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PRODUCTION OF CALCIUM- AND IRON-BINDING PEPTIDES BY PROBIOTIC STRAINS OF *Bacillus subtilis*, *B. clausii* AND *B. coagulans* GBI-30

PRODUCCIÓN DE PÉPTIDOS FIJADORES DE CALCIO Y HIERRO POR CEPAS PROBIÓTICAS DE *Bacillus subtilis*, *B. clausii* Y *B. coagulans* GBI-30

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Abstract

Some strains of *Bacillus subtilis*, *B. clausii* and *B. coagulans* are commercially used as probiotic bacteria and their proteolytic activity is well known. The aim of this work was to evaluate their capacity to produce calcium- and iron-binding peptides when grown in culture media with different nitrogen sources. The fermentation media included broths containing tryptic soy (TS), casein peptone (CP), soy peptone (SP), and a crude phycobiliprotein extract (CPE). Cell-free supernatants (CFS) were prepared from the fermented media after 24, 48 and 72 h of fermentation and, tested for degree of hydrolysis (DH). Calcium- and iron-binding activities were determined as well. When the inocula were prepared in a medium designed for the production of alkaline proteases (AP), the degree of hydrolysis and the mineral-binding activity in the CFS were higher. The best results for calcium-binding activity and DH were obtained when *B. subtilis* and *B. coagulans* grew in a CPE-containing medium. Analogous results were found for the iron-binding activity; nevertheless, this bioactivity was also high for *B. clausii* when grown in broths containing TS-, CP- and, SP.

Keywords: bioactive peptides, *Bacillus subtilis*, *Bacillus clausii*, *Bacillus coagulans*, calcium-binding, iron-binding, proteolysis, spirulina.

Resumen

Algunas cepas de *Bacillus subtilis*, *B. clausii* y *B. coagulans* se utilizan comercialmente como probióticos y su actividad proteolítica es de sobra conocida. El objetivo de este trabajo fue la evaluación de estos microorganismos debido a su capacidad para producir péptidos fijadores de calcio y hierro cuando son cultivados en medios con diferentes fuentes de nitrógeno. Los medios para la fermentación incluyeron caldos con soya y triptona (ST), peptona de caseína (PC), peptona de soya (PS) y, un extracto crudo de ficobiliproteína (ECF). Se obtuvieron sobrenadantes libres de células (SLC) a partir de los caldos de fermentación y se determinó el grado de hidrólisis (GH) y la bioactividad fijadora para calcio y hierro. El GH y la actividad fijadora de minerales fue mayor cuando se emplearon inóculos preparados a partir de un medio diseñado para la producción de proteasas alcalinas (AP). El mejor resultado para el GH y la bioactividad fijadora de calcio se obtuvo con *B. subtilis* y *B. coagulans* cultivados en caldo con ECF como fuente de nitrógeno. Se observaron resultados similares para la actividad fijadora de hierro, sin embargo, también se obtuvieron buenos resultados con *B. clausii* cuando se utilizaron para su crecimiento los caldos ST, PC y PS.

Palabras clave: péptidos bioactivos, *Bacillus subtilis*, *Bacillus clausii*, *Bacillus coagulans*, fijadores de calcio, fijadores de hierro, proteólisis, spirulina.

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1 Introduction

The role of proteins as biofunctional compounds has been studied in several works. Many of these proteins show their functional activities directly or after enzymatic hydrolysis either *in vitro* or *in vivo*. Proteolytic enzymes have many applications in food science and technology (Salazar-Leyva *et al*., 2014) and their action provides a rich source of biological active peptides whose sequences are inactive inside the precursor protein. They are released through the action of proteolytic enzymes from different origins such as microorganisms, digestive system, etc (Korhonen, 2009; Korhonen and Philanto, 2006). Starter and non-starter bacteria used in the manufacture of fermented foods (Mayo *et al*. 2010; Gonzáles-Olivares *et al*., 2011; Choi *et al*., 2012; Ramírez-Romero *et al*., 2013) can thus, generate bioactive peptides. Bioactivities such as mineral-binding have a positive impact on human health. The deficiency in the intake of some minerals like calcium and iron is a worldwide health problem. Iron deficiency is estimated to affect about 30% of the world population (Figueroa *et al*., 2012). Iron supplementation in the form of tablets and food fortification has not been successful in developing countries, and iron deficiency is still the most important deficiency related to malnutrition. Dietary factors have been shown that affect iron intake and some milk-derived peptides, in particular caseinophosphopeptides (CPP) produced during enzymatic digestion enhance iron absorption (Jung *et al*., 2007; Bouhallab *et al*. 2002). Calcium is an essential mineral in the human body since it regulates many cellular processes such as nerve response, bone growth, muscle contraction, cardiac functions and many more (Sussman *et al*., 1989; Bass *et al*. 2006; Choi *et al*., 2012). An inadequate intake of calcium can generate osteoporosis in middle-aged people (Choi *et al*., 2012). Osteoporosis affects 20% of postmenopausal women in the USA and 7.8 million women in the world (Figueroa *et al*., 2012). CPP capable to bind minerals, improve the stability, absorption and bioavailability of calcium (Bao *et al*., 2007; Choi *et al*., 2012; Figueroa *et al*. 2012). Other protein sources like soybean have been shown to increase the intestinal calcium absorption in women (Bao, 2007). Although, there are several protein sources that have not been explored for the production of bioactive peptides. Among these protein sources is spirulina (*Arthospira sp*.), a cyanobacteria (blue green algae) that has been harvested for centuries from the Texcoco Lake in Mexico to be used as a food. Protein content up to 64% (dry basis) can be reached under the right culture conditions (Pandey and Tiwari, 2010). Within the most important proteins, phycobiliproteins are possibly, the components with the highest commercial value. Due to their protein nature, unique color, and other properties with a wide range of promising applications including the production of bioactive peptides (Simeunović *et al*., 2012).

The genus *Bacillus* includes moderately thermophilic, aerobic or facultative anaerobic, proteolytic spore-forming rods. Some strains have been catalogued as probiotics and can produce a broad spectrum of bioactive peptides, with great potential for biotechnological and biopharmaceutical applications (Cutting, 2011; van Dijl *et al*., 2013, Seyedeh *et al*., 2007). The aim of this study was the evaluation of the capacity of three probiotic strains of *Bacillus* to produce calcium- and iron-binding peptides when grown in culture media with different nitrogen sources, a crude extract of phycobiliproteins included. Two different growth media were used to explore their effect on the production of proteases: the first was an alkaline media for optimal growth (AM) and the second, a medium to activate proteases (AP).

2 Materials and methods

2.1 Microorganisms

The *Bacillus* strains were isolated from commercial probiotic-containing products: *Bacillus coagulans* GBI-30 from Digestive Advantage®, *Bacillus clausii* from Enterogermina®, and *Bacillus subtilis* from Salvacolon®. Spore germination was induced by a heat treatment at 70°C for 30 min (Vepachedu *et al*., 2004). The three strains were grown on Alkaline Medium (AM) pH 10.5 containing (g L^{-1}): glucose - 10, casein peptone - 5, yeast extract - 5, KH_2PO_4 - 1, $MgSO_4$ - 0.2, Na_2CO_3 - 10 as reported by Horikoshi (1971). Stock cultures were prepared and then preserved by storage in 30% glycerol at -70°C.

2.2 Inocula preparation

The first inoculum was prepared in AM (pH 10.5) and the second in AP (medium for alkaline protease production) which contained (g L^{-1}): sucrose - 11, yeast extract - 5, KNO_3 - 5.2, K_2HPO_4 - 4, trisodium citrate - 4, $CaCl_2$ - 0.002, $MgSO_4 \cdot 7H_2O$ - 0.5, Na_2CO_3 - 10. After autoclaving and cooling, the medium was mixed with 10 ml of a sterile trace element

solution containing (g L^{-1}): trisodium citrate - 10, $(NH_4)_6MO_7O_{24}$ - 0.1, $FeSO_4 \cdot 7H_2O$ - 2, $CuSO_4 \cdot 5H_2O$ - 0.2, $ZnCl_2$ - 0.2. The cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of sterile inoculation medium at 35 °C with orbital shaking (350 rpm) for 16 h (Seyedeh *et al*., 2008). After reaching an optical density of 2 at 600 nm, 10 % (v/v) of the culture was used to inoculate the fermentation flasks.

2.3 Fermentation

The fermentation conditions were 35°C and 350 rpm orbital shaking in 250 ml Erlenmeyer flasks with 50 ml of sterile medium. The incubation time was 72 h and the culture media included different nitrogen sources at a 2% concentration and pH 10. Tryptic soy broth (TS) containing (g L^{-1}) casein peptone - 17, soy peptone - 3, NaCl - 5, K_2HPO_4 - 2.5, and dextrose - 2.5 was used as the standard commercial comparison medium. The other three culture media included only one nitrogen source at a 2% level: soy peptone (SP), casein peptone (CP), and a crude phycobiliprotein extract (CPE). Samples were taken every 24 h and centrifugated at 10000xg for 20 min at 4°C. Cell-free supernatants (CFS) were prepared by syringe filtering (0.22 μm). Protein content of CFS were determined by the Lowry method modified by Hartree (1972).

The CPE was obtained by aqueous extraction of dry spirulina (*Arthrospira platensis*) biomass purchased from Química Farmacéutica Esteroidal (Mexico City, Mexico). The suspension was subjected to cycles of freezing and thawing and centrifuged at 15000xg at 4ºC for 30 min. The blue color in the supernatant was indicative of a correct phycobiliprotein extraction (Patil *et al*., 2008; Hemlata *et al*. 2011). Finally, the extract was filtered through a 0.22 μm membrane. Protein, lipids and ash contents were determined in the CPE by Kjeldahl (NX6.25), AOAC 920.39, and AOAC 923.03 methods respectively.

2.4 Proteolytic activity

The release of free amino groups in the CFS during the proteolytic reactions was determined spectrophotometrically by the 2,4,6-trinitrobenzenesulphonic acid (TNBS) method as described by Adler (1979). Two mL of phosphate buffer pH 8.2 was mixed with 250 μL of CFS samples, and then the mixture was stirred vigorously and mixed with two mL of a 0.1% solution of trinitrobenzenesulfonic acid (Sigma-Aldrich, USA). The reaction mixture was stirred and dark-incubated at 50°C for 60 min. The reaction was stopped by addition of four mL of 0.1 N HCl and the absorbance read at 340 nm. A standard curve was prepared using a 3 mM solution of glycine containing 1% SDS. Transformation of the measured glycine amino equivalents to a degree of hydrolysis was carried out by means of a standard curve for each particular protein substrate as reported by Adler (1979). Tricine-SDS-PAGE (Schägger, 2006) was used to separate proteins in the MW range of 1-100 kDa and was also used to determine the extent of protein hydrolysis.

2.5 Iron-binding capacity

The iron binding capacity of the CFS was determined by the methodology reported by Hwang *et al*. (2001) with minor modifications. An aliquot of 0.1 ml of CFS was mixed with 0.6 mL of deionized water and 0.1 mL of 0.2 mM $FeCl_2$-$4H_2O$. The samples were incubated for 30 s at room temperature and mixed with 0.2 mL of 1 mM Ferrozine (Sigma-Aldrich, USA) solution. The mixture was stirred and incubated for 10 min at room temperature. Absorbance was read at 520 nm. A blank was also prepared in the same way but water was used instead of the CFS. The bound iron was determined with Eq. 1.

$$\text{Bound iron (\%)} = \frac{100(C_0 - C_1)}{C_0} \qquad (1)$$

where:
C_0 = total iron concentration
C_1 = unbound iron concentration

2.6 Calcium-binding capacity

Calcium-binding assay was performed using a modification of the method of Charoenphun *et al*. (2013). A four mL sample of CFS (diluted 1:10) was mixed with 5 mM $CaCl_2$ in 0.5 mM sodium phosphate buffer (pH7.8). The mixture was stirred at 25°C for 30 min and the pH was adjusted to 5 with 10 M HCl. The sample was filtered through Whatman filter paper No 1. The calcium content in the filtrate was determined by means of LAQUAtwin Compact Calcium Ion Meter (Horiba, Ltd., Japan).

The amount of bound calcium was calculated with Eq. 2:

$$\text{Bound calcium} = \text{Total calcium} - \text{ACFS calcium} \qquad (2)$$

where:

Total calcium = $CaCl_2$ concentration before the addition of CFS (mg L^{-1})

ACFS calcium = $CaCl_2$ concentration after the addition of CFS (mg L^{-1})

3 Results and discussion

3.1 *Proteolysis*

The extent of proteolysis during fermentation was generally quantified as the degree of hydrolysis (DH), referred to the percentage of the total peptide bonds cleaved by the enzyme system. TNBS, as a reagent for free amino groups, is adequate for this objective. Basically, it is a spectrophotometric assay which measures the absorbance of a trinitrophenylated amino-sulfite complex at 340 nm under alkaline conditions (Wang *et al*., 2013).

During the fermentation of the media containing, as a nitrogen source, TS, SP, CP, and CPE, the DH of the protein sources varied depending on the strain, time of fermentation and inoculums as shown on Table 1. The highest DH values (%) were observed in CPE fermented by *B. subtilis*, previously grown in AP after 48 and 72 h (99.24 and 98.49 % respectively) and *B. coagulans*, also previously grown in AP, after 48 and 72 h (92 and 97% respectively). In the case of TS broth, *B. clausii* previously cultivated in AP after 72 h reached a DH of 51.7%. In the case of SP, *B. coagulans* inocula produced in AM and AP showed the highest DH values of 27% after 72 h of fermentation. The extensive hydrolysis of CPE was confirmed by Tricine-SDS-PAGE (Fig. 1), where the production of peptides in a range of 18 000 to 307 Da can be observed.

Many studies on proteolysis have been performed with pure enzymes. Some of them have shown that, when casein is hydrolyzed with trypsin, a DH of 50-35% can be reached along with an increase in the amount of peptides with MW of 20 kDa (Wang *et al*., 2013). When chymotrypsin was used, a DH value of 65% was obtained with casein as substrate (Srinivas *et al*., 2010). Other experiments have shown that when soy protein is hydrolyzed with a *Bacillus* protease (Benardi Don *et al*., 1991), Flavorzyme (Moure *et al*., 2006), a microbial neutral protease (Achouri *et al*., 1998) and trypsin (Kim *et al*., 1990), values of DH of up to 17, 63.4, 8 and, 20% respectively were obtained. In this study, higher values of DH could be obtained, indicating that the *Bacillus* strains used in this research could be good sources of proteolytic enzymes. In the CFS, the protein concentration decreased, as expected, during fermentation (TS: from 13.67 to 5.14, SP: from 13.11 to 4.82, CP: from 8.12 to 0.42, CEP: from 2.38 to 0.013 mg protein ml^{-1}).

3.2 *Calcium-binding activity*

The properties of the peptides contained in the CFS are mainly dependent on the nitrogen source, strain, and the medium used to produce the inoculum. In this study, the CFS obtained from the fermentation of TS, SP, CP and CPE, by the three strains of *Bacillus*, were evaluated for their ability to produce bioactive peptides with Calcium-binding capacity (Table 1). According to the results of this study, the higher the DH, the higher the Calcium-binding capacity of the produced peptides (R = 0.8118). The best producer of Calcium-binding peptides in CPE was *B. subtilis* (previously grown in AP) with 12.67 and 5.35 mmol Ca^{2+} mg $prot^{-1}$ after 48 and 72 h of fermentation respectively. Also, in CPE medium, AP-grown *B. coagulans* produced 10.77 and 7.23 mmol Ca^{2+} mg $prot^{-1}$ after 48 and 72 h of fermentation. In the case of the media with CP and TS, peptides with calcium-binding capacities of 3.35 and 1.37 mmol Ca^{2+} mg $prot^{-1}$ respectively, were produced by AP-grown *B. coagulans* and *B. clausii* after 72 h of cultivation.

Many studies about generating calcium-binding peptides have been performed with different enzymes or extracts and substrates. One of them involved α_s - y β -caseins hydrolyzed by glutamic acid-specific endopeptidases (GSE) from a commercial extract of *B. licheniformis*; the hydrolysates had a MW from 3.6 to 10.8 kDa, and showed binding capacities in the range of 0.24- 0.14 mmol Ca^{2+} mg $prot^{-1}$ (Park, 1998). Another study involved the production of calcium-binding peptides produced by *Lactococcus lactis* subsp. *cremoris* NCFB 712 during milk fermentation with control of pH; in this case, the peptides had a binding capacity of 0.28 mmol Ca^{2+} mg $prot^{-1}$ and a good correlation with the DH (Figueroa *et al*., 2012). The results related to the calcium binding capacities for the SP controls in this work were similar to those studies above mentioned. All the controls (non-fermented culture media) in this study included, with the exception of CPE, nitrogen sources which were partially hydrolyzed proteins with calcium-binding activity. Calcium-binding peptides are usually present in fermented products like yogurt and cheese (Dimitrov, 2009; Figueroa *et al*., 2012).

In the case of SP media, the CFS obtained from *B. clausii* when inocula were produced in AP, generated

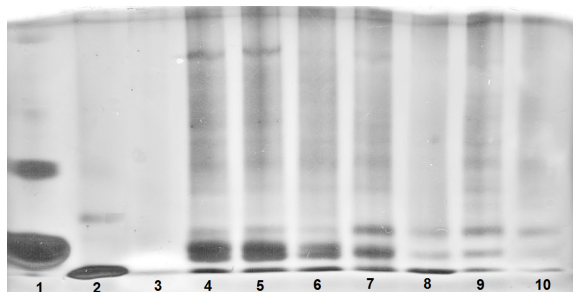

Fig. 1. Tricine SDS-PAGE from different protein preparations. 1 - β-Lactoglobulin 18 kDa, 2 Lysozyme 14 kDa, cell-free supernatants from the fermented broths with crude extracts of phicobiliproteins: 4, 5, and 6 - unfermented control, 7 - *B. subtilis* inoculum from AM after 24 h, 8 - *B. subtilis* inoculums from AM after 48 h, 9 - *B. subtilis* inoculums from AM after 72 h, 10 - *B. subtilis* inoculums from AP after 24 h. A higher degree of hydrolysis can be observed when strains previously grown in AP medium are used as inoculums (see lanes 7 and 10).

peptides with Calcium-binding activity in the range of 0.66-0.94 mmol Ca^{2+} mg prot^{-1} with the activity increasing with the fermentation time. In other study, soybean proteins hydrolyzed with protease M generated peptides with a Ca binding capacity of 66.9 mg Ca^{2+} g^{-1} and MW in the range of 14.4 to 8-9 kDa (Bao, 2008). In this study, an increase in the production of low MW peptides (<14 kDa) can be observed in the electrophoresis gels (Fig. 1). Calcium-binding occurs between the carboxyl group of acidic amino acids and the imidazole group of histidine (Bao *et al*., 2007). The considerable amounts of Asp, Glu, and His and the overall negative charge of the soybean protein derived peptides surely play an important role in calcium-binding (Don-Wong *et al*., 2012; Bao *et al*., 2007). Bao *et al*. (2008) reported that partial hydrolysis might loosen the tight structure of soybean globulins and increase the availability of calcium binding sites, while further hydrolysis reduces the MW and might lead to a decrease in binding sites and a decrease in the calcium-binding capability of the peptides. Their results suggest that soybean protein fragments with the highest calcium binding capacity have average MW in the range of 8 to 14.4 kDa.

The highest binding activity was obtained from CFS when grown in CPE-containing media. These CFS also had the highest DH (see Table 1) and the lowest MW peptides (see Fig. 1). It is possible that the extensive peptide bond cleavage had exposed some of the carboxyl groups of acidic amino acids (Glu, Asp) improving the calcium-binding activity.

3.3 *Iron-binding activity*

The results in this study indicated that the iron-binding activity of the CFS increased with the time of fermentation. The highest activity was observed in the CFS where AP-produced inocula were used. In the case of TS-, SP-, and CP-containing media, the highest activities were produced by *B. clausii* CFS (96.23, 95.89, and 95.01 % respectively) after 72 h of fermentation. When the CPE-containing medium was used. The highest iron-binding activity was observed in the CFS from *B. subtilis* and *B. coagulans* after 72 h of cultivation (96.87 and 97.14 % respectively).

Figueroa *et al*. (2012) observed 60% of iron-binding activity in milk fermented with *L. lactis* and Kim *et al*. (2007) found an activity of 97.56% in whey protein hydrolyzed with alcalase from *Bacillus licheniformis*. These results are similar to those found in this study. Storcksdieck and Hurrell (2007) found that low MW (< 10 kDa) peptides in pepsin digests of myofibrillar proteins were the major responsible peptides of iron binding. This effect could not be observed in the case of casein, egg albumin, and most sarcoplasmic proteins after pepsin digestion. Chaud *et al*. (2002) suggested that the iron-binding capacity is related to the net charge of the peptide. The phosphopeptides derived from α_s-caseins and β-casein (CPP) have negative charges, so they efficiently bind to divalent cations. Iron binding usually takes place at the free γ- and δ-carboxyl groups of aspartic and glutamic acid residues (Chaud *et al*., 2002; Storcksdieck *et al*., 2007). Both acidic amino acids are known to form very stable iron chelates through the formation of a possible tridentate structure (Storcksdieck *et al*., 2007). The ε-amino nitrogen of lysine, the guanidinium nitrogen of Arg, and the imidazole nitrogen of His may also be involved in iron binding (Lee *et al*. 2009; Storcksdieck *et al*., 2007). L-Tyr appeared to form complexes with iron through phenolate oxygen (Chaud *et al*., 2002). At a neutral pH, L-Cys reduces iron (III) and complexes with the generated iron (II) through sulfur bonds (Storcksdieck *et al*., 2007). Most reported peptides with iron binding activity have low MW (Huang *et al*., 2012; Kim *et al*., 2007; Lee *et al*., 2009).

Table 1. Calcium-binding activity and degree of hydrolysis determined in the CFS prepared from fermented broths containing different nitrogen sources and inoculated with probiotic strains previously grown in two culture media

CFS			TS		SP		CP		CPE	
	I	T (h)	CBA (mmol Ca^{2+} mg $prot^{-1}$)	%DH	CBA (mmol Ca^{2+} mg $prot^{-1}$)	%DH	CBA (mmol Ca^{2+} mg $prot^{-1}$)	%DH	CBA (mmol Ca^{2+} mg $prot^{-1}$)	%DH
C	-	0	0.436 ± 0.036	17.16	0.254 ± 0.051	10.381	0.485 ± 0.052	37.721	0.525 ± 0.020	9.855
		24	0.626 ± 0.044	22.969	0.406 ± 0.050	20.409	0.905 ± 0.095	38.013	0.929 ± 0.005	7.196
	AM	48	0.386 ± 0.045	20.179	0.291 ± 0.045	21.115	1.057 ± 0.072	33.813	1.922 ± 0.006	32.731
Bs		72	0.801 ± 0.077	24.786	0.309 ± 0.036	22.353	1.015 ± 0.055	45.189	1.700 ± 0.005	19.746
		24	0.371 ± 0.051	19.373	0.537 ± 0.083	18.327	0.774 ± 0.057	41.312	4.488 ± 0.016	75.565
		48	0.661 ± 0.045	28.722	0.377 ± 0.058	16.624	1.169 ± 0.118	40.981	12.670 ± 0.046	99.243
	AP	72	0.772 ± 0.061	22.375	0.635 ± 0.075	21.414	1.556 ± 0.083	30.291	5.352 ± 0.011	98.490
		24	0.430 ± 0.042	24.129	0.350 ± 0.054	19.680	1.027 ± 0.072	39.965	0.789 ± 0.028	25.607
	AM	48	0.557 ± 0.039	21.453	0.462 ± 0.054	22.114	1.217 ± 0.101	33.506	1.597 ± 0.005	22.416
Bcl		72	0.489 ± 0.048	24.039	0.367 ± 0.064	21.492	0.770 ± 0.091	25.269	2.129 ± 0.004	27.400
		24	0.783 ± 0.055	42.954	0.664 ± 0.063	26.298	1.098 ± 0.087	46.109	0.565 ± 0.029	31.942
	AP	48	0.689 ± 0.043	25.773	0.890 ± 0.098	23.081	0.784 ± 0.071	46.122	0.525 ± 0.023	30.519
		72	1.367 ± 0.070	51.701	0.938 ± 0.064	21.834	1.361 ± 0.093	49.091	0.680 ± 0.030	39.315
		24	0.597 ± 0.032	19.318	0.341 ± 0.052	26.752	0.465 ± 0.071	37.969	5.015 ± 0.012	69.368
	AM	48	0.274 ± 0.029	20.290	0.322 ± 0.049	24.592	2.600 ± 0.811	63.424	2.355 ± 0.007	40.838
Bco		72	0.513 ± 0.036	24.318	0.322 ± 0.053	27.178	0.652 ± 0.076	50.056	1.905 ± 0.004	40.297
		24	0.287 ± 0.063	23.926	0.433 ± 0.051	27.688	0.751 ± 0.074	61.737	1.601 ± 0.007	27.608
		48	0.362 ± 0.042	27.068	0.427 ± 0.045	26.204	2.837 ± 0.331	64.788	10.767 ± 0.054	92.086
	AP	72	0.809 ± 0.037	25.566	0.422 ± 0.049	27.521	3.346 ± 0.328	67.053	7.229 ± 0.024	97.040

CFS Cell Free supernatant, C Control, Bs *B. subtilis*, Bcl *B. clausii*, Bco *B. coagulans*, I inoculum, T time (h), TS trypticasein-soy broth, SP soy peptone-containing broth, CP casein peptone-containing broth, CPE crude extract of phycobiliprotein-containing broth, CBA Calcium-binding activity (mmol Ca^{2+} mg $prot^{-1}$) %DH Degree of hydrolysis (%)

Conclusions

This study has shown that some probiotic strains of *Bacillus subtilis*, *B. clausii* and *B. coagulans* can be considered as good producers of bioactive peptides when grown in culture media with different nitrogen sources. When the inocula were prepared in a medium optimized for the production of alkaline proteases (AP) the degree of hydrolysis and the mineral-binding activity of the generated peptides were higher. The best results for calcium-binding activity and degree of hydrolysis were obtained from of *B. subtilis* and *B. coagulans* growing in CPE-containing broth. A good positive correlation was found between these two responses. Similar results were observed for the iron-binding activity. However, this last activity was also high in the case of *B. clausii* grown in TS-, CP-, and SP-containing broths.

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