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# MICROENCAPSULATION OF BAYO BEAN (*Phaseolus vulgaris*) PROTEIN HYDROLYSATE WITH INHIBITORY ACTIVITY ON ANGIOTENSIN-I CONVERTING ENZYME THROUGH FREEZE-DRYING

# MICROENCAPSULACIÓN DE UN HIDROLIZADO PROTEÍNICO DE FRIJOL BAYO (*Phaseolus vulgaris*) CON ACTIVIDAD INHIBITORIA SOBRE LA ENZIMA CONVERTIDORA DE ANGIOTENSINA-I MEDIANTE LIOFILIZACIÓN

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**Abstract**

The aim of this study was to obtain microcapsules of a bayo bean protein hydrolysate (BBPH) with antihypertensive activity using Eudragit® L 30 D-55 as wall material (EGLD) through freeze-drying processing. The BBPH was obtained using sequential pepsin-pancreatin enzymatic system. The ACE-I inhibitory activity was measured using tripeptide hippuryl-histidyl leucine (HHL) as model peptide. Three microcapsule formulations were prepared containing BBPH and EGLD at ratios of 1:20, 1:4 and 1:1, respectively. The physicochemical characteristics of microcapsules were evaluated by optical (OM) and scanning electronic microscopy (SEM), confocal laser scanning microscopy (CLSM), differential scanning calorimetry (DSC), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR). ACE-I inhibitory activity of BBPH was $IC_{50}$=0.42 mg/mL. All microcapsules showed irregular shapes. The BBPH was distributed homogeneously in all formulations. The DSC and XRD analysis revealed a uniform dispersion of the BBPH and partially crystalline structures of EGLD and BBPH. The FT-IR confirmed the chemical stability of BBPH in the microcapsules. In conclusion, the EGLD microcapsules containing BBPH were prepared successfully by freeze-drying processing.

*Keywords*: bayo bean, protein hydrolysate, Eudragit® L 30 D-55, microcapsules, freeze-drying.

**Resumen**

El objetivo de este estudio fue obtener microcápsulas de un hidrolizado proteínico de frijol bayo (BBPH) con actividad antihipertensiva utilizando Eudragit® L 30 D-55 como material de pared (EGLD) por el método de liofilización. El BBPH se obtuvo usando el sistema enzimático secuencial pepsina-pancreatina. La actividad inhibidora sobre la ECA-I se midió utilizando hipuril-histidil leucina (HHL) como péptido modelo. Se prepararon tres formulaciones conteniendo BBPH y EGLD en proporciones de 1:20, 1:4 y 1:1, respectivamente. Las características fisicoquímicas de las microcápsulas se evaluaron por microscopía óptica (OM) y microscopía electrónica de barrido (SEM), microscopía confocal de barrido láser (CLSM), calorimetría diferencial de barrido (DSC), difracción de rayos X (XRD) y espectroscopía infrarroja con transformada de Fourier (FT-IR). La actividad inhibitoria del BBPH sobre la ACE-I presentó un valor de $IC_{50}$=0.42 mg/mL. Todas las formulaciones mostraron microencapsulados con forma irregular. El BBPH se distribuyó homogéneamente en todas las formulaciones. El análisis por DSC y XRD mostró que EGLD y BBPH presentaron una distribución uniforme del BBPH y estructuras parcialmente cristalinas. El análisis FT-IR confirmó la estabilidad del BBPH en las microcápsulas. En conclusión, las microcápsulas de EGLD conteniendo BBPH fueron preparadas satisfactoriamente por liofilización.

*Palabras clave*: frijol bayo, hidrolizado proteico, Eudragit® L 30 D-55, microcápsulas, liofilización.

## 1 Introduction

Angiotensin-I converting enzyme (ACE-I; EC 3.4.15.1) play an important role in regulating blood pressure. This enzyme catalyzes the conversion of angiotensin-I (DRVYIHPFHL) into a potent vasoconstrictor, the angiotensin-II (DRVYIHPF) and inactive the vasodilatory peptide bradykinin

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(RPPGFSPFR). Inhibition of ACE-I activity is thus considered to be a pivotal therapeutic approach for treating hypertension (Udenigwe and Mohan, 2014). ACE-I synthetic inhibitors such as captopril, lisinopril and enalapril are used as treatment to control high blood pressure. However, prolonged use of such drugs can produce side effects, for instance vertigo, cough, agiodemas and decreased kidney function (Regulska *et al*., 2014). Currently the bioactive peptides isolated from different protein sources of animal or vegetable origin may inhibit the activity of ACE-I, becoming an alternative for the prevention and control of hypertension (Hernández-Ledesma *et al*., 2011).

Protection of bioactive peptides from physiological changes is essential in the translation of *in vitro* activity into animal and human models; therefore, microencapsulation could be used as an alternative to minimize factors that might interfere with their bioavailability. According to Nesterenko *et al*., (2013) microencapsulation is a process by which an encapsulated substance (solid, liquid or gaseous), also called active compound, active, internal or core phase, is covered by embedded in another substance called encapsulating coating, membrane, wall material, external phase or matrix; resulting in the formation of microcapsules which aim to provide a protective barrier to the active compound against factors that may affect its activity or structure.

The correct choice of wall material directly affects the encapsulation efficiency, stability and degree of protection of the active compound. Materials commonly used are synthetic polymers and copolymers, carbohydrates (starch, maltodextrin, gum arabic), fats, waxes, animal (gelatin, casein) and plant (soy protein) proteins, petroleum-based polymers used commonly in pharmacy and medicine as a matrix (polystyrenes, polyamides, polyurethanes, polyacrylates, and polyethylene glycols), among others (Nesterenko *et al*., 2013; Ray *et al*., 2016). Eudragits®, as the well-known pharmaceutical excipients, have been widely used for the formation of different sustained and controlled release formations. Eudragit® L 30 D-55 is an anionic copolymer based on methacrylic acid and ethyl acrylate. The ratio of the free carboxyl groups to the ester groups is approximately 1:1. It is characterized by a fast dissolution in the upper Bowel (duodenum) to pH 5.5 (El-Malah and Nazzal, 2008).

There are many techniques available to carry out the production of microencapsulates, such as spray-drying, spray-cooling/chilling, freeze-drying, fluidized bed, coacervation/phase separation, gelation, solvent evaporation, supercritical fluid expansion, interfacial polymerization (polycondensation), emulsion polymerization and extrusion (Nesterenko *et al*., 2013). The choice of the convenient microencapsulation technique depends on size, biocompatibility and biodegradability of desired microcapsules, the physicochemical properties of the active compound and the wall material, the proposed mechanism for the release of the active compound and process costs. Freeze-drying is used for water removal with a minimum of associated heat damage to the solid matrix and is based on the sublimation of the ice fraction of the product. Mass transfer occurs by diffusion of vapour through the dry layer of the specimen under the action of a pressure difference (Viveros-Contreras *et al*., 2013). From the phenomenon of sublimation, a process of successful lyophilization retains most of the initial properties of raw materials such as shape, size, appearance, taste, color, texture and biological activity (Ceballos *et al*., 2012). Based on this, the aim of this study was to microencapsulate a bayo bean protein hydrolysate with ACE-I inhibitory activity using Eudragit® L 30 D-55 as wall material through freeze-drying processing.

# 2 Material and methods

## *2.1 Material*

*Phaseolus vulgaris* L. com. var. Bayo seeds crop 2012, were donated by CEVAMEX (Campo Experimental Valle de México) at Santa Lucía, Texcoco, Estado de México, México. Pepsin from porcine gastric mucosa (P7125), Pancreatin from porcine pancreas (P1750) and Angiotensin Converting Enzyme (ACE) from rabbit lung (A6778) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Eudragit® L 30 D-55 was provided by HELM (Naucalpan de Juárez, Edo. de México, México) and was used as received. All other reagents were analytical grade and purchased from J.T. Baker (Phillipsburg, NJ, USA), Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA) and Bio-Rad (Bio-Rad Laboratories, Inc. Hercules, CA, USA).

## *2.2 Preparation of protein concentrate*

Bayo bean protein concentrate (BBPC) was prepared according to the method described by Betancur-Ancona *et al*., (2004). Whole seeds were ground to

powder and passed through a 0.2 mm mesh sieve. The resulting flour was mixed with distilled water (1:10 w/v) adjusted to pH 11 (NaOH 1 N) and stirred for 1 h. This suspension was allowed to sediment for 1 h at room temperature to recover the starch and protein fractions. The protein fraction was adjusted to their isoelectric point (pH 4.5) with HCl 1 N in order to precipitate proteins. The precipitated proteins were recovered by centrifugation at 1,317 g for 15 min and freeze-dried at -47 ºC and 1.3 Pa. The protein content was analyzed according to the method 954.01 from AOAC (AOAC, 1997).

## 2.3 Enzymatic hydrolysis

Enzymatic hydrolysis was performed with the sequential pepsin-pancreatin system according to Megías *et al*., (2004) with modifications. The BBPC was mixed with distilled water to produce a protein solution to 5% (w/v) which was equilibrated at optimum temperature (37 °C for both enzymes) and pH (2.0 for pepsin and 7.5 for pancreatin) for each protease before adding the respective enzyme. Protease was then added to the protein solution at a ratio 1:10 (enzyme/substrate). The hydrolysis with the sequential pepsin-pancreatin system was done for 60 min: predigestion with pepsin for 30 min followed by incubation with pancreatin for 30 min. The hydrolysis reaction was stopped by heating at 80 °C for 20 min, followed by centrifuging at 10,000 g for 30 min to remove the insoluble portion. The supernatants (protein hydrolysate) were freeze-dried at -47 ºC and 1.3 Pa.

## 2.4 Degree of hydrolysis

Degree of hydrolysis (DH) was calculated by determining free amino groups with O-phthaldialdehyde following as described by Nielsen *et al*., (2001) using Ec. (1).

$$DH = \frac{h}{h_{tot}} \times 100 \quad (1)$$

Where $h$ is the number of hydrolyzed bonds, and $h_{tot}$ is the total number of peptide bonds per protein equivalent.

## 2.5 ACE-I inhibitory activity

Angiotensin-I converting enzyme inhibitory activity in the samples was determined by following the method described by Hayakari *et al*., (1978). Hippuryl-L-histidyl-L-leucine (HHL) is hydrolyzed by ACE-I to yield hippuric acid and histidyl-leucine. This method relies on the colorimetric reaction of hippuric acid with 2,4,6-trichloro-triazine (TT) in a 0.5 mL incubation mixture containing 40 $\mu$M potassium phosphate buffer (pH 8.3), 300 $\mu$M sodium chloride, 3% HHL in potassium phosphate buffer (40 $\mu$M, pH 8.3), and 100 mU/mL of ACE-I. The mixture was incubated at 37 ºC for 45 min and the reaction finished by adding TT (3%, v/v) in dioxane and 3 mL of potassium phosphate buffer (0.2 M, pH 8.3). After centrifuging the reaction mixture at 10,000 g for 10 min, enzymatic activity was determined in the supernatant by measuring absorbance at 382 nm. ACE-I inhibitory activity was quantified by a regression analysis of ACE-I inhibitory activity (%) versus protein hydrolysate concentration and defined as an $IC_{50}$ value, that is, the protein hydrolysate concentration (mg protein/mL) required to produce 50% ACE-I inhibition under the described conditions.

## 2.6 Amino acid composition

Protein sample (2 mg) was hydrolysed with HCl 6 N (4 mL) at 110 ºC for 24 h in tubes sealed under nitrogen. After derivatization with diethyl ethoxymethylenemalonate, amino acids were determined by high-performance liquid chromatography (HPLC) according to the method described by Alaiz *et al*., (1992) using D,L-$\alpha$-aminobutyric acid as an internal standard and a Novapack C18 reverse phase column (300 x 3.9 mm i.d., 4 $\mu$m film thickness; Waters, Milford, MA, USA). Tryptophan was analyzed by HPLC after basic hydrolysis according to Yust *et al*., (2004).

## 2.7 Preparation of microcapsules

The microcapsules were prepared in the following way: Eudragit® L 30 D-55 as wall material (EGLD) was dissolved in 50 mL of phosphate buffer at pH 4.2 with constant stirring for 30 minutes. Similarly, the protein hydrolysate (active compound) was dissolved and stirred under the same experimental conditions. These solutions were mixed at 13,000 rpm for 1 min using an Ultra-Turrax® (T25Basic, IKA Labortechnik, Wilmington, Delaware, USA). The Eudragit® L 30 D-55-protein hydrolysate dispersions were processed for two cycles through a M-110Y microfluidizer (Microfluidics®, Newton, Massachusetts, USA) with two interaction

chambers: the primary one of the type "Y" (F20Y, $\phi$=75 $\mu$m) and the secondary one of type "Z" (H30Z, $\phi$ =200 $\mu$m). The air chamber of the equipment operated at room temperature and 70 MPa. Finally, the dispersions obtained were frozen at -80 °C for 24 h and lyophilized (Virtis, Consol 255L, France) to -47 °C and 1.3 Pa. Three formulations were prepared from protein hydrolysate and EGLD with the following ratios: 1:20, 1:4 and 1:1, respectively.

## 2.8 Characterization of microcapsules

### 2.8.1. Efficiency of encapsulation (EE)

The encapsulation efficiency (EE) was determined according to the protein content in 10 mg of sample determined by the colorimetric method of Biuret using a calibration curve for BSA (0.5-10 mg/mL, $R^2$=0.998). The EE was calculated in agree the Ec. (2).

$$RE = \frac{CPE}{CPT} \times 100 \quad (2)$$

Where $CPE$ corresponds to experimental protein content and the content $CPT$ theoretical protein.

### 2.8.2. Optical (OM) and scanning electronic (SEM) microscopy

The shape and surface morphology of the samples were observed with an optical microscope Nikon Eclipse 50i (Tokyo, Japan), a ring light microscope Amscope Led-144 (CA, USA) was used as lighting. Images were acquired at 150x of total amplification. SEM was also used (EVO LS10, ZEISS, Munich, Germany) at an accelerating voltage of 6 kV. It was used an EDS detector of the brand Bruker QUANTAX 200. The samples were fixed on an aluminum support and processed in environmental mode. Images were acquired at 500x of total amplification.

### 2.8.3. Confocal laser scanning microscope (CLSM)

The samples were mounted on glass slides and viewed under CLSM (LSM 710, Carl Zeiss, Germany). The laser wavelengths excitation were 405, 488 and 561 nm. A spectral canal was used to detect autofluorescence signals, which comes from microencapsulated components, in where the measurements of fluorescence intensity were performed using the built ZEN software of the LSM 710. The intensity peak characteristic of fluorescence emission signal was 463 nm for EGLD and 472 nm for BBPH.

### 2.8.4. Differential scanning calorimetry (DSC)

The thermal properties of the samples were studied with a Perkin-Elmer Differential scanning calorimeter (DSC). Samples (5 mg) were weighed and sealed in an aluminum DSC pan. Scanning was performed from 30 to 200 °C at a heating rate of 10 °C/min with a nitrogen flow of 40 mL/min. The temperature at the onset ($T_o$), the temperature at peak ($T_p$), the temperature at the end ($T_f$), and the enthalpy ($\Delta H$) were determined.

### 2.8.5. X-ray powder diffraction (XRD)

The samples were analyzed in an X-ray diffractometer (MiniFlex 600, Rigaku, Tokyo, Japan) equipped with a CuK$\alpha$ radiation. The X-ray tube was run at a power of 40 kV and 15 mA. The scanning was operated in the range between 5 and 70° 2$\Theta$ with a step angle of 0.02° at a scan rate of 1°/min.

### 2.8.6. Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra of samples were obtained using a Fourier transform infrared (FT-IR) spectrometer (Perkin-Elmer, Waltham, Massachusetts, USA) in the region of 400 to 4000 $cm^{-1}$. Each spectrum was obtained at a resolution of 1 $cm^{-1}$.

## 2.9 Statistical analysis

Results were presented as mean ± standard deviation. A Duncan multiple range test was used to estimate significant differences among the mean values at $2\alpha$ = 0.05 significance level. All analyses were processed with the Minitab 17 software.

# 3 Results and discussion

## 3.1 Enzymatic hydrolysis

The protein content in the concentrate obtained from bayo bean seeds was 63.18 g/100 g of sample, this value is similar to that reported by Valdez-Ortiz *et al*., (2012) for sulfured bean protein concentrates (61-66 g/100 g sample), but lower than that reported by Guzmán-Méndez *et al*., (2014) for *P. vulgaris* (76.66 g/100 g of sample) and P. lunatus (71.88 g/100 g of sample) concentrate. According to Torruco-Uco *et al*., (2009) the protein content of BBPC is suitable to be enzymatically hydrolyzed and produce bioactive peptides with possible antihypertensive activity.

The hydrolysis of BBPC with sequential pepsin-pancreatin system produced a DH value of 24.29%, which was lower than 35.74% in 90 min reported for a V. unguiculata protein hydrolysate (Segura-Campos *et al*., 2010), and to 28.47% for a hardened bean protein hydrolysate produced in 90 min (Betancur-Ancona *et al*., 2014), both obtained with the same sequential enzyme system. According to Pedroche *et al*., (2002) the sequential use of proteases with different or same catalytic activity can generate protein hydrolysates having a degree of hydrolysis greater than 10%. These hydrolysates may be useful to obtain biologically active peptides for the treatment or prevention of chronic degenerative diseases. Furthermore, Segura-Campos *et al*., (2010) reported that the production of protein hydrolysates with sequential pepsin-pancreatin system produces a group of peptides similar to those generated during gastrointestinal digestion in vivo. Therefore, the resulting peptides are resistant to these enzymes, suggesting that can be absorbed by the digestive epithelial cells in the small intestine and reach the blank site exerting its biological activity.

## *3.2 ACE-I inhibitory activity*

The $IC_{50}$ value of BBPH produced in 60 min with sequential pepsin-pancreatin system was 0.42 mg/mL. This value is within the range reported for other protein hydrolysates obtained with the same enzyme system, and from vegetable protein sources, such as chickpea (0.14-0.23 mg/mL), pea (0.16 mg/mL) (Barbana and Boye, 2010), lentil (0.44 mg/mL) (Boye *et al*., 2010), azufrado beans (0.06-0.32 mg/mL) (Valdez-Ortiz *et al*., 2012), lupin (0.23-0.27 mg/mL) and soybean (0.22 mg/mL) (Boschin *et al*., 2014).

Table 1. Amino acid composition of bayo bean protein hydrolysate (BBPH).

| Amino acids | BBPH | FAO/WHO (1991) |
|---|---|---|
| Asp+Asn | 12.5 ± 0.01 | |
| Glu+Gln | 15.8 ± 0.01 | |
| Ser | 6.6 ± 0.01 | |
| His* | 3.0 ± 0.07 | 1.9 |
| Gly | 4.8 ± 0.01 | |
| Thr* | 4.2 ± 0.14 | 3.4 |
| Arg | 5.8 ± 0.07 | |
| Ala | 4.5 ± 0.01 | |
| Pro | 5.0 ± 0.35 | |
| Tyr* | 2.8 ± 0.07 | |
| Val* | 5.1 ± 0.01 | 3.5 |
| Met* | 0.9 ± 0.01 | 2.5 |
| Cys* | 0.5 ± 0.01 | |
| Ile* | 4.5 ± 0.01 | 2.8 |
| Trp* | 1.5 ± 0.01 | 1.1 |
| Leu* | 8.7 ± 0.07 | 6.6 |
| Phe* | 6.2 ± 0.07 | 6.3 |
| Lys* | 8.1 ± 0.07 | 5.8 |
| Amino acid distribution | | |
| Hydrophobic | 36.4 | |
| Hydrophilic | 45.2 | |
| Neutral | 18.9 | |

Values correspond to mean ± standard deviation of two replicates and expressed in g/100g of sample on dry base. *Essential amino acids, FAO/WHO: Met±Cys, Phe±Tyr. Hydrophobic (Ala, Val, Met, Phe, Leu, Ile, Pro, Trp) Hydrophilic (Arg, Asp, His, Lys, Glu) Neutral (Ser, Gly, Thr, Tyr, Cys)

Table 2. Values of encapsulation efficiency (EE) of all microcapsule formulations.

| Formulation | BBPH (mg) | EGLD (mg) | Ratio | EE (%) |
|---|---|---|---|---|
| EGHP01 | 100 | 2000 | 1:20 | 35.95 ± 3.50$^a$ |
| EGHP02 | 250 | 1000 | 1:4 | 79.34 ± 2.55$^b$ |
| EGHP03 | 500 | 500 | 1:1 | 96.83 ± 1.93$^c$ |

Values correspond to mean ± standard deviation of three replicates.

The $IC_{50}$ value obtained in the present research indicates that the hydrolysis with sequential pepsin-pancreatin system was efficient to release ACE-I inhibitory peptides. However, Betancur-Ancona *et al*., (2014) indicated that the ability of a protein hydrolysate to inhibit the activity of ACE-I cannot be completely attributed to the value of $IC_{50}$, since it is mainly due to the amino acid composition of the peptides in the hydrolysate.

The amino acid analysis showed that BBPH contains a high proportion of hydrophobic amino acids (Table 1). A study on the structure-activity relationship of ACE-I inhibitory peptides have confirmed that tripeptides composed of hydrophobic amino acids at their C- and N-terminal positions have potent ACE-I inhibitory activity (Ondetti and Cushman, 1982). Hwang and Ko (2004) mention that tripeptides and peptides with high inhibitory activity have Trp, Phe, Tyr or Pro in position C-terminal, and basic amino acids (Lys, Hys, Arg) in position N-terminal. Other authors have suggested that amino acids as Leu, Ile and Val can contribute to increased ACE-I inhibition activity (Ruiz *et al*., 2004). BBPH has a high proportion of some of the above amino acids that may be contributing to inhibition of the activity of ACE-I.

## *3.3 Characterization of microcapsules*

### *3.3.1. Encapsulation efficiency (EE)*

Table 2 shows the values of EE, being the EGHP03 formulation the one with the highest efficiency (96.83%), a value similar to that reported for diclofenac sodium-Eudragit® RS100 nanoparticles and solid dispersions prepared by single-emulsion solvent diffusion and solvent co-evaporation. The diclofenac sodium encapsulation efficiency for all prepared nanoparticles and solid dispersions was over 99.8% (Barzegar-Jalali *et al*., 2012). According to the results obtained in this study the proportion of EGLD and BBPH are directly related to the encapsulation efficiency. Similar behavior was reported by Sansone *et al*., (2011), the authors studied the encapsulation of naringin and quercetin in a phythalate cellulose acetate emulsion system and the encapsulation efficiency ranged 62-94% depending on the combination used. Also, Joshi *et al*., (2013) found that the encapsulation efficiency depends on the nature of polymer used in the microsphere preparation with Eudragit RS100 and RL100.

### *3.3.2. Optical (OM) and scanning electronic (SEM) microscopy*

The shape and surface morphology of all microcapsule formulations determined by OM and SEM are presented in Fig. 1. The images obtained by optical microscopy (Fig. 1A-C) show microcapsules with irregular shapes and sizes greater than 100 $\mu$m. Furthermore, compact structures, formation of agglomerates with subparticles of about 1 $\mu$m were observed in the SEM images (Fig. 1D-F). Generally, the microencapsulates had similar morphological characteristics despite of the concentration of EGLD and BBPH used in each formulation. The conservation of a uniform size of microencapsulated after freeze drying process is considered a good indication of a successful freeze drying cycle. Molina Ortiz *et al*., (2009) reported a similar behavior when preparing microcapsules of a casein hydrolysate by spray drying with soybean protein isolate as wall material.

### *3.3.3. Confocal laser scanning microscope (CLSM)*

Fig. 2 shows the spatial distribution of BBPH (green fluorescence) inside the microcapsules EGLD (red fluorescence) obtained by freeze-drying and analyzed by CLSM. Regarding the images corresponding to EGHP01 (Fig. 2-1) and EGHP02 (Fig. 2-2) formulations, it was observed that EGLD and BBPH were homogeneously distributed in the microcapsules, the EGLD fluorescence intensity was greater compared to the obtained for BBPH. This behavior indicates that the greatest amount of active compound was within the microcapsules.

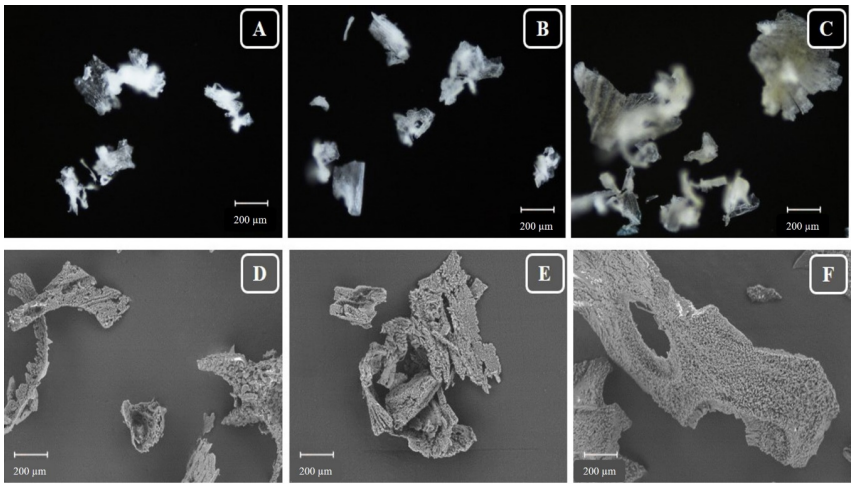


Fig. 1: Micrographs corresponding to EGHP01 (A), EGHP02 (B) and EGHP03 (C) obtained by optical microscopy (150x), and micrographs of EGHP01 (D), EGHP02 (E) and EGHP03 (F) obtained by SEM (500x).

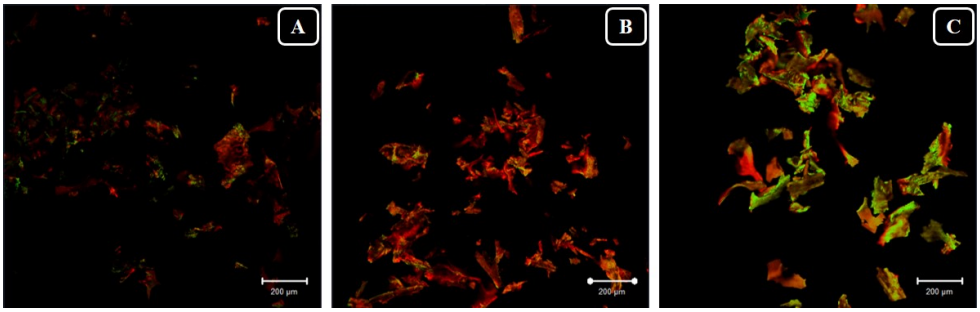


Fig. 2: Micrographs corresponding to EGHP01 (A), EGHP02 (B) and EGHP03 (C) obtained by CLSM (150x).

Nevertheless, in the EGHP03 formulation (Fig. 2-3) it was observed accumulation of BBPH on the microcapsule surface, indicated by an increased fluorescence of the active compound.

### *3.3.4. Differential scanning calorimetry (DSC)*

The thermal properties of EGLD, BBPH and EGHP03 determined by DSC are shown in Table 3. Results indicate that EGLD had a melting enthalpy of 220.41 J/g, which was higher than the BBPH. For EGHP03 formulation, the melting enthalpy was 162.44 J/g. According to Attama *et al*., (2006), a high 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. The Eudragit® L 30 D-55 is an anionic copolymer based on methacrylic acid and ethyl acrylate (1:1). Its linear structure gives certain crystalline characteristic while BBPH being a denatured protein (enzymatic hydrolysis) crystalline structure becomes amorphous. The results obtained in the EGHP03 formulation indicate an interaction of the components, and that the active compound (BBPH) is uniformly dispersed in the EGLD microcapsules. Similar behavior was reported by Xu *et al*., (2015) in microspheres of clarithromycin-Eudragit® L-100.

### *3.3.5. X-ray pattern diffraction (XRD)*

The X-ray pattern diffraction (XRD) of EGLD, BBPH and EGHP03 showed in Fig. 3 reported greater intensity peaks at 2°Θ: 11.5° and 29.8°, and weaker signals to 2°Θ: 9.0°, 11.5°, 17.1°, 19.2°, 22.6°, 25.2°, 27.1°, 28.3°, 31.1°, 32.7°, 33.7°, 35.7° and 36.7°. These results reflect a partially crystalline structure of wall material and active compound. XRD of EGHP03 formulation (Fig. 3-3) showed a decrease in the

Table 3. Thermal properties of EGLD, BBPH and EGHP03.

| Samples | $T_o$ (°C) | $T_p$ (°C) | $T_f$ (°C) | $\Delta H$ (J/g) |
|---|---|---|---|---|
| EGLD | 57.77 | 63.32 | 66.82 | 220.41 |
| BBPH | 55.58 | 58.27 | 60.11 | 205.88 |
| EGHP03 | 53.46 | 58.28 | 61.01 | 162.44 |

$T_o$: onset temperature; $T_p$: peak temperature; $T_f$: final temperature; $\Delta H$: gelatinisation enthalpy.

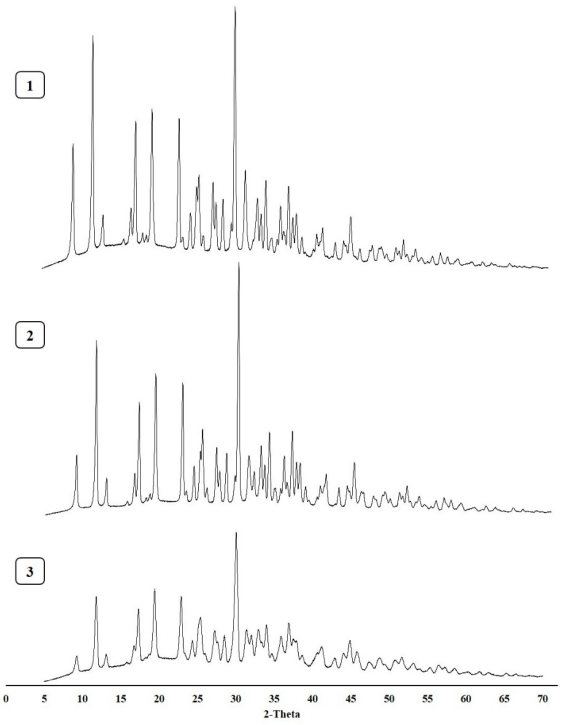


Fig. 3: X-ray diffraction patterns of EGLD (1), BBPH (2) and EGHP03 (3).

intensities of BBPH typical peaks. This behavior indicates that the active compound in the formulation exhibits an amorphous structure. Barzegar-Jalali *et al*., (2012) reported a similar behavior in solid dispersions and diclofenac sodium-Eudragit® RS100 nanoparticles. The XRD analysis was consistent with the DSC results.

### *3.3.6. Fourier transform infrared spectroscopy (FT-IR)*

The results of FT-IR spectra of EGLD, BBPH and EGHP03 are shown in Fig. 4. In the FT-IR spectrum of EGLD (Fig. 4-1) characteristic bands of the polymer structure functional groups can be observed: 1705 $cm^{-1}$ corresponds to vibrations C=O of carboxylic acid; 1250-1270 $cm^{-1}$ to ester groups.

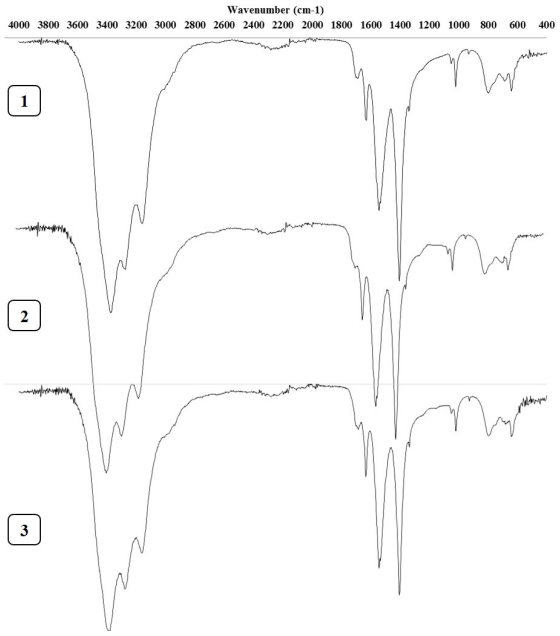


Fig. 4: FT-IR spectra of EGLD (1), BBPH (2) and EGHP03 (3).

The bands in the range of 2.500-3.500 $cm^{-1}$ are characteristic of OH vibration, while vibration signals at 1385, 1450, 1475 and 2940-2990 $cm^{-1}$ belong to groups C-H. These results are consistent with those reported by Hao *et al*., (2013) in the preparation of a novel emulsion diffusion method, enteric Eudragit® L100-55 nanoparticles by ultrasonic dispersion and diffusion solidification.

In the FT-IR spectrum BBPH (Fig. 4-2), a broad band of approximately 3100 $cm^{-1}$ relates to OH vibrations while amida-I groups are characterized to 1650 $cm^{-1}$. This peak may correspond to vibrations C=O and N-H. Among 1480 and 1575 $cm^{-1}$ there are signals, corresponding to vibrations NH and CN (amide-II). Cian *et al*., (2015), who assessed bioactive properties (ACE-I inhibition and antioxidant capacity) from wheat gluten peptides hydrolysate fractionated by pH (4.0, 6.0 and 9.0), to determine peptide action mechanism, and to relate it to the secondary structure and functional groups of peptides, reported similar

results.

In the FT-IR spectrum corresponding to the EGHP03 formulation (Fig. 4-3) it is showed that the intensity of some peaks was higher or lower compared to those obtained individually by EGLD and BBPH. According to Comunian *et al*., (2013), this behavior can be related to the ratio of wall material and active compound used in the formulation EGHP03. Also Barzegar-Jalali *et al*., (2012) observed in FTIR spectrum of solid dispersions and nanoparticles of diclofenac sodium-Eudragit® RS100 a reduction in peak intensity which possibly is due to weak electrostatic interactions between carboxyl groups of diclofenac sodium and ammonium groups Eudragit® RS100. This type of interaction may take place between the carboxyl groups of EGLD and the amino groups of BBPH in the EGHP03 microcapsules.

# Conclusions

The bayo bean protein hydrolysate microencapsulated with Eudragit® L 30 D-55 at a 1:1 ratio, exhibited high encapsulation efficiency. Analysis by microscopy showed irregular structures and a homogeneous distribution of BBPH in the microcapsules. DSC and XRD analysis confirmed a decrease of crystallinity of BBPH in the microcapsules of the EGHP03 formulation. The intermolecular interaction between EGLD and BBPH were detected in the FT-IR spectrum of EGHP03 formulation. The interactions may take place between the carboxyl groups of EGLD and the amino groups of BBPH. The preparation of microcapsules of protein hydrolysates through freeze-drying processing, using Eudragit® L 30 D-55 as wall material, constitute a new route for the development of innovative new delivery systems and/or functional food products.

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