



Antihypertensive and Antioxidant Properties from Whey Protein Hydrolysates Produced by Encapsulated *Bacillus subtilis* Cells

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Abstract

The proteolytic action from encapsulated *Bacillus subtilis* cells into polymeric system based in sodium alginate-chitosan-poly (ethylene glycol) (SA/CS/PEG) was evaluated to produce whey protein hydrolysates with antihypertensive and antioxidant properties. Polymeric system SA/CS/PEG resulted in an adequate composite material for the encapsulation of *B. subtilis* cells. The capsules showed porosity, resistance to agitation and they did not show material leakage and cells leakage. The proteolysis from encapsulated *B. subtilis* was comparable with the hydrolysis by whole free *B. subtilis* cells, achieving approximately 40% of hydrolysis of whey proteins after of 6 h. In addition, the proteolysis was maintained during various cycles of hydrolysis, without a drastic reduction of the enzymatic activity. The hydrolysate products included two peptidic fractions; peptides up 11 kDa and those of smaller size than 3 kDa. Peptides than 3 kDa showed high antihypertensive and antioxidant activity (82.33 and 76.54% respectively), and a great contained of amino acids, which were associated to these biological properties.

Keywords Angiotensin · Antioxidant · *Bacillus subtilis* · Encapsulation systems · Whey protein

Introduction

Whey protein hydrolysates are a source of amino acids fractions with different biological activities and health benefits; mainly, whey derived peptides have been identified as molecules able to inhibit angiotensin converting enzyme (ACE); thus they may be usefulness in the prevention and treatment of hypertension (Muro-Urista et al. 2011; Iwaniak et al. 2014). Also, whey peptides are recognized by their important role as antioxidant substances (Corrêa et al. 2014; Rocha et al. 2017) and other biological effects, which include antimicrobial, anticancer and immunomodulatory action.

These properties have supported their study and application in different research areas, particularly in the production of functional foods, nutraceuticals, cosmetics and as therapeutic fragments with clinical approaching (Muro-Urista et al. 2011).

Whey peptides are often obtained by enzymatic processes; generally commercial and selective enzymes are utilized to carry out the protein hydrolysis to produce potential bioactive substances with different amino acid sequences (Dullius et al. 2018). In this case, the enzymes are selected according to the substrate to achieve protein hydrolysates with defined biological characteristics. However, the application of enzymes presents some disadvantages, such as, high cost, because the enzymes are regularly extracted and purified from different sources; enzyme chain presents also instability, denaturation and they may give low efficiency (up 25–35%) in the hydrolysis; while, enzymes require high temperatures or changes of pH for their inactivation. Thus, these extreme conditions reduce the nutritional properties and biological action of released peptides (Aggei et al. 2016). Additionally, the presence of inactivated enzyme in

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the hydrolysates may produce allergenic effects in the product and unwanted flavors (Nielsen et al. 2017; Ivens et al. 2017).

All these reasons have allowed us to look for other alternatives to obtain hydrolysates by enzymatic processes that could conduce to better results; one of them is the use of encapsulated living cells from proteolytic microorganisms. The encapsulated cells are highly efficient because they produce multiple enzymes, but also is controlled their release. While, the encapsulation allows protecting cells from harsh environment and controlling their surrounding as well as their concentration (Meunier et al. 2010). Therefore encapsulating systems improve the enzymes production and contribute to make more effective the enzymatic process.

This alternative allows also a better manipulation of the cells, and it makes more favorable their separation from the products; hence the contamination by biomass is minimized or is avoided altogether. Application in continuous fixed-bed operation is also achieved by encapsulation, as cells can be used in repeated and continues processes (Mohamad et al. 2015). In consequence, the costs of the hydrolysis is lower against the use of purified enzymes y probaly against free cells.

The achieved benefits by encapsulated microorganisms have conducted to development of different studies on this topic; however, there are still several challenges that have to be addressed. In relation to whey protein hydrolysis, there are little information on the effect of cells encapsulation to produce therapeutic molecules with defined biological properties. Encapsulation cells alternative has been applied principally to improve protease enzymes production, acelerate the processes of cheese ripening, to preserve the cualities of probiotics in fermented products, as source of prebiotics, and to forme healthy and functional molecules with adecuated properties during cheese maturing (Albenzio et al. 2010; Santillo et al. 2012; Moumita et al. 2017). The most common species utilized for this purpose have been *Bacilli* and *Lactobacilli*, since they produce heat stable enzymes which remain active in milk and cheese even after the bacteria are killed by pasteurization. Specifically *Bacillus subtilis* have been identified as a proteolytic bacteria capable of produce various rennet-like enzymes which may coagulate milk proteins. However, nowadays there are various reports that reveal the potential activity, which has allowed its manipulation to produce alkaline enzymes at great scale, as starter cultures in fermented food, and to obtain products with nutraceutical and therapeutic characteristics (Moayedid et al. 2018). Chiefly it has been identified that enzymes from *B. subtilis* can increase the digestibility of hydrolysates and they can produce active peptides with ACE-inhibitory activity. In addition, these enzymes have been utilized to hydrolyze allergenic peptides by degradation of products from β -Lg and α -La, which in turn are generated during

cheese ripening by trypsin/chymotrypsin enzymes. Other studies have also confirmed that proteases from *B. subtilis* on casein fractions and whole milk (CN) casein samples, produce an intense hydrolysis to obtain active fractions with several properties (Albillos et al. 2007). Specially, Uluko et al. (2014) found that the hydrolysis of milk protein concentrate (MPC) by Neutrase, produces ACE inhibitor and antioxidant peptides of size 0.2–8 kDa. Nevertheless to date, no work has been done to elucidate the action of encapsulated *B. subtilis* cells on WPC hydrolysis for the obtaining active peptides.

The aim of this study was to investigate the antioxidant and ACE-inhibitory activities of fermented whey protein hydrolysates by encapsulate *B. subtilis* cells into a polymeric system based in sodium alginate-chitosan-poly (ethylene glycol). Encapsulation efficiency and degree of hydrolysis were analized, as wella as, the hydrolysates fractions were characterised by their peptide distribution (SDS-PAGE) and aminoacid profile.

Materials and Methods

Chemicals and Microorganism

Bovine cheese whey was supplied by a dairy industry from Mexico. *B. subtilis* (free-living strain 05) was obtained from the Research Laboratory of Environmental Engineering from the Technological Institute of Toluca, Mexico. The *B. subtilis* was cultivated in Man, Rogosa y Sharpe (MRS) broth and incubated for 24 h at 50 °C in a rotary shaker.

The following materials and reagents were supplied by Merck Chemicals: sodium alginate (SA), viscosity of 20,000–40,000 cps, chitosan (CS) with an acetylation degree of 70–80%, viscosity of 200–800 cps, poly (ethylene glycol) (PEG), average molecular weight of 400 g mol⁻¹, and calcium chloride (CaCl₂). By Sigma-Aldrich Inc. (USA): tris hydrochloride (Tris/HCl) molecular weight 157,60, sodium dodecyl sulfate solution (SDS), acetic acid, reagent, $\geq 99.7\%$, ammonium hydroxide solution, reagent, 28–30% NH₃ basis, acrylamide $\geq 99\%$ (HPLC) powder, *N,N'*-ethylene bisacrylamide for electrophoresis $\geq 98\%$ powder, agarose for molecular biology, tris-glycine-SDS buffer 10× concentrate, sodium dodecyl sulfate, for electrophoresis, $\geq 98.5\%$ (GC), 2-mercaptoethanol, for electrophoresis, glycerol, for electrophoresis, $\geq 99\%$, bromophenol blue sodium salt, for molecular biology, for electrophoresis, *N*-Hippuryl-His-Leu hydrate powder, $\geq 98\%$ (HPLC), angiotensin-converting enzyme (ACE) from rabbit lung ≥ 2.0 U protein g⁻¹, ethyl acetate anhydrous, 99.85%, potassium phosphate dibasic, reagent grade, $\geq 98.0\%$, (K₂HPO₄) and ascorbic acid as antioxidant standard. By AMRESCO: 2, 2'- azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

Cells Encapsulation of *B. subtilis*

The polymeric encapsulation system was prepared with SA, CS, and PEG. The proportion of the polymers and the standard conditions used were as follows: 5% (w/v) of CS was added to an aqueous dissolution of SA 3% (w/v) (Branson Ultrasonic 1800) during 30 min to obtain a homogenized matrix of SA/CS. After PEG was incorporated by agitation for 15 min to obtain the composite material SA/CS/PEG.

Bacillus subtilis cells (10 mL) were later dispersed in the composite SA/CS/PEG. In this experiment, slow agitation was applied in a magnetic stirrer Cimarec Thermo Scientific for 20 min. Next, the mixture polymer-cells was dropped into a CaCl_2 solution 30% (w/v), and *B. subtilis* encapsulates of SA/CS/PEG, was thus obtained. The capsules remained in the CaCl_2 solution for 24 h at 4 °C; at the end of this time, the capsules were separated by decantation and washed several times with distilled water in sterile conditions and stored in distilled water until their use in whey protein hydrolysis.

The encapsulated of *B. subtilis* were analyzed to determine properties like morphology, porosity, material leakage, structural integrity, cell density and cell leakage, before and after their use in the process of whey proteins degradation. The morphology was determined through scanning electron microscopy (SEM) with a JEOL JSM-6610LV microscope. The porosity was evaluated by specific surface areas (BELSORP-aqua3, BEL Japan, Inc). While cell release was evaluated by measuring the cell density in the medium after 24 h of immersion.

Whey Protein Hydrolysis by Encapsulated *B. subtilis* Cells

Sweet whey samples from dairy industry were previously degreasing. Fat was removed from 250 mL of whole milk whey by centrifugation at 5000×g, for 30 min, at 10 °C. The product of whey protein concentrated (WPC) was obtained by ultrafiltration (UF) process. The UF was performed by a cross-flow polymeric MICROZA membrane of cut-off 15 kDa. The analysis of proteins concentration from WPC was determined by the Biuret colorimetric method. WPC hydrolysis processes were carried out in an Erlenmeyer flask in individual experiments. The tests included WPC hydrolysis by encapsulated *B. subtilis* cells, and WPC hydrolysis process by *B. subtilis* as whole free cells (FC), which was utilized as hydrolysis control. The flasks were capped and put under orbital stirring at 150×g at 50 °C, at the hydrolysis time of 2, 4, 6, 8 and 10 h. The proteolytic action was stopped by separating the encapsulated capsules or FC, according to each experiment. Hydrolyzed products (supernatants) were separated and identified as WPC hydrolysate products (WPCH). Each product was frozen and stored at −10 °C until further analysis. Hydrolysis experiments were

performed in triplicate. The enzymatic activity from encapsulated of *B. subtilis* cells and FC was measured through of the degree of hydrolysis as a percentage (DH %), which was determined by quantifying of the protein concentration of the supernatant sample before and after of the hydrolysis.

Encapsulated cells reusability was studied by recycling hydrolysis process in a batch system by measuring proteolytic cells activity after five operational cycles. The capsules were utilized subsequently on new samples of WPC, to complete the five cycles of hydrolysis. The proteolytic action was determined by DH % on WPC samples and compared with the initial activity.

WPC Hydrolysates Fractionation

Products, WPCH were fractionated by a polymeric MICROZA membrane with molecular weight cut-off 3 kDa. Hydrolysate permeates fractions (WPCHP) and the retentate fractions (WPCHR) were collected and they were analyzed by molecular size, amino acid composition, and biological properties, as it is described below. Protein molecular size was measured by gel electrophoresis (SDS-PAGE). The running buffer contained 0.124 M Tris/HCl (pH 6.8) and 0.959 M Glycine. The separating gel was prepared with, 0.375 M Tris/HCl (pH 8.8) and 0.1% SDS. The markers utilized were, of high range, between 10 and 250 kDa. initial voltage was 80 V for 20 min, and after was increased to 100 V. Gel slabs were fixed and stained simultaneously using Bio-Rad Coomassie Blue R-250 stain solution (40% methanol, 10% acetic acid, 0.1% Coomassie Blue R-250) for 5 h with 2–3 changes of the de-staining solution.

Determination of Amino Acid Composition and Biological Activity in Hydrolysate Fractions

Amino acid profiles of WPCHR and WPCHP fractions were revealed by liquid chromatography HPLC analysis using a Waters 1525 chromatograph. 50 µL of sample (2 mg/mL) was previously filtered with Millipore filters (0.45 µm) and later was injected in Hi-Pore RP-318 250×4.6 mm (Bio-Rad) column. Moving faces A and B consisted of: A 0.1% trifluoroacetic acid (TFA) and acetonitrile, and B TFA (0.1%), respectively. The flow rate was 1 mL/min. The gradient was linear from 0 to 50% of solvent B in 60 min. Detection was carried out at 214 and 280 nm by 17 amino acids standards.

Biological properties from WPCHR and WPCHP fractions were measured as antihypertensive and antioxidant activity. The antioxidant activity was determined by the colorimetric method by 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and Ascorbic acid as antioxidant standard (Abdel-Hamid et al. 2017). The antihypertensive activity was analyzed by Angiotensin-converting enzyme

(ACE) inhibitory action according to the colorimetric method found in Corrêa et al. (2014). The test included HHL, dibasic potassium phosphate and ACE reagents. Both tests were carried out by a spectrophotometer LAMBDA 35 Perkin Elmer.

Statistical Analysis

The obtained data were expressed as mean $\pm$ standard deviation (SD) of three determinations. Statistical comparison was performed by using one-way analysis of variance (ANOVA). Differences between means at the 5% ($p < 0.05$) level were considered significant.

Results and Discussion

B. subtilis Cells Encapsulation

SEM images of encapsulating systems of SA/CS/PEG-containing entrapped *B. subtilis* cells are shown in Fig. 1. *B. subtilis* cells encapsulation gave as result SA/CS/PEG microcapsules with solid structure and porosity, as well as a high mechanic resistance. The average pore diameter of the microcapsules was of 4.64×10^{-2} nm, indicating an adequate pore size to make out an efficient mass transport of oxygen, nutrients and enzymes. It is due to the porosity and pore size induce of entrance of water into the capsule (Wischke and Schwendeman 2008), which may increase the transport of substances and may also accelerate the secreted enzyme pore-diffusion and release.

Encapsulating systems similarly provided a remarkable microenvironment for the *B. subtilis* to proliferate; cells density after 24 h was 9.0×10^8 cfu/g. This result was mainly linked to the properties of the porous and rugose matrix, as well as, the attraction forces of *B. subtilis* for adheres in

the walls of material, since through of the pores is possible the ingress of oxygen and nutrients, which it is essential to facilitate the cells survival. In addition, the interstitial networks formed between the materials, most likely allowed cell proliferation and cell interactions.

Furthermore, leakage of the encapsulation material, leakage of cells and clogging problems were not seen in these encapsulating SA/CS/PEG systems; this fact was also attributed to the structural integrity of the material and probable interactions of *B. subtilis* cells. The networks could also minimize the risk of further encapsulation matrix shrinkage and thus the stress exerted on the biological species could also be reduced.

The structural integrity of the polymeric material and porosity properties of the encapsulated made of SA/CS/PEG was recognized to the inclusion of PEG, which improved cell adhesion and prevented cell leakage.

It is due to individual materials SA and CS or in combined form, SA/CS; the encapsulated have shown fragility and friability with high pore size, which causes fractures, disintegration, and leakage of cells during their use as encapsulating systems (Baysal et al. 2013). Recent studies have been showed that the addition of other materials as filler improve the mechanical properties of these encapsulating systems, and they increment the ionic interactions between the microorganisms and enzymes with the support. Chan et al. (2011) utilized SA for encapsulating the probiotic *L. casei*. In this case, the addition of starch as filler improved the physical properties of SA capsules. In addition, less porous and less hygroscopic properties resulted in higher cell viability.

WPC Hydrolysis by Encapsulated *B. subtilis* Cells

The obtained WPC product presented approximately 8.182 g/L of proteins, against 10.18 g/L in whey, achieving

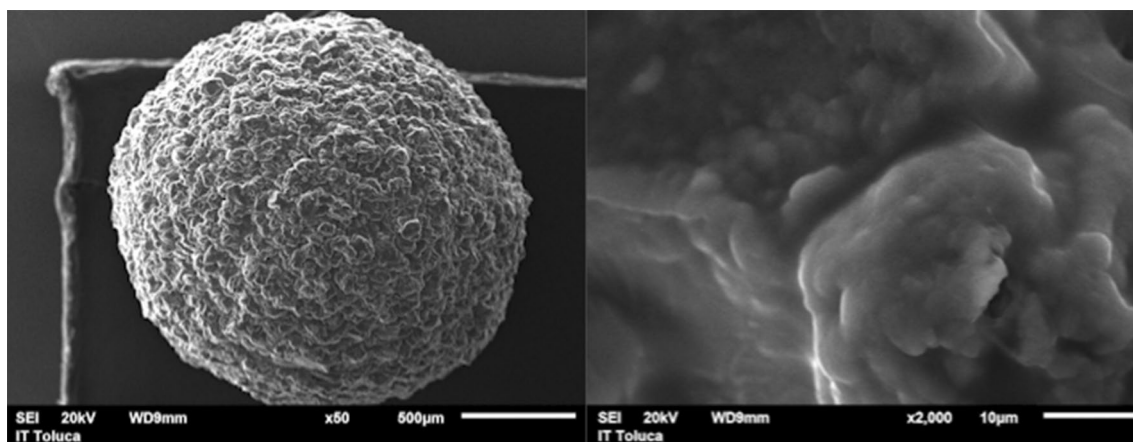


Fig. 1 SEM images of the encapsulating system of the *B. subtilis* cells made of SA/CS/PEG, at a magnification of $\times 50$ and $\times 2000$

80.37 of yielding. Regarding WPC hydrolysis, proteolytic action from encapsulated *B. subtilis* cells reached the maximum DH (38.5%) for 8 h. This result was comparable with the control; WPC hydrolysis by FC showed 40.3%. After that time, both experiments showed slow degradation for 10 h more, getting a stable state. Data on hydrolysis kinetics of WPC by FC and encapsulated cells of *B. subtilis* in SA/CS/PEG system can be seen in Fig. 2. According to this information, it may be deduced that the proteolysis of encapsulation of *B. subtilis* was similar to the FC. Therefore the encapsulating systems did not restrict the enzymes production and allow its transference to the substrate; thus, SA/CS/PEG was established as an effective and suitable encapsulation system for *B. subtilis* cells. The hydrolysis capacity from *B. subtilis* was analogous to the information reported by Corrêa et al. (2014). In this work, authors utilized a protease from *Bacillus* sp. P7 for the hydrolysis of sheep cheese whey. They achieved the maximum hydrolysis of DH as 55% during 4–6 h. This data was similar to that reported by Pant et al. (2015); those authors showed that entrapped *B. subtilis* presented a higher alkaline protease production when cells were entrapped in SA/gelatin. The enzymatic secretion reached a maximum level during 6 h of incubation; after this time, enzyme production decreased, which indicates a comparable behavior of entrapped cells.

Concerning reusability study of encapsulated cells, Fig. 3 shows the proteolytic action from encapsulated *B. subtilis* up to 5th hydrolysis cycle. It was observed, that proteolysis was maintained in subsequent hydrolysis processes up to 4th cycle. After of this cycle a slight decrease of hydrolysis percentage was detected, which was mainly attributed to the natural dead cells, because *B. subtilis* was used in subsequent hydrolysis for 5 weeks. The loss of proteolytic activity

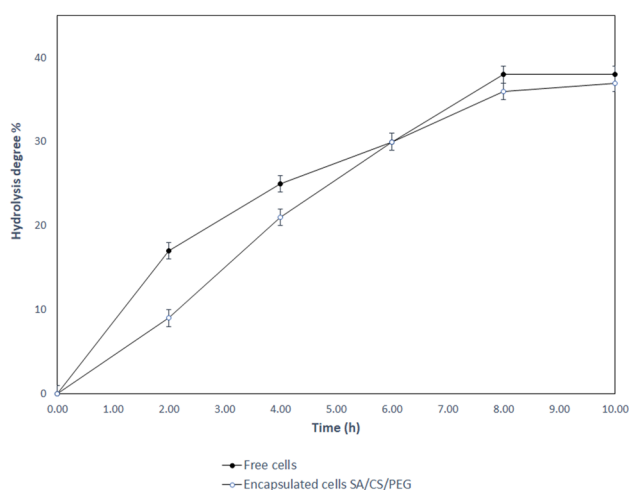


Fig. 2 Kinetics of the hydrolysis of WPC by FC and encapsulated *B. subtilis* cells into SA/CS/PEG, after 2–10 h of incubation time (error bars represent $\pm$ standard deviations, $n=3$)

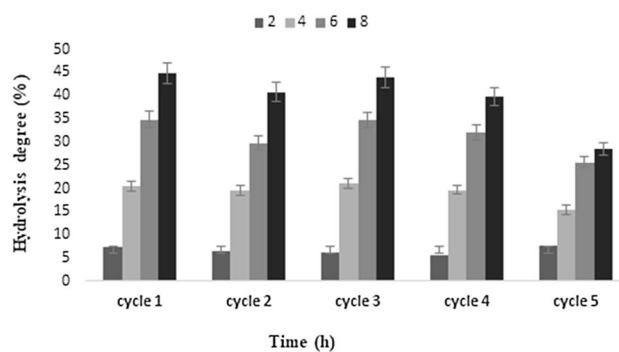


Fig. 3 WPC hydrolysis by encapsulated *B. subtilis* cells up to 5th hydrolysis cycle (error bars represent $\pm$ standard deviations, $n=3$)

can also be recognized as enzyme denaturalization, which is also presented in a natural form in FC. Other factors as denaturation of secreted enzymes and diffusional limitations could also be presented in these results.

Consequently, SA/CS/PEG was recognized as promising material as encapsulating matrix of *B. subtilis* cells. It means that encapsulated systems do not limit the enzyme production, so, encapsulated cells were found to be a better alternative for carrying out the hydrolysis of WPC as repeated batch systems and for reusing. Furthermore, it is an advantageous proposal, because such biocatalysts display improved operational stability, and cells from encapsulating matrix had a significant increase in the proteolytic action; which was attributed to the covalent binding of the microbial biomass or enzymes to entrapment matrix. Thus, encapsulating systems of microbial cells can be more economic processes than those that are made of purified enzymes.

An important evidence on this topic was also reported by Adinarayana et al. (2005); authors tested SA, k-carrageenan, agar, polyacrylamide, and gelatin as encapsulating materials for *B. subtilis* cells. They used these encapsulation systems to promote the production of enzymes, establishing that these biopolymers, allow to produce a greater amount of enzymes. The authors also found that a gradual reduction of up to 50% of enzyme activity is observed in the ninth cycle.

WPC Hydrolysates Fractionation

Molecular size from hydrolysate permeate fractions (< 3 kDa peptide fraction) and the retentate (> 3 kDa peptides) was confirmed by results of SDS-PAGE. Figure 4 shows the electrophoresis diagram of these fractions. In this diagram, it is seen that WPCH (6 h) product from encapsulated *B. subtilis* presented a molecular size to approximately 40 kDa. Bands of WPCHR and WPCHP also demonstrate hydrolysate fractions from retentate up to 11 kDa and permeate < 3 kDa, probably with the presence of peptides from β -Lg and α -La. The occurrence

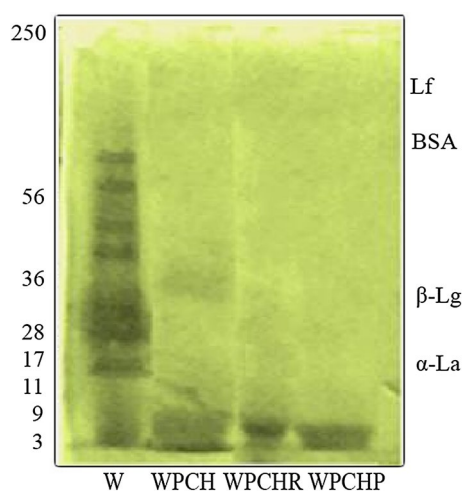


Fig. 4 Electrophoresis diagram of hydrolysate fractions. Band M links to marker, WPCH corresponds to hydrolysate product after 6 h of hydrolysis and bands WPCHR and WPCHP are relatives to hydrolysate fractions retentate and permeate respectively from membrane 3 kDa

of these fractions also possibly confirmed the hydrolysis of β -Lg and α -La. Comparable results were found in Farvin et al. (2010); the authors analyzed fractions from yogurt > 30 kDa and 10–30 kDa in LC-MS/MS, which revealed peaks among m/z 2026 and 1773; where these peaks confirmed the degradation of α -La.

Amino Acid Composition and Biological Activity in Hydrolysate Fractions

Table 1 presents amino acids profile in WPCH and WPCHR (between 3 and 11 kDa) and WPCHP (< 3 kDa) fractions from WPC hydrolysis by FC and encapsulated cells. Moreover, biological properties, antioxidant and ACE-inhibitory activity from these products are shown in Table 2.

The data disclosed that the amount of amino acids present in fractions from encapsulated cells WPCHP (5.16 mg/100 mL sample) was greater in comparison with WPCHR (4.48 mg/100 mL sample). While fractions from FC shown 4.18 mg/100 mL sample in WPCHP and 1.61 mg/100 mL sample in WPCHR, which means that a higher content of short peptides < 3 kDa are present in fractions of WPCHP from both hydrolysis systems.

The existence of short peptides and certain amino acids were linked to hydrolysates fractions from proteins β -Lg and α -La. On this subject, information on ACE-inhibitory property showed that WPCHP from the encapsulated system presented strongest activity (82.33%) than FC (68.89%). In the same way, the tests also confirmed that large peptides contained in WPCHR and WPCH cause a lower activity.

The obtained results of ACE-inhibitory activity and the presence of amino acids such as valine, leucine, tyrosine, phenylalanine, proline, arginine and isoleucine, indicated that WPCHP may be identified as ACE-inhibitory substance.

High levels of ACE inhibitors are usually considered between 70–90%, and they are associated with short

Table 1 Profile of amino acids from WPCH (6 h), fractions WPCHR and WPCHP by FC and encapsulating cells

Amino acid	Symbol	Molecular size (Da)	WPCHP (mg/100 mL of sample) FC	WPCHR (mg/100 mL of sample) FC	WPCHP (mg/100 mL of sample) encapsulating cells	WPCHR (mg/100 mL of sample) encapsulating cells
Threonine	T	119.1	ND	ND	ND	0.25
Aspartic acid	D	133.1	0.10	0.15	0.03	0.06
Serine	S	105.0	0.05	0.03	ND	ND
Glutamic acid	E	147.1	2.56	0.30	3.01	3.68
Glycine	G	75.0	0.19	0.16	0.06	0.09
Alanine	A	89.0	0.11	0.05	0.10	0.21
Cysteine	C	121.1	ND	ND	ND	0.01
Valine	V	117.2	ND	ND	0.18	0.06
Methionine	M	149.2	ND	ND	ND	ND
Isoleucine	I	131.2	0.54	0.12	ND	ND
Leucine	L	131.2	ND	ND	0.28	0.07
Tyrosine	Y	181.2	0.15	0.21	0.18	0.05
Phenylalanine	F	165.2	0.33	0.36	0.46	0.63
Lysine	K	146.2	ND	ND	ND	ND
Histidine	H	155.1	ND	ND	ND	ND
Arginine	A	174.2	0.15	0.23	0.19	0.05
Proline	P	115.1	ND	ND	0.15	0.03

Table 2 Antioxidant and ACE-inhibitory activity from hydrolysate products

Hydrolysis system	Whey hydrolysate fraction	Amino acid concentration (mg/100 mL)	Antioxidant activity %	ACE-inhibitory activity %
Encapsulating cells	WPCHP	5.16 ± 0.10	76.54 ± 0.11	82.33 ± 0.20
	WPCHR	4.48 ± 0.12	54.32 ± 0.15	63.37 ± 0.15
	WPCH	9.86 ± 0.21	39.79 ± 0.21	40.27 ± 0.12
FC	WPCHP	4.18 ± 0.15	59.32 ± 0.15	68.89 ± 0.15
	WPCHR	1.61 ± 0.13	35.21 ± 0.13	23.75 ± 0.21
	WPCH	5.79 ± 0.10	32.35 ± 0.10	34.65 ± 0.20

peptides < 8 amino acids containing proline (cyclic amino acids) at the C-terminal end. Mainly, structural characteristics of ACE inhibitors contains hydrophobic side chains; such as, tyrosine and phenylalanine. Amino acids structures having aliphatic side-chains with a branch (a central carbon atom bound to three or more carbon atoms), like glycine, leucine, isoleucine and valine, are also identified as ACE inhibitors (FitzGerald et al. 2004; Vermeirssen et al. 2004). Recent results have also revealed that hydrophobic amino acid with a polar functional group in the C-terminus also form part of ACE inhibitory substances (Iwaniak et al. 2014).

Regarding the antioxidant property, all fractions showed high scavenging activity, consequently, protein hydrolysis by *B. subtilis* was also effective for producing peptides with antioxidant action.

Short peptides exhibited the strongest radical quenching effect (peptides < 3 kDa); specifically, WPCHP fractions shown the maximum antioxidant activity. This result was also associated with peptides with short chains from β -Lg; while high scavenging activity was also attributed to the existence of tyrosine, glycine, phenylalanine, proline and isoleucine, of which some are identified as hydrophobic amino acid residues producing the antioxidant property.

Another remarkable data was the presence of glutamic acid; all fractions revealed a high concentration of this amino acid, which was also indirectly associated with the high antioxidant action, because the glutamic acid is utilized to prevent the oxidation of brain cells (Dutta et al. 2013).

In general, the whey peptide fractions containing the above mentioned amino acids are also indirectly linked to antioxidant therapeutic molecules for the preventing and treatment of properoxidative stress and metabolic/neurodegenerative diseases (Bhat et al. 2015), besides of cardiovascular, diabetes mellitus, cancer and Alzheimer diseases (Valko et al. 2007).

As discussion of these last results, in this work was determined that the enzymatic action from *B. subtilis* cleaved preferably on aromatic/hydrophobic amino acids, which could be related to its serine protease enzymes. Accordingly, the encapsulated system made of SA/CS/PEG did not

limit the enzymatic action of *B. subtilis*, and this matrix promoted the secretion of the enzymes, which favored the whey hydrolysis and peptides release with high antihypertensive and antioxidant properties. In consequence, this alternative may have a low cost in comparison with the use of purified enzymes, and probably also against the use of the free cells in fermented processes.

Analogous data are found in diverse research; however, the nearest to our results are those of Corrêa et al. (2014). They evaluated the enzymatic action from a purified protease preparation from *B. subtilis* sp. P7 on sheep cheese whey. Larger DH (90%) was found in 4 h; the proteolytic action was attributed to neutrase, the principal enzyme produced by *B. subtilis*. In this study, the author indicated that maximal ACE-inhibition values from hydrolyzed fraction were found to be 55%, although 42% inhibition was already observed after 1 h hydrolysis. Other important results were found in Tavares et al. (2011), authors utilized an enzymatic extract for the hydrolysis of WPC. Authors revealed that peptides with a molecular size below 3 kDa were mainly responsible for the ACE-inhibitory effect of the hydrolysates. In addition, the most potent ACE-inhibitory effect was detected in peptides from β -Lg. Amino acid sequences of these peptides also showed the presence of hydrophobic and aliphatic amino acids.

Cicero et al. (2013) also revealed that α -La and β -Lg are the major source of ACE-inhibitory peptides, and it may be comparable to that of captopril, an antihypertensive drug. Mainly, the known peptides as α -lactorphin (TLF), β -lactorphin (YLLF), β -lactosin B (ALPM) and tripeptide IPA are originated from β -Lg, and their therapeutic applications have confirmed that they exert a strong antihypertensive activity (Ibrahim et al. 2017).

The antioxidant property of the whey peptides also represents a recent class of biological substances with strong radical quenching effects and therapeutic approach for oxidative stress associated diseases (El-Desouky et al. 2017). Amino acids including leucine, isoleucine, valine and cysteine have been associated to antioxidant activity. (Rocha et al. 2017). These peptides are often derivate from β -Lg, α -La, Lactoferrin and Lactoperoxidase. Predominantly, peptides with

residues of proline and phenylalanine have been identified as molecules hydrogen donors and also as a direct radical scavenger.

Sequences as LAFNPTQLEGQCHV was found the hydrolysate of sheep cheese whey displaying the higher ABTS radical scavenging activity (74.4%), which was recognized as a derived sequence from β -Lg. Moreover, three new antioxidant peptides from buffalo milk (YPSG, HPFA, and KFQ) found by Abdel-Hamid et al. (2017) confirmed this information; whereas, a study carried out by Jemil et al. (2016), also established that *B. subtilis* is able to produce antioxidant peptides by hydrolysis of sardinelle proteins; the highest action was ranged from 30 to 48%. However, no work has been done to elucidate the action of encapsulated *B. subtilis* cells on the hydrolysis of whey proteins to obtain active peptides.

Conclusions

In this study, we successfully prepared encapsulated of *B. subtilis* cells into a polymeric system SA/CS/PEG to obtain whey hydrolysate products of smaller chains with superior biological activity. Polymeric system SA/CS/PEG resulted in an adequate material for this purpose. In addition, this encapsulating system can be utilized during various cycles of hydrolysis, without a drastic reduction of the performance of the processes. Furthermore, enzymes from *B. subtilis* allowed the obtaining of hydrolysate products with excellent antioxidant and antihypertensive properties. Consequently, the use of encapsulated of *B. subtilis* cells could be economically viable to produce peptides from whey proteins for efficient control of the blood pressure and to act as an antioxidant in human therapy.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Abdel-Hamid M, Otte J, De Gobba C, Osman A, Hamad E (2017) Angiotensin I-converting enzyme inhibitory activity and antioxidant capacity of bioactive peptides derived from enzymatic hydrolysis of buffalo milk proteins. *Int Dairy J* 66:91–98. <https://doi.org/10.1016/j.idairyj.2016.11.006>
- Adinarayana K, Jyothi B, Ellaiah P (2005) Production of alkaline protease with immobilized cells of *Bacillus subtilis* PE-11 in various matrices by entrapment technique. *AAPS Pharm Sci Technol* 6:E391–E397. <https://doi.org/10.1208/pt060348>
- Aggei D, Ongkudon CM, Weib CY, Chan AS, Danquah MK (2016) Bioprocess challenges to the isolation and purification of bioactive peptides. *Food Bioprod Process* 98:244–256. <https://doi.org/10.1016/j.fbp.2016.02.003>
- Albenzio M, Santillo A, Caroprese M, Marino R, Trani A, Faccia M (2010) Biochemical patterns in ovine cheese: influence of probiotic strains. *J Dairy Sci* 93:3487–3496. <https://doi.org/10.3168/jds.2009-3036>
- Albillos SM, Busto MD, Perez-Mateos M, Ortega N (2007) Analysis by capillary electrophoresis of the proteolytic activity of a *Bacillus subtilis* neutral protease on bovine caseins. *Int Dairy J* 17:1195–1200. <https://doi.org/10.1016/j.idairyj.2007.02.003>
- Baysal K, Aroguz AZ, Adiguzel Z, Baysal BM (2013) Chitosan/alginate crosslinked hydrogels: preparation, characterization, and application for cell growth purposes. *Int J Biol Macromol* 59:342–348. <https://doi.org/10.1016/j.ijbiomac.2013.04.073>
- Bhat AH, Dar KB, Anees S, Zargar MA, Masood A, Sofi MA, Ganie SA (2015) Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. *Biomed Pharmacother* 74:101–110. <https://doi.org/10.1016/j.biopha.2015.07.025>
- Chan ES, Wong SL, Lee PP, Lee JS, Ti TB, Zhang Z, Yim ZH (2011) Effects of starch filler on the physical properties of lyophilized calcium–alginate beads and the viability of encapsulated cells. *Carbohydr Polym* 83:225–232. <https://doi.org/10.1016/j.carbpol.2010.07.044>
- Cicero AF, Aubin F, Azais-Braesco V, Borghi C (2013) Do the lactotripeptides isoleucine–proline–proline and valine–proline–proline reduce systolic blood pressure in European subjects? A meta-analysis of randomized controlled trials. *Am J Hypertens* 26:442–449. <https://doi.org/10.1093/ajh/hps044>
- Corrêa APF, Daroit DJ, Fontoura R, Meira SM, Segalin J, Brandelli A (2014) Hydrolysates of sheep cheese whey as a source of bioactive peptides with antioxidant and angiotensin-converting enzyme inhibitory activities. *Peptides* 61:48–55. <https://doi.org/10.1016/j.peptides.2014.09.001>
- Dullius A, Goettertb MI, Volken de Souza CF (2018) Whey protein hydrolysates as a source of bioactive peptides for functional foods—biotechnological facilitation of industrial scale-up. *J Funct Foods* 42:58–74. <https://doi.org/10.1016/j.jff.2017.12.063>
- Dutta S, Ray S, Nagarajan K (2013) Glutamic acid as anticancer agent: an overview. *Saudi Pharm J* 21:337–343. <https://doi.org/10.1016/j.jsps.2012.12.007>
- El-Desouky WI, Mahmoud AH, Abbas MM (2017) Antioxidant potential and hypolipidemic effect of whey protein against gamma irradiation induced damages in rats. *Appl Radiat Isot* 129:103–107. <https://doi.org/10.1016/j.apradiso.2017.07.058>
- Farvin KS, Baron CP, Nielsen NS, Otte J, Jacobsen C (2010) Antioxidant activity of yogurt peptides: part 2—Characterization of peptide fractions. *Food Chem* 123:1090–1097. <https://doi.org/10.1016/j.foodchem.2010.05.029>
- FitzGerald RJ, Murray BA, Walsh DJ (2004) Hypotensive peptides from milk proteins. *J Nutr* 134:980S–988S. <https://doi.org/10.1093/jn/134.4.980S>
- Ibrahim HR, Ahmed A, Miyata T (2017) Novel angiotensin-converting enzyme inhibitory peptides from caseins and whey proteins of goat milk. *J Adv Res* 8:63–71. <https://doi.org/10.1016/j.jare.2016.12.002>
- Ivens KO, Baumert JL, Hutkins RL, Taylo SL (2017) Effect of proteolysis during Cheddar cheese aging on the detection of milk protein residues by ELISA. *Dairy Sci* 100:1629–1639. <https://doi.org/10.3168/jds.2016-11649>
- Iwaniak A, Minkiewicz P, Darewicz M (2014) Food-originating ACE inhibitors, including antihypertensive peptides, as preventive food components in blood pressure reduction. *Compr Rev Food Sci Food Saf* 13:115–134. <https://doi.org/10.1111/1541-4337.12051>

- Jemil I, Mora L, Nasri R, Abdelhedi O, Aristoy MC, Hajji M, Nasri M, Toldrá F (2016) A peptidomic approach for the identification of antioxidant and ACE-inhibitory peptides in sardinelle protein hydrolysates fermented by *Bacillus subtilis* A26 and *Bacillus amyloliquefaciens* An6. *Food Res Int* 89:347–358. <https://doi.org/10.1016/j.foodres.2016.08.020>
- Meunier CF, Dandoy P, Su BL (2010) Encapsulation of cells within silica matrixes: towards a new advance in the conception of living hybrid materials. *J Colloid Interface Sci* 342:211–224. <https://doi.org/10.1016/j.jcis.2009.10.050>
- Moayedi A, Mora L, Aristoy MC, Safari M, Hashemi M, Toldrá F (2018) Peptidomic analysis of antioxidant and ACE-inhibitory peptides obtained from tomato waste proteins fermented using *Bacillus subtilis*. *Food Chem* 250:180–187. <https://doi.org/10.1016/j.foodchem.2018.01.033>
- Mohamad NR, Marzuki NHC, Buang NA, Huyop F, Wahab RA (2015) An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnol Equip* 29:205–220. <https://doi.org/10.1080/13102818.2015.1008192>
- Moumita S, Goderska K, Johnson EM, Das B, Indira D, Yadav R, Jayabalan R (2017) Evaluation of the viability of free and encapsulated lactic acid bacteria using in-vitro gastrointestinal model and survivability studies of symbiotic microcapsules in dry food matrix during storage. *LWT-Food Sci Technol* 77:460–467. <https://doi.org/10.1016/j.lwt.2016.11.079>
- Muro-Urista C, Álvarez-Fernández R, Riera-Rodríguez F, Arana-Cuenca A, Téllez-Jurado A (2011) Review: production and functionality of active peptides from milk. *Food Sci Technol Int* 17:293–317. <https://doi.org/10.1177/1082013211398801>
- Nielsen SD, Jansson T, Le TT, Jensen S, Eggers N, Rauh V, Sundekilde UK, Sørensen J, Andersen HJ, Bertram HC, Larsen LB (2017) Correlation between sensory properties and peptides derived from hydrolysed-lactose UHT milk during storage. *Int Dairy J* 68:23–31. <https://doi.org/10.1016/j.idairyj.2016.12.013>
- Pant G, Prakash A, Pavani JVP, Bera S, Deviram GVNS., Kumar A, Prasuna RG (2015) Production, optimization and partial purification of protease from *Bacillus subtilis*. *J Taibah Univ Sci* 9:50–55. <https://doi.org/10.1016/j.jtusci.2014.04.010>
- Rocha G, Rocha F, Kise F, Rosso MB, Parisi MG (2017) Potential antioxidant peptides produced from whey hydrolysis with an immobilized aspartic protease from *Salpichroa origanifolia* fruits. *Food Chem* 237:350–355. <https://doi.org/10.1016/j.foodchem.2017.05.112>
- Santillo A, Albenzio M, Bevilacqua A, Corbo MR, Sevi A (2012) Encapsulation of probiotic bacteria in lamb rennet paste: effects on the quality of Pecorino cheese. *J Dairy Sci* 95:3489–3500. <https://doi.org/10.3168/jds.2011-4814>
- Tavares T, del Mar Contreras M, Amorim M, Pintado M, Recio I, Malcata FX (2011) Novel whey-derived peptides with inhibitory effect against angiotensin-converting enzyme: in vitro effect and stability to gastrointestinal enzymes. *Peptides* 32:1013–1019. <https://doi.org/10.1016/j.peptides.2011.02.005>
- Uluko H, Zhang S, Liu L, Li H, Cui W, Xue H, Lv J (2014) Pilot-scale membrane fractionation of ACE inhibitory and antioxidative peptides from ultrasound pretreated milk protein concentrate hydrolysates. *J Funct Foods* 7:350–361. <https://doi.org/10.1016/j.jff.2014.01.025>
- Valko M, Leibfritz D, Moncola J, Cronin MTD, Mazura M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39:44–84. <https://doi.org/10.1016/j.biocel.2006.07.001>
- Vermeirssen V, van der Bent A, Van Camp J, van Amerongen A, Verstraete W (2004) A quantitative in silico analysis calculates angiotensin I converting enzyme (ACE) inhibitory activity in pea and whey protein digests. *Biochimie* 86:231–239. <https://doi.org/10.1016/j.biochi.2004.01.003>
- Wischke C, Schwendeman SP (2008) Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. *Int J Pharm* 364:298–327. <https://doi.org/10.1016/j.ijpharm.2008.04.042>