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Enzymatic hydrolysis combined to membranes for upgrading seafood by-products

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Abstract:

This review reports some interesting works on the recovery and fractionation by membrane techniques of marine compounds resulting from hydrolysis of fish by-products such as proteins or peptides, lipids or fatty acids, and carbohydrates. Ultrafiltration (UF) appears as the most widely used technique for such applications.
It must be noted that only a few works deal with the optimization or the enhancement of processes for the recovery of added value molecules. Indeed, most of works use UF as a convenient and simple tool for solute fractionation, for this reason the influence of operating parameters on membrane separation efficiency is unfortunately seldom considered. When attention is paid in a study to the process development, it is specified in this review.

1. Introduction

Recently, more than 130 million tons of fishing products are produced worldwide [1]. Among the fishing products, a large part of fish is not used and can be recovered during production by fish traders, food-packers. The fish by-products represent 30 to 60% of whole fish. Beside fish, marine macroalgae also represent a resource insufficiently upgraded.
Up to now, the majority of by-products are upgraded in low added value products such as oil, meal, minced fish, and protein hydrolysates [2]. However, these by-products are rich in specific lipids, proteins, or polysaccharides which can find better upgrading owing to their bio-activities (anti-viral, anti-tumour, antioxidation, immunological regulation…) and nutritional properties (nutrient supplement).
The conventional extraction techniques of these molecules (distillation, solvent extraction, chemical hydrolysis…) are generally high energy or chemical consumers and lead sometimes to low extraction yields.
An emerging way for by-products upgrading is the extraction (concentration, purification and fractionation) of valuable molecules by membrane technology [3, 4, 5, 6]. Membrane operations can be used alone or combined with a bioreactor or other processes. They are safe, easy to implement, preserve structural integrity of molecules and require moderate energy. Moreover, membrane technology allows working in a continuous mode (reuse of enzymes, separation of enzymes / hydrolysate).
In the present chapter, a review on upgrading of fish by-products using simultaneously hydrolysis and membrane techniques is given. Emphasis is laid on protein, lipid and carbohydrate extraction.

2. Protein hydrolysates, peptides

Several recent experimental studies are presented below. Most of them concern the use of membranes (UF) to fractionate hydrolysates with biological activities. Other works are related to the improvement of the organoleptic quality or to the clarification and colour removal of hydrolysates. Finally some interesting works are presented on the coupling of enzymatic digestion with membranes for proteins and DNA extraction.

Ultrafiltration for fractionation of hydrolysates with biological activities

Most of works were carried out at the Department of Chemistry at the Pukyong National University in Pusan, South Korea. These works rely on a common experimental methodology that consists in passing a hydrolysate through a series of UF membranes (up to 5) from molecular weight ranging from 1 to 30 kDa and to compare the specific activity of each fraction with that of the initial hydrolysate, with the aim to identity the most active fractions. Hydrolysates from different substrates have been fractionated in this way: hot washing waters of fresh fishes [7], cod frame proteins [8], Alaska pollack frame proteins [4, 9], jumbo squid skin gelatin [10], giant squid muscle [11], hoki frame proteins [9, 12], and conger eel muscle [13]. Activities tested are most of the time antioxydant and antihypertensive ones, but radical scavenging activities and foaming or emulsifying properties have been also considered. Table 1 gathers for each work the activity tested, the molecular weight cut-off of the membranes used and the main conclusions. Most of works reported that highest antioxidant and antihypertensive activities were found in lower molecular weight fractions.
Similar works have been also reported by other teams (table 1). Neves *et al.* [14] studied the impact of enzyme source and hydrolysis conditions on the molecular weight distribution of a hydrolysate of brackish water minced fish and minced shrimps. Hydrolysates were fractionated by ultracentrifugation using Centricon concentrator equipped with membrane with MWCO of 30, 10 and 3 kDa to isolate four fractions (> 30 kDa, 30-10 kDa, 10-3 kDa et <

3 kDa). Amino acid profile of each fraction and the ratio between the concentration of branched chain and aromatic amino acids were evaluated with the aim to determine the fractions that could be useful for dietetic management of patients with chronic liver diseases. Peptides smaller than 3 kDa seem to be suitable for use in hypoallergenic formulas. Sumaya-Martinez [15] fractionated a shrimp frame hydrolysate by successive microfiltration (0.45 µm) and ultrafiltration (30 kDa, 5 kDa) steps. Three peptidic fractions were isolated (0.45 µm-30 kDa, 30-5 kDa, < 5 kDa) and the highest DPPH radical scavenging activity was linked to the fraction lower than 5 kDa. Finally, He *et al.* [16] compared angiotensin I converting enzyme (ACE) inhibitory activity and hydroxyl radical scavenging activity of shrimp hydrolysate filtrated or not through 3 kDa UF membrane.

Kim *et al.* [17] produced Alaska pollack skin gelatin hydrolysate using a *three-step recycling membrane reactor* to isolate fractions of defined molecular weights and to measure their antioxidative activity. The three reactors were equipped with hollow fibre UF membranes with decreasing molecular weight cut-off. In the first reactor gelatin was digested with Alcalase and the hydrolysate fractionated through a 10 kDa UF membrane before being hydrolysed with Pronase E in the second-step membrane reactor. The second hydrolysate was fractionated through a 5 kDa UF membrane and then hydrolysed with collagenase in the third-step membrane reactor. The third hydrolysate was fractionated through a 1 kDa UF membrane. Major peaks of the first, second and third hydrolysates were located at 6-8 kDa, 2-4 kDa and 0,5-2 kDa. The highest antioxidant activity was observed in the second step reactor (fraction 2-4 kDa), which exhibited about 58% inhibition of linoleic acid peroxidation.

Most of works aims only to refine a hydrolysate and to increase its specific activity in the perspective of industrial upgrading of byproducts to produce ingredients for human food or animal feed. However, hydrolysates are sometimes processed by a succession of separation steps including ultrafiltration and chromatography in order to isolate and identify one peptide with a high level activity. Do [18] isolated in this way ACE inhibitory peptides from mackerel muscle proteins, and the amino acid sequence of purified peptides was found to be Tyr-Val-Ala. Similar works are reported by Je *et al.* [4] and Je *et al.* [9] who isolated from an Alaska pollack frame protein hydrolysate a new hypertensive peptide (Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala) and a new antioxidative peptide (Leu-Pro-His-Ser-Gly-Tyr, M = 672 Da).

By the end, it should be noticed that Ruttanapornvareesakul *et al.* [19] evaluated the functional properties of a shrimp head protein hydrolysate whose manufacturing process included a stage of clarification with 30 kDa UF membrane (plane, Millipore®).

Table 1: Main investigations on fractionation by UF of fish or shellfish hydrolysates with biological activities or functional properties

References	Substrates	Activity or functional property	Membrane series	Main conclusions
Department of Chemistry at the Pukyong National University in Pusan, South Korea				
Yoon *et al.* [7] (1998)	Fresh fish hot washing waters (carp, snakehead, eel, Israeli carp)	Antioxidant	3 – 1 kDa	Highest activity in the fraction < 1 kDa for raw waters without hydrolysis, or in the 1-3 kDa fraction for hydrolysed waters
Jeon *et al.* [8] (1999)	Cod frame proteins	Emulsifying and foaming properties Antioxidant, antihypertensive[2]	30 – 10 – 5 – 3 kDa (Minitan®, Millipore)	Foam stability increased in 30 kDa and 10 kDa permeates Best emulsifying properties in the 10 kDa permeate at any pH Highest antioxidant activity in the 5 kDa permeate (twofold higher than the original hydrolysate) Highest antihypertensive activity in the 3 kDa permeate
Kim *et al.* [17] (2001)	Alaska pollack skin gelatin	Antioxidant[3]	Succession of 3 EBR[2] with hollow fiber UF membranes: 10 – 5 – 1 kDa A/G Technology (10 and 5 kDa) and Romicon (1 kDa)	Highest activity on the second reactor (fraction 2-4kDa)
Buyn *et al.* [21] (2001)	*idem*	Antihypertensive[1]	*idem*	Highest activity on the second reactor
Je *et al.* [4] (2004)	Alaska pollack frame proteins	Antihypertensive[1]	30 – 10 – 5 – 3 – 1 kDa	Purification and identification of a new antihypertensive peptide (Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala)
Je *et al.* [9] (2005)	*idem*	Antioxidant	*idem*	Highest antioxidant activity in the fraction < 1 kDa Purification and identification of a new antioxidant peptide (Leu-Pro-His-Ser-Gly-Tyr, M = 672 Da)
Mendis *et al.* [10] (2005)	Jumbo squid skin gelatine	Antioxidant[3]	10 – 5 – 3 kDa	Highest activity in the 3 kDa permeate
Rajapaske *et al.* [11] (2005)	Giant squid muscle	*idem*	*idem*	*idem*
Je *et al.* [20] (2005)	Hoki frame proteins	Antioxidant[3]	10 – 5 – 3 – 1 kDa	Highest activity in the 1 – 3 kDa fraction
Kim *et al.* [12] (2007)	*idem*	Radical scavenging	*idem*	*idem*
Ranathunga *et al.* [13] (2006)	Conger eel muscle	Antioxidant[3]	5 – 3 – 1 kDa	The four collected fractions have similar activities

Other works				
Do [18] (2000)	mackerel muscle proteins	Antihypertensive[1]	Succession of UF and chromatography steps	The most active peptides have been purified and sequenced (Tyr-Val-Ala)
Sumaya-Martinez [15] (2004)	Shrimp frame hydrolysate	DPPH Radical scavenging	MF 0.45 μm – 30 kDa – 5 kDa Plane membrane, Millipore® PVDF (0.45 μm) or regenerated cellulose (30 and 5 kDa)	Highest antihypertensive activity in the fraction < 5 kDa
Neves *et al* [14] (2005)	brackish water minced fish and minced shrimps	Hypoallergic and dietetic formulas	30 – 10 – 3 kDa Centricon concentrator	Peptides smaller than 3 kDa seem suitable for hypoallergenic formulas
He *et al.* [16] (2006)	Shrimp hydrolysates	Antihypertensive[1] Hydroxyl radical scavenging activity	3 kDa	Antihypertensive activity is fourfold higher in permeate than in the original hydrolysate Radical scavenging activity is significantly higher in the permeate than in the original hydrolysate (67.9 % against 42.4 %)

[1] Angiotensin Angiotensin I converting enzyme (ACE) inhibitory a
[2] Enzymatic Membrane Reactor
[3] Iinhibition of linoleic acid peroxidation

Ultrafiltration: a means to improve organoleptic quality of fractions

Bae *et al.* [22] studied the manufacturing of fermented and dried sauces from hydrolysate of underutilized fishes such as hair tail, gizzard shad, and kangdale and showed that 100 and 500 Da UF membranes were very efficient for bitter taste removing.
Park *et al.* [23] showed that the elimination of the peptides of high molecular weight by UF enhanced sweetness, umami, sourness and bitterness of the Vietnamese sauce of fish called Nuoc-Mâm. The peptides with small molecular weights (< 500 Da) due to prolonged fermentation of the sauce contributed to its complete taste.
Jao and Ko [24] recovered and concentrated, by UF and reverse osmosis, free amino acids and small peptides from the hydrolysis of proteins contained in tuna cooking juice to estimate the sensory profile of this condiment. The fractionation was performed on polysulfone membrane (AF-30-1812-T, 10 kDa, 0.2 m², AMT, San Diego) at ambient temperature up to a concentration factor equal to 6.

Membranes for the clarification and colour removal of hydrolysates

Ye and Xu [25] ultrafiltered a hydrolysate of degreased mussel powder in order to eliminate the colour, the proteins which were not hydrolysed and other macromolecules.
Liaset *et al.* [26] produced a salmon frame hydrolysate by an enzymatic hydrolysis combined with ultrafiltration. The phase separation allowed isolating insoluble, lipid, intermediate (emulsion) and aqueous fractions. The aqueous fraction was clarified on 100 kDa UF membrane (FP100, PCI, 2.65 m²). UF allowed reduction of lipid content from 16 grams by kilogram of dry sample to less than 1 gram by kilogram of dry sample. The physicochemical characterization of the aqueous fraction and the ultrafiltrate showed that total amino acids composition and free amino acid content were not modified. However, more than one third of the ammonia content was lost during filtration due to interactions with retained particles or adsorption on membrane material.
We should notice a study led by Wang and co-workers [27] on the influence of filtration operating conditions to the hydraulic performances of membranes during the filtration of scallop skirt enzymatic hydrolysate. They studied the influence of pressure, temperature, pH and volume concentration ratio on the permeate flux through a hollow fibre membrane and on the selectivity. A high separation efficiency was found for an operating pressure kept at 0.5 - 1 bar. The temperature should be kept at 40 - 43°C and the hydrolysate pH at around 7.0. Compared to the initial hydrolysate, the UF permeate had an acceptable colour and flavour and a high clarification degree. The retention of nitrogenous amino acids was about 92 %. The same team in 2002 conducted a study on washing and regeneration of ultrafiltration hollow fibre membranes after scallop (*Chlamys livida*) hydrolysate treatment [28]. The impact of the various cleaning agents, the influence of cleaning agent concentration, the temperature and the duration of the cleaning and the use of physical methods were investigated.

Interest of coupling enzymatic digestion with membranes for proteins and DNA extraction from seaweeds

The enzymatic hydrolysis of the red seaweed cell wall was used to improve the extraction of molecules with a biological or an industrial interest such as proteins or DNA.
The simultaneous application of xylanases and cellulases on the red alga *Palmaria palmata* significantly increases the recovery yield of proteins, especially the yield of R-phycoerythrin (factor 4, 7 in regard to the blank) [29].
In the similar context, the preliminary enzymatic digestion of cell wall with β-Glucorinidases (Ultraflow), β-Glucanases (Finizym), Xylanases (Shearzyme), Cellulases (Celluclast) or Agarase allows to extract a DNA with a high purity degree (1.8-1.9) and the recovery yield is improved to a factor of 5 from the red seaweeds belonging to species *Palmaria palmata* or *Gracilaria verrucosa* [30]. Additional studies will be conducted by this research team by coupling such a

specific hydrolysis with ultrafiltration or nanofiltration in order to increase the concentration and the purity of the targeted molecules.

3. Lipids

Fish oils have been the focus of much attention in the past years due to their high content of n-3 polyunsaturated fatty acids (PUFAs), such as EPA (eicosapentaenoic acid) and DHA (docosahexanoic acid) that confer to them an interesting role for human health and useful properties for functional ingredient manufacturing. Because PUFAs are thermally unstable, membrane separations appear well-suited for the concentration and purification of these fatty acids. However, in a review on the use of membrane technology to process agricultural fats and oils, Snape and Nakajima [31] quoted only one team having worked in Japan on the concentration and purification of fish oils by membrane processes. The following description of this work below is borrowed from Snape and Nakajima.

Sahashi *et al.* [32, 33] combined a solvent extraction and a membrane separation whose role was the separation of two phases. A 75 % ethanol aqueous solution was added to the fish-oil containing both triglycerides having PUFAs and free saturated fatty acids, and several flat-sheet membranes were tested for their ability to separate the oil from the solvent which contained free fatty acids. A hydrophilic polyimid UF membrane (Nitto Denko, MWCO 20 kDa) was found the most suitable for the separation of solvent phase as a permeate. A rotating disk membrane was effective in a large-scale operation for getting PUFA-rich glycerides without oil quality deterioration.

Xu *et al.* [34] produced structured lipids in an ultrafiltration membrane reactor through a reaction between medium chain triacylglycerols (MCT) and n-3 polyunsaturated fatty acids previously concentrated from fish oil by ultrafiltration. The membrane reactor was a stirred cell equipped with flat membranes (Dow, ETNA 01A and 10A), the biocatalyst being Lipozyme IM. The membranes were used to separate selectively the released medium chain fatty acids selectively from the mixture during the reaction. Factors such as fluxes of various compounds under different pressures, and selectivity characteristics under different conditions were investigated. The authors found that the incorporation of polyunsaturated fatty acids into MCT was increased by about 15% over 80 h by using a membrane bioreactor.

Linder *et al*. [35] fractionated lipids obtained by a selective enzymatic hydrolysis of fish oil extracted salmon by-products. They used a hydrophilic UF membrane (regenerated cellulose, Amicon YM 10 membrane, MWCO 10 kDa) in order to retain in the retentate most of the saturated fatty acids. This step allowed a significant increase of polyunsaturated fatty acids (PUFA) from 39.2% in the crude oil to 43.3% in the permeate

Dumay [36] combined various steps of enzymatic hydrolysis and ultrafiltration to add value to sardine (*Sardina Pilchardus*) by-products. Various oily and aqueous fractions were obtained, the later ones containing peptides and also lipids of interest, phospholipids in particular. UF has been used in particular to separate phospholipids from peptides by concentrating phospholipids in the retentate. As in previous works, bests results were obtained with membrane made of a highly hydrophilic material (regenerated cellulose).

Relying on previous works on the fractionation of triglycerides from fish oil with supercritical CO_2 after esterification, some authors propose to couple supercritical (SC) extraction with membrane processes.

Sarrade *et al.* [37] from the French “Commissariat à l’Energie Atomique” patented a two-step process for fractionating a solid or liquid phase, comprising an extraction with a supercritical fluid followed by a nanofiltration, the permeate stream of which containing light compounds as the retentate retained the heavy compounds. The process is claimed to be applicable in particular to fish oils with the aim to fractionate the fatty substances.

The same team presented then two new tubular nanofiltration membranes adapted to SC operating conditions [38]. The first one was a multilayer composite membrane with a macroporous alumina layer, a mesoporous titanium oxide layer and an organic top layer in Nafion®, and the second one was a strictly ceramic membrane. These membranes were tested in particular for the fractionation of fish oil triglycerides, for which the composite membrane allowed a significant concentration of EPA and DHA in the retentate stream and of short-chained fatty acids in the permeate.

4. Carbohydrates

Polysaccharides:

A major use of cross-flow ultrafiltration for the recovery of material in sea waters (and more generally in bulk natural waters including lakes and rivers) has been to concentrate dissolved organic matter or colloids produced by micro-organisms, especially polysaccharides. There has been a large amount of works on this topic. UF has been used here again as a separation tool, without production objectives, before compound analysis and characterization for environmental studies [39, 40, 41]. We will not quote anymore these works, although most of them can probably have industrial applications in aquaculture, for instance for the prevention and control of micro-organism blooms in growing ponds.
The Asahi Kasei Kogyo Kabushiki Kaisha Company, Kosaka, and Takeo [42], took out in 1983 a Japan patent for a process of concentration and purification of high-molecular polysaccharides extracted from seaweed such as carrageen, agar-agar or alginic acid derivatives. The innovative part of the process consisted in using ultrafiltration to remove impurities such as seaweed residue and colorants from the aqueous solution of polysaccharide (probably in a diafiltration mode although not stated explicitly). A highly pure product which cannot be obtained by a conventional process has been produced at a concentration up to about 3.0 to 4.5 %.
Lignot *et al.* [43] described a low-cost two-steps, non denaturing process for the production of chondroitin sulfate (CS), a glycosaminoglycan well known for its chondroprotective effect, from skate cartilage, a fishery by-product. The process consisted in an enzymatic extraction with a protease (Lypaine) in order to destroy the collagen matrix, followed by a membrane separation to concentrate the CS and refine it in a diafiltration mode. The performances of various inorganic UF and MF membranes were compared in terms of flux and selectivity. A UF 0.1 µm-pore size membrane appeared to be the most efficient one to separate CS from the other compounds, salts and peptides.
Kim *et al.* [3] prepared laminarin polysaccharides from *Laminaria japonica*, a marine brown alga with potential biological activities, by hot water extraction, ultrafiltration and gel chromatography. Oligosaccharides were then obtained by enzymatic hydrolysis and some biological activities were tested

Oligosaccharides:

Some works reported oligosaccharides fractionation mainly to study their biological properties.
Most of these works were carried out at the Department of Chemistry at the Pukyong National University in Pusan, South Korea, following the same methodology as for proteins hydrolysates.
Jeon et al. [44] reported the use of a membrane enzymatic reactor system with a MWCO 3,000 ultrafiltration membrane to produce chitosan oligosaccharides (COSs), with the aim to study their biological properties (COSs are involved in in-vivo calcium metabolism).
Jeon and Kim [45] have designed a membrane enzymatic reactor (MER) to hydrolyse chitosan in order to produce chitosan oligosaccharides with antibacterial activity. They indicated that at least 11 batches of substrate could thus be hydrolysed for the same quantity of enzyme (chitosanase). In another work, this membrane reactor was used to fractionate chitosan hydrolysates into three kinds of chitooligosaccharides with relatively higher, medium, or lower

molecular weights [46]. The authors identified in this way that the molecular weight of chitooligosaccharides is critical for micro-organism inhibition and required higher than 10,000 Da. Antitumour activity of these chitosan oligosaccharides was also tested on tumour cell-bearing mice by Jeon and Kim [47]. Among these COSs, those with medium molecular weight ranging from 1.5 to 5.5 kDa showed the better activity (indeed, they effectively inhibited the growth of tumour cells and the mice treated with MMWCOS showed improved survival rate).The same idea was taken up by this team to test radical scavenging [48] and antimicrobial effects [49]. The results revealed that the antimicrobial effects of hetero-chitosans and their oligosaccharides against *V. parahaemolyticus* depend on the degree of deacetylation, their molecular weights, and strains tested.
In the same way, Mou *et al.* [50] used ultrafiltration to recover low-molecular-weight carrageenans, and to determine the anti-tumour activity of these compounds after a chemical modification.

Conclusion

Although few works have been done compared to dairy or agricultural hydrolysates (soja, colza, wheat, …), UF has been reported to be used to recover and (sometimes) to fractionate marine solutions obtained by enzymatic hydrolysis. The majority of studies concern peptides and oligosaccharides with *bioactive activities* intended to be used in ingredients for human food or animal feed. It can be noted that no work is related to the production of oligomers with *functional properties* (foaming, emulsifying, …) whereas there is some literature on this topics for dairy [1] or agricultural [2] products. However, it will not be probably true any more for a long time since some works have recently been published on the functional properties of fish hydrolysates [51].

UF is often used as a convenient tool to fractionate peptides and oligosaccharides use UF for analytic purposes. Hydrolysates are then processed by a succession of separation steps including ultrafiltration and analytic methods (chromatography in general) to isolate and identify one or a few oligomer(s), *i.e.* peptides or oligosaccharides: only a few works discuss the performance of the fractionation from an engineering point of view, in terms of permeation fluxes or fractionation selectivity.

At the opposite, works on lipids are fewer but more oriented towards a process development. Indeed, all of the works reported here describe an integrated process (membrane enzymatic reactor, membrane separation with an extraction solvent) or a combined one (successive steps of ultrafiltration and enzymatic hydrolysis) to recover fish oils or lipids.

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