Shrimp (*Penaueus vannamei*) cooking wastewater as a source of astaxanthin and bioactive peptides

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ABSTRACT

BACKGROUND: Ultrafiltration has led to the recovery of valuable products from seafood processing wastes. However, the use of crustacean process wastewaters as a source of bioactive compounds with application in the chemical, pharmaceutical and food industries has been scarcely studied to date.

RESULTS: This study reports the recovery of high concentrations of astaxanthin (10-13 μg mL^{-1}) by 300 kDa ultrafiltration of shrimp cooking wastewater, indicating astaxanthin is somehow associated to high MW retained proteins. Individual UF with 300, 100 and 30 kDa MWCO membranes and the sequential UF 100→30 kDa were the most effective configurations for protein concentration. Besides, hydrolysates from these three protein-concentrated fractions showed very potent ACE-inhibitory (1.98, 9.87 and 23.10 μg mL^{-1} IC_{50} values) and ß- carotene bleaching activities compared to hydrolysates from other fish and seafood species.

CONCLUSIONS: According to these results, shrimp cooking wastewater is a good source of astaxanthin and bioactive peptides. This approach based on the ultrafiltration of shrimp cooking wastewaters contributes towards the depuration of this by-product, while enabling the recovery and production of high value-added products.

Keywords:

Ultrafiltration; enzymatic proteolysis; bioactive peptides; seafood wastewater; shrimp; astaxanthin.

Abreviations:

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.

1. Introduction

The shrimp processing industry produces large amounts of industrial wastes. These residues are mostly solid by-products consisting of body carapace and head [1] that are rich in proteins, chitin and carotenoids. Among these products, chitin has focused scientific interest because its N-deacetylated derivative, chitosan, is soluble in acid solutions and has many different applications. The recovery of carotenoids such as astaxanthin from shrimp solid by-products has also been widely studied [1,2] due to its application in the pharmaceutical, chemical, food and feed industries because, among others, to its colouring and antioxidant properties. Astaxanthin is bound to lipoproteins from the shell matrix of crustaceans [3]. The removal of free astaxanthin from these sources implies the using of thermal treatment [1], enzymatic hydrolysis [3,4] or fermentation with lactic acid bacteria [2], followed by the extraction with organic solvents [4,5] or edible oils [1,6,7]. Although the yield of carotenoids recovered from these shrimp solid wastes depends on their treatment, in general, organic solvents are more efficient than oil extraction [4]. However the latter are preferred as an environmentally-friendly alternative compatible with food applications. Recently, alternative processes like solid-phase [8] and supercritical CO_2 [9] extraction have also been proposed for astaxanthin recovery from shrimp by-products.

In addition to solid wastes, shellfish cooking plants produce highly pollutant wastewaters [10]. Although the effluent composition varies depending on the ratio product/water, the animal species and the cooking duration, they usually show a high chemical oxygen demand due to their high organic load [11]. At present, effluents are either directly discarded in the environment or sent to a public wastewater treatment plant according to their pollutant load [10]. Conventional seafood wastewater processing methods were traditionally activated sludge and aerated lagoon system, but more recently new processes offering improved capability, lower cost

performance, and better care for the environment have been implemented [12]. Some alternatives are the use of anaerobic treatment or hybrid systems combining anaerobic and aerobic processes with anaerobic pre-treatment [12]. However, the efficiency of biological treatment of wastewaters with high saline content by conventional techniques is poor due to sodium toxicity to microorganisms [13]. The low depuration of cooking effluents containing a high saline content [11] leads to poor effluent quality and therefore alternative methods must be explored.

Besides, the costs of wastewater processing are generally high [11] due to the need of pre-treatement and the complexity of the different phases involved. It has been claimed that the recovery of the residual organic material present in crustacean cooking water could reduce the cost of depolluting treatments, and it would also lead to their valorisation by searching for potentially bioactive molecules [14]. In this context, the use of membrane technology is a promising alternative allowing the achievement of both objectives in a single stage or combined with the hydrolysis of the retained protein. Actually, the application of ultrafiltration for the separation, concentration and purification of valuable compounds from seafood by-products has been successfully applied to different sources. Li et al. [15] reported the recovery of the enzymes trypsin and chymotrypsin from yellowfin tuna spleen extract by microfiltration followed by continuous-batch concentration using a 0.10 μm hollow fiber membrane. On the other hand, Murado et al. [16] obtained highly pure chondroitin sulphate (99%) by chemical hydrolysis, selective precipitation and ultrafiltration–diafiltration of Thornback skate (*Raja clavata*). Also the use of ultra- and nanofiltration has proven to be effective for the recovery of aroma [11,17], proteins [18, 19] and bioactive peptides [19] from seafood wastewaters. The latter, mainly antioxidant and ACE-inhibitory peptides, have received special attention due to their economical interest as potential alternatives to synthetic compounds. Recently, Pérez-Santín et al. [14] reported the

production of a functional concentrate with antioxidative and antihypertensive (ACE-inhibitory) properties from shrimp (*Penaeus* spp.) cooking juice by centrifugation. These activities were strongly related to the presence of small peptides and other antioxidants, such as free astaxanthin (cis and trans isomers) and derived esters.

However, to the best of our knowledge, there are no reports on the use of ultrafiltration (UF) for the recovery of astaxanthin neither for producing antioxidants and ACE-inhibitory hydrolysates from shrimp cooking wastewaters.

In this paper we propose an integrated method for producing protein hydrolysates with high ACE-inhibitory and/or antioxidant activity from shrimp cooking wastewaters, by combining UF and enzymatic hydrolysis. Also a protein and astaxanthin-concentrated fraction with potential application as additive for aquaculture feeding can be recovered by membrane technology. This approach can be an interesting alternative for reducing the costs of wastewater treatment and their pollutant effects.

2. Materials and Methods

2.1. Shrimp cooking wastewater

In this study we utilised cooking wastewater from the industrial manufacturing of shrimp (*Penaueus vannamei*) that was kindly provided by Bajamar Séptima S.A. (Arteixo, Galicia, Spain). The wastewater was received under refrigeration, decanted to discard particulate matter such as small meat and shell pieces, sampled for analytical determinations and stored at -18ºC until further use.

2.2. Analytical methods

The content of ashes, protein, total nitrogen, total and reducing sugars was determined. Solid residue and ashes were quantified by heating and calcination at 106 and 550ºC, respectively. Total nitrogen was determined by the method of

Havilah et al. [20]. Soluble proteins were measured using the method of Lowry et al. [21], total sugar content by the phenol-sulphuric acid method, according to Strickland and Parsons [22], and reducing sugars were quantified by means of 3,5-dinitrosalicylic reaction [23]. 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 [24], being the mean compositions given in Table 1.

To determine astaxanthin concentration, 1 volume of the 300 kDa retentate was combined with 3 volumes of sunflower oil at 30ºC and the mixture stirred for 18 h [25]. Then the solution absorbance was measured at the λ_{max} (487 nm: A487) and the carotenoid yield as astaxanthin (μg mL^{-1}) was calculated using the following equation [1]:

$$Y = \frac{A_{487} \times V_{oil} \times 10^6}{100 \times V_w \times E} \quad (1)$$

where,

Y is the astaxanthin yield per volume of bulk liquid (μg mL^{-1}); V_{oil} is the volume of recovered pigmented oil; V_w the volume of retentate and E the specific extinction coefficient.

2.3. Ultrafiltration-diafiltration process

The cooking wastewater was subjected to ultrafiltration-diafiltration (UF-DF) using a single or sequential UF steps with membranes (Prep/Scale-TFF cartridges, Millipore Corporation, Bedford, MA, USA) of 300, 100, 30, 10 and 1 kDa molecular weight cut-off (MWCO). 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 40ºC with total recirculation of retentate was performed, immediately followed by a diafiltration (DF) step. 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. The final retentate (after DF) was lyophilized and stored at 4ºC for further analysis. Permeate of the UF step was stored at -18 °C until the following UF-DF was carried out and permeate from the DF phase was discarded.

For modelling the membrane process, we assumed a DF with constant volume (filtration flow = water intake flow), were the concentration (or the total amount) of a permeable solute in the retentate followed first order kinetics [19]:

$$R = R_f + R_0 \exp\left[-(1-s)D\right] \qquad (2)$$

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 equation (2) the value of parameter D by 3.

2.4. Preparation of enzymatic hydrolysates

Each lyophilized protein concentrate was suspended in 5 mM Britton-Robinson buffer pH 9.0 at a final concentration of 100 g L^{-1} and hydrolysed using a 0.4 % (v/w) of

alcalase 2.4 L from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). These suspensions were divided into individual experimental units of 2 mL, each corresponding to a time of hydrolysis and the enzymatic hydrolysis carried out in a water bath at 55ºC under agitation. Just after adding the enzyme, a sample was taken (t=0) and then after 15, 30, 60, 120, 240 and 480 min of hydrolysis. Samples were boiled for 5 min to inactivate the protease and then cooled in an ice-water bath. Hydrolysates were centrifuged at 5000 *g* for 15 min in an Allegra X-12R centrifuge (Beckman Coulter, Inc., Miami, USA) and supernatants were stored at -18ºC until further analysis of their degree of hydrolysis, ACE inhibitory and antioxidant activities.

2.5. Degree of hydrolysis determination

The degree of hydrolysis (DH) at each sampling time was defined as the ratio of the released tyrosine to the maximal tyrosine in the sample [19,26], according to the following expression:

$$DH = \frac{C_t - C_0}{C_m} \times 100 \quad (3)$$

where, C_t is the tyrosine concentration (g L^{-1}) of the hydrolysate at time *t*, C_0 is the tyrosine concentration (g L^{-1}) of the sample at time zero and C_m is the maximal tyrosine concentration (g L^{-1}) of the sample. Tyrosine was measured by the method described in Barker and Worgan [27]. The kinetic profiles of DH (%) were described using the von Bertalanffy equation [19]:

$$DH = K\left[1 - \exp(-rt)\right] \quad (4)$$

where, K is the maximum DH (%) or the limit of the function when $t \to \infty$, r is the specific maximum rate of hydrolysis (min^{-1}) and t the time of hydrolysis (min). Furthermore, we have defined the maximum rate of hydrolysis (v_m in % min^{-1}) as $v_m = Kr$.

2.6. Angiotensin I-converting enzyme (ACE) inhibition assay

The antihypertensive activity of the hydrolysates was determined using N-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate [28] as adapted by Estévez et al. [29]. Briefly, 10 µL of supernatant hydrolysate were mixed with 10 µL of ACE solution (0.5 U mL^{-1}) in each well of a 96-well microtiter plate. The reaction was started by adding 150 µL of substrate (0.88 mM FAPGG in 50 mM Tris-HCl, pH 7.5, 0.3 M NaCl) preheated at 37°C. The absorbance at 340 nm (A_{340}) was recorded at time intervals of 30 seconds for 30 minutes. The control consisted of samples containing 10 µL of buffer (50 mM Tris-HCl, pH 7.5, 0.3 M NaCl) instead of protein hydrolysate.

Hydrolysates ACE-inhibitory capacity (I_{ACE}) was calculated as a function of the average slope of decrease in absorbance with time and expressed as percent inhibition of the enzyme, according to the following expression:

$$I_{ACE}(\%) = \left[1 - \frac{\rho A_h}{\rho A_c}\right] \times 100 \qquad (5)$$

where, I_{ACE} is the ACE-inhibitory capacity (%), ρA_h is the slope of decrease in A_{340} in the presence of inhibitor (hydrolysate) and ρA_c is the slope of decrease in A_{340} in the absence of inhibitor (control).

For the calculation of the protein concentration causing a 50% ACE inhibition (IC_{50}), dose-response curves were obtained assaying different concentrations of hydrolysates. IC_{50} values were calculated by fitting the dose-response curves of I_{ACE} *vs.* protein concentration to a sigmoidal model defined by the Weibull equation [19,29]:

$$I_{ACE} = K\left\{1-\exp\left[-\ln 2\left(\frac{C}{m}\right)^{a}\right]\right\} \quad (6)$$

where, K is the maximum I_{ACE} (%), C is the protein concentration (μg mL^{-1}), m is the concentration for semi-maximum I_{ACE} (IC_{50} in μg mL^{-1}) and a is the form parameter related to the maximum slope of the function (*dimensionless).*

2.7. Antioxidant activity determination

2.7.1. β *-carotene bleaching assay*

The β-carotene bleaching assay adapted to use with microplate spectrophotometer [30] was utilized. Reactions were performed combining in each well of a 96-well microplate, 50 *μ*L of antioxidant (BHT at 6-30 mg L^{-1} or hydrolysate sample) with 250 *μ*L of β-carotene/linoleic emulsion. The absorbance at 470 nm was recorded during 180 min in a Multiskan Spectrum Microplate Spectrophotometer (Thermo Scientific) at 45ºC.

All reaction mixtures were performed in triplicate. For each series, reversed curves were obtained by subtracting the absorbance at time *t* from the absorbance value at time *0*. The area under the curves (AUC) can be approximated by the following function:

$$AUC = \frac{\Delta t}{2}\left(y_0 + 2\,y_1 + 2\,y_2 + \cdots + 2\,y_{n-2} + 2\,y_{n-1} + 2\,y_n\right) \quad (7)$$

where y_0 to y_n are the n+1 y-values defining the curve, and Δt is the sampling interval (min).

Calculated areas were plotted against BHT concentrations and fitted by linear regression. Then antioxidant activities were calculated as μM BHT equivalent mg^{-1} of sample by interpolation of the calculated areas for each sample in the BHT curve obtained for each assay.

2.7.2. 1,1-Diphenyl-2-picryhydrazyl (DPPH) radical-scavenging capacity

The antioxidant activity as radical-scavenging capacity was determined with DPPH as a free radical, using an adaptation to microplate of the method described by Brand-Williams et al. [31]. For the modified procedure, hydrolysate supernatants containing the antioxidant activity (10 µl) were added to 200 µl of a 60 µM solution of DPPH in 50% ethanol. The decrease in absorbance was followed at 515 nm every 5 min until the reaction reached a plateau. The activity was expressed as BHT equivalents (μM BHT equivalent mg^{-1}), calculated as described above.

2.8. Numerical and statistical methods

Fitting procedures and parametric estimations calculated from the experimental data were carried out by minimising the sum of quadratic differences between the observed and model-predicted values, using the non-linear least-squares (quasi-Newton) method provided by the macro *Solver* of the Microsoft Excel spreadsheet. The confidence intervals for the parametric estimates (t-Student test; α = 0.05), consistency of the mathematical models (Fisher's F test; α = 0.05) and covariance and correlation matrices were calculated using the '*SolverAid*' macro, which is freely

available from Levie´s Excellaneous website: http://www.bowdoin.edu/~rdelevie/exellaneous/.

3. Results and Discussion

3.1. Protein and astaxanthin concentration by UF-DF

The shrimp cooking wastewater had rather low protein content and COD (Table 1) compared to values previously reported for seafood cooking juices [11,17]. The composition of the effluents varies depending on the ratio product/water, species to be treated and processing time [32] and so the low protein concentration must be due to a low ratio shrimp/water and/or to a short time of cooking.

The protein concentration kinetics of four examples from all the UF cut-offs and sequences studied are shown in Figure 1 (additional UF-DF kinetics can be seen in the Appendix, Figures 1-3). Since equation (2) accurately simulated the DF process and parameter determinations were always significant (Student's t test, α = 0.05), the estimated protein rejection (Q_{3D}) was calculated using this equation.

Ultrafiltration with 300, 100 and 30 kDa MWCO membranes led to the best results of protein recovery, reaching a maximum of 27.12 ± 0.16 g L^{-1} protein content after 30 kDa UF (Table 2). In general, the using of a single membrane produced better results in terms of protein recovery (>80%) than following sequential UF. Nonetheless, 100→30 kDa and 300→100→30 kDa UF sequences resulted in a 90% and 75% of protein retention while in all other cases, protein rejection varied from 33% to 66% (Table 2) revealing a moderate to low efficiency of these UF sequences for protein concentration. These results suggest the major shrimp proteins in cooking wastewater are in a range of molecular weights within 100 and 30 kDa.

Comparing UF performances with reported values in the literature is not always simple because experimental conditions are diverse in terms of wastewater source,

concentration methodology and parameter determination (usually chemical oxygen demand or nitrogen content). But, taking into account that most of organic compounds in the cooking wastewater are protein (Table 1), COD reductions above 80% were achieved leading to comparable depuration efficiencies than those reported for the recovery of marine flavours from seafood cooking waters by nanofiltration and reverse osmosis [10] and by nanofiltration of fish meal waters [18]. Also COD depurations were higher than those reported by Ferjani et al. [33] for cuttlefish wastes with greater organic load.

The UF-DF process at 300 kDa surprisingly showed a high retention of astaxanthin despite the low molecular weight (597 Da) of the pigment. In fact, during the UF phase, the initial permeates showed slight yellowish coloration, completely disappearing after DF and leading to an intense colored (pink-orange) retentate. A 13-fold concentration factor was achieved after 300 kDa UF-DF, reaching astaxanthin concentrations between 10 and 13 μg mL^{-1} in the retentate according to spectrophotometric analysis (Equation 1). Astaxanthin is found naturally in the shell matrix of crustaceans mainly esterified or complexed with proteins [3]. So, this result suggests astaxanthin is retained because it must be forming polymeric aggregates and/or bound to macromolecules, mainly proteins, that are also retained during ultrafiltration using a 300 kDa membrane.

3.2. Enzymatic hydrolysis of protein concentrates

The concentration of shrimp hydrolysable protein in the concentrates recovered by UF-DF was found to be very different (Table 3). Surprisingly, the protein content before hydrolysis was significantly lower for those fractions resulting from the UFs with the highest performances (300, 100 and 30 kDa). Since all fractions were normalized in dry matter content upon lyophilisation, the low protein concentration

indicated a relatively higher content in other compounds such as lipids, sugars and salts. Hydrolysis of shrimp protein concentrates recovered by UF-DF were performed with alcalase using experimental conditions (pH 9.0, 55ºC) previously utilised for the production of cuttlefish hydrolysates [19]. As in that case, alcalase was chosen due to the broad substrate specificity and low-cost compared to other proteases commercially available.

Shrimp protein concentrates recovered by UF using different MWCO membranes provided a wide variety of hydrolysis profiles (Figure 2). Proteolytic kinetics were modelled using the von Bertalanffy equation (Equation 4) obtaining statistically significant parameters ($p<0.05$) for all of them (Table 3). As previously reported for shrimp by-products [4] and other fish species [34,35], alcalase efficiently hydrolysed shrimp proteins recovered from cooking wastewaters. According to the parametric estimations, the highest levels of hydrolysis (K) were achieved for protein concentrates obtained from individual rather than sequential UF and decreased when lower MWCO membranes were utilised (Table 3). Thereby, retentates of 300 and 100 kDa provided maximal K values (80 and 70%, respectively) whereas the DH dropped to 35 and 43% when UF was performed using 30 and 1 kDa membranes. Also maximal hydrolysis rates (v_m) for these samples were 10-80 times higher than those of protein concentrates recovered from sequential UF, indicating retentates from UF at 300, 100, 30 and 1 kDa were the fastest and highly hydrolysable (Table 3). An identical trend was observed in the hydrolysis of cuttlefish proteins recovered by UF from four industrial effluents [19] and support the preference of alcalase for high molecular weight substrates.

The protein content might have an effect on the degree and rate of hydrolysis but, if time enough is given to the hydrolysis mixture to react, a higher E/S ratio would increase the rate of hydrolysis rather than the maximum degree of hydrolysis. Since

almost all hydrolysis carried out reached a steady-state phase (data not shown), other factors might have a greater influence on the efficiency of the hydrolysis process, including the presence of other compounds and the MW and composition of the main proteins in the UF fractions.

3.3. ACE inhibitory activity of shrimp hydrolysates

ACE-inhibitory activity (I_{ACE}) varied from 24.5 to 92.4% (Table 3). Our results revealed ACE-inhibitory activity increased ($p<0.05$) up to a maximum after 1-2 h of hydrolysis, remaining stable after 8 h (data not shown). Therefore and for comparative purposes, only the activities before and after 8 h of hydrolysis are summarised (Table 3). The higher ACE-inhibitory values after hydrolysis revealed the efficacy of alcalase in releasing active peptides from shrimp protein concentrates.

Due to its broad specificity, alcalase extensively hydrolyses peptidic bonds and so it is more likely to produce active peptides. For this reason it has been widely employed to digest different marine organisms [19,34,37] to produce ACE inhibitors. Peptides containing hydrophobic amino acids in the C-terminal sequence are particularly suitable for their binding to ACE and consequently better inhibitors of the enzyme [36]. On the contrary, the hydrolysis of the 300→1 kDa protein concentrate did not significantly increase ACE-inhibitory activity, suggesting a further hydrolysis of the active peptides or a formation of less inhibitory peptides [29]. Nevertheless, further analysis in terms of size characterization and amino acid composition of the different fractions is needed to confirm this hypothesis.

However, the calculation of IC_{50} values is the best way to compare ACE-inhibitory activity because this parameter only depends on intrinsic characteristics of the sample. In our study, at least seven concentrations of each protein hydrolysate were assayed to calculate their IC_{50} values. Dose-response curves were constructed

plotting ACE-inhibitory activity (%) as a function of protein concentration (μg mL^{-1}). Data of samples showing different dose-response profiles (Figure 3) were modelled using equation (6) in order to compare their ACE-inhibitory potency. This model has been used to fit dose-response curves in different research areas [38,39] and recently has also been satisfactorily applied for IC_{50} calculation of protein hydrolysates [19,29]. Equation 6 accurately fitted the experimental data (Figure 3) providing in all cases high coefficients of determination (R^2> 0.98). Moreover, this equation was consistent (Fisher's F test; p < 0.05) and parameter estimations were always significant (Student's t test, α = 0.05) (data not shown).

Since the IC_{50} value is the protein concentration causing a 50% ACE inhibition, this means that samples providing identical inhibitory level at lower protein concentration are more active, *i.e*. have a lower IC_{50} value. But this does not imply samples with different protein content would necessarily have different IC_{50} values. Actually, a sample with higher protein content could have a lower IC_{50} value depending on the composition, MW and the ratio of active/ total peptides. For instance, 8h hydrolysates of the samples 300→100→30 and 100→30 kDa have higher protein content than 30→10→1 kDa and yet their IC_{50} value is lower (Table 3).

Our results indicated that UF-fractionation followed by proteolysis of shrimp protein concentrates produced hydrolysates with very different IC_{50} values (Table 3). ACE inhibitory potencies varied from 1.98 to 618 μg mL^{-1}, suggesting that this methodology can provide peptide mixtures with highly diverse compositions based on the MWCO of the UF membranes utilized. The analysis of IC_{50} values clearly showed that samples hydrolysed to a higher extent were the most active, *i. e.* with lower IC_{50} values. Therefore, hydrolysates from protein concentrates recovered by UF at 300, 100, 30 and 1 kDa had the lowest IC_{50} values, although the activity of the latter was from 9 to 100-fold lower than the others. Balti et al. [34] also observed a direct

relationship between ACE inhibitory activity (%) and DH of cuttlefish muscle protein hydrolysates prepared by treatment with various bacterial proteases.

The IC_{50} values of the 300, 100, 30 kDa hydrolysates were as low as 1.98, 9.87 and 23.10 μg mL^{-1}, revealing a very potent ACE-inhibitory activity of these peptide mixtures. These ACE-inhibitory capacities were higher than those recently reported for a concentrate recovered from shrimp (*Penaeus* spp.) cooking juice by centrifugation [14]. These authors found IC_{50} values of 4004 and 1700 μg mL^{-1} for the liquid supernatant (CJ-L) and the concentrate (CJ-S) separated after passing a thawed cooking juice (CJ) through a centrifugal separator. Also higher IC_{50} values than those of our four most active fractions were reported for *Acetes chinensis* muscle hydrolysates at laboratory [40] and pilot scale [41] production before (980 μg mL^{-1}) and after (220 μg mL^{-1}) of 3 kDa UF. However, closer ACE-inhibitory potencies to those reported in our work were obtained for alcalase (100–200 μg mL^{-1}) and protamex (70 μg mL^{-1}) hydrolysates produced from solid wastes of cooked shrimps (*Pandalopsis dispar*) [42].

3.4. Antioxidant activity of shrimp hydrolysates

Results of *in vitro* antioxidant activity support the fact that the most bioactive hydrolysates are those prepared from 300, 100 and 30 kDa protein concentrates (Table 4). In contrast to ACE-inhibitory activity, although in general our results showed increased antioxidant capacity along proteolysis progress, in some cases, the effect was not significant or the activity decreased after 8h of alcalase hydrolysis. In fact, the scavenging on DPPH of the most active fractions significantly ($p<0.05$) decreased (300 and 100 kDa) or was maintained (30 kDa) after proteolysis (Table 4). Although several investigations have shown a positive correlation between high degree of hydrolysis and antioxidant activity for different fish [43,44] hydrolysates, other

studies have also reported a decrease of scavenging activity after protein hydrolysis [45]. Hence these results agree in suggesting that, in addition to peptide length, other factors such as amino acid composition, presence of free amino acids and differences in test conditions also determine antioxidant activity of protein hydrolysates. A recent study dealing with the contribution of individual or groups of amino acids to antioxidant activities of food protein hydrolysates [46], concluded that sulfur-containing, acidic and hydrophobic amino acids had strong positive effects on scavenging on DPPH. In contrast, positively- charged amino acids negatively contributed to scavenging of this stable radical. On the other hand, the presence of these residues, such as arginine and lysine, in C-terminal peptide sequences contribute towards improved ACE inhibitory activity [42]. Then, if alcalase hydrolysis was likely to produce a high proportion of peptides containing this residues in the C-terminus position, this would explain the observed increase in ACE-inhibitory activity and the loss of DPPH scavenging activity after extensive hydrolysis with this protease. Many peptides containing at least one of these residues in the C-terminal sequence were identified in most of the major ACE-inhibitory fractions isolated from a cuttlefish hydrolysate [47], prepared using the same conditions reported in this work. Nevertheless, to confirm this hypothesis the composition of the most active fractions must be analysed.

The highest antioxidant activity was observed in the 300 kDa retentate due to the high astaxanthin concentration in this fraction (Table 4). This carotenoid is known to have strong antioxidant activity because of its ability to quench singlet oxygen and to scavenge free radicals as a consequence of the presence of conjugated double bonds in this compound. According to Pérez-Santín et al. [14], other antioxidant compounds in shrimp cooking juice might be principally peptides, reducing sugars,

citrates or phenolic compounds, being the latter also reported to occur in shrimp shell wastes.

The 300 kDa UF process leading to the recovery of a protein and astaxanthin concentrated fraction from shrimp cooking wastewater is schematically represented in Figure 4. This flowchart also summarizes the protein concentration, ACE-inihibitory and antioxidant activity of the 300, 100, 300→100→30→10 →1 and 100→30→10 →1 kDa, as two examples of sequential UFs using all the MWCO membranes utilised in this work.

4. Conclusions

Ultrafiltration of shrimp cooking wastewater with 300, 100, 30 and 100→30 kDa MWCO membranes is an effective resource to protein concentration, indicating the main soluble proteins in this effluent are within 100 and 30 kDa. Hydrolysates prepared from 300, 100 and 30 kDa protein-concentrated fractions have remarkable antihypertensive (IC_{50} values of 1.98, 9.87 and 23.10 μg mL^{-1}, respectively) and antioxidant activities.

In addition, concentrations of 10-13 μg mL^{-1} astaxanthin can be recovered after UF at 300 kDa due to its aggregation to high MW proteins. For these reasons, shrimp cooking wastewater is a promising source of valuable compounds for the formulation of functional foods while allowing wastewater depuration.

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TABLE CAPTIONS

Table 1. Average composition of shrimp industrial wastewater. TS: total sugars, RS: reducing sugars, DR: dry residue, TP: total soluble Lowry-protein, COD: chemical oxygen demand, SS: suspended solids. Confidence intervals for α=0.05.

Table 2. Initial (C_i), final (C_f) protein concentration and Q_{3D} parameter in the retentates for each UF-DF process with different molecular weight cut-off (MWCO) membranes are shown.

Table 3. Parameters of the hydrolysis kinetics (equation (4)) from shrimp protein concentrates produced by UF with different MWCO membranes. Total protein (TP) and ACE-inhibitory activity (%) of these hydrolysates before and after 8 h hydrolysis, and IC_{50} values of 8 h hydrolysates or the final retentate, when hydrolysis was not carried out, are also shown.

Table 4. β-carotene bleaching assay (βC) and DPPH radical scavenging activities (μM BHT equivalent mg^{-1} of sample) of shrimp hydrolysates determined before and after 8h hydrolysis. ND: not activity detected.

FIGURE CAPTIONS

Figure 1. Selection of four different UF-DF process from shrimp cooking wastewater using the following molecular weight cut-offs membranes: A) 300 kDa; B) 300→100 kDa; C) 300→100→30 kDa and D) 300→100→30→10 kDa. Left: concentration of retained protein in linear relation with the factor of volumetric concentration (fc) showing experimental data (points) and theoretical profiles corresponding to a completely retained solute (discontinuous line). Right: progress of protein (○) and nitrogen (●) retention with the increase of diavolume from DF process (D). For clarity, confidence intervals (in all cases less than 5% of the experimental mean value; α = 0.05; n = 2) were omitted. Equation (2) was used to fit the experimental data.

Figure 2. Proteolytic kinetics of shrimp protein concentrates obtained by ultrafiltration using different molecular weight cut-off membranes. Left: ●, 30 kDa; ○, 300→100→30 kDa; ▼, 100→30 kDa. Middle: ●,300→100→30→10 kDa; ○, 30→10 kDa. Right: ●, 1 kDa; ○, 300→1 kDa; ▼, 100→30→10→1 kDa; △, 30→10→1 kDa. Experimental data (points) were fitted to equation (4) (continuous lines).

Figure 3. Experimental data (symbols) for ACE-inhibitory activity of hydrolysates prepared from different shrimp protein concentrated fractions recovered by ultrafiltration (UF). The dose-response curves (lines) were obtained according to equation (6). Keys in A: ●, 300 kDa; ○, 100 kDa; ▲, 30 kDa UF retentates. Keys in B: ○, 100→30→10→1 kDa; ●, 300→100→30→10→1 kDa and ▲ 30→10→1 kDa UF retentates.

Figure 4. Flowchart summarizing different alternatives for the recovery of astaxanthin and the production of bioactive peptides from shrimp cooking wastewaters.