to note that these methods may lead to structural changes and potential loss of certain peptides (Sharma et al., 2023b).

Applications of FPH

Bioactive peptides find applications across diverse industrial sectors including food, cosmetics, and pharmaceuticals. In the food industries, many dairy merchandise fortified with bioactive peptides have been already marketed (Nirmal et al., 2022; Sasidharan & Venugopal, 2020). FPH has been explored for integration into a range of products including fish meat items, cereals, crackers, and desserts. Several brands have successfully introduced products containing these hydrolysates (Chalamaiah et al., 2012). Antioxidant peptides, crucial for retarding lipid oxidation, have gained significant attention in food packaging, as seen in products like sauces, meats, fish, and nuts (Gómez-Estaca et al., 2014). In Thailand, Japan and other countries, various products featuring FPH and peptides are available in the market, advertised as functional foods like powdered soups, dietary supplements, beverages, etc. Over sixty peptides have received FDA approval for medicinal application, with numerous preclinical studies underway (Rivero-Pino et al., 2020).

Food Applications

Protein hydrolysates have attracted substantial interest in recent years because of their improved functionalities and high nutritional value resulted from the generation of free amino acids and small peptides through hydrolysis (Benjakul et al., 2014). FPH have been documented as a superior source of protein for human nutrition due to their balanced amino acid composition and high absorption in gastrointestinal tract (Cunha & Pintado, 2022). High solubility of FPH across a wide range of pH renders them highly versatile for various food applications (Dinakarkumar et al., 2022).

FPH has been extensively used in food products. Their excellent interfacial properties make them become valuable emulsifying agents in products such as dressings, margarine, and meat batter. They also contribute to the creation and stabilization of foam-containing products like mousse, meringues, and whipped cream (Nirmal et al., 2022). Khan et al. (2003) employed FPH as a cryoprotectant to inhibit denaturation of proteins in lizardfish surimi during frozen stage. Damodaran (2007) showed the ability of gelatin hydrolysate ability to hinder ice crystallization in ice cream. Additionally, FPH from mince obtained from gutted and headed red hake or frame from red hake produced with Flavozyme at natural pH (6.8) and a fish /water ratio of 5:2 were employed as a natural flavor stock (Imm and Lee 1999). Beverage fortified with FPH from blue whiting (*Micromesistius poutassou*) (Egerton

et al., 2018), instant coffee added with galactose-fish skin gelatin hydrolysate (Karnjanapratum & Benjakul, 2017), and protein-rich ice cream fortified with FPH from saithe (*Pollachius virens*) (Shaviklo et al., 2011) have been documented. Bighead carp (*Hypophthalmichthys nobilis*) hydrolysate was prepared using alcalase and hydrolytic enzymes extracted from *Oncorhynchus mykiss*. The resulting hydrolysate could be used in the development of active packaging as antimicrobial and antioxidant components (Naghdi et al., 2023).

Pharmaceutical and Nutraceutical Applications

The potential of FPH in lowering the risk of various illnesses has been confirmed, such as reducing plasma cholesterol levels associated with cardiovascular diseases and lowering cell proliferation in cell lines of human breast cancer (Picot et al., 2006). FPH from various fish species demonstrated higher inhibition of cell lines in human breast cancer, MCF-7/6 and MDA-MB-231 (Picot et al., 2006). Furthermore, calcium-binding bioactive peptides obtained from Alaska pollock (*Theragra chalcogramma*) via pepsin hydrolysis and hoki (*Johnius belengerii*) frame offer a potential alternative for individuals with lactose intolerance, with calcium rich foods or calcium added fruit juices serving as dairy substitute (Jung & Kim, 2007). Peptides from FPH have also demonstrated the ability to enhance calcium absorption (Jung et al., 2006a; Kim & Mendis, 2006a). Moreover, clinical studies recommend that the ingestion of FPH can alleviate pain in osteoarthritis patients (Wu et al., 2004). Collagen hydrolysate combined with a calcitonin-rich diet was more effective in reducing breakdown of bone collagen compared to a calcitonin-rich diet alone (Moskowitz, 2000). Fish collagen hydrolysate ingestion has also been augmented with the synthesis of extracellular matrix macromolecules by chondrocytes (Bello & Oesser, 2006). Additionally, fish skin collagen hydrolysates from salmon and trout have been found to influence metabolism and lipid absorption in rat models. (Saito et al., 2009). FPH from Asian seabass skin also showed wound healing properties bone strengthening and skin nourishment effects in *in vitro* cell line studies (Benjakul et al., 2018a; Chotphruethipong et al., 2021). While FPH presents numerous potential benefits, there are currently limited commercial products containing FPH existing for human consumption. Nonetheless, products with health-promoting properties derived from aquatic resources, mainly FPH, hold promising potential in the market.

Production and Characteristics of Fish Biocalcium

Fish bone and scale, which are important raw material for biocalcium, contain non-collagenous constituents, including meat proteins and lipids, etc. Pretreatments with single or several steps are applied to diminish the undesirable components before processing to augment the purity of produced fish biocalcium. The first step of pretreatment is the remaining meat removal and thorough cleaning prior to process in further pretreatments (Figure 3) (Benjakul et al., 2017b). Softening and reduction of material size are also important for facilitating the efficacy of fish biocalcium production. Pretreatment with alkaline solution such as sodium hydroxide (NaOH) is normally used for removal of non-collagenous protein, pigments and lipids. Increasing alkaline concentration and pretreatment time are able to augment proteins solubilization or unfolding. NaOH at concentration of 2 M has been used for pretreatment of fish bones and scales (Benjakul et al., 2017b; Nilsuwan et al., 2023). Benjakul et al. (2018b) cleaned the pre-cooked skipjack tuna bone with high pressure water jet at 120 bar for 2 min, followed by treatment with NaOH solution (2 M) at temperature of 50 °C for 30 min with bone/solution ratio of 1:10 (w/v). The aforementioned alkaline pretreatment is able to remove the remaining meat and lipids, leading to the augmenting Ca and P contents in biocalcium powder (Benjakul et al., 2018b). Benjakul et al. (2017a) found that biocalcium powders from pre-cooked tongol (*Thunnus tonggol*) and yellowfin (*Thunnus albacores*) tuna bones, pretreated with NaOH solution (2 M) at temperature of 50 °C for 30 min with bone/solution ratio of 1:10 (w/v), had Ca contents of 26.76 and 26.73% and P contents of 12.7 and 12.74%, respectively. The Ca:P ratio was nearby 2:1, indicating compatibility to that found in human bones. Idowu et al. (2020) found that biocalcium powder from salmon (*Salmo salar*) frame pretreated with 2 M NaOH for 40 min at 50 °C with a bone/solution ratio of 1:10 (w/v) had 27.32% Ca and 13.22% P with the Ca/P mol ratio of 1.60. The uses of NaOH solutions (0.5 - 1.0 M) and ethanol (40 - 60%, v/v) for processing of bones and skeletons from Indian oil sardine (*Sardinella longiceps*) and small-headed ribbonfish (*Trichiurus savala*) yielded the powders with calcium content ranging from 18.91% to 32.73%. (Logesh et al., 2012). The NaOH solution (2 M, 10 min) can also remove proteins effectively and minimize the loss of collagen as indicated by high hydroxyproline content in biocalcium from Asian sea bass (*Lates calcarifer*) scales (Nilsuwan et al., 2023). In addition, the alkaline pretreatment process can diminish undesirable volatile compounds in biocalcium. Idowu et al. (2019a) documented that lower abundance of volatile compounds such as 2-propanone and 2-butanone was obtained for biocalcium powders from salmon frame after treatment with 2 M NaOH for 40 min at 50 °C with a bone/solution ratio of 1:10 (w/v). However, strong condition should be obviated. Fish frame leftover from filleting process, still contains flesh. This meat rich in protein can be removed by enzymatic hydrolysis (Idowu et al., 2019a). The enzymatic hydrolysis using protease is widely implemented to separate non-collagenous protein from fish frame or trimming (Figure 3) (Idowu et al., 2020; Sinthusamran et al., 2020a). The enzymatic

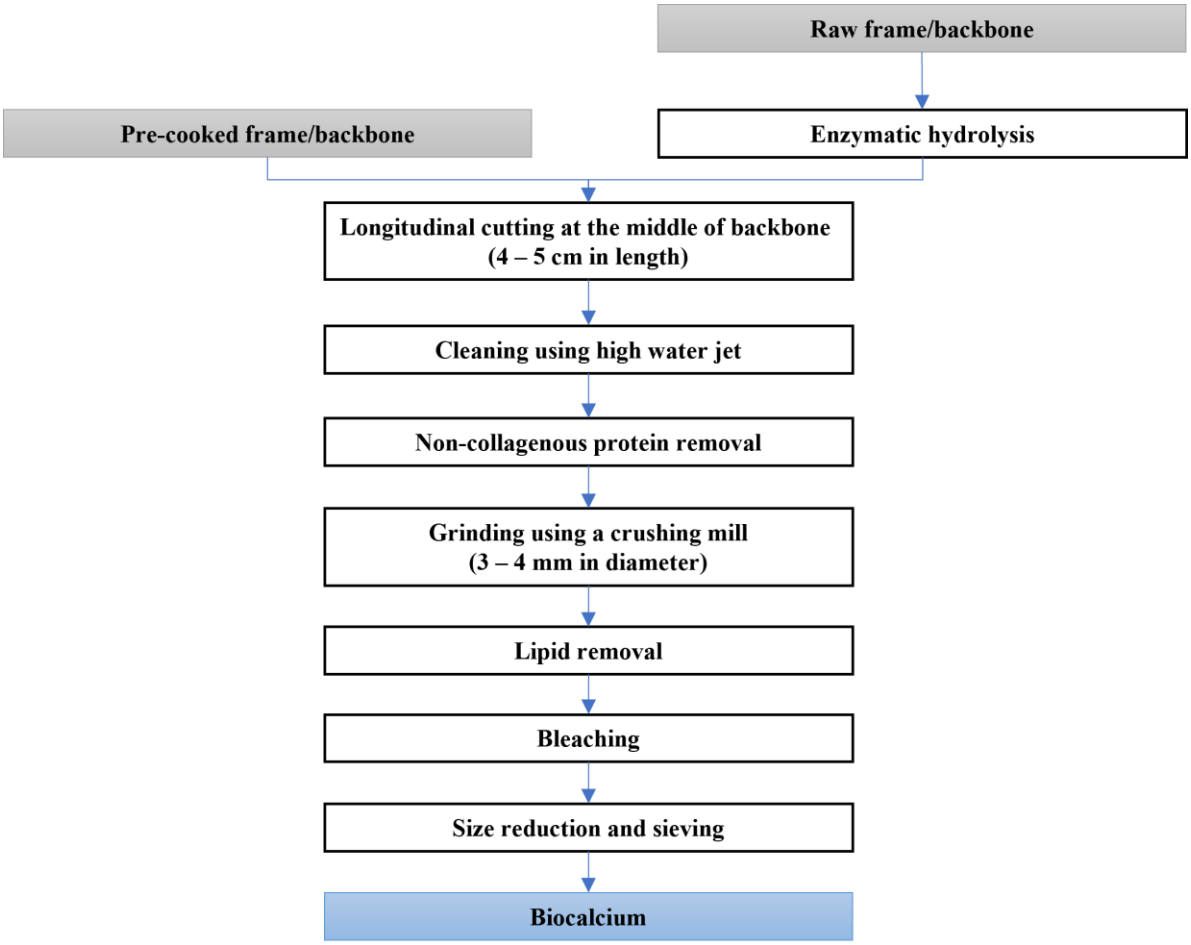

Figure 3. Flowchart for production of biocalcium from pre-cooked and raw frame/backbone

hydrolysis is able to hydrolyze the myofibrillar proteins into hydrolysate solution, which can be separated from the bone solid easily (Idowu et al., 2020).

Regarding fat removal, the solvents are commonly used for treatment of raw materials. Hexane is generally employed for fat removal from biocalcium, which can reduce fat in raw material effectively (Benjakul et al., 2017a; Benjakul et al., 2017b; Idowu et al., 2020). Lipid content of biocalcium powders from pre-cooked tongol tuna bone, pre-cooked yellowfin tuna bone, and salmon frame bone defatted with hexane were 0.26, 0.19 and 0.33%, respectively (Benjakul et al., 2017a; Idowu et al., 2020). Moreover, mixed solvents can also be used for defatting process. Pudtikajorn et al. (2023) documented that biocalcium powder with lipid content of 0.09% was obtained from skipjack tuna eyeball scleral cartilage defatted with the mixture of hexane and isopropanol (1:1) for 6 cycles. The efficiency of defatting process with the hexane and isopropanol mixture (1:1) was 99.63% for lipid reduction (Pudtikajorn et al., 2023).

Whiteness of biocalcium powder is related to customer acceptability. Bleaching using sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) alone or combination can improve the whiteness of biocalcium. *L**-value of biocalcium powders from pre-cooked tongol and yellowfin tuna bones bleached with 2.5% (v/v) NaOCl for 30 min followed by 2.5% (v/v) H_2O_2 for 60 min was 93.92 and 92.52, respectively (Benjakul et al., 2017a). Moreover, the highest *L**-value (92.26) was observed when H_2O_2 alone was used as bleaching agent for production of Asian sea bass (*Lates calcarifer*) backbone biocalcium (Wijayanti et al., 2021b).

Grinding is another crucial step for making fish biocalcium powder. Since the bone matrix is strong and compact in structure, heating under high pressure could assist softening of bone. This could facilitate the size reduction process. Softening process can enhance grinding efficiency of raw materials into powders. Wijayanti et al. (2021a) documented that high pressure heating with the aid of autoclave for 90 min lowered hardness of Asian sea bass backbone from 57,000 g force to around 3000 g force. Treated samples could be ground into the powder with ease. Choi et al. (1998) reported that the autoclaving at 40 min resulted in Alaska pollock (*Theragra chalcogramma*) and cod (*Gadus chalcogrammus*) powders having appreciable soluble calcium content. Longer time of autoclave had no impact on the mineral yield and soluble calcium ratio (Choi et al., 1998). Apart from steaming, different acids have been used for treatment of Alaska pollock frame prior to extraction of calcium. In general, coarse ground particles can provide sandiness of mouth feel. Ball milling process has been widely used for production of fish biocalcium from tuna bone, salmon bone and

seabass bone (Benjakul et al., 2017b; Idowu et al., 2020; Wijayanti et al., 2021b). Particle size of biocalcium lower than 75 µm was generally obtained when the biocalcium powders from tuna eyeball scleral were sieved using screen having mesh no. 200 (Pudtikajorn et al., 2023).

Bioavailability of Fish Biocalcium

The exploitation of fish bone as functional materials is less, though it contains nutraceutical and bioactive molecules. This was due to its low solubility (Kim & Jung, 2012). The solubilization is required for the absorption of calcium in the body. Calcium salt is mostly precipitated in the ileum caused by alkaline conditions, however some calcium ions are present in solution (Kim & Jung, 2012). Typically, calcium is rarely absorbed in the intestine. Gastric contents after calcium is dissolved in the stomach move into the small intestine, where calcium is absorbed. Bicarbonate (HCO^{-3}) is secreted into the intestine to neutralize the contents. The concentration of calcium available for absorption in the jejunum and ileum, which maintain a pH of approximately 7, is therefore expected to be lower than that dissolved in the stomach (Goss et al., 2010). The absorption of calcium is complicated determined by local solubility, the sojourn time in the particular intestinal segment, and the rate of transepithelial movement (Duflos et al., 1995). Malde et al. (2010) reported that young healthy men had absorption of calcium from cod and salmon bones at 21.9 and 22.5%, respectively. Tuna bone powder exhibited higher calcium availability (53.93%) than most calcium salts including calcium carbonate, calcium lactate, calcium citrate and milk powder (Nemati et al., 2016). Based on the the amount of calcium transported across the Caco-2 monolayer, Idowu et al. (2019a) documented that bioavailability of biocalciums obtained from salmon frames treated with and without alkaline solution were 43.02 and 38.13%, respectively. Hake fish bone (HFB) powder exhibit non cytotoxic effect on the cell line (SaOS-2 cells) (Flammini et al., 2016). HFB powder also had comparable efficacy to commercial calcium tablets on rat bone mineralization (Flammini et al., 2016). Additionally, peptides act as calcium carrier, which enhance calcium absorption at small intestine (Goss et al., 2010). Jung et al. (2006a) found that peptides could bind with calcium and enhance solubility of calcium salts at neutral pH in *In vitro* study. The ovariectomized rats supplemented with calcium binding peptides had higher calcium retention and lower loss of bone mineral (Jung et al., 2006a). Oligophosphopeptides, which are generated via enzymatic hydrolysis of hoki (*Johnius belengerii*) frames by heterogeneous enzyme extracted from the intestine of bluefin tuna, could help solubilization of calcium and improve calcium bioavailability (Jung et al., 2006a).

Fortification of Biocalcium in Drink and Foods

Fish biocalcium has been applied as dietary supplement such as commercial tablets. Fish biocalcium can also be incorporated or fortified in various foods. Tuna bone peptide (TBP) and tricalcium phosphate (TCP) were added into bakery products i.e. cookies and bread as a calcium supplement (Nemati et al., 2016). A higher *in vitro* bioavailability was found for cookies fortified with TBP, compared to that of bread fortified with TBP. Cookies fortified with TBP and TCP had calcium bioavailability at 38.9% and 39.5%, respectively, while bioavailability of bread containing TBP and TCP were 36.7% and 37.4%, respectively. Cookies and bread containing TBP or TCP had similar overall acceptability scores in the range of 7.76 – 7.80 and 7.30 – 7.48, respectively (Nemati et al., 2016). In addition, Idowu et al. (2019a) prepared biocalcium powders from salmon (*Salmo salar*) frames via hydrolysis with alcalase prior to treatment with NaOH solution (2 M, 50°C, 40 min). The obtained biocalcium (BC) was fortified in whole wheat crackers along with protein hydrolysate (PH) at wheat flour substitution level of 16.67%. Fortification of BC and PH powders had a profound impact on compositions of resulting whole wheat cracker. Increases by 17- and 8-fold were obtained for calcium and phosphorus contents, respectively, compared to those found in the control (without BC and PH). This was associated with higher ash content of the BC/PH fortified sample (Idowu et al., 2019a). Furthermore, Karnjanapratum & Benjakul (2018) developed coconut oil based cookie fortified with tuna bone biocalcium. The coconut oil was used to substitute the shortening at 65% and 100%. Biocalcium from tuna bone was added into the batter at 8.5%, 12.0% and 15.5% (w/w). Addition of biocalcium powder at 12.0% had no effect on sensory properties of cookie containing coconut oil as shortening replacer. The developed cookie showed higher calcium and phosphorus contents as well as higher saturated fat, ash, and protein contents but lower energy value, compared to that of cookie without biocalcium and coconut oil. The significant increases in protein and ash contents in developed cookie were related to high protein (24.26%) and ash (72.20%) of tuna biocalcium powder (Benjakul et al., 2017b). It is worthnoting that tuna bone biocalcium powder was effectively used for calcium fortification in cookie. Nevertheless, the replacement of shortening by coconut oil showed higher content of saturated fat with lower mono- and poly-unsaturated fats in cookie. In general, coconut oil is abundant in medium chain saturated fatty acids (MCFAs) such as lauric acid (C12:0) and myristic acid (C14:0) (Karnjanapratum & Benjakul, 2018).

Biocalcium can be used in surimi products to increase nutritional value and improve surimi gel properties. Wijayanti et al. (2021c) incorporated biocalcium powder prepared from bone of Asian sea bass (ASBB) in threadfin bream (*Nemipterus* sp.) surimi gel at levels of 0 – 10% (w/w). The addition of 8% (w/w)

ASBB possessed high breaking force and high overall likeness score in resulting surimi gel. The 8% (w/w) ASBB added surimi gel also had higher ash, calcium, and phosphorus content as well as higher compactness and finer microstructure, compared to the control without ASBB (Wijayanti et al., 2021c). Additionally, the addition of biocalcium along with other calcium salts can enhance the improvement of surimi gel properties. Wijayanti et al. (2021d) prepared surimi gel from threadfin bream added with fish bone biocalcium (8%) in combination with calcium chloride (CC) or calcium lactate (CL) at different levels (0.1–0.6%, w/w). The highest breaking force along with the lowest expressible moisture content and higher whiteness were obtained for gel added with 8% biocalcium together with 0.6% CL. The aforementioned gel also had elastic texture as confirmed by the highest G′ and denser microstructure (Wijayanti et al., 2021d). Furthermore, fish tofu produced from threadfin bream surimi fortified with 7.5% (w/w) skipjack tuna eyeball scleral cartilage biocalcium (SCBC) also had a denser microstructure with greater ash and calcium contents than the control without SCBC incorporation (Pudtikajorn et al., 2022).

Biocalcium has also been fortified into food emulsion products. Asian sea bass bio-calcium (ASBC) fortified mayonnaise had the enhanced firmness, consistency and cohesiveness with augmenting ASBC levels (0%–10%). Mayonnaise sample added with ASBC showed greater G′, G″, viscosity and shear stress value. The increases in lightness (L^*) and total color difference (ΔE^*), but decreases in a^* and b^*-values were found in mayonnaise with increasing ASBC amount. Lower creaming index of mayonnaise added with ASBC was noted as indicated by lower droplet size and higher zeta potential. Dense and small droplet sizes were observed for mayonnaise fortified with ASBC at 2.5 with higher protein, ash and calcium content than the control (without ASBC) (Wijayanti et al., 2023). Biocalcium was also added into fish spread. Wijayanti et al. (2022a) prepared the Indian mackerel fish spread incorporated with Asian sea bass bone biocalcium (BC) (0–10%) and potato starch (2–4%). The obtained fish spreads were generally increased in firmness, consistency and cohesiveness as BC level was augmented (Wijayanti et al., 2022a). Shear thinning flow behaviour was generally found in all fish spreads. Fish spread containing 10% BC and 4% starch had higher acceptability without sandy mouth feel. Fish spread had higher ash content with less moisture, protein and lipid contents than those without BC. After *in vitro* gastrointestinal tract system of fish spread fortified with 10% BC, calcium solubility was augmented and higher solubility of calcium was obtained, than that of BC (Wijayanti et al., 2022a).

Nevertheless, biocalcium can also be added into fruit juice. Wijayanti et al. (2022b) prepared the 4% (w/v) soluble Asian sea bass bone bio-calcium (SBC) in 9% (w/v) citric acid prior to fortify into sterilized apple juices. The apple juice samples had varying in pH, turbidity, brown color and calcium content. Lower pH along with higher turbidity, brown color and calcium content was observed as the levels of SBC was increased. No impact of SBC fortification on likeness score for all quality attributes as well as sandy mouthfeel. Nevertheless, apple juice fortified with excessive SBC possessed lower flavor, taste and overall likeness. Calcium content of apple juice fortified with SBC up to 1% was augmented by 6.13-fold with no detrimental effects on sensory property (Wijayanti et al., 2022b).

Conclusion

Value-added products, especially functional ingredients or nutraceuticals can be produced from fish processing leftovers, which are generally wasted or used for production of low value products. For better utilization of bone or frame, they can be used for production of protein hydrolysate with bioactivities as well as biocalcium, which has higher availability with absorption. Both protein hydrolysate and biocalcium could be fortified in drink or food products, in which food quality could be improved. More importantly, the functional ingredient could play a major role in health promotion/benefit due to their nutraceutical property.

Ethical Statement

Ethics approval was not required for this research.

Funding Information

This study was funded by Prachayacharn Grant from Prince of Songkla University, Hat Yai, Thailand (Grant no. AGR6602079N). This research was also supported by the Postdoctoral Fellowship from Prince of Songkla University to Dr. Umesh Patil.

Author Contribution

Umesh Patil: Conceptualization, Visualization and Writing-original draft; Krisana Nilsuwan: Conceptualization, Writing-review and editing; Soottawat Benjakul: Supervision, Writing-review and editing. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors gratefully acknowledge the International Center of Excellence in Seafood Science and Innovation, Faculty of Agro-Industry, Prince of Songkla University for all support.

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