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Research paper

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Received 20/01/2010; accepted 03/02/2010; published on-line 26/03/2010
Oceanraise © MS 02022010-02

Abstract

In a global context of marine biological resource overexploitation, a better upgrading of fish and shellfish biomass is a challenge for the 21st century. One of the main and promising issues will be the production of marine bio-ingredients using enzymatic hydrolysis. This paper presents the key steps in the production of enzymatic hydrolysates, such as (i) enzymatic treatment for the bioconversion of solid discards, and more particularly, use of proteases, (ii) quantification of the proteolysis extend and procedures of quality-control and (iii) identification of biological activity, using *in vitro* and *in vivo* methods. In the last part, examples of marine, commercially available functional foods or nutraceutical ingredients carrying bioactive properties are presented in order to demonstrate the interest of biotechnological exploitation of marine resources.

Key words: marine by-products; enzymatic process; degree of hydrolysis; bioactive peptides; functional food; nutraceutical ingredients.

Résumé

Dans un contexte global de surexploitation des ressources biologiques marines, la valorisation des biomasses de poissons, mollusques et crustacés représente un enjeu majeur pour le 21ème siècle. Une des voies les plus prometteuses sera la production de bio-ingrédients marins utilisant l'hydrolyse enzymatique. Ce document présente les étapes principales de la production des hydrolysats enzymatiques, telles que (i) le traitement enzymatique pour la bioconversion des déchets solides et, plus particulièrement, l'utilisation des protéases, (ii) la mesure des taux de protéolyse et les procédures de contrôle-qualité, et (iii) l'identification des activités biologiques à l'aide de méthodes de dosage *in vitro* et *in vivo*. Dans la dernière partie sont présentés des exemples d'aliments fonctionnels d'origine marine commercialisés ou d'ingrédients nutraceutiques porteurs de propriétés bioactives, démontrant ainsi l'intérêt de l'exploitation biotechnologique des ressources marines.

Mots clefs: Co-produits marins; process enzymatiques; degré d'hydrolyse; peptides bioactifs; aliments fonctionnels; ingrédients nutraceutiques.

1. Introduction

Out of the estimated 140 million tons of fish and shellfish produced each year worldwide, only one part is used for direct human consumption. This part varies from 50 to 70 % according to the type of species and the possibilities of processing. The remainder includes carcasses, frames, heads, intestinal organs and also a large amount of edible fish. In the past, these by-products have often been dumped or used without treatment for animal feed or as fertilizer. However, due to the worldwide decline of fish stocks, a better use of by-catch and by-products is deemed necessary.

Today, directing by-products more to fish hydrolysates and active ingredients is the 'big dream' of marine biotechnology industry: these products are low in volume but with high value, and there is a tremendous potential for these innovative molecules (Thorkelsson *et al.,* 2008).

Indeed, enzymatic processes applied to protein and lipid recovery in fish or shellfish discards render it possible to produce a large and diversified range of products for different applications (Guérard, 2007). Among the published works, many of them deal with enzyme technology (Aspmo *et al.,* 2005; Batista *et al.,* 2009; Chabeaud *et al.,* 2009) or fractionation of hydrolysates using membrane processes (Bourseau *et al*., 2009). A review by Kristinsson & Rasco (2000) pointed out the development of enzyme technologies for producing new feeding stuffs, similar to that already obtained by enzymatic hydrolysis of milk and plant proteins. In addition, recent studies have revealed the wide range of FPH applications, as peptone ingredient in microbial growth media, or as new sources of bioactive peptides related to immune defence, opioid, antioxidant and antihypertensive effects.

Strategic and technical considerations for enzymatic bioconversion of solid discards

When generating peptide fractions from marine by-products using an enzyme-assisted hydrolysis, 3 main factors appear to be the most important on the release of peptides with biological activity:

- The nature of the matrices
- The choice of protease
- The extend of hydrolysis

Before dealing with the choice of enzyme, it is advisable to specify that among the marine by-products, numerous substrates are potential candidates to be transformed into FPH, in conditions whereby (i) they are available in sufficient quantity for a transformation at industrial scale and (ii) the by-products are of as good quality as that of the products intended for human consumption in particular in terms of freshness and weak microbial contamination (Guérard *et al.,* 2008). Concerning the choice of enzyme, there is no standard methodology, however this step is very important if the product has to exhibit predetermined properties such as functional or bioactive properties. Because numerous enzymatic preparations are commercially available, it is neither easy nor realistic to predict which enzyme should be 'the best one' to generate peptide fractions with targeted biological activities. Table 1 summarises information on the characteristics of the most popular enzymes used in many documented studies for by-product solubilisation and suggests treatment times for inactivation at given pH and temperatures. All of them are endopeptides (*e.g*. Alcalase®, Neutrase,® Protamex®) except Flavourzyme® which is a mixture of exopeptidase/endopeptidase. The introduction of new proteases capable of degrading bitter peptides (such as Flavourzyme® from Novozymes) has contributed to eliminate the problem of bitter hydrolysate bitterness that often occurs with moderate enzymatic hydrolysis.

Table 1. *Characteristics of most popular proteases used for by-product solubilisation (adapted from www.novozymes.com and www.dsm.com).*

			Treatment times for inactivation at given pH and temperature			
	Optimum pH	Optimum temperature (℃)	pH	T(℃)	Time	Max. % DH
Alcalase® 2,4L	6.5 - 8.5	60	4-8	50-85	30-10	15-25
Flavourzyme®	5.0 - 7.0	50-55	6-8	90	10	-
Neutrase ®	5.5 - 7.5	45-55	4-7	50-80	30-4	10-15
Protamex™	7 - 8	50	4-8	50-85	30-10	10-20

Figure 1 outlines the main steps of the process in the enzymatic solubilisation of marine by-products. Briefly, solid discards are ground and suspended in water, then pH is adjusted before enzyme is added to the slurry. In some cases, the raw material is first heated in order to denature the endogenous proteases. The reaction is allowed to proceed from under one hour to several hours, depending on the activity of the enzyme employed, temperature of the process and other factors. The added enzymes are then inactivated by pH or heat treatment at the end of the batch reaction. After separation of solids, the aqueous layer is clarified, and then dryied or concentrated. Finally, biochemical assays are performed on the resulting hydrolysate. In some cases, a continuous process including enzymatic hydrolysis coupled with membrane technology will allow a repeated use of enzymes in the bioreactor.

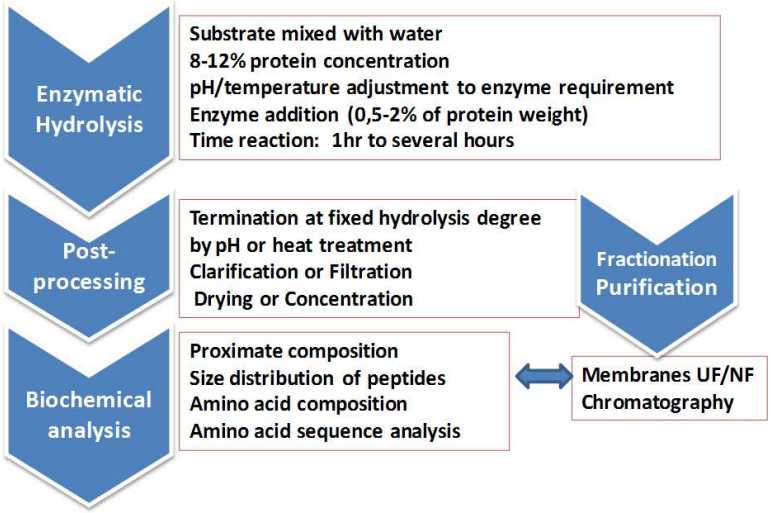

***Figure 1**. Main steps of the process in the enzymatic solubilisation of marine by-products.*

2.Quantification of the proteolysis extent

The hydrolysis reaction should be carefully controlled in order to maintain a uniform quality of the end products. The Degree of Hydrolysis (DH), which is defined as the percentage of cleaved peptide bonds, is commonly used to describe hydrolysis of food protein and serves as the controlling parameter for the hydrolysis reaction. Several methods have been applied to the quantification of proteolysis extent but one of the most popular methods is based on the evaluation of DH according to the pH-stat method (for a review, see Guérard *et al.,* 2005; Kristinsson, 2007). The pH-stat technique consists in adding base for titration of the released α-amino groups, thus maintaining the pH constant (at pH values above 6.5). Equation (1), which relates the DH to alkali consumption, is as follows (Alder-Nissen, 1982):

$$\%DH = B\ xNb\ x\frac{1}{\alpha}\ x\frac{1}{MP}\ x\frac{1}{h_{tot}}\ x\,100 \qquad (1)$$

Where B is the volume (mL) of base added, Nb is the normality of the base (mM), α is the average degree of dissociation of the α-NH2 groups, MP is the gram of protein in the reaction mixture, h_{tot} is the total number of peptide bonds (meq g^{-1}). The principle of the pH-stat method has been used by many workers for kinetic studies and the DH gives a measurement of the enzyme hydrolytic efficiency.

3. Control of the reproducibility of the enzymatic hydrolysis based on the study of molecular weight distribution

In the generation of peptide fractions with biological activities using an enzyme-assisted hydrolysis, a crucial question is: How to guarantee a consistent composition of hydrolysate peptide fractions in all batches? Protein hydrolysates can be characterized according to the size of peptides released during enzymatic hydrolysis. This procedure using Size Exclusion Chromatography (SEC) is complementary to the methods implemented for the quantification of DH. In our opinion, it is not used as frequently as it should be. SEC is a useful tool when it is necessary to check that hydrolysates can be produced in a repeatable manner (*e.g*. for the production of peptides with biological activities), whatever the production scale is (lab scale, or pilot/industrial scale). In our lab, we have selected the Superdex® Peptide HR 10/300 (Amersham Biosciences) which is characterized by a fractionation range corresponding to most of peptides exhibiting biological activity, ranging from 100 to 7000 Da. The separation conditions were optimized and resulted in using a mixture 30% (ACN): 70 % (H2O-TFA 0.1 %)

as mobile phase (Guérard *et al.,* 2001). However, the fractionation range values can only serve as guidelines, especially because the elution behaviour of peptides in non-dissociating media is influenced by adsorption and aggregation and because of the underestimation of small peptides and free amino acids. The advantage of working in FPLC mode is the rate of fractionation, as chromatographic separation is performed in less than one hour compared to several hours when using low pressure chromatography (data not shown).

4. Examples of commercially available hydrolysates carrying bioactive properties

The potential of bioactive peptides derived from dietary proteins has aroused a lot of scientific interest and attention. Bioactive peptides have been defined as specific protein fragments that may exert regulative activities on body functions, in particular, reducing the risk of disease or enhancing a certain physiological function (Meisel, 2007). They are low in molecular weight, and easily absorbable and digestible. However, the presence of bioactive peptides in marine by-product hydrolysates is less documented than bioactive peptides derived from milk or soy protein. Recent studies have identified a number of biological activities in hydrolysates from marine substrates such as fish bone, skin, internal organ, shellfish and crustacean. Researchers have identified that FPH possessed hormonal-like peptides to accelerate calcium absorption and provide satiety such as calcitonin/CGRP-like peptides and cholecystokinin-like peptides (Cudennec *et al.,* 2008) ; (Martinez-Alvarez *et al.,* 2007) and other biological activities such as immunostimulant (Yang *et al.,* 2009), antioxidant (Ranathunga *et al.,* 2006; Klompong *et al.,* 2007; Klompong *et al.,* 2008) anti-hypertensive (He *et al.,* 2007 ; Qian *et al.,* 2007; Nii *et al.,* 2008), antimicrobial effects or could be used as calcium supplement. It can be observed that among scientific publications related to biological activities of marine by-product hydrolysates, anti-hypertensive and antioxidant peptides are by far the best documented.

4.1. Lowering blood pressure

For example, hypertension is a major problem threatening people health in the world: about 20 % of the world's adult population is suffering from hypertension. Therefore, any substance such as captopril (a synthesis chemical drug) or natural ACE inhibitory peptides can finally lead to the drop of blood pressure. There are many scientific publications showing that peptides obtained from enzymatic treatment of marine substrates such as shrimp hydrolysates (He *et al.,* 2008) and Alaska Pollack hydrolysates (Byun & Kim, 2001) are able to inhibit ACE. Peptide sequences are known, ranging from 2 to 15 amino acid residues. Some commercial products containing fish protein hydrolysates able to reduce blood pressure have been approved by authorities, such as Japanese authorities in the case of the Katsuobushi oligopeptide made by hydrolysis of bonito muscle (a Thermolysin digest: Leu-Lys-Pro-Asn-Met). Several human clinical trials have confirmed this formula's efficacy with no adverse effects. These products can be used on their own or with other supplements and prescription medications.

4.2. Reducing oxidative stress

Some protein hydrolysates have been reported to exhibit antioxidant properties (*i.e*. the capacity to reduce oxidative stress). The term antioxidant is defined as 'any substance that, when present at low concentration compared to that of oxidizable substrate, significantly delays or inhibits oxidation of that substrate'. Antioxidant can act at different levels in an oxidative sequence by (i) retarding the formation of free radicals (preventive antioxidants) or (ii) by introducing substances that compete for the existing radicals and remove them from the reaction medium (chain breaking antioxidants). As a matter of fact, antioxidative activities result from a complex peptidic mixture including inactive, antioxidant and/or prooxidant compounds and represent the average activity of all peptides within the hydrolysate. Peptide sequences of antioxidant peptides are well-documented, ranging from 5 to more than 15 amino acid residues, from substrates such as Tuna backbone hydrolysed using trypsin (Je *et al.,* 2007), Hoki skin gelatine treated with trypsin (Mendis *et al.,* 2005).

Many commercial products for nutraceutical applications are available, such as:

- Stabilium 200, an Atlantic fish autolysate (www.yalacta.com) and PROTIZEN®, a white fish hydrolysate (www.copalis.fr), both carrying relaxing effect.
- Nutripeptin®, a cod hydrolysate, for lowering glycemic index (www.copalis.fr).
- Seacure®, a fish fillet hydrolysate obtained by fermentation using a marine microorganism, mainly composed of dipeptide and tripeptide, for improving gastrointestinal health (www.propernutrition.com (USA)).
- Fortidium LIQUAMEN®, a fish autolysate of white fish (*Molva molva*) (www.biothalassol.com) exhibiting multi effects such as Reducing oxidative stress, Lowering glycemic index and Anti-stress. This product also contains fish oil and vegetal oil.

6. Conclusion

The upgrading of seafood by-products is currently a rapidly developing area as shown by the numerous scientific papers published worldwide on this topic for the past ten years. This can be explained with the increasing awareness that by-products contain valuable components which can be utilized for conversion into useful and high-value products. In this review, we have attempted to demonstrate that enzymatic processes applied to marine by-product solubilisation offer a rapid and reproducible method for the recovery of high valuable compounds.

However, the first biggest challenge is ensuring good quality of raw material in terms of low microbial charge and low contaminant content such as dioxin, PCB, heavy metals, good stability to oxidation, and consistent composition in all batches resulting from enzymatic treatment of marine biomasses. The second one is documenting and verifying health claims. According to Thorkelsson *et al.,* (2008) the focus must be on more *in vivo* animal and eventually clinical human studies. The fact that most companies in this area are small makes this a difficult task because costs can be very high for those types of trials.

How to succeed?

- By setting up a fully integrated chain for the production of value added compounds derived from marine resources, embracing the total seafood production chain system from stocks to market.
- By helping the companies to take advantage of the use of modern biotechnological tools and to contribute to a diversification of the activities derived from marine biomass exploitation.
- By developing transfer of knowledge from research centres to enterprises.

Some of these actions are currently being undertaken within the European Union ERDF - Atlantic Area Programme", Biotecmar project (contract n°: 2008-1/032) which objective is to help SMEs of the Atlantic Area in making the necessary steps for supporting a fully-integrated and environmentally compatible chain for underexploited Atlantic marine resources.

Acknowledgements

This work is performed within the GESTION-DURABLE and the BIOTECMAR projects (contract n°:2008-1/032). It is co-financed with the support of the program PSDR Grand Ouest (and about Regional Development) and with the support of the European Union ERDF - Atlantic Area Programme, BIOTECMAR project n°: 2008-1/032".

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