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IRON BINDING AND ANTITHROMBOTIC PEPTIDES RELEASED DURING THE FERMENTATION OF MILK BY *Lactobacillus casei* SHIROTA

LIBERACIÓN DE PÉPTIDOS ACARREADORES DE HIERRO Y ANTITROMBÓTICOS DURANTE LA FERMENTACIÓN DE LECHE CON *Lactobacillus casei* SHIROTA

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Abstract

Fermented milks have been associated with health benefits for a long time, which could arise from indirect effects such as the production of bioactive peptides released by lactic acid bacteria from milk proteins. In this work, the production of iron binding and antithrombotic peptides from milk fermented with *Lactobacillus casei* Shirota under different fermentation conditions of pH and temperature was investigated. *Lactobacillus casei* Shirota produced both iron binding and antithrombotic peptides in the different fermentations. The highest iron binding activity (4.62×10^{-4} mmol Fe^{2+}/mg protein) and the highest antithrombotic activity (clot inhibition of 79.1%), were observed at pH 6.0 at 39.5 °C at 12 h. The results here obtained are useful for the adequate selection of fermentation milk conditions to produce bioactive peptides by *L. casei* Shirota.

Keywords: bioactive peptides, iron binding activity, antithrombotic activity, *Lactobacillus casei* Shirota.

Resumen

Las leches fermentadas se han asociado con beneficios para la salud durante mucho tiempo, que podrían provenir de efectos indirectos, como la producción de péptidos bioactivos liberados por bacterias ácido - lácticas a partir de proteínas de la leche. En este trabajo, se investigó la producción de péptidos acarreadores de hierro y antitrombóticos de leche fermentada con *Lactobacillus casei* Shirota bajo diferentes condiciones de fermentación de pH y temperatura. *Lactobacillus casei* Shirota produjo péptidos antitrombóticos y acarreadores de hierro en las diferentes fermentaciones. La mayor actividad acarreadora de hierro (4.62×10^{-4} mmol de Fe^{2+}/mg de proteína) y la mayor actividad antitrombótica (inhibición de coágulo de 79.1%) se observaron a pH 6.0 a 39.5 °C a las 12 h. Los resultados aquí obtenidos son útiles para la selección adecuada de las condiciones de fermentación de leche para producir péptidos bioactivos por *L. casei* Shirota.

Palabras clave: péptidos bioactivos, actividad acarreadora de hierro, actividad antitrombótica, *Lactobacillus casei* Shirota.

1 Introduction

In the last three decades, the production of bioactive peptides released by lactic acid bacteria from milk proteins has been studied. Milk proteins have been widely studied as a source of bioactive peptides (Korhonen, 2009; Domínguez-González *et al*., 2014; Toldrá *et al*., 2018). In particular, these proteins contain within their structure a variety of peptides that can affect different systems: nervous, immune, nutritional and cardiovascular (Silva and Malcata, 2005). These peptides may be released via milk fermentation by lactic acid bacteria. *Lactobacillus casei* is used in fermented food and as a probiotic (Figueroa-González *et al*., 2011; Rojas-Ronquillo *et al*., 2012). Although it has been shown that this microorganism produces bioactive peptides during fermentation, there are few reports that demonstrated the generation of these compounds, especially peptides with antithrombotic and iron binding activity (Pihlanto *et al*., 2010; Domínguez-González *et al*., 2014). Some peptides

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derived from intestinal digestion of αs_1-casein (?s1-CN) and β-casein (β-CN), known as casein phosphopeptides (CPP) are small peptides that contains phosphorylated serine, therefore, they contain a large amount of negative charges that allow them bind strongly divalent cations (Fe, Mg, Mn, and Cu), forming soluble complexes (Erdmann *et al*., 2008; Huang *et al*., 2011). Some iron-binding peptides have been found in whey protein isolate, hydrolyzed with pancreatin, and in goat milk treated with trypsin (Caetano-Silva *et al*., 2018; Smialowska *et al*., 2018). Particularly, some peptides obtained by hydrolysis of milk whey protein, e.g. lactoferricin, are capable of binding di and trivalent cations, this attribute could be used to increase iron bioavailability in anemic patients (Kim *et al*., 2004).

Antithrombotic peptides are characterized by their ability to inhibit platelet aggregation and fibrinogen binding. The reported antithrombotic peptides derived from milk proteins are casoplatelin (MAIPPKKNQDDK) from κ-casein, peptide YQEPVLGPVRGPFPIIV from β-casein and KRDS peptide from lactoferrin (Rutherfurd and Gill, 2000; Erdmann *et al*., 2008; Rojas-Ronquillo *et al*., 2012).

The proteolytic activity of lactic acid bacteria has been studied and reviewed by several authors. The main components of this system are the cell envelope proteinase (CEP); these enzymes are responsible for extracellular hydrolysis of milk proteins during fermentation (Kojic *et al*., 1991; Lozo *et al*., 2011). The specificity of CEP varies according to the species, and with the conditions of fermentation, such as temperature, pH, and incubation time; which establishes the release of bioactive peptides and biological activity of fermented milk (Guo *et al*., 2009; Pan and Guo, 2010). Few studies have devoted to the characterization of the CEP of *Lactobacillus casei*; Brandsaeter and Nelson (1956) reported that the highest enzymatic activity for *Lactobacillus casei* strain 25 CEP was obtained at 42 °C and pH 6.0, while Xing *et al*. (2012) reported optimal activity at pH 7.0 and 37 °C for *Lactobacillus casei* DI-1. In addition to the release of bioactive peptides, it is important to highlight that *Lactobacillus casei* Shirota is a recognized probiotic (Dorantes-Alvarez *et al*., 2018), and the use of dairy products, enriched with probiotic bacteria, is an attractive option for consumers, with potential benefits to health (Borrás-Enríquez *et al*., 2018). The aim of this work was to study the conditions of milk fermentation by *Lactobacillus casei* Shirota necessary for obtaining antithrombotic and iron binding peptides.

2 Materials and methods

2.1 Microorganism

Lactobacillus casei Shirota was kept at 4 °C on skim milk medium (Difco, Detroit, USA). The inoculum was pre-cultured in MRS broth (Difco, Detroit, USA) for 24 h at 37 °C.

2.2 Fermentation

Fermentations were performed in a BioFlow II® fermenter (New Brunswick, USA) with 1 L of skim milk Svelty® (100 g/L). The medium was pasteurized at 115 °C for 5 min. Milk was inoculated with 10 mL of *L. casei* Shirota (optical density of 0.327 absorbance units at 650 nm). The fermentations were performed according to Table 1, and pH was maintained by addition of 2 N NaOH. Samples were taken at 12 and 20 h. Samples pH were adjusted to 9.5 (to solubilize casein) and centrifuged at 12000 x g for 30 min at 4 °C (Beckman Coulter Inc., USA); the supernatants were recovered to determine iron binding and antithrombotic activity.

2.3 Iron binding activity determination

The iron binding activity was determined as described by Farvin *et al*. (2010) with minor modifications. At 3.7 mL of sample (at concentration of 0.2 mg protein/mL) was added 0.1 mL of 2 mM $FeCl_2$ and incubated for 30 min at room temperature. Then 0.2 mL of 5 mM ferrozine (Sigma-Aldrich, USA) was added and incubated for 10 min at room temperature. Absorbance was read at 562 nm with a spectrophotometer (Shimadzu 160A, Japan). A blank without sample was prepared in a similar manner. The bound iron was determined according to Equation 1:

$$\text{Iron binding activity}\left(\frac{\text{mmol}Fe^{2+}}{\text{mg protein}}\right) = \frac{[Fe_i]-[Fe_f]}{[protein]} \quad (1)$$

where $[Fe_f]$ is unbound iron, $[Fe_i]$ is total iron, [protein] is protein concentration in sample. Protein concentration was determined by the technique of Lowry *et al*. (1951).

2.4 Antithrombotic activity determination

The antithrombotic activity was determined by the inhibition of fibrin cross-linking which is responsible for the clotting activity, using the method reported by Zhang *et al*. (2008) with minor modifications. A microplate reader was set at a wavelength of 405 nm, at 37 °C (Beckman ELx800, USA). Fibrinogen, thrombin and the sample were prepared in 0.05 M Tris-HCl buffer at pH 7.2 containing 0.12 mM NaCl. Afterward 140 μL fibrinogen solution (1 g/L) and 40 μL of sample were added into the plate wells, incubated at 37 °C for 10 min, and then the absorbance of the sample blank (SB) was measured. Next 10 μL thrombin solution (12 U/mL) was added to the wells to start the reaction of thrombin-catalyzed coagulation of fibrinogen. After incubation at 37 °C for 10 min, the absorbance of the sample (S) was measured. Additionally, in other wells 140 μL fibrinogen solution (1 g/L) and 40 μL of Tris-HCl buffer (instead of the sample)

were added into the plate wells, incubated at 37 °C for 10 min, and then the absorbance of the control blank (CB) was measured. Next 10 μL thrombin solution (12 U/mL) was added to the wells to start the reaction of thrombin-catalyzed coagulation of fibrinogen. After incubation at 37 °C for 10 min, the absorbance of the control (C) was measured. The antithrombotic activity was determined according to Equation 2:

$$\text{Antithrombotic activity (\% clot inhibition)} = \frac{(C-CB)-(S-SB)}{C-CB}\times 100 \quad (2)$$

Table 1. Experimental design for produce antithrombotic and iron binding peptides with *L. casei* Shirota in milk.

Fermentation[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
pH	6	6	6	6.2	6.2	6.2	6.5	6.5	6.5	6	6	6	6.2	6.2	6.2	6.5	6.5	6.5
T (°C)[b]	37	39.5	42	37	39.5	42	37	39.5	42	37	39.5	42	37	39.5	42	37	39.5	42
Time (h)	12	12	12	12	12	12	12	12	12	20	20	20	20	20	20	20	20	20

a Fermentation number
b Temperature

2.5 *Statistical analysis*

All fermentations were carried out in triplicate, and results are expressed as the mean with standard deviations. To assess significant differences in the iron binding and antithrombotic activity, analysis of variance (ANOVA) and Tukey's tests were performed using the SPSS 19.1 software (SPSS Inc., Chicago, IL) using $\alpha = 0.05$ as the threshold of statistical significance

3 Results and discussion

3.1 *Iron binding activity*

The iron binding activity is shown in Fig. 1, where it can be observed that fermentation 2 (pH 6.0, 39.5 °C; Table 1) presented the highest activity ($p \leq 0.05$), demonstrating that these conditions favored the production of iron binding peptides. This temperature may have favored the activity of *L. casei* Shirota CEP, in agreement with that reported by Brandsaeter and Nelson (1956) who studied *L. casei* strain 25. Furthermore, the second highest value of iron binding activity was found in fermentation 3 (pH 6.0, 42 °C; Table 1); both fermentations carried out at a time of 12 h. In contrast, the lowest value of activity was found in fermentation 11 (pH 6.0, 39.5 °C; Table 1), carried out at 20 h, which confirms the importance of fermentation time to produce this type of peptides.

There are few studies about iron binding peptides derived from milk proteins. Farvin *et al*. (2010) reported an iron binding activity of 7.7% in samples of yogurt. Figueroa-Hernández *et al*. (2012) have reported that hydrolysates of milk obtained by fermentation with the *Lactococcus lactis* subsp. *cremoris* NCFB 712, showed iron binding

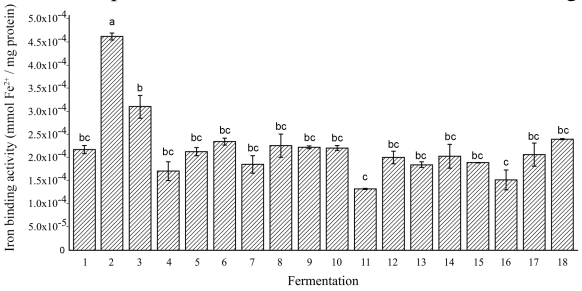

Fig. 1. Iron binding activity by generated peptides from different fermentations with *L. casei* Shirota. Data represent average values from three independent experiments. Significant differences ($p \leq 0.05$) among fermentations are represented with different letters.

values between 30 and 60%. In order to compare the results of the present work with those from previous reports, iron binding activity was calculated in percentage units. The values range from 48.8 to 88.7% of bound iron and are higher than those reported by the above-mentioned researchers. It should be noted that bacterial species studied in this work are different from *L. casei* Shirota, which confirms the importance of the species used for the production of iron binding peptides (Korhonen, 2009). In fact, the use of peptidases from different sources may influence the biological activity of the protein hydrolysates, since these enzymes show different peptide patterns (Toldrá *et al*., 2018). Recently, Caetano-Silva *et al*. (2018) found iron binding activity in a pancreatic hydrolysate from whey protein isolate, bioactive peptides had a molecular mass < 5 kDa. Smialowska *et al*. (2017) studied the iron binding ability of peptides derived from tryptic-digested goat milk proteins and found an iron binding activity of 54.37 ± 0.50 mg of Fe^{2+}/g of protein (9.7×10^{-4} mmol Fe^{2+} / mg protein).

3.2 *Antithrombotic activity*

The antithrombotic activity is shown in Fig. 2, where it can be observed that fermentation 2 (pH 6.0, 39.5 °C; Table 1) presented the highest activity, with a value of 79.1%. On the other hand, fermentations 1 and 6 did not show antithrombotic activity, probably the combined effect

of temperature, pH and time in these treatments did not stimulate the release of this type of peptides. Moreover in the present work it was found that at 37 °C, pH 6.5 and 20 h (fermentation 16) 58.2 % of inhibition was obtained, which is close to that reported by Rojas-Ronquillo *et al.* (2012) who studied the antithrombotic activity of a casein hydrolysate with *L. casei* Shirota at 37 °C, finding a 50% inhibition after 20 h. Domínguez-González *et al.* (2014) studied a commercial product fermented by *L. casei* and *Streptococcus thermophilus*, and found an antithrombotic activity of 57%. Differences may be because in the present work, milk was used as a fermentation medium. This means that a variety of proteins and other components in milk may favor the production of antithrombotic peptides.

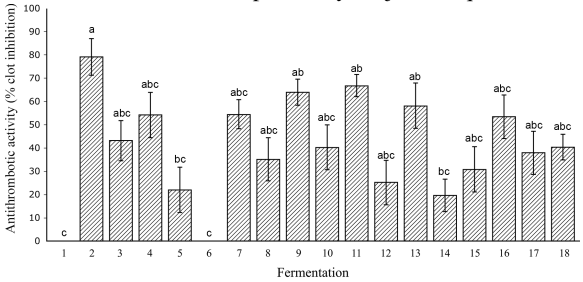

Fig. 2. Antithrombotic activity by generated peptides from different fermentations with *L. casei* Shirota. Data represent average values from three independent experiments. Significant differences ($p \leq 0.05$) among fermentations are represented with different letters.

Rutherfurd and Gill (2000) reported that κ-casein glycomacropeptide is capable of inhibiting thrombin by 50%. Similarly, other peptides such as the peptide KRDS from human lactoferrin are capable of inhibiting platelet clot formation by 55%. These two peptides are also reported by several authors (Fiat *et al.*, 1993; Erdmann *et al.*, 2008). There are few reports in literature of other peptides derived from milk with thrombin inhibiting properties; however, it has been found activity in peptides derived from κ-casein with sequences KNQDK and IAIPPKKIQDK (Chabance *et al.*, 1995). It is noteworthy that both in the iron binding activity and in the antithrombotic activity, highlights fermentation 2 (pH 6.0, 39.5 °C, 12 h) which shows the highest activity in both cases, this could indicate the presence of a peptide with both bioactivities. Some authors have reported peptides with multiple functions, Rojas-Ronquillo *et al.* (2012) reported a peptide (YQEPVLGPVRGPFPIIV) with antithrombotic and antihypertensive function from the β-CN; Domínguez-González *et al.* (2014) reported two peptidic fraction sharing ACE and antithrombotic peptides.

Conclusions

Lactobacillus casei Shirota was able to produce antithrombotic and iron binding peptides under different fermentation conditions studied. It was found that the combination of pH, temperature and fermentation time has an influence on the production of these kinds of peptides. Highest iron binding and antithrombotic activities were found at pH 6.0 at 39.5 °C at 12 h. It is important to note that the employment of *L. casei* Shirota for release of antithrombotic and iron-biding peptides has been few reported, so it opens an interest field of work. The release of bioactive peptides from milk proteins, represents an additional contribution to well-known benefits to health derived from the employment of a probiotic strain such as *L. casei* Shirota. In future, in vivo tests will be performed with the peptides generated.

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