LWT - Food Science and Technology 59 (2014) 841–848

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Production and characterization of encapsulated antioxidative protein hydrolysates from Whitemouth croaker (*Micropogonias furnieri*) muscle and byproduct

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ARTICLE INFO

Article history:
Received 3 February 2014
Received in revised form
28 April 2014
Accepted 7 May 2014
Available online 15 May 2014

Keywords:
Antioxidant
Encapsulation
Hydrolysate
Fish
Protein

ABSTRACT

The objective of this study was to produce encapsulated protein hydrolysates from Whitemouth croaker (*Micropogonias furnieri*) muscle and its industrialization byproduct. The protein hydrolysates were prepared from the muscle (MPH) and byproduct (BPH) from croaker by enzymatic hydrolysis using Flavourzyme®. The hydrolysates were encapsulated using phosphatidylcholine as the wall material of the capsules. The capsules were evaluated for particle size, polydispersity, encapsulation efficiency, zeta potential, morphology, thermal properties, Fourier transform infrared (FTIR) spectroscopy and antioxidant activity. The average size of the capsules for both MPH and BPH liposomes range between 266 and 263 nm with low polydispersity. The capsules showed high encapsulation efficiency of around 80%. The FTIR analysis allowed suggesting that there was an effective ionic complexation between phosphatidylcholine and hydrolysate peptides. The antioxidant activity of the hydrolysates and capsules containing MPH and BPH was similar to the activity of α-tocoferol, but lower than that of vitamin C.

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1. Introduction

Enzymatic hydrolysis proteins are an efficient way to produce potent bioactive peptides (Thiansilakul, Benjakul, & Shahidi, 2007). Various food protein sources including fish, milk, egg, soybean, wheat and zein, among others, have been exploited to produce antioxidative protein hydrolysates and peptides (Samaranayaka & Li-Chan, 2011). Protein hydrolysates are breakdown products of enzymatic conversion of proteins into smaller peptides. Generally, protein hydrolysates are small fragments of peptides that contain 2–20 amino acids. These protein hydrolysates are produced by the enzymatic hydrolysis of native proteins. Protein hydrolysis decreases peptide size, thereby making hydrolysates the most available amino acid source for various physiological functions of the human body (Neklyudov, Ivankin, & Berdutina, 2000).

Proteins are sources of bioactive peptides that are inactive and are activated during the digestive process or during food processing. Once released, peptides exert diverse physiological functions such as anti-ulcer, anticarcinogenic, antihypertensive, and antioxidant activity (Korhonen & Pihlanto, 2003). Antioxidant activity is associated with certain peptides present in protein sequences, released after enzymatic hydrolysis. Antioxidants are compounds that can act as hydrogen donors, stabilizing free radicals that are formed naturally in cell metabolism and are responsible for many degenerative diseases such as cardiovascular diseases, diabetes, and Alzheimer's disease (Chanput, Theerakulkait, & Nakai, 2009; Harnedy & FitzGerald, 2012).

The antioxidant properties of hydrolysates have been investigated and have been demonstrated by the hydrolysis of several proteins by gastrointestinal enzymes or by acid hydrolysis. The exact mechanism of antioxidant activity is not well understood, but several studies show that hydrolysates are lipid oxidation inhibitors

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http://dx.doi.org/10.1016/j.lwt.2014.05.013
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which are capable of scavenging free radicals and chelating metal ion activity (Qian, Jung, & Kim, 2008; Rajapakse, Mendis, Jung, Je, & Kim, 2005). According to Moosman and Behl (2002), hydrolysates with a high degree of hydrolysis have a higher amount of low—molecular-weight peptides, and thus higher potential for oxidation inhibition, compared to hydrolysates with a low degree of hydrolysis.

The antioxidant properties of peptides are reported for their composition, structure, and hydrophobicity. The amino acids tyrosine, tryptophan, methionine, lysine, cystine, and histidine are examples that may act as antioxidants because they have aromatic residues that donate protons to free radicals. The antioxidant activity of peptides containing histidine is related to the ability of donating hydrogen and ability of chelating metal ions from the imidazole group (Rajapakse et al., 2005). In addition, as described by Qian et al. (2008), the SH group in cysteine has an important antioxidant activity due to direct interaction with the radicals.

Fish are a protein source rich in essential amino acids (lysine, methionine, cystine, threonine, and tryptophan). The fish muscle proteins are made up of several groups of proteins: the fraction forming the sarcoplasm, which performs biochemical functions in the cells; myofibrillar proteins of the contractile system; and connective tissue proteins (stromal proteins), mainly responsible for the integrity of muscles. In addition to their functional, technological, and nutritional properties, some fish proteins may exhibit antioxidant activity, which is associated with certain bioactive peptides present in protein sequences. Qian et al. (2008), who used fish proteins and proteolytic enzymes, obtained peptides with excellent free-radical scavenging activity that are potent lipid peroxidation inhibitors and can be used in food preservation.

The encapsulation of compounds protects a sensitive substance within the capsule, physically isolating it from the external environment. This barrier can provide protection against various agents, such as oxygen, water, and light, allows for a controlled release of the substance, and prevents contact with other components in a mixture. One of the benefits of encapsulation is the ability to control the release of chemical compounds incorporated and deliver them to a specific target at an appropriate time. The controlled release of ingredients can improve the efficiency of food additives and ensure optimal dosage. Antioxidant encapsulation can be used to protect the nutritional and sensory quality of food and/or to protect the body against chronic diseases related to aging. In general, antioxidants are subject to degradation and, when administered by the body in their free form, they can not pass through the cell membranes and are rapidly eliminated in the circulation (Mozafari et al., 2006). The use of liposomes has been studied in the encapsulation of proteins and other food ingredients and different methods for preparation of liposomes have been proposed in the literature. Thus, depending on the method and on the characteristics of the sample, several types of structures can be designed with different features and encapsulation efficiencies. Therefore, the encapsulation of protein hydrolysates can protect its antioxidant activity until its release in the food or in the human body.

The biological activity of the peptides can be used in food preservation by incorporating these peptides into food products or biodegradable packaging. This incorporation can be done through encapsulated materials for the antioxidant activity to remain active and controlled throughout the shelf life of the product. The high protein content in fish waste has encouraged studies on the recovery of these proteins in the form of isolated and hydrolysed protein with excellent functional properties. Whitemouth croaker (*Micropogonias furnieri*) is a fish of low commercial value which can potentially be used in the production of protein hydrolysates. Based on this, the objective of this study was to produce encapsulated protein hydrolysates from croaker muscle and the byproduct of its industrialization.

2. Materials and methods

2.1. Material

Whitemouth croaker *(M. furnieri*) fish captured in southern Brazil were provided by Pescal Industry in Rio Grande, Brazil. The fish were washed in chlorinated water (5 mg/kg) at 4 °C and subsequently filleted to separate the muscle. After these operations, the guts were removed and the carcasses were processed in a meat/bone separator (High Tech, model HT250, Chapecó, Brazil) to obtain the byproduct. The raw materials (muscle and byproduct) were placed in plastic containers and stored in a freezer at −18 °C.

2.2. Enzymatic hydrolysis of croaker proteins

In order to obtain the muscle protein hydrolysate (MPH) and the byproduct protein hydrolysate (BPH), the croaker muscle and the byproduct were subjected to a hydrolysis reaction with microbial protease for obtaining protein hydrolysates. The enzyme used in the reaction was Flavourzyme® 1000L (enzyme of microbial origin), provided by Novozymes Latin America Ltda, at a 1:5 substrate/buffer ratio, while the other reaction conditions were: 2 g/100 g enzyme/substrate, pH 7, and 120 min of reaction at 50 °C. Flavourzyme® 1000L is an exopeptidase with an activity of 1 LAPU/g. An LAPU (leucine aminopeptidase unit) is the amount of enzyme which hydrolyses 1 μmol of leucine-ρ-nitroanilide per minute. The hydrolysates were inactivated by heating in a water bath at 95 °C for 15 min and then freezing at −80 °C for 24 h in an ultrafreezer for subsequent lyophilisation.

2.3. Hydrolysate encapsulation

The encapsulation process of hydrolysates in the form of liposomes was carried out through the lipid film hydration method, as described by Malheiros, Micheletto, Silveira, and Brandelli (2010). The source of lipids used to prepare the liposomes was purified phosphatidylcholine. In order to obtain purified phosphatidylcholine, the wall material of the capsules, crude soy lecithin, was purified according to the method described by Mertins, Sebben, Schneider, Pohlmann, and Silveira (2008). First, 1 g of phosphatidylcholine was dissolved in 10 mL of chloroform in a 250 mL glass flask. After its complete dispersion, the organic solvent was removed in a rotary evaporator at 50 °C until the formation of a lipid film deposited on the walls of the flask. The chloroform traces were removed by storing the glass flask for 18 h in a vacuum desiccator at room temperature. The resulting lipid film was dispersed into 20 mL of phosphate buffer pH 7.0, 0.2 mol/L containing 0.2 g of lyophilised hydrolysate. The mixture in the flask was subjected to heating at 60 °C for 3 min and slow stirring so the dispersion did not form foam. After that, the liposomes underwent rapid stirring in a vortex for 1 min, were left to stand for 3 min, and finally heated at 60 °C for 2 min; this operation was repeated 3 times. After this step, the suspension was subjected to 10 cycles of sonication for 1 min and kept in cold water for 3 min, using an ultrasonic bath (USC-800, Unique Group). A control was performed under the same encapsulation process conditions, but with no hydrolysate sample, called control capsule.

2.4. Degree of hydrolysis (DH)

The DH was determined at intervals of zero, 30, 60, 90, and 120 min. An aliquot was removed, mixed with trichloroacetic acid (TCA 6.25 g/100 g) to inactivate the enzymes and subjected to filtration. The filtrate was used to determine the concentration of soluble proteins. The degree of hydrolysis was calculated as the

ratio between the amount of total protein present in the substrate by the Kjeldahl method (AOAC, 1997) and the amount of soluble proteins calculated by the method of Lowry, Rosenbrough, Farr, and Randall (1951).

2.5. Particle size and polydispersity

To calculate the average particle size and polydispersity, the dynamic light scattering technique was used (Malvern 4700MW, model Spectra-physics 127) according to the method described by Teixeira, Santos, Silveira, and Brandelli (2008) at a wavelength of 632.8 nm, coupled to a BI-200M version 2.0 goniometer and BI-9000AT digital correlator from Brookheaven Instruments. Polydispersity evaluates the size distribution of particles, showing the suspension's degree of homogeneity. The liposomes were filtered through 0.45 μm paper filter and two drops of the sample dissolved in 8 mL of phosphate buffer pH 7.0 0.2 mol/L were used for analyses.

2.6. Encapsulation efficiency

The encapsulation efficiency, i.e., the mass of liposome-encapsulated protein hydrolysate, was assessed through the indirect method. First, 0.5 mL of the liposomes was placed in a centrifuge tube with 1 mL of acetone, since phosphatidylcholine is insoluble in this solvent. The samples were centrifuged at 5000 *g* for 30 min at 3 °C, separating into two phases. The supernatant containing the non-encapsulated sample was withdrawn and placed in an oven at 60 °C until complete evaporation of the solvent. The remaining dried material was resuspended with 5 mL of distilled water and the protein concentration was determined through method by Lowry et al. (1951), indirectly calculating the amount of non-encapsulated sample which was solubilized in acetone. A 0.5 mL aliquot of the initial sample was withdrawn and 1 mL of 0.06 g/100 g Triton was added to determine the total protein in the sample. The material was then homogenized in a vortex (Phoenix AP56) until complete solubilization of phosphatidylcholine. The encapsulated material was calculated by the weight difference between the total and non-encapsulated material. The encapsulation efficiency was calculated by ratio between the amount of encapsulated material and the total weight.

2.7. Capsule suspension stability

The suspension stability of the capsules was evaluated through zeta potential using the equipment Zetasizer Nanoseries Nano-Z (Malvern Instruments) at 20 °C and 90° angle. The suspensions were stored at 4 °C, protected from light and oxygen.

2.8. Capsule transmission electron microscopy (TEM)

The morphology of the capsules containing muscle and byproduct hydrolysates was evaluated in a Zeiss- EM900 transmission electron microscope (TEM). The sample was fixed with Karnovsky's fixative. Liquid samples were centrifuged at 8944 × *g* for 10 min, the supernatant was removed and the fixer was added and stirred by vortexing. The material was removed from the fixative solution and placed in pH 7.2, 0.2 mol/L sodium cacodylate buffer (1 mL/sample). Thereafter, the second fixing was taken, which was placed in osmium tetroxide for 2 h. The material was successively subjected to dehydration in ethanol (30, 50, 70, 90, 95, and 100 mL/100 mL) and impregnated in epoxi resin. The samples were cut into slides for evaluation in the transmission electron microscope.

2.9. Capsule thermal properties

The thermal properties of the capsules were determined using differential scanning calorimetry (Shimadzu DSC 60, TA Instruments, New Castle, USA). Samples (approximately 2.5 mg, dry basis) were weighed directly in an aluminium pan with distilled water. The pan was hermetically sealed and then heated from 40 to 200 °C at a rate of 10 °C/min. An empty pan was used as reference. The temperature at the onset (T_o), the temperature at peak (T_p), the temperature at the end (T_f), and the enthalpy (ΔH) were determined. The temperature range was calculated as T_f–T_o.

2.10. Capsule Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of the capsules were obtained using a Fourier transform infrared (FTIR) spectrometer (Prestige-21, Shimadzu, Japan), in the region of 4000–400 cm^{-1}. Pellets were created by mixing the sample with KBr at a ratio of 1:100 (sample:KBr). Ten scans were collected at a resolution of 4 cm^{-1}.

2.11. Antioxidant activity by the linoleic acid peroxidation inhibition method

The lipid peroxidation inhibition activity of hydrolysates and capsules was measured in a linoleic acid emulsion system according to the method of Osawa and Namiki (1985) with some modifications. In short, the sample (5.0 mg), standard α-tocopherol, or vitamin C was dissolved in 10 mL of 50 mmol/L phosphate buffer (pH 7.0), and added to a solution of 0.13 mL linoleic acid and 10 mL of 99.5 g/100 g ethanol. The mixture was homogenized and the final volume was adjusted to 25 mL with deionized water. A control reaction was prepared using 50 mmol/L phosphate buffer (pH 7.0). The mixture was incubated in screw-cap tubes at 40 ± 1 °C in the dark. The degree of linoleic acid oxidation was measured after seven days. A 0.1 mL aliquot of the incubated solution was mixed with 4.7 mL of 75 mL/100 mL ethanol, 0.1 mL of 30 g/100 g ammonium thiocyanate, and 0.1 mL of 0.02 mol/L ferrous chloride in 3.5 mL/100 mL hydrochloric acid. After 3 min, the degree of colour development, which represents the oxidation of linoleic acid, was measured by reading the absorbance at 500 nm on a spectrophotometer (Biospectro UV, SP-22, Brazil) and calculated according to Eqn. (1).

$$\text{inhibition}\ (\%) = [1 - (\text{sample absorbance}/\text{control absorbance})] \times 100 \quad (1)$$

2.12. Statistical analysis

Analytical determinations for the samples were performed in triplicate, and standard deviations were reported, except for the results of thermal properties. A comparison of the means was ascertained by Tukey's test at 5% significance level by analysis of variance (ANOVA) using the software Statistica 7.0.

3. Results and discussion

3.1. Degree of hydrolysis

The extent of protein degradation by protease was measured by assessing the degree of hydrolysis (DH), which is the most widely used indicator to compare different protein hydrolysates. According to preliminary tests, the degree of hydrolysis of the proteins was evaluated at 30, 60, 90, 120, and 150 min of reaction and it was

observed that, after 120 min of reaction, the degree of hydrolysis remained constant. Therefore the production of hydrolysates was obtained at 120 min of reaction. The degree of hydrolysis at 120 min for muscle proteins (28.5%) did not differ statistically from the degree of hydrolysis of byproduct proteins (27.0%). Thiansilakul et al. (2007), who produced protein hydrolysates from *Decapetrus maruadsi* with the enzyme Flavourzyme® and 60 min of reaction, found higher values for degree of hydrolysis of proteins with average of 60%. The difference in the values of degree of hydrolysis between different raw materials can be related to the arrangement of amino acids present in the protein, and the different enzyme activity.

According to Vioque, Clemente, Pedroche, Yust, and Millán (2001), hydrolysates can be categorized into three main groups based on their degree of hydrolysis, which determines their application: hydrolysates with low DH improve the functional characteristics of proteins, hydrolysates with average DH are generally used as flavourings, and hydrolysates with high DH are used as nutritional supplements and in special diets in medicine. Vioque et al. (2006) classified products depending on the degree of hydrolysis as partially hydrolysed (DH $< 10\%$) and highly hydrolysed (DH $> 10\%$). Therefore, the products obtained in this study are considered highly hydrolysed and can be used in foods for nutrient availability and as bioactive compounds.

3.2. Encapsulation efficiency, size, polydispersity, and stability of the capsules

Table 1 shows the results of encapsulation efficiency, average particle size, polydispersity and stability of the capsules in the form of liposomes from muscle and byproduct hydrolysates. These data were compared with the control capsule (without sample). The capsules, also known as liposomes, showed high encapsulation efficiency, with no statistical differences between the samples from muscle and byproduct (Table 1). The capsules from croaker muscle and byproduct hydrolysates showed an encapsulation efficiency higher than that found by Were, Bruce, Davidson, and Weiss (2004), who used the lipid film hydration method and sonication for encapsulation of an antimicrobial peptide (nisin) and obtained encapsulation efficiency of approximately 54%.

The choice of encapsulation method must ensure molecule stability and the retention of its biological activity, because there is great difficulty in choosing a system nucleus and capsule wall that enables a suitable encapsulation efficiency. In accordance with the method used in this study, encapsulation by liposomes has been widely studied by researchers in the healthcare area due to their potential use as drug coating surrounding bioactive macromolecules (Malheiros et al., 2010).

Gómez-Hens and Fernández-Romero (2005) classified the liposomes according to their structure into giant unilamellar vesicles (>1 μm), multilamellar vesicles (>400 nm), large unilamellar vesicles (80 nm–1 μm) and small unilamellar vesicles (20–80 nm). The average size of the hydrolysate capsules did not differ, but were higher than the control capsule that was made only with the wall material (Table 1). The size of the capsules is related to various factors such as the stirring speed used in the encapsulating process, composition, concentration, and type of polymer present in the formulation (Moinard-Chécot, Chevalier, Briançon, Beney, & Fessi, 2008).

The polydispersity index value found for the capsules was approximately 0.2 (Table 1), which indicates the presence of monodisperse particle populations or a narrow size range. The low polydispersity value indicates the homogeneity in particle size distribution. The capsule containing MPH showed higher polydispersity compared with the capsule of BPH (Table 1). Changes in the distribution of their diameters may indicate a tendency to particle aggregation and sedimentation in the system, which did not happen in this study.

The suspension stability of the capsules was evaluated by the zeta potential of the MPH and BPH capsules. Almost all particles in contact with a liquid acquire an electric charge on their surface. The electric potential at the shear plane is called zeta potential. This is an important and useful indicator for predicting and controlling the stability of lipid capsules. The lipid capsules have a negative surface charge due to the negative charge of the phospholipid molecules (Manconi et al., 2003). But the zeta potential can be influenced by different factors such as particle composition, the dispersing environment, pH, and ionic strength in the solution.

The zeta potential had negative values (Table 1), allowing to keep the particles away thereby avoiding the formation of aggregates. The MPH capsules showed higher zeta-potential modulus as compared to BPH capsules, thus indicating increased stability (Table 1). The relatively high values of zeta-potential modulus are important for good physical and chemical stability of formulations, because the repulsive forces tend to prevent aggregation due to incidental collisions of adjacent particles. According to Mohanraj and Chen (2006), nanoparticles that have zeta potential values near $\pm$ 30 mV have good colloidal stability in solution. The values of the zeta potential in the range of -2.2 and -5.8 (Table 1) indicate low stability of the particles.

Table 1
Average particle size, polydispersity, encapsulation efficiency and suspension stability as measured by zeta potential of the capsules obtained from croaker protein hydrolysates.

Sample[a]	Average size (nm)	Polydispersity index	Encapsulation efficiency (%)	Zeta potential (mV)
Control capsule	208.1b	0.228b	–	−5.8a
Muscle hydrolysate capsule	266.8a	0.298a	80.4a	−5.5a
Byproduct hydrolysate capsule	263.9a	0.197b	79.3a	−2.2b

[a] Data in the same column with different letters are significantly different ($p \leq 0.05$).

3.3. Capsule transmission electron microscopy (TEM)

TEM allows for a qualitative understanding of the internal structure, spatial distribution, and dispersion of the particles within the polymer matrix, and views of the defect structure through direct visualization. Fig. 1 shows the micrographs of encapsulated and non-encapsulated hydrolysates. Fig. 1a is the external surface of the control capsule, which has no sample inside. Based on Fig. 1b and c, it can be seen that the capsules are spherical and that they do contain material inside. The encapsulation can be seen by comparing the empty liposomes (Fig. 1a) and MPH and BPH liposomes (Fig. 1b and c, respectively).

The micrograph of BPH liposome (Fig. 1c) shows a more homogeneous dispersion size when compared with the MPH liposome (Fig. 1b), this statement can be related to the results of polydispersity because the BPH liposomes showed lower polydispersity index as compared to the MPH liposomes (Table 1).

In the micrographs of non-encapsulated hydrolysates (Fig. 1d and e) there is a small amount of spherical particles, as these samples were not subjected to the encapsulation process, we suggest that these particles are fat globule as lipid residue present in the protein hydrolysates.

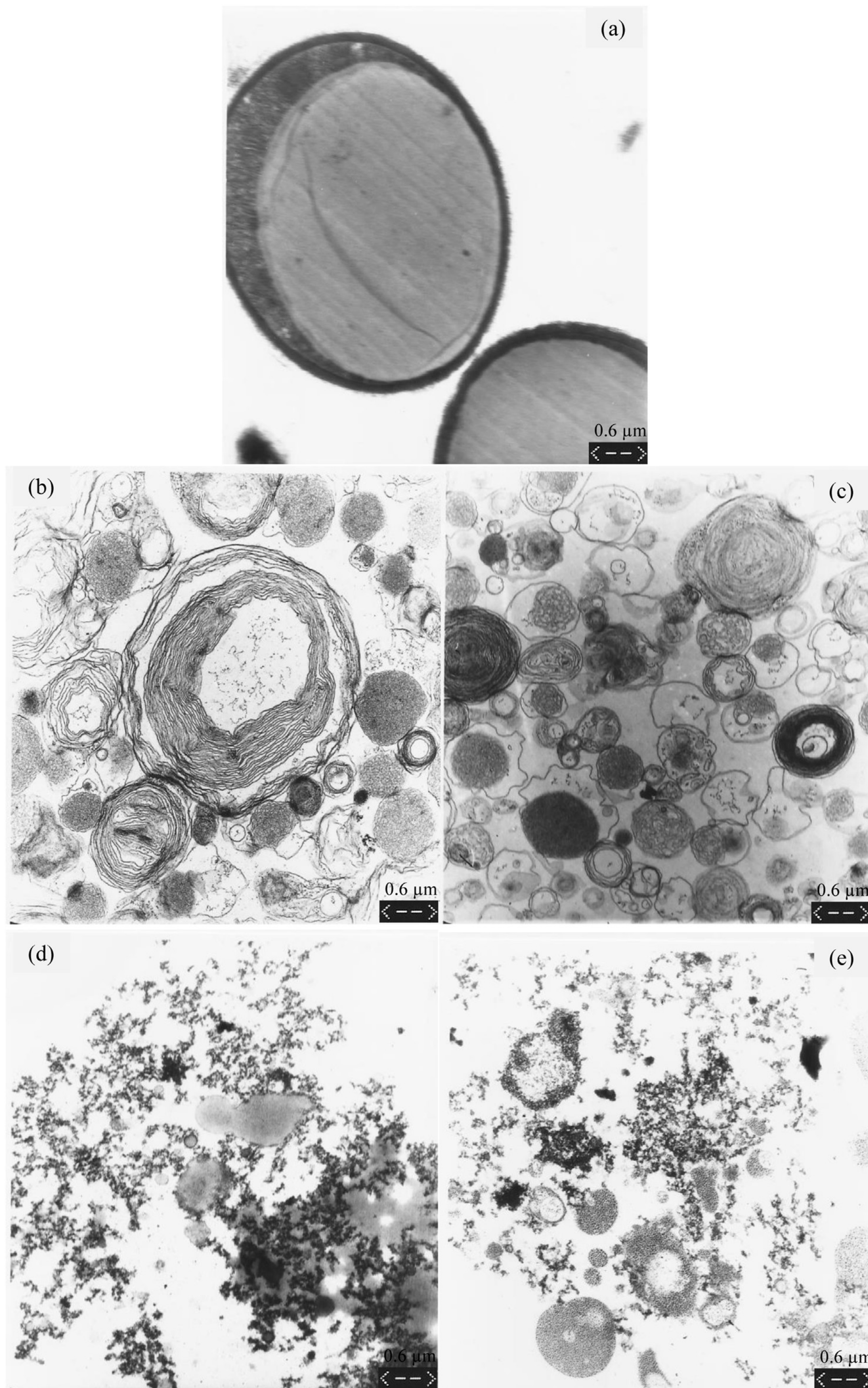

Fig. 1. TEM of the control capsule (a), muscle hydrolysate capsule (b), byproduct hydrolysate capsule (c), non-encapsulated muscle hydrolysate (d), and non-encapsulated byproduct hydrolysate (e).

3.4. Capsule thermal properties

The thermal properties of MPH and BPH capsules are shown in Table 2. The DSC technique is very important in the physical characterization of particles since it provides information about their crystallinity. It is known that the degree of crystallinity of a particle is extremely important because it will affect encapsulation efficiency, the release rate of the active compound, and the release of the active compound during the storage process. The DSC analysis also provides the fusion temperature and enthalpy of particles. The encapsulated hydrolysate showed high values of enthalpy (Table 2). According to Attama, Schicke, and Muller-Goymann (2006), a high

Table 2
Thermal properties of capsules obtained from croaker protein hydrolysates.

Sample[a]	T_o (°C)	T_p (°C)	T_f (°C)	ΔT (T_f–T_o)	ΔH (J/g)
Control capsule	186.3	187.7	191.5	5.2	–52.6
Muscle hydrolysate capsule	177.5	179.4	185.5	8.0	–118.2
Byproduct hydrolysate capsule	166.6	172.3	180.6	13.9	–124.8

[a] T_o: onset temperature; T_p: peak temperature; T_f: final temperature; ΔH: gelatinisation enthalpy; ΔT: gelatinisation temperature range.

value of melting enthalpy suggests high organization in the crystalline reticulum, because the fusion of a highly organized crystal requires more energy to rupture the forces of cohesion of the crystalline reticulum than the fusion of a slightly ordered or amorphous crystal. Moreover, those authors reported that through this technique it is possible to observe the crystallization behaviour of the particles, which influences whether or not the active compounds will be expelled.

The control capsule, which contains only phosphatidylcholine, had a higher melting temperature (187.7 °C), but showed a lower melting enthalpy when compared with the hydrolysate capsules (Table 2). Marcato (2009) reported that in testing the release kinetics it is possible to evaluate if a compound in contact with the lipid system in an aqueous environment is able to migrate or diffuse through the solid nanoparticles and dissolve into the lipid phase. If such migration occurs, it is detected by a change in the shape of a calorimetric peak due to the increased amount of the compound within the particles. This behaviour is seen in Fig. 2, which show peaks in different ways, i.e., the peak of the control capsule was narrower when compared with the peaks from the MPH capsules and the BPH capsules. The figures also show that the values of temperature difference (ΔT) were lower than those of the control capsule (Table 2). This change in peak shape is due to the presence of the active compounds within the capsules.

3.5. Capsule Fourier transform infrared (FTIR) spectroscopy

The capsules were analysed by absorption spectroscopy in the infrared region in order to verify the presence of interaction between phosphatidylcholine and hydrolysates (Table 3). FTIR analysis was used to monitor small changes in the structure of the lipid and active compound by analysing the frequency, intensity, and changes in the bandwidth of the different vibrational modes that represent the acyl chains, the interfacial region, and the surface region.

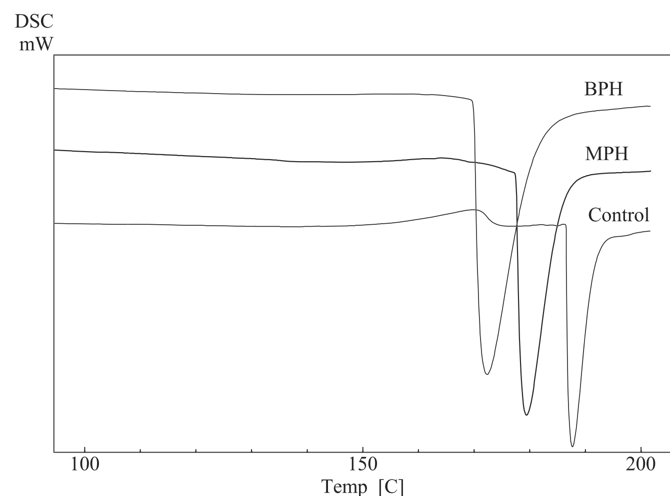

Fig. 2. DSC curves of the control capsule (Control), muscle hydrolysate capsule (MPH), and byproduct hydrolysate capsule (BPH).

Table 3
Wavenumber (ν in cm^{-1}) of spectra of encapsuled hydrolysates by FTIR analysis.

Stretching	Control capsule	MPH capsule[a]	BPH capsule[b]
PO_2	1082	1082	1084
CH_2	2852	2852	2850
C=O	1645	1637	1656
CH_3	2924	2924	2922
OH	3440	3433	3439
N^+CH_3	989	989	991

[a] MPH: muscle protein hydrolysate.
[b] BPH: byproduct protein hydrolysate.

In the liposome, an orientation occurs of the lipophilic part to the middle of the lipid bilayer and the polar part is directed towards the inside of the capsule and to its external surface. Theoretically, the FTIR technique measures the asymmetric axial stretching of the phosphate group's (PO_2) double bond, the axial stretching of the carbonyl group (C=O), and the axial stretching of the CH_2 groups of liposomes (Toyran & Severcan, 2003).

Table 3 shows the bands present in the spectra and that are specific for each functional group of the capsules. The Fig. 3 presents the FTIR spectra of control capsule and capsules containing protein hydrolysate. The capsules exhibited bands near 3440 cm^{-1} (Table 3), characteristic of OH stretch. Ferraresi, Ferreira, Silva, and Neto (2012) also reported that, in the first part of spectra, various signals observed are due to intense OH and NH vibrations, molecules that are hydrogen bonded with each other (broader band), and those that do not have this type of interaction (acute bands).

According to Bai et al. (2011), the vibration frequency of the CH_2 stretching reflects the structural information about the interior of the lipid bilayer of the liposome. There was a shift of the band at 2852 cm^{-1} which represents stretching of the CH_2 of the BPH capsule to 2850 cm^{-1}, demonstrating that the peptides had an influence in the interior of the bilayer capsules. Thus, the results obtained in this study suggest an effective ionic complexation between phosphatidylcholine and peptides from protein hydrolysates.

It is observed that the stretching of the choline (N^+CH_3) group, present in the polar part of phosphatidylcholine, had a band shifted from 989 cm^{-1} to 991 cm^{-1} in the BPH capsule, which indicated the presence of peptides in the polar region of phosphatidylcholine, i.e., at the region within the liposome. However, it did not affect the

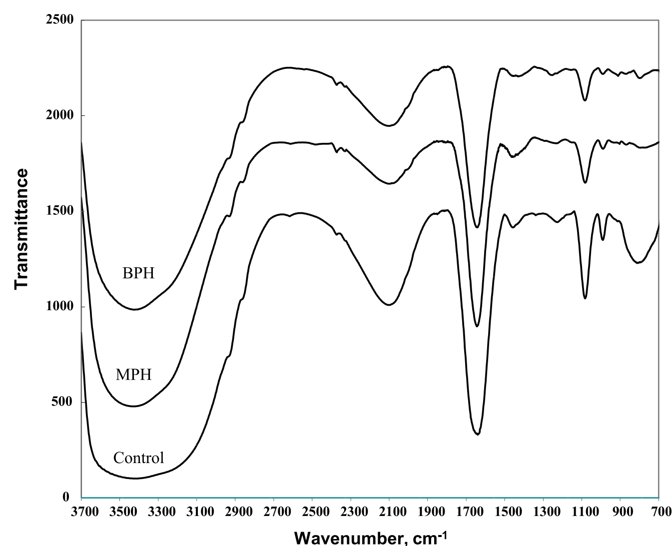

Fig. 3. Spectra of the control capsule (Control), muscle hydrolysate capsule (MPH), and byproduct hydrolysate capsule (BPH) by FTIR analysis.

band of the MPH capsule. According to Bai et al. (2011), the position of the peak of the choline group was confirmed with the band at 949 cm^{-1} and was shifted to 986 cm^{-1} after the molecules interact with the surface of the carboxymethyl group of phosphatidyl choline.

The frequency of the phosphate group is useful to monitor the hydration status of the polar groups of phospholipids. An increase in frequency will correspond to the dehydrated phosphate group, while a decrease in frequency corresponds to the hydrous phosphate group (Toyran & Severcan, 2003). The frequency of this band determines the presence or absence of hydrogen bonds between the phosphate group and the hydrogen atoms of water or biological macromolecules (Kan-Zhi et al., 1996).

In BPH capsules, a shift from 1082 cm^{-1} to 1084 cm^{-1} was observed in the band of phosphate from the phosphatidylcholine grouping (Table 3). There was a reduction in peak intensity (FTIR spectra) of the phosphate grouping of capsules containing protein hydrolysate compared to the control capsule. The absorption of the stretching vibration of the C═O band shifted from 1645 cm^{-1} to 1637 cm^{-1} in the MPH capsule and to 1656 cm^{-1} in the BPH capsule (Table 3) and reduced intensity after encapsulation. According to Bai et al. (2011), this shift of the bands of the C═O stretching indicates interactions with hydrogen bonds between the carbonyl region of the liposome bilayer and the active compound, which in this case are peptides. The FTIR spectra indicate interactions of the peptides contained in the hydrolysates with the C═O and PO_2 groups of phosphatidylcholine, indicating the location of these peptides in the lipid polar region, possibly forming hydrogen bonding with the PO_2 group.

3.6. Antioxidant activity of hydrolysates and capsules

Protein hydrolysates produced from the muscle and byproduct with the same protease and under the same reaction conditions may have peptides of different sizes and different amino acid sequences. The antioxidant activity of hydrolysates was determined using the method of linoleic acid peroxidation inhibition method.

The measurement of lipid peroxidation inhibition is calculated by measuring the inhibition of lipid oxidation in a system. Hydroperoxides generated during linoleic acid oxidation react with ferrous sulphate, producing ferric sulfate and later, with the addition of ferric thiocyanate, forms a blood-red coloured complex detected at 500 nm. After the reaction starts, the oxidation process becomes autocatalytic and only ends when the reserve of unsaturated fatty acids and oxygen is depleted (Halliwell & Gutteridge, 2007, pp. 268–340).

As shown in Table 4, the linoleic acid oxidation was inhibited by the protein hydrolysates and by the encapsuled hydrolysate. MPH and BPH showed no significant difference in their antioxidant activities, as well as the MPH and BPH capsules. They did not differ significantly regarding the inhibition of α-tocopherol. However, vitamin C showed the highest inhibition of linoleic acid oxidation compared with the other samples. For comparison purposes, a control containing only the wall material of the capsules was also performed. If the lipid peroxidation inhibition from MPH and BPH capsules is subtracted from the inhibition value of the control capsule, inhibition values of 14.2% and 13.6%, respectively, are obtained. These values are lower than those of MPH (27.0%) and BPH (31.9%) (Table 4) due to the low proportion of protein hydrolysate as the nucleus of the capsule when compared with the amount of wall material (phosphatidylcholine), because one gram of wall material contains only 0.2 g of hydrolysate.

Table 4
Lipid peroxidation inhibition of the hydrolysed, encapsulated hydrolysed, α-tocoferol and vitamin C.

Sample[a]	Lipid peroxidation inhibition (%)
Muscle hydrolysate	27.0bc
Byproduct hydrolysate	31.9b
Control capsule	8.0d
Muscle hydrolysate capsule	22.2c
Byproduct hydrolysate capsule	21.6c
α-tocoferol	25.7bc
Vitamin C	43.2a

[a] Data in the same column with different letters are significantly different ($p \leq 0.05$).

Centenaro, Mellado, and Prentice-Hernández (2011) found higher values in a study that evaluated linoleic acid oxidation inhibition by protein hydrolysates from fish and chicken bones using different proteases (Flavourzyme®, α-chymotrypsin, and trypsin). Those authors concluded that, among the fish bone hydrolysates, those hydrolysed with Flavourzyme® had the highest inhibition (77.3%) after 7 days of evaluation and, among the chicken bone hydrolysates, the ones hydrolysed with Flavourzyme® also had the highest inhibition power (61.6%). According to that study, such results indicate that these hydrolysates might contain antioxidant peptides.

4. Conclusion

It was possible to develop capsules using phosphatidylcholine as wall material and protein hydrolysate as the active compound. The capsules exhibit high encapsulation efficiency at approximately 80% and the FTIR analysis shows that there was an effective ionic complexation between phosphatidylcholine and peptides present in the hydrolysates. Moreover, the capsules maintained the antioxidant activity of the hydrolysates.

The protein hydrolysates produced from croaker muscle and its industrialization byproduct showed similar antioxidant activity, close to the activity of α-tocopherol, indicating a potential use for the production of bioactive compounds from raw material of low commercial value.

References

AOAC. (1997) (16th ed.). *Association of Official Analytical Chemists. Official methods of analysis* (Vols. 1–2) Arlington.

Attama, A. A., Schicke, B. C., & Muller-Goymann, C. C. (2006). Further characterization of theobroma oil beeswax admixtures as lipid matrices for improved drug delivery systems. *European Journal of Pharmaceutics and Biopharmaceutics, 64*, 294–306.

Bai, C., Peng, H., Xiong, H., Liu, Y., Zhao, L., & Xiao, X. (2011). Carboxymethylchitosan-coated proliposomes containing coix seed oil: characterisation, stability and in vitro release evaluation. *Food Chemistry, 129*, 1695–1702.

Centenaro, G. S., Mellado, M. S., & Prentice-Hernández, C. (2011). Antioxidant activity of protein hydrolysates of fish and chicken bones. *Advance Journal of Food Science and Technology, 3*, 280–288.

Chanput, W., Theerakulkait, C., & Nakai, S. (2009). Antioxidative properties of partially purified barley hordein, rice bran protein fractions and their hydrolysates. *Journal of Cereal Science, 49*, 422–428.

Ferraresi, T. M., Ferreira, E. P. B., Silva, W. T. L., & Neto, L. M. (2012). Aplicação da espectroscopia no infravermelho próximo e médio na avaliação da biomassa microbiana do solo. *Boletim de Pesquisa e Desenvolvimento, 38*, 1–35.

Gómez-Hens, A., & Fernández-Romero, J. M. (2005). The role of liposomes in analytical processes. *Trends in Analytical Chemistry, 24*, 9–24.

Halliwell, B., & Gutteridge, J. M. C. (2007). *Free radicals in biology and medicine* (4th ed.). Oxford University Press.

Harnedy, P. A., & FitzGerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: a review. *Journal of Functional Foods, 4*, 6–24.

Kan-Zhi, L., Jackson, M., Sowa, M. G., Haisong, J., Dixon, I. M. C., & Mantsch, H. H. (1996). Modification of the extracellular matrix following myocardial infarction monitored by FTIR spectroscopy. *Biochimica et Biophysica Acta, 1315*, 73–77.

Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides: opportunities for designing future foods. *Current Pharmaceutical Design, 9*, 1297–1308.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Malheiros, P. S., Micheletto, Y. M. S., Silveira, N. P., & Brandelli, A. (2010). Development and characterization of phosphatidylcholine nanovesicles containing the antimicrobial peptide nisin. *Food Research International, 43*, 1198–1203.

Manconi, M., Aparicio, J., Vila, A. O., Pendas, J., Figueruelo, J., & Molina, F. (2003). Viscoelastic properties of concentrated dispersions in water of soy lecithin. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 222*, 141–145.

Marcato, P. D. (2009). Preparação, caracterização e aplicações em fármacos e cosméticos de nanopartículas lipídicas sólidas. *Revista Eletrônica de Farmácia, 6*, 1–37.

Mertins, O., Sebben, M., Schneider, P. H., Pohlmann, A. R., & Silveira, N. P. (2008). Caracterização da pureza de fosfatidilcolina da soja através de RMN de ^{1}H e de ^{31}P. *Química Nova, 31*, 1856–1859.

Mohanraj, V. J., & Chen, Y. (2006). Nanoparticles 447 – a review. *Tropical Journal of Pharmaceutical Research, 5*, 561–573.

Moinard-Chécot, D., Chevalier, Y., Briançon, S., Beney, L., & Fessi, H. (2008). Mechanism of nanocapsules formation by the emulsion–diffusion process. *Journal of Colloid and Interface Science, 317*, 458–468.

Moosman, B., & Behl, C. (2002). Secretory peptide hormones are biochemical antioxidants: structure activity relationship. *Molecular Pharmacology, 61*, 260–268.

Mozafari, M. R., Flanagan, J., Matia-Merino, L., Awati, A., Omri, A., Suntres, Z. E., et al. (2006). Review: recent trends in the lipid-based nanoencapsulation of antioxidants and their role in foods. *Journal of the Science of Food and Agriculture, 86*, 2038–2045.

Neklyudov, A. D., Ivankin, A. N., & Berdutina, A. V. (2000). Properties and uses of protein hydrolysates. *Applied Biochemistry and Microbiology, 36*, 452–459.

Osawa, T., & Namiki, M. (1985). Natural antioxidants isolated from Eucalyptus leaf waxes. *Journal of Agricultural and Food Chemistry, 33*, 777–780.

Qian, Z. J., Jung, W., & Kim, S. (2008). Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, *Rana catesbeina* Shaw. *Bioresource Technology, 99*, 1690–1698.

Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y., & Kim, S. K. (2005). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Research International, 38*, 175–182.

Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: a review of their production, assessment, and potential applications. *Journal of Functional Foods, 3*, 229–254.

Teixeira, M. L., Santos, J., Silveira, N. P., & Brandelli, A. (2008). Phospholipid nanovesicles containing a bacteriocin-like substance for control of *Listeria monocytogenes*. *Innovative Food Science and Emerging Technologies, 9*, 49–53.

Thiansilakul, Y., Benjakul, S., & Shahidi, F. (2007). Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). *Food Chemistry, 103*, 1385–1394.

Toyran, N., & Severcan, F. (2003). Competitive effect of vitamin D_2 and Ca^{2+} on phospholipid model membranes: an FTIR study. *Chemistry and Physics of Lipids, 123*, 165–176.

Vioque, J., Clemente, A., Pedroche, J., Yust, M. M., & Millán, F. (2001). Obtencion y aplicaciones de hidrolizados proteicos. *Grasas Aceites, 52*, 132–136.

Vioque, J., Pedroche, J., Yust, M. M., Lqari, H., Megías, C., Girón-Calle, J., et al. (2006). Peptídeos bioativos em proteínas vegetais de reserva. *Brazilian Journal of Food Technology*, 99–102. III JIPCA.

Were, L. M., Bruce, B., Davidson, P. M., & Weiss, J. (2004). Encapsulation of nisin and lysozyme in liposomes enhances efficacy against *Listeria monocytogenes*. *Journal of Food Protection, 67*, 922–927.