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Production of antihypertensive and antioxidant activities by enzymatic hydrolysis of protein concentrates recovered by ultrafiltration from cuttlefish processing wastewaters

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ABSTRACT

This work focuses on the production of antihypertensive and antioxidant activities using enzymatic hydrolysis of protein concentrates recovered by ultrafiltration of different wastewaters from the industrial processing of cuttlefish (*Illex argentinus*). The effluents were produced in the processes of thawing (E1), softening (E2), boiling (E3) and gelation (E4). Our results showed that membranes with cut-off at 100, 30 and 10 kDa were an effective resource to protein concentration of E2 and E3 but limited for E1 and E4. In addition, E2 and E3 retentates led to remarkable antihypertensive and antioxidant activities, further improved by enzymatic hydrolysis. Also sequential ultrafiltration revealed the enrichment of these protein concentrates in peptides with high angiotensin-converting enzyme (ACE)-inhibitory activity. Thereby, UF-fractionation followed by proteolysis of protein concentrates from cuttlefish wastewaters offers new opportunities for the development of bioactive hydrolysates with application in the food industry. In addition, this approach contributes to an improved depuration of industrial wastewaters, reducing the treatment costs and leading to a decrease in its contaminating effect.

1. Introduction

Canning and seafood industries generate several wastewaters (washing, boiling, thawing, *etc.*) that imply problems of pollution and environment health. Among these effluents, generally boiling water has the largest concentration of salt and organic matter [1]. Although the composition of the effluents varies depending on the ratio product/water, species to be treated and time of process, it usually shows a high chemical oxygen demand [2]. Consequently these wastewaters must be depurated to eliminate their pollutant concentration, representing an increase in the cost of the industrial process.

An environmentally friendly alternative to reduce these costs is the recovery of high added value products such as proteins, aromas and flavours [3], using ultrafiltration (UF) processes. The application of membrane technology as the main method of separation, concentration and purification [4] of valuable compounds from industrial waste materials has been recently developed and successfully applied to diverse sources. Some examples include fish meal [5] and palm oil mill effluents [6], and solid byproducts of the brewing industry [7]. Among the compounds with economical interest that can be obtained from wastewaters and which have recently gained special attention, are peptides with biological activity, mainly antioxidant and antihypertensive.

Lipid oxidation has been long considered a main concern for the food industry because oxidative changes in foods may lead to loss of nutritive value and flavour deterioration. Commercial antioxidants such as butyl hydroxytoluene (BHT) and butyl hydroxyanisole (BHA) are commonly used as feed additives, being an effective strategy for preventing and reducing lipid oxidation in foods. However, and despite their high antioxidant activity, these compounds may constitute a potential health hazard for consumers [8] and consequently, search on naturally occurring compounds with antioxidant activity has increased dramatically in the last years. Several substrates have been used for obtaining such peptides, being recently fish muscle [9] and seafood byproducts [10] some of the most utilized.

Among antihypertensive peptides, those showing angiotensin-converting enzyme (ACE)-inhibitory activity are currently been widely studied and characterized due to their proven positive effect on cardiovascular health [11]. One of the most commonly used synthetic ACE inhibitors is captopril, which has been reported to have very low IC_{50} value *in vitro* [12] and it is mainly used in

Abbreviations: ACE, angiotensin-converting enzyme; BHA, butyl hydroxyanisole; BHT, butyl hydroxytoluene; DF, diafiltration; DPPH, 1,1-diphenyl-2-picryhydrazyl; FAPGG, *N*-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine; IC_{50}, inhibitory concentration causing a 50% ACE inhibition; MWCO, molecular weight cut-off; UF, ultrafiltration.

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Table 1
Mean composition of cuttlefish industrial wastewaters, E1: thawing effluent; E2: softening effluent; E3: boiling effluent; E4: gelation effluent. TS: total sugars, RS: reducing sugars, DR: dry residue, TP: total soluble Lowry-protein, COD: chemical oxygen demand and SS: suspended solids. Confidence intervals for $\alpha = 0.05$. nd: non-detected and ndd: non-determined.

	E1	E2	E3	E4
pH	7.17 ± 0.15	8.37 ± 0.04	8.25 ± 0.25	5.41 ± 0.09
TS ($g\,L^{-1}$)	0.06 ± <0.01	0.02 ± <0.01	0.07 ± 0.01	nd
RS ($g\,L^{-1}$)	0.03 ± <0.01	0.02 ± <0.01	0.03 ± 0.01	nd
DR ($g\,L^{-1}$)	5.70 ± 1.21	13.04 ± 1.86	10.11 ± 1.36	0.93 ± 0.02
Ashes ($g\,L^{-1}$)	1.74 ± 0.01	6.89 ± 1.07	5.55 ± 0.41	0.68 ± 0.10
TP ($g\,L^{-1}$)	2.81 ± 0.83	3.66 ± 1.68	3.45 ± 1.33	0.13 ± 0.02
COD ($mg\,O_2\,L^{-1}$)	ndd	165 ± 75.7	332 ± 128	ndd
SS ($mg\,L^{-1}$)	ndd	88.5 ± 40.7	300 ± 117	ndd

the treatment of essential hypertension. However, researchers are now investigating natural sources of ACE inhibitors as a milder but effective alternative for the control of blood pressure [13]. In this regard, ACE-inhibitory peptides have been obtained from various food sources such as milk [14], gelatin [15,16] and different meat and fish proteins [17].

Enzymatic proteolysis can be applied to improve and/or increase the functional and nutritional properties of food proteins [18] and to obtain value-added ingredients or additives with interest for the food industry such as pigments [19]. More recently, however, most protein hydrolysis studies focused on bioactive functionality. Among these investigations, those using fish as a protein source usually handled fish muscle as starting material for the hydrolysis and subsequent recovery of bioactive peptides [17]. Besides, during the last years, the using of ultrafiltration [5,20–22] and nanofiltration [23,24] systems for the concentration of protein from seafood wastewaters has been widely studied. This methodology allows the recycling of valuable proteins into the fish meal process, while treated water can be discharged into the sea or reused in the plant [20]. Nonetheless, to our knowledge there are no studies using protein recovered by ultrafiltration of seafood wastewaters for the production of activities with biological interest.

Therefore, the objective of this study was initially to depurate cuttlefish wastewaters and subsequently to optimize the production of peptide mixtures with antihypertensive and antioxidant activities by means of UF-fractionation followed by hydrolysis of cuttlefish processing wastewaters. For this purpose, UF using 100, 30, 10 and 1 kDa molecular weight cut-off (MWCO) membranes were performed with four effluents produced in the processes of thawing, softening, boiling and gelation of cuttlefish. Then, hydrolysates were prepared from protein-concentrates recovered by UF using alcalase, and their *in vitro* antihypertensive and antioxidant activities were calculated and compared taking into account the influence of MWCO, UF sequence and hydrolysis time. In addition, this study also analyzed how this approach contributes to a depuration of wastewaters from the seafood industry.

2. Methods

2.1. Industrial wastewaters

Wastewaters from the industrial manufacturing of Argentinus cuttlefish (*Illex argentinus*) were kindly provided by Frinova S.A., Pescanova Group (Porriño, Galicia, Spain). Four waters corresponding to thawing (E1), softening (E2), boiling (E3), and gelation (E4) processes were previously decanted in order to discard the particulate matter, sampled for analytical determinations and finally stored at −18 °C until further use.

2.2. Analytical methods

Contents of ashes, protein, total nitrogen, total and reducing sugars of the four wastewaters, were determined from subsamples taken previous to storage. Solid residue and ashes were quantified by heating and calcination at 106 and 550 °C, respectively. Total nitrogen was determined by the method of Havilah et al. [25]. Soluble proteins were measured using the method of Lowry et al. [26], total sugar content by the phenol-sulphuric acid method, according to Strickland and Parsons [27], and reducing sugars were quantified by means of 3,5-dinitrosalicylic reaction [28]. Further analysis of chemical oxygen demand (COD) and suspended solids (SS) were carried out using the APHA Standard Methods 5220 C and 2540 B, respectively [29], being the mean compositions given in Table 1.

2.3. Ultrafiltration–diafiltration process

This step consisted on performing different cascades of ultrafiltration–diafiltration using waste effluents. For this purpose, Prep/Scale-TFF cartridges (Millipore Corporation, Bedford, MA, USA) of 100, 30, 10 and 1 kDa molecular weight cut-off (MWCO) were used. According to the manufacturer, cartridges were made of polyethersulfone, except for 1 kDa, which was from regenerated cellulose.

The operation mode was the following: an initial phase of ultrafiltration (UF) at 40 °C with total recirculation of retentate was performed, immediately followed by a diafiltration (DF) step (Fig. 1). During UF, the inlet pressure remained constant to determine the drops of flow rate due to the increased concentration of the retentate and to possible adhesions to the membrane. In each case, the final retentate (after DF) was lyophilized and stored at 4 °C for further analysis. The permeate obtained in the UF step was stored at −18 °C until the following UF–DF was carried out, according to the sequential scheme shown in Fig. 1. The permeate from the DF phase was discarded.

For modelling the membrane process, we assumed that in the DF with constant volume (filtration flow = water intake flow), the concentration (or the total amount) of a permeable solute in the retentate followed first order kinetics [4]:

$$R = R_f + R_0 \exp[-(1 - s)D] \quad (1)$$

where, R is the concentration of permeable protein in the retentate (% from the level at initial DF), R_0 the initial concentration (%), R_f is the final and asymptotic concentration (%), D is the relative diavolume (volume of added water/constant retentate volume) and s is the specific retention of protein with variation between 0 (the solute is filtered as the solvent) and 1 (the solute is totally retained). Thus, using normalized values (%): $R_0 + R_f = 100$, with $R_0 = 0$ if all protein is permeable. In addition, the percentage of protein eliminated by three diavolumes (Q_{3D}) was calculated by substituting in Eq. (1) the value of parameter D by 3.

2.4. Preparation of enzymatic hydrolysates

Each lyophilized retentate was suspended in 5 mM Britton–Robinson buffer pH 9.0, at a final concentration of 100 $g\,L^{-1}$. These suspensions were divided into individual experimental