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Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties

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Abstract:

BACKGROUND: Numerous studies have demonstrated that in vitro controlled enzymatic hydrolysis of fish and shellfish proteins leads to bioactive peptides. Ultrafiltration (UF) and/or nanofiltration (NF) can be used to refine hydrolysates and also to fractionate them in order to obtain a peptide population enriched in selected sizes. This study was designed to highlight the impact of controlled UF and NF on the stability of biological activities of an industrial fish protein hydrolysate (FPH) and to understand whether fractionation could improve its content in bioactive peptides.

RESULTS: The starting fish protein hydrolysate exhibited a balanced amino acid composition, a reproducible molecular weight (MW) profile, and a low sodium chloride content, allowing the study of its biological activity. Successive fractionation on UF and NF membranes allowed concentration of peptides of selected sizes, without, however, carrying out sharp separations, some MW classes being found in several fractions. Peptides containing Pro, Hyp, Asp and Glu were concentrated in the UF and NF retentates compared to the unfractionated hydrolysate and UF permeate, respectively. Gastrin/cholecystokinin-like peptides were present in the starting FPH, UF and NF fractions, but fractionation did not increase their concentration. In contrast, quantification of calcitonin gene-related peptide (CGRP)-like peptides demonstrated an increase in CGRP-like activities in the UF permeate, relative to the starting FPH. The starting hydrolysate also showed a potent antioxidant and radical

scavenging activity, and a moderate angiotensin-converting enzyme (ACE)-1 inhibitory activity, which were not increased by UF and NF fractionation.

CONCLUSION: Fractionation of an FPH using membrane separation, with a molecular weight cut-off adapted to the peptide composition, may provide an effective means to concentrate CGRP-like peptides and peptides enriched in selected amino acids. The peptide size distribution observed after UF and NF fractionation demonstrates that it is misleading to characterize the fractions obtained by membrane filtration according to the MW cut-off of the membrane only, as is currently done in the literature.

Keywords: Fish protein hydrolysate (FPH), ultrafiltration (UF), nanofiltration (NF), membrane separation, fractionation process, bioactive peptide

INTRODUCTION

Peptides resulting from natural digestion or controlled enzymatic hydrolysis of food proteins exhibit not only nutritional but also biological activities of dietary or pharmacological interest [1, 2]. Following ingestion, proteins are hydrolyzed in the stomach by HCl and pepsins and, at a lesser extent, in the upper part of the intestine by trypsin and chymotrysin. The resulting enzymatic hydrolysate mostly consists in a mixture of free amino acids and dipeptides, able to bind gastric receptors on mucosal cells, and to be resorbed via a transcellular transport across the intestinal brush border cells, as demonstrated using synthetic oligopeptides[3, 4]. Beyond this classical paradigm, it was demonstrated that oligopeptides can also cross the intestinal border following a transepithelial route or oligopeptides transporters[5,6]. Some factors such as the intraluminal hypertonicity increase this process[7]. *In vitro* and *in vivo* studies have shown that enzymatic hydrolysis of dietary proteins leads to bioactive peptides such as growth factors[8], immunomodulators[9], antimicrobial[10], antithrombotic[11], ACE-1 inhibitors[12], opiates or antiproliferative peptides[13]. Research focused on peptides contained in fish protein hydrolysates (FPH) demonstrated they also constitute promising health benefits molecules for nutritional or pharmaceutical applications[14-16]. Only few studies were performed to confirm the *in vivo* biological activity of FPH peptides, but convincing data were obtained on relevant animal models, such as spontaneously hypertensive or diabetic rats. For instance, *in vitro*[17-19] and *in vivo* studies on hypertensive rats[20-22] demonstrated that pollack flesh or tuna muscle peptides inhibit ACE-1 and decrease blood pressure. The *in vivo* hypotensive activity of a short peptide identified in a sardine muscle hydrolysate was also demonstrated in humans, by a dietary study on mild hypertensive subjects [23].

Fish and shellfish peptides could also contribute to free radicals scavenging and pathologies prevention[24]. Peptides obtained from shrimp by-products[25], hoki frames[26], tuna backbone proteins[27], jumbo squid skin gelatine [28,29], conger eel muscle [30] and yellow stripe trevally[31] exert a significant *in vitro* antioxidant activity. Most antioxidant peptides identified are short (5 to 16 amino acids), contain hydrophobic amino acids, Val and Leu, at the N-terminus and Pro, His or Tyr in their sequence[14]. Tyrosines substantially contribute to the scavenging of free radicals because their phenolic lateral chains act as potent electron donors [32, 28, 29], thus allowing the termination of the radical chain reaction[31, 33].

FPH have also been identified as a source of hormonal peptides [34-37, 41-42], particularly peptides able to bind gastrin / cholecystokinins (CCK), and CGRP receptors. FPH peptides also cross-react with specific antibodies directed against these hormones. Gastro-intestinal secretagogue peptides such as Gastrin/CCK [34-36] exhibit a large spectrum of activities ranging from the stimulation of protein synthesis[37], the control of intestinal mobility and the secretion of digestive enzymes[38]. Involvement of CCK-8 in the satiety mechanisms controlling food intake in humans is well documented[39, 40], and the gastric localization of gastrin and CCK receptors suggests that dietary peptides acting as agonists on these receptors could be of interest as satietogenic ingredients in functional food. CGRP is a neuropeptide synthesized by alternative splicing of the calcitonin gene [41] and regulating a large number of physiological functions such as vasodilatation, gastric acid secretion and cardiac metabolism[42, 43]. Binding of CGRP to its receptors reduces acidic secretion and the risk of ulceration, suggesting that peptides acting as CGRP receptors agonists could be of pharmacological interest [44,45].

Several studies refer to the use of UF in order to refine hydrolysates and to increase their specific activity in the perspective of industrial upgrading of by-products to produce bioactive ingredients for human food or animal feeding. The specific activity of UF fractions is then compared with that of the initial hydrolysate, with the aim of identifying the most active fractions. Several hydrolysates from different substrates have been fractionated in this way at the Pukyong National University in Pusan, South Korea: hot washing waters of cod frame proteins[46], Alaska pollack frame proteins[47], jumbo squid skin gelatine[28], giant squid muscle[48], hoki frame proteins[26], and conger eel muscle [30]. Activities tested are generally antioxidant and antihypertensive, but radical scavenging activities and foaming or emulsifying properties have also been considered. Similar works have been reported by other teams. Neves[49] 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. Sumaya-Martinez [50] fractionated a shrimp frame hydrolysate by successive microfiltration (0.45 µm) and ultrafiltration (30 kDa, 5 kDa). Unfortunately, only few details are given in these works concerning the operating conditions of the fractionation process, except the molecular weight cut-off (MWCO) of the membranes used. For instance, the volume reduction factor (VRF) is rarely precised so that the peptidic population whose activity is tested is not precisely known. It is however recognized that short peptides, below 3 or 4 kDa, usually harbour bioactive properties and the peptide size is a physico-chemical parameter – although not the unique one – controlling peptide activities. Some studies discuss however the impact of ultrafiltration on peptidic

populations on the basis of size-exclusion chromatograms [51, 52, 53, 54]. This study was then designed to understand if UF and NF of a bioactive FPH, performed in controlled conditions, represent innovative industrial processes to obtain fractions defined by a strict range of peptide size and enriched in bioactive peptides.

MATERIALS AND METHODS

Fish protein hydrolysate (FPH)

The FPH selected for this study was PROLASTIN, a commercial product from the french SME Copalis. PROLASTIN is an elastin hydrolysate obtained by controlled proteolysis of skins from North Atlantic lean fishes (gadidae, mostly cod and pollack) followed by purification steps based on sieving, centrifugation, and discolouration. Proteolysis was performed in the SME COPALIS, using an industrial bacterial endopeptidase at optimized time, pH and E/S ratio. PROLASTIN is composed of polypeptides with a low molecular weight (1000 to 5000 Da), which makes it soluble and very digestible. Its NaCl content is very low (0,79 %, w:w), which allows a relevant study of the biological activity of the starting hydrolysate and related fractions. It is sold as a functional ingredient for dietary complements, helping to give elasticity to tissues and limit their ageing. It is produced at industrial scale and the reproducibility of the process and final product was confirmed before fractionation.

UF and NF of the FPH

PROLASTIN was fractionated successively by UF and NF, the UF permeate being used as the feed solution for the NF step (Figure 1). Experiments were carried out on a Microlab40 pilot plant (VMA Industrie) with a maximum capacity of 5 L (launching tank 4.3 L + dead volume 0.7 L). The pilot plant was equipped with tubular organic membranes (PCI Ltd), diameter 12 mm, surface area 0.033 m²: a NF membrane in Polyamid/Polyethersulfon (60 % retention in $CaCl_2$, ref. AFC40) and a UF membrane in modified Polyethersulfon (MWCO 4 kDa, ref. ESP04). The ESP04 UF membrane was chosen in the PCI range according to the molecular weight distribution of the hydrolysate. It was recently shown that a nominal MWCO between the molecular weight of the biggest and the medium sized peptides is a good choice for the fractionation of a hydrolysate[55]. The AFC40 NF membrane was selected as it has the poorest salt retention in the PCI range, which makes it the most appropriate to fractionate the UF permeate. A MWCO of about 300 Da has been estimated by the membrane supplier for the fractionation of organic molecules onto this NF membrane.

A 10 L volume of solution was prepared by mixing about exactly 100 g of hydrolysate powder in warm pure water. A 1 L-aliquot was reserved for analysis. So, 9 L of the initial solution were filtered with the 4 kDa membrane. At the end of the concentration process, volumes of about 8 L of permeate and 1 L of retentate were recovered. All retentate was kept for analysis, as well as a sample of about 1 L of permeate. The

remaining permeate (7 L) constituted the initial solution filtered by NF in the second step of the fractionation cascade. Six liters of permeate and 1 L of retentate were obtained and kept for analysis. At the end of each filtration step, a sample of 10 to 50 mL of "instantaneous" permeate was reserved in order to estimate the retention factor at this end of the operation. Filtrations were carried out with the following operating conditions: tangential velocity V = 2.5 m/s, temperature T = 55°C, transmembrane pressure ΔP = 30 bar for UF or 35 bar for NF. The selected values for V and ΔP were the maximum allowed by the pilot and the membrane, in order to minimize the filtration time. However, filtration times were also quite long due to the high reduction volumic factors that were reached. Thus, a fairly high temperature was chosen in order to maximize permeation fluxes, as well as to limit bacterial growth.

Previously unused UF and NF membranes were conditioned before being used according to the following sequence of washing: Water – Ultrasil 11 – water – nitric acid – water. Recovery rate in peptides in UF or NF fractions were computed as the nitrogen content of the fraction considered (retentate or permeate) divided by the sum of nitrogen contents in retentate and permeate. Nitrogen content of samples was determined by the Kjeldahl method following the norm NF EN 25663 ISO 5663 (1994).

Physical and biochemical characterization of FPH, UF and NF fractions

Colour, dry matter, pH, NaCl content, protein content and protein recovery rates were measured or calculated following normalized procedures. NaCl contents were determined using a coulometric method (chlorimeter 926, Corning). Protein contents were calculated as 6.25 times the total Kjeldahl nitrogen amount. Aminogram of PROLASTIN was determined using ten milligrams of freeze-dried sample hydrolyzed for 24h at 110°C in 200 µL of HCl 6N in vacuum sealed vials. The hydrolyzed sample was dried under N2, diluted with 2.5 mL of deionized water and aminoacids composition was determined using the EZ:faast™ procedure (Phenomenex, USA), consisting in a SPE step followed by derivatization and liquid-liquid extraction. The organic phase containing derivatized aminoacids was analyzed by GC-FID (Perkin Elmer Autosystem XL).. Aminoacids were identified according to their retention time and quantified using a calibration curve and an internal standard (Norvaline 200 µM added to each sample). The MW distributions of the native hydrolysate and UF/NF fractions were analyzed by size exclusion chromatography in FPLC mode using a Superdex Peptide® HR 10/300 column (Amersham, fractionation range: 7000-100 Da) according to Guérard[25]. Samples were diluted so that the mass injected was the same for each sample. The resulting normalized chromatograms obtained in this way are representative of the peptide distribution, both in terms of molecular weight and mass composition, and are more expressive than raw chromatograms to analyze the impact of a membrane fractionation. Total areas of the chromatograms were integrated and separated into five molecular weight ranges expressed as the percentage of the total surface (> 7000, 7000–3000, 3000–1000, 1000–300, and < 300 Da).

Radioimmunoassay of gastrin/CCK-like peptides

Radioimmunoassay (RIA) of gastrin/CCK-like peptides was conducted in triplicate assays using rabbit antiserum, synthetic iodine 125-radiolabeled gastrin-17 as tracer and synthetic gastrin-17 as a standard (Gask-PR, Cis bio international). The principle of the assay is based on the binding competition between ^{125}I-gastrin-17 with gastrin contained in the standards or gastrin/CCK-like peptides in the samples, on a given limited number of specific binding sites on anti-gastrin antibodies. At the end of the incubation period, the amount of radiolabeled gastrin bound to the antibody is inversely proportional to the amount of non-radiolabeled gastrin/CCK originally present in the assay. The method proposed for the separation of the free and bound fractions uses an immunoprecipitant in which a second antibody has been preprecipitated in excess[36]. Three parameters could be examined after the analysis of the raw values: the slope coefficient of the regression line obtained with different dilutions of the FPH in comparison with the slope coefficient of the standard, the ED_{50} and the amount of peptide-like/mg of dry powder. Parallel slopes indicate the specific binding of peptides to antibodies. ED_{50} is the amount of peptides occupying 50% of the binding sites. This parameter reflects the affinity of the peptides for the antibody. The last parameter is the amount of hormone-like peptides in the dry sample. All RIA were performed in triplicate independent assays on all fractions.

Radioreceptorassay of CGRP-like peptides

The amount of CGRP-like molecules was measured using a specific radioreceptorassay. This assay used the ability of CGRP like molecules present in the hydrolysates to interact with CGRP binding to its receptors present in specific target tissues. This assay was developed using rat liver membranes and ^{125}I labeled human CGRP. Liver membranes were prepared using male Wistar rats according to the method of Neville until step 11[56]. Proteins were quantified by the method of Folin-Lowry using Bovine Serum Albumine (BSA) as standard[57]. Incubations, in a 400 µL final volume, were performed at 22°C for 1 hour[58]. At the end of the incubation, bound and free ligands were separated by centrifugation in a solution containing 2% BSA. Each batch was tested at four increasing protein concentrations, and only the straight lines presenting slopes similar to that obtained with the standard hormone (0.01-1 ng/assay) were considered as positive. The receptor binding ability of each purified fraction was determined in triplicate independent assays and used to calculate the quantity (pg) of CGRP like activity per mg of protein. Data were also expressed as the amount of protein (mg) that induced a 50% inhibition of the initial CGRP binding to rat liver membranes (ED_{50}).

Antioxidant activity

DPPH radical scavenging activity

Radical scavenging activity was measured according to the procedure reported by Morales and Jimenez-Perez [59]. An aliquot of sample (200 µL) was added to 1 mL of a daily-prepared solution of 1,1-Diphenyl-2-Picryl-

Hydrazyl ($DPPH^{\bullet}$) at a 74 $mg.L^{-1}$ concentration in ethanol. The mixture was shaken for 1 hour at 25°C. The sample was centrifuged at 10 000 x g for 5 min and absorption of the supernatant was measured at 520 nm. The $DPPH^{\bullet}$ concentration in the reaction medium was calculated from the calibration curve, determined by linear regression:

$$[DPPH]_t = 0.0241\ (A_{520\ nm}) + 0.022\ (r^2=0.9995)$$

The radical scavenging activity of the sample was expressed as percentage disappearance of $DPPH^{\bullet}$,

$$\text{DPPH radical scavenging activity (\%)} = (1 - ([DPPH]_t/[DPPH]_{H2O})*100)$$

where $[DPPH]_{H2O}$ is the concentration of DPPH in the presence of water instead of hydrolysate. *In vitro* DPPH radical scavenging activity was determined in triplicate independent assays and expressed as AC_{50}, corresponding to the concentration of hydrolysate ($mg.mL^{-1}$ protein determined according to the Kjeldahl method) able to scavenge 50% of DPPH radical.

Quantification of antioxidant activity using the beta-carotene/linoleate model system

The antioxidant activity was determined using the beta-carotene/linoleate model system as described by Marco[60] with some slight modifications. Five mL aliquots of a beta-carotene-linoleate emulsion were transferred to glass tubes, containing 200 µL of sample at several protein concentrations in MilliQ water. All samples were stirred and incubated simultaneously for 2 hours at 50°C. Absorbance values at 470 nm of samples and controls (water and BHA) were recorded every 30 min with a microplate reader (SPECTRAmax PLUS 384, Molecular Devices). The antioxidant activity gives an estimate of the relative protection of each sample against the oxidation of linoleate, observed by the bleaching extent of the beta-carotene-linoleate emulsion. The antioxidant activity was calculated as:

$$\text{Antioxidant activity} = (OD_{t=60min} / OD_{t=0min}) * 100$$

In vitro antioxidant activity was determined in triplicate independent assays and expressed as AC_{50}, corresponding to the concentration of hydrolysate ($mg.mL^{-1}$ protein determined according to the Kjeldahl method) inducing 50% of antioxidant activity.

ACE-1 inhibition assay

In vitro inhibition of ACE-1 was assayed following the spectrophotometrical Holmquist method [61]. Inhibition was calculated from triplicate independent assays and expressed as IC_{50} and maximal % inhibition. IC_{50} corresponds to the concentration of hydrolysate or fraction inducing 50% inhibition of ACE-1 activity. Captopril $21.7.10^{-3}$ $\mu g.mL^{-1}$ (0.1 µM) was used as a reference inducing 100% inhibition.

RESULTS AND DISCUSSION

Physical, chemical and biochemical properties of FPH and fractions

Colour, dry matter, pH, NaCl content, protein content, protein recovery rates and aminograms of PROLASTIN and related UF and NF fractions are given in Tables 1 and 2. Aminograms of PROLASTIN and related fractions revealed a balanced composition and confirmed their high nutritional value (Table 2). It is of interest to note that peptides containing PRO, HYP, ASP and GLU were concentrated in the UF and NF retentates compared to the unfractionated hydrolysate and UF permeate respectively (Table 2), suggesting that the presence of specific amino-acids may influence the interactions of peptides with the peptide layer in contact with the membranes.

Peptidic profiles and impact of the fractionation on MW distributions

PROLASTIN is a highly hydrolyzed peptide mix, where 98.6% (in mass) of peptides had a molecular weight lower than 4,000 Da (Table 3). Ultrafiltration of the crude hydrolysate, as well as nanofiltration of the ultrafiltrated permeate constituted very efficient processes to desalt the peptide fractions (Table 1). The impact of the 2-steps UF-NF process on the peptidic populations of PROLASTIN, in term of yield obtained in the various fractions and peptide size repartition relatively to the MWCO of the membranes, was described in detail in a previous publication [55]. The objective here was to process crude extracts in conditions representative of an industrial operation to compare the biological activities of the fractions relatively to their peptide population content. Thus, fairly concentrated solutions were processed (about exactly 100 $g.L^{-1}$) at a high volume reduction factor (VRF) in order to obtain four fractions with compositions as different as possible (Table 3):

- the UF retentate, enriched in peptides above 4,000 Da;
- the UF permeate, poor in peptides above 4,000 Da;
- the NF retentate, poor in peptides above 4,000 Da and rich in peptides above *ca* 300 Da;
- the NF permeate, poor in peptides above *ca* 300 Da.

This study demonstrated that membranes do not carry out sharp separations, some MW classes being found in several fractions. Thus, the NF retentate still contained about 22 % of peptides below 300 Da. More dramatically, the UF retentate contained a large amount of peptides (93 % in weight) below 4,000 Da (the nominal MWCO of the membrane) even though high VRF were reached. NF and UF permeate fluxes are given on Figure 2 as a function of time in order to illustrate the effect of dynamic cake formation on filtration behaviour. Fluxes values and their similar decrease in the course of time are discussed elsewhere[55]. One important result of this study is that it is clearly misleading to characterise the fractions obtained by membrane filtration according only to the MW cut-off of the membrane, as it is currently done in the literature (> 4,000 Da for the UF retentate, 300–4,000 Da for the NF retentate, and so on).

Presence of gastrin/CCK-like peptides in FPH and fractions

Dosage of gastrin/CCK-like peptides in the fractions is presented in Table 4. Gastrin/CCK-like peptides were identified in the unfractionated hydrolysate and in all UF and NF fractions. The curve slope measured with the unfractionated hydrolysate was very close to that of standard ^{125}I-gastrin, indicating the presence of peptides binding specifically to gastrin antibodies. The amount of gastrin/CCK-like peptides present in the unfractionated hydrolysate was in the range of data previously published for fish and shellfish hydrolysates[36], the best activity (about 4 pg of gastrin-like peptides) being obtained with a shrimp hydrolysate fraction containing peptides between 4000 and 1000 Da. The ED_{50} value of the unfractionated hydrolysate was very high, which is characteristic of a low binding affinity. UF and NF of PROLASTIN resulted in variations in the curve slope and dilution of gastrin/CCK-like peptides, suggesting that these processes do not allow an enrichment in peptides of interest. Interestingly, the ED_{50} measured for UF and NF retentates was much lower than that of the unfractionated hydrolysate, suggesting that the gastrin antibody binding affinity of peptides present in these fractions was increased. However, the main conclusion of this dosage is that UF or NF fractionations do not allow to concentrate gastrin/CCK-like peptides in selected fractions.

Presence of CGRP-like peptides in FPH and fractions

The dosage of CGRP-like peptides in the fractions demonstrated an important increase (10 fold) of the CGRP-like activities in the UF permeate, relatively to the crude extract (Table 5). Nanofiltration of the UF permeate did not allow a higher concentration of CGRP-like peptides. The highest activity found in the UF permeate was also confirmed by the ED_{50} values (Table 5). The best interaction was observed with the UF permeate with only 3.6 mg of dry weight sample necessary to obtain a 50% inhibition of the maximal binding of radiolabelled CGRP; this value corresponded to a 5.2 fold affinity increase compared to the unfractionated hydrolysate. Moreover, this ED_{50} value could not be increased by a subsequent NF process. Therefore, it appears that UF allows to concentrate CGRP-like peptides in the UF permeate, with an important increase of affinity for CGRP receptors, as measured using the binding displacement of ^{125}I-CGRP.

Antioxidant activities

The *in vitro* antioxidant activities of the PROLASTIN hydrolysate and fractions are presented in Table 6. Before fractionation, PROLASTIN showed a high DPPH radical scavenging activity with an AC_{50} of 24.7 $mg.mL^{-1}$. This result suggests that PROLASTIN contains peptides reacting with free radicals to form more stable products. PROLASTIN also showed a potent antioxidant activity, with an AC_{50} of 0.12 $g.L^{-1}$. Fractionation of PROLASTIN induced a weak loss of antioxidant and DPPH radical scavenging activities.

According to Pihlanto[62], the structure-activity relationship of the antioxidant mechanism of protein hydrolysates is not yet fully understood. The antioxidant activity seems to be inherent to the characteristic amino acid sequences of the peptides, depending both on the amino acid composition of the substrate and on the specificity of the protease used in the process. We in fact observed a different result fractionating a cod protein hydrolysate, as the antioxidant activity of UF fractions was superior to that of the unfractionated hydrolysate (data not shown as the NaCl content of this hydrolysate was > to 5% w:w). A stepwise fractionation of a protein hydrolysate using membrane separation by molecular weight cut-off adapted to the peptide composition of hydrolysate may thus provide an effective mean to concentrate antioxidative peptides, but the mechanisms allowing a concentration in antioxidant peptides have yet to be clarified and the process optimized. Changing the selectivity of the membrane, not only in term of peptide size but also in term of aromatic selectivity, could be of interest to concentrate antioxidant peptides.

ACE-1 inhibitory activity of FPH and fractions

In vitro ACE-1 inhibitory activities of PROLASTIN and fractions are summarized in Table 7. PROLASTIN, UF and NF fractions exerted a moderate ACE-1 inhibition when compared to control pharmacological inhibitors (captopril, 100% inhibition at $21.7.10^{-3}$ µg.mL^{-1} (100 nM), IC_{50} = 4.34. 10^{-3} µg.mL^{-1} (0.02 µM). ACE-1 inhibition was in the range of activities previously measured with enzymatic hydrolysates obtained from other fish proteins (IC_{50} ranging from 52 to 63 µg of peptides per mL for unfractionated and fractionated PROLASTIN, IC_{50} = 44 µg peptides per mL for ultrafiltrated (< 10 kDa) Pacific hake peptides[63]). These data confirm that FPH constitute interesting sources of ACE-1 inhibitory peptides and reinforce the common view that the *in vitro* ACE inhibitory activity is mostly dependent on peptide size rather than on fish species source. Ultrafiltration of PROLASTIN did not result in drastic changes in ACE inhibitory activity, contrary to previous observations obtained on Pacific hake peptides. Several hypotheses can be drawn to explain why UF had only a weak impact on ACE inhibitory activities. The presence of a large amount of peptides < 4 kDa in the UF retentate demonstrates that the unfractionated hydrolysate was already rich in low MW peptide, hardly separated by UF as only peptides with a MW < 400 Da can freely cross the UF membrane. For peptides ranging from 400 Da to 4 kDa, the ability to cross the UF membrane is directly dependent upon their MW, the lower crossing easier. It can thus be considered that the UF permeate contained a peptide population with a MW distribution centred on low MW, and that the short peptides were not all removed from the UF retentate, in spite of a high UF volumic reduction factor. In the same way, the NF retentate still contained an important proportion of peptides < 300 Da. The possibility of synergistic ACE-inhibitory effects between peptides with different MW, and the potential oxidative degradation of peptides during the UF process, performed at 55°C, can not also be excluded. NF only weakly enhanced the activity of ultrafiltrated hydrolysates, with no important optimization regarding the development costs of an industrial NF process.

CONCLUSION

This study was designed to highlight the impact of controlled UF and NF on the stability of biological activities of an industrial FPH, and to understand if fractionation could improve its content in bioactive peptides. One important result arising from UF and NF fractionation performed in this study is that it is misleading to characterise the fractions obtained by membrane filtration according only to the MW cut-off of the membrane, even when high volume reduction factor are achieved, as some MW classes can be found in different fractions. We also interestingly observed that fractionation allowed the concentration of peptides containing glutamate, aspartate, proline and hydroxyproline in the UF and NF retentates. The data obtained in this study suggest that UF and NF fractionation allow to obtain fractions rich in bioactive peptides, depending on the composition of the starting hydrolysate and the activity studied. In our study, UF had a positive impact on the presence of CGRP-like peptides in the fractions but not on gastrin/CCK-like peptides. Our data demonstrated that fractionation of a FPH exerting a potent antioxidant activity does not systematically allow an increase of this activity, but changing the selectivity of the membrane, not only in term of peptide size but also in term of selectivity for aromatic amino-acids, may be of interest to concentrate antioxidant peptides. It was described in the first part of this article that PROLASTIN is a highly hydrolyzed peptide mix and this could explain the weak impact of fractionation on the biological activities. In this view, it could be of high interest to prepare a weakly hydrolyzed PROLASTIN sample, to fractionate it and compare the data to those obtained in this study. Our data demonstrated that the knowledge of the MW distribution is of high importance to select the MW cut-off of the membranes but insufficient to define a strategy to obtain fractions enriched in bioactive peptides. In this view, defining the relations existing between the physico-chemical properties of a hydrolysate (charge, sequence, peptides size, and MW distribution) and its biological activities, as well as the influence of the fractionation process (MW cut-off, vrf, delta P, ...) on the distribution of peptide populations will be of great interest for industrials developing functional ingredients[64].

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FIGURES

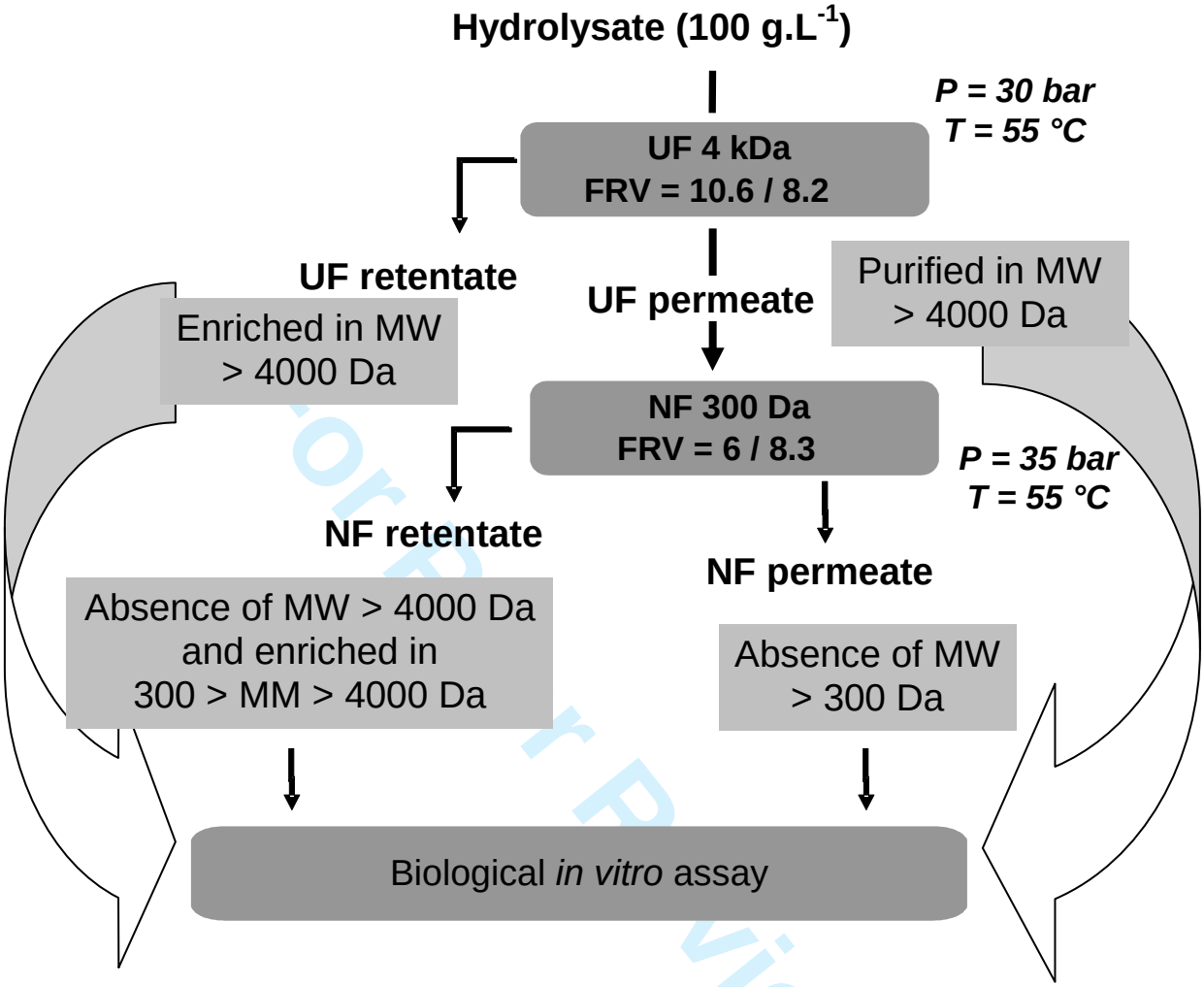

Figure 1. The ultrafiltration–nanofiltration sequence.

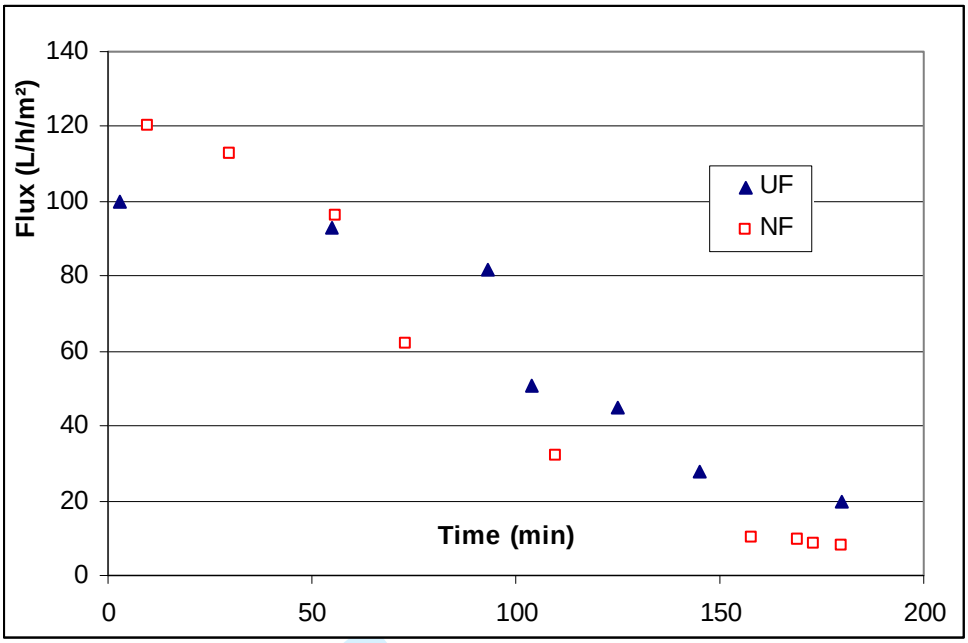

Figure 2. Flux evolution with time for the UF and NF steps, PROLASTIN hydrolysate (from Bourseau et al., Desal. 2009 [55])

	Colour	Dry matter (DM) $g.L^{-1}$	pH	NaCl/ DM (%)	Protein/ DM (%)	Protein recovery rate % *
Unfractionated hydrolysate	Yellow orange	111.50	8	0.79	88.5	
Retentate UF 4 Kd	Yellow orange	355.25	7.9	0.06	90.9	31
Permeate UF 4 Kd	Yellow	85	7.9	1.06	81.6	69
Retentate NF 300 Da	Yellow	325	7.9	0.09	91.3	77
Permeate NF 300 Da	Yellow	25.55	8.1	3.60	71.1	23

Table 1. Physical properties of the PROLASTIN hydrolysate and its UF and NF fractions. * Protein recovery rate was determined in reference to the unfractionated hydrolysate for the ultrafiltration and in reference to the UF permeate for the NF fractions.

	mmol.g^{-1} of dry sample																
	ALA	**GLY**	**VAL**	**LEU**	**ILE**	**THR**	**SER**	**PRO**	**ASP**	**MET**	**HYP**	**GLU**	**PHE**	**LYS**	**HIS**	**HLY**	**TYR**
Unfractionated hydrolysate	0.71	1.83	0.25	0.35	0.18	0.29	0.46	0.56	0.40	0.13	0.22	0.43	0.14	0.12	0.05	0.00	0.04
Retentate UF 4 Kd	0.65	**2.16**	0.26	0.27	0.20	0.30	0.47	**0.78**	**0.64**	0.11	**0.40**	**0.64**	0.12	0.14	0.06	0.02	0.04
Permeate UF 4 Kd	0.72	1.62	0.29	0.37	0.22	0.29	0.44	0.49	0.43	0.12	0.20	0.56	0.13	0.15	0.06	0.01	0.05
Retentate NF 300 Da	0.70	1.85	0.31	0.33	0.23	0.31	0.44	**0.62**	**0.52**	0.12	**0.27**	**0.68**	0.13	0.16	0.07	0.01	0.04
Permeate NF 300 Da	0.88	1.25	0.28	0.51	0.19	0.27	0.47	0.27	0.21	0.11	0.04	0.25	0.16	0.13	0.06	0.01	0.05

Table 2. Amino-acid composition of unfractionated PROLASTIN and related UF and NF fractions. (ALA : alanine, GLY : glycine, VAL : valine, LEU : leucine, ILE : isoleucine, THR : threonine, SER : serine, PRO : proline, ASP : aspartic acid, MET : methionine, HYP : hydroxy proline, GLU : glutamic acid, PHE : phenylalanine, LYS : lysine, HIS : histidine, HLY : hydroxy lysine , TYR : tyrosine).

Molecular weight (Da)	> 7000	7000 - 4000	4000 - 1000	1000 - 300	< 300
Unfractionated hydrolysate	0.23	1.18	23.28	45.78	29.54
Retentate UF 4 Kd	3.98	3.48	41.15	41.55	9.84
Permeate UF 4 Kd	0.02	0.08	13.9	49.66	36.33
Retentate NF 300 Da	4.85	0.09	17.49	55.78	21.78
Permeate NF 300 Da	0	0	0.74	17.79	81.47

Table 3. Molecular weight distribution of peptides contained in PROLASTIN and related UF and NF fractions (% repartition)

FRACTION	Slope	pg of gastrin/CCK-like peptides per mg dry weight	ED_{50} (mg dry weight)
^{125}I-G17	-41.99	-	-
Unfractionated hydrolysate	-38.87	1.30	11.03
Retentate UF 4 Kd	-58.86	0.98	5.97
Permeate UF 4 Kd	-48.83	0.93	9.03
Retentate NF 300 Da	-64.61	0.76	5.99
Permeate NF 300 Da	ND	ND	ND

Table 4. Secretagogue activities (gastrin/CCK-like peptides) in the PROLASTIN hydrolysate and related UF and NF fractions (ND: not determined).

FRACTION	pg of CGRP-like peptides per mg dry weight	ED_{50}, mg dry weight
Unfractionated hydrolysate	4	19
Retentate UF 4 Kd	ND*	ND*
Permeate UF 4 Kd	42	3.6
Retentate NF 300 Da	21.5	6.1
Permeate NF 300 Da	26.6	6.9

Table 5. CGRP-like peptides in the PROLASTIN hydrolysate and related UF and NF fractions (*ND: not detectable as the slope was very different from the CGRP standard slope).

FRACTION	Radical scavenging activity (DPPH$^{\bullet}$ test) AC_{50} ($mg.mL^{-1}$)	Antioxidant activity (β-carotene test) AC_{50} ($mg.mL^{-1}$)
Unfractionated hydrolysate	24.7	0.12
Retentate UF 4 Kd	39.3	0.24
Permeate UF 4 Kd	40	0.36
Retentate NF 300 Da	32.7	0.24
Permeate NF 300 Da	ND	< 0.12

Table 6. Radical scavenging and antioxidant activities of the PROLASTIN hydrolysate and related UF and NF fractions. AC_{50} corresponds to the concentration of hydrolysate ($mg.mL^{-1}$ protein) able to scavenge 50% of DPPH$^{\bullet}$ radical or induce 50% of antioxidant activity.

FRACTION	ACE-1 IC_{50} ($\mu g.mL^{-1}$)	ACE-1 max inhibition (%*)
Captopril (control ACE-1 inhibitor)	$4.34\ 10^{-3}$	100
Unfractionated hydrolysate	63	67
Permeate UF 4 Kd	62	62
Retentate UF 4 Kd	63	60
Permeate NF 300 Da	52	64
Retentate NF 300 Da	55	65

Table 7. ACE-1 inhibitory activity of the PROLASTIN hydrolysate and related UF and NF fractions.* Captopril $21.7.10^{-3}$ $\mu g.mL^{-1}$ (100 nM) was used as a reference inducing 100% ACE-1 inhibition.