Angiotensin-converting enzyme inhibitory activity of milk fermented by wild and industrial *Lactococcus lactis* strains

J. C. Rodríguez-Figueroa, R. Reyes-Díaz, A. F. González-Córdova, R. Troncoso-Rojas, I. Vargas-Arispuro, and B. Vallejo-Cordoba¹

Centro de Investigación en Alimentación y Desarrollo A.C. (CIAD, A.C.). Carretera a la Victoria Km. 0.6, Hermosillo, Sonora, 83000, Mexico

ABSTRACT

Angiotensin I-converting enzyme inhibitory (ACEI) activity was evaluated and compared in <3 KDa water-soluble extracts (WSE) isolated from milk fermented by wild and commercial starter culture *Lactococcus lactis* strains after 48 h of incubation. The highest ACEI activities were found in WSE from milk inoculated with wild *L. lactis* strains isolated from artisanal dairy products and commercial starter cultures. On the other hand, the lowest ACEI activities were found in WSE from milk inoculated with wild strains isolated from vegetables. Moreover, the IC₅₀ values (concentration that inhibits 50% activity) of WSE from artisanal dairy products were the lowest, indicating that these fractions were the most effective in inhibiting 50% of ACE activity. In fact, a strain isolated from artisanal cheese presented the lowest IC₅₀ (13 µg/mL). Thus, it appears that wild *L. lactis* strains isolated from artisanal dairy products and commercial starter cultures showed good potential for the production of fermented dairy products with ACEI properties.

Key words: angiotensin I-converting enzyme inhibitory activity, *Lactococcus lactis*, fermented milk

INTRODUCTION

Hypertension is estimated to affect one-third of the Western population and is a risk factor for cardiovascular disease and stroke (López-Fandiño et al., 2006). The long-term regulation of blood pressure is associated with the renin-angiotensin system. The conversion of angiotensin I into angiotensin II, a potent vasoconstrictor octapeptide, by angiotensin-converting enzyme (ACE; EC 3.4.15.1) has long been known (Skeggs et al., 1956). Hence, the inhibition of ACE can reduce high arterial blood pressure through ACE-inhibitory drugs.

It is accepted that food proteins may act as precursors of biologically active peptides with different physiologi-

cal effects. Among these biological activities of peptides, inhibition of ACE is one of the most comprehensively studied (Hernández-Ledesma et al., 2005). It has been reported that an effective way to increase the amount of bioactive peptides in fermented dairy products is to ferment milk with highly proteolytic strains of lactic acid bacteria (LAB; López-Fandiño et al., 2006). Growth of LAB in milk is dependent on its proteolytic system to partially degrade casein and generate free amino acids and free peptides. These peptides are further hydrolyzed to amino acids by the combined action of an assortment of peptidases (Hugenholtz, 2008). However, for the generation of bioactive peptides, the strains should present a balance between proteolytic activity and the right specificity of the proteinases and peptidases for the generation of ACE-inhibitory (ACEI) peptides (López-Fandiño et al., 2006).

Angiotensin-converting enzyme-inhibitory peptides obtained from the hydrolysis of milk proteins by LAB showed antihypertensive activity (Gobbetti et al., 2000; Quirós et al., 2007; Nielsen et al., 2009), although most of the studies focused on lactobacilli strains. In fact, antihypertensive milk fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Nakamura et al., 1995) has been commercialized in Japan (Calpis, Calpis Co. Ltd., Tokyo, Japan). In addition, LAB such as *Enterococcaceae* strains have been evaluated. Indeed, milk inoculated with *Enterococcus faecalis* strains showed ACEI activity (Quirós et al., 2007). Similarly, in another study, different LAB isolated from raw milk were screened and selected based on high ACEI activity. *Enterococcus faecalis* strains stood out as producers of fermented milk with potent ACEI activity, followed by lactobacilli strains (Muguerza et al., 2006).

Lactococcus lactis is the LAB most used in the manufacture of fermented dairy products because of its fast lactose fermentation and flavor production (Kuipers, 2001). Wild lactococci strains have been associated with the generation of unusual flavors, including higher amounts of certain volatile compounds, compared with those produced by commercial starter cultures (Ayad et al., 1999). Thus, increased interest exists in exploring new strains of *L. lactis* for the improvement of the sen-

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¹Corresponding author: vallejo@ciad.mx

Table 1. Codification and sources of isolation of *Lactococcus lactis* strains (Gutiérrez-Méndez et al., 2008)

Strain code	Source of isolation
B7	Beetroot (<i>Beta vulgaris</i>)
J6	Green beans (<i>Phaseolus vulgaris</i>)
M1	Corn (ears) (<i>Zea mays</i>)
M5	Corn (leaves) (<i>Zea mays</i>)
M7	Corn (leaves) (<i>Zea mays</i>)
C1	Dairy starter cultures of undefined mesophilic lactococci ¹
Z1	Dairy starter cultures of undefined mesophilic lactococci ¹
Z2	Dairy starter cultures of undefined mesophilic lactococci ¹
E1	Dairy starter cultures of undefined mesophilic lactococci ¹
E3	Dairy starter cultures of undefined mesophilic lactococci ¹
P4	Dairy starter cultures of undefined mesophilic lactococci ¹
K1	Dairy starter cultures of undefined mesophilic lactococci ¹
K3	Dairy starter cultures of undefined mesophilic lactococci ¹
K5	Dairy starter cultures of undefined mesophilic lactococci ¹
R1	Whey ²
R7	Whey ²
Q1	Chihuahua cheese ²
Q2	Chihuahua cheese ²
Q3	Curd ²
Q5	Chihuahua cheese ²

¹Commercial lyophilized starter cultures.²Raw milk dairy products.

sory characteristics of fermented dairy products. In a previous study, strains of *L. lactis* isolated from different ecosystems (vegetables, commercial starter cultures, or artisanal dairy products) presented marked differences in aroma production capacities during their growth in milk (Gutiérrez-Méndez et al., 2008). However, their ACEI activity was not explored. In fact, a comparison of ACEI activities of milk fermented exclusively with *L. lactis* isolated from different sources has not been reported in the literature. Thus, the objective of this study was to evaluate and compare the ACEI activities of water-soluble extracts from milk fermented by *L. lactis* strains isolated from different sources as a screening in the search of useful strains for the production of potentially antihypertensive dairy foods. Additionally, the relationship between proteolytic and ACEI activities was investigated.

MATERIALS AND METHODS

Substrates and Chemicals

Lactose and M17 broth were obtained from Difco (Sparks, MD). Nonfat dry milk (USDA organic grade A) was from Organic Valley (La Farge, WI). *o*-Phthalaldehyde (**OPA**) was from Fluka (Linz, Austria), and TCA, sodium borate, SDS, 2-mercaptoethanol, ACE (EC 3.4.15.1, from rabbit lung powder; 5U), and hippuryl-L-histidyl-L-leucine (Hip-His-Leu) were obtained from Sigma Chemical Co. (St. Louis, MO).

Culture Propagation

All *L. lactis* strains were obtained from the dairy laboratory of CIAD (Centro de Investigación en Alimentación y Desarrollo, A.C., Hermosillo, México) culture collection (Gutiérrez-Méndez et al., 2008). These strains were isolated from vegetables (**VG**), artisanal dairy products (**ADP**), and commercial starter cultures (**CSC**; Table 1). *Lactococcus lactis* strains were inoculated individually in 10 mL of sterile M17 broth and incubated for 24 h at 30°C. This procedure was repeated twice to obtain fresh cultures. Finally, every *L. lactis* strain was grown until reaching 10⁶ to 10⁷ cfu/mL as enumerated on lactose M17 agar.

Production of Fermented Milk

Nonfat dry milk was reconstituted (10%, wt/wt) and sterilized at 100°C for 20 min. Precultures of every *L. lactis* strain were obtained by inoculating sterilized milk with a stock culture having an initial bacterial population of 10⁶ to 10⁷ cfu/mL. The inoculated milk was incubated at 30°C for 12 h. Precultures (3% vol/vol) were added to sterilized milk to obtain the different fermented milk batches. Incubation was performed at 30°C and culture was stopped at 5, 24, or 48 h by pasteurization at 75°C for 1 min. pH measurements were directly taken from the fermented milk using a pH meter (Orion 4 Star, Singapore). Measurements were taken in duplicate.

Proteolytic Activity

For proteolysis determination, the OPA method, which measures free NH_3 groups, was used (Church et al., 1983). Distilled water (0.5 mL) and 5 mL of 0.75 *N* TCA were added to 2.5 mL of fermented milk and vortexed for 1 min. The suspension was filtered after 10 min using Whatman #2 paper. All filtrates were frozen at -80°C until analysis. The OPA reagent was prepared daily. A 20- μL sample aliquot containing TCA-soluble peptides were added to 400 μL of the OPA reagent. After 2 min at 20°C , absorbance was measured using a spectrophotometer (Cary 50 bio, Varian, Palo Alto, CA) at 340 nm. Unfermented milk was used as a control, and measurements were taken in duplicate.

ACE-Inhibitory Activity Assay

Samples of *L. lactis* fermented milk obtained after 48 h of incubation at 30°C and pasteurized were used for ACEI activity assays. Aliquots were stirred vigorously and centrifuged at $35,000 \times g$ (J2-21 rotor, Beckmann, Fullerton, CA) for 40 min. The corresponding supernatants were ultrafiltered through 3-kDa cut-off membranes (Pall Life Sciences, Port Washington, NY) at $9,800 \times g$ for 6 min (J2-21 rotor, Beckmann). The pH of the WSE were adjusted to 8.3 using 10 *N* NaOH and frozen at -80°C until analysis.

For ACEI activity of whey fractions, the method of Cushman and Cheung (1971) was applied with some modifications. The pH of the WSE was adjusted to 8.3 using 10 *N* NaOH. The buffered substrate solution was 5 mM hippuryl-L-histidine-L-leucine (substrate) in 100 mM sodium borate buffer solution containing 300 mM NaCl adjusted to pH 8.3 at 37°C . Angiotensin-converting enzyme at 0.1 U/mL was used and 4 microtubes were prepared: A = 100 μL of buffered substrate solution + 40 μL of distilled water + 20 μL of ACE; B = 100 μL of buffered substrate solution + 20 μL of distilled water + 40 μL of WSE; C = 100 μL of buffered substrate solution + 40 μL of whey fraction + 20 μL of ACE; and D = 100 μL of buffered substrate solution + 60 μL of distilled water.

All samples were incubated at 37°C for 35 min. The reaction was stopped by adding 250 μL of 1 *M* HCl. Ethyl acetate (1 mL) was added to every sample for the extraction of released hippuric acid. Samples were stirred vigorously for 20 s and centrifuged at $1,500 \times g$ for 10 min. An aliquot of 750 μL of the organic phase was evaporated at 75°C for 30 min. The residue was dissolved in 1 mL of distilled water and stirred vigorously. The absorbance was measured in 400 μL samples at 228 nm.

Angiotensin converting enzyme-inhibition was calculated as follows:

$$\text{ACEI activity (\%)} = [1 - (C - B/A - D)] \times 100.$$

Angiotensin converting enzyme inhibitory activity can also be expressed as the IC_{50} , which is the peptide content ($\mu\text{g/mL}$) necessary to inhibit ACE activity by 50%. Peptide content ($\mu\text{g/mL}$) in every WSE was determined by Bradford's method (Bradford, 1976). Bovine serum albumin was used as standard protein. The IC_{50} was calculated using graphical extrapolation by plotting ACE inhibition as a function of peptide content (Donkor et al., 2007). Every sample was adjusted at least to 3 levels of peptide concentration by standard dilution volume. Measurements were taken in duplicate.

Statistical Analysis

The experiment was based on the fermentation of sterile milk by each *L. lactis* strain. Every experiment was repeated 3 times and data were analyzed by GLM ANOVA. Differences between means were assessed by Tukey-Kramer's test and were considered significant when $P < 0.05$. The NCSS 2007 statistical program (NCSS Inc., Kaysville, UT) was used to process the results.

RESULTS AND DISCUSSION

pH Changes

Acidifying activity of *L. lactis* strains isolated from different sources such as VG, CSC, and ADP was evaluated and compared by monitoring the pH of inoculated milk during the experiment. Milk fermented by wild strains isolated from VG presented significantly ($P < 0.05$) less acidifying activity than milk fermented with strains from CSC and milk fermented by wild ADP; milks inoculated with CSC and ADP were not significantly ($P > 0.05$) different (Figure 1). Moreover, the acidifying activity of *L. lactis* strains within the same group was strain dependent (Figure 1). For example, one strain (Q3) from ADP reached its lowest pH (4.8) after only 5 h of incubation, whereas others (Q5 and R1) took 48 h to reach their lowest pH (6.3 and 5.4, respectively).

Milk is an excellent medium of nutrients including lactose (Walstra et al., 1999), and lactose utilization by LAB determines lactic acid production. Thus, *L. lactis* strains isolated from CSC or wild ADP may easily utilize lactose as a carbon source for their growth.

Figure 1. pH from each sample of milk fermented by *Lactococcus lactis* strains isolated from different sources at 5, 24, and 48 h of incubation. Strain C = control (unfermented milk); VG = vegetables; CSC = commercial starter culture; ADP = artisanal dairy products. Mean $\pm$ SD ($n = 3$).

In a previous study, the use of lactose by these *L. lactis* strains was markedly influenced by the source of isolation. Indeed, wild strains isolated from VG presented the slowest growth rates when lactose was used as a carbohydrate source in M17, whereas strains isolated from CSC or wild ADP presented the fastest growth rates (Gutiérrez-Méndez et al., 2010).

In this study, milk fermented by wild *L. lactis* strains Q3, Q2, and R7 isolated from ADP showed high acidifying activity in 24 h (Figure 1), although it has been reported that the overall acidifying activity of wild lactococci strains is rather low (Ayad et al., 2004).

Lactococcus lactis strains are widespread in nature and it is believed that the plant environment is the natural niche of lactococci. In fact, Bardowski et al. (1994) found that lactococci strains were able to assimilate β -glucosides such as cellobiose, salicin, arbutin, and esculin, which are plant carbohydrates. Technological implications of changing the native environments of *L. lactis* to an enriched nutrient medium such as milk are not clear (Ayad et al., 2004); however, the data obtained in this study showed that wild ADP strains adapted as well to milk as did CSC strains.

Proteolytic Activity

Figure 2 shows proteolytic changes assessed at 5, 24, and 48 h of incubation of all *L. lactis* strains within each group or isolation source, once the control was subtracted. Wild strains isolated from VG were significantly ($P < 0.05$) less proteolytic than CSC or wild ADP strains at all incubation times. Although proteolysis at 24 h was not significantly different ($P > 0.05$) for milk fermented with CSC or wild ADP strains (Figure 2), the latter showed significantly ($P < 0.05$) higher proteolytic activity at 48 h. In general, proteolysis increased until the bacteria reached the stationary phase at 24 h of incubation, with an average number of viable cells in the fermented milk of 10^8 to 10^9 cfu/mL. After this time, wild ADP and strains isolated from VG maintained constant growth, whereas growth for CSC strains started to decline (Gutiérrez-Méndez et al., 2008).

Similarly, Ayad et al. (1999) reported that nondairy wild strains isolated from grass, silage, and soil were not proteolytic. Boekhorst et al. (2004) suggested that comparative genomics revealed some differences

Figure 2. Proteolytic activity (according to the *o*-phthalaldehyde method) in fermented milk by *Lactococcus lactis* strains isolated from vegetables (VG), commercial starter culture (CSC), and artisanal dairy products (ADP). The control was subtracted from each sample. Mean $\pm$ SD ($n = 3$).

among the proteolytic systems of LAB strains, which are thought to reflect the influence of environmental niches.

Because the proteolytic activity of *L. lactis* strains may be a key point for the generation of ACEI activity in fermented milk (Fuglsang et al., 2003), the most proteolytic wild ADP strains (Q1, Q2, Q3, and Q5) in this study offer good potential for use in the production of fermented milk with potent ACEI activity.

ACEI Activity

All WSE obtained from milk inoculated with *L. lactis* strains isolated from different groups or isolation source showed ACEI activity (%) at 48 h of incubation (Figure 3). In general, ACEI was significantly ($P < 0.05$) higher for WSE-ADP and WSE-CSC strains than for WSE-VG (Figure 3).

Water-soluble extracts from fermented milk inoculated with wild *L. lactis* ADP strains (Q1, Q2, and Q5) presented the highest ACEI activities (90–98%); in fact, these were strains with high proteolytic activity. In general, a significant ($P < 0.05$) correlation was found between proteolytic activities for wild ADP strains and ACEI activities of WSE-ADP ($r = 0.55$). On the contrary, a significant ($P < 0.05$) negative correlation was found among proteolytic activities for wild strains isolated from VG and ACEI activities of WSE-VG ($r = -0.54$). Although results presented significant correlations, the values obtained suggested a weak relationship. Thus, from these results, it appears that the relationship between ACEI and proteolytic activity may depend on the isolation source. Similarly, a weak correlation ($r = 0.499$) was reported between ACEI activities and proteolysis by different lactic acid bacteria (Fuglsang et al., 2003).

Figure 3. Angiotensin-converting enzyme (ACE) inhibitory activity (%; ♦) of water-soluble extracts obtained from milk fermented by wild (artisanal dairy products, ADP, and vegetables, VG) and commercial starter culture (CSC) *Lactococcus lactis* strains. The concentration that inhibits 50% activity (IC₅₀, µg/mL) is indicated by bars. Mean ± SD (n = 3).

It has been reported that the composition of the proteolytic system of wild *L. lactis* influences ACE inhibition; in fact, negative mutants for peptidase activities presented higher ACEI activities than did their wild counterparts (Algaron et al., 2004). Thus, the results in this study suggest that the proteinases and peptidases involved in the proteolytic system of wild ADP strains differ substantially from those present in wild strains isolated from VG.

In general, WSE isolated from milk fermented by the *L. lactis* strains studied presented high ACEI activity, because ACEI was >50% for all *L. lactis* strains except for 3 (B7, M7, and E3). On the other hand, all WSE-ADP presented ACEI activities from 74 to 98%. Thus, it appears that ACEI activity may be strain dependent and may be related to the isolation source. In one study, the highest ACEI activities reported for WSE from milk fermented with *L. lactis* strains were 25 and 33% (Nielsen et al., 2009), whereas in another study, an ACEI activity as high as 92.8% was reported (Muguerza et al., 2006).

The WSE-ADP presented the lowest ($P < 0.05$) IC₅₀ of all wild strains, which means that these fractions were the most effective in inhibiting 50% of ACE activ-

ity (Figure 3). Similarly, WSE-CSC presented low IC₅₀ except those showed by strains C1 and K5.

The WSE-ADP presented IC₅₀ from 13 to 50 µg/mL, with Q1 and Q2 strains presenting the lowest IC₅₀ values (20 and 13 µg/mL, respectively). One study reported an IC₅₀ of 520 µg/mL for whey fractions produced by *L. lactis* ssp. *cremoris* isolated from raw cow milk after 48 h incubation in reconstituted skim milk powder. However, in that work the extract evaluated was obtained by centrifugation of fermented milk without further fractionation (Muguerza et al., 2006).

Additionally, the <3 kDa whey fraction obtained from milk fermented with strains Q1 or Q2 presented greater ACEI activities than those in milk fermented with *Enterococcus faecalis* (IC₅₀ of 28 ± 2 µg/mL; Quirós et al., 2007), including the water-soluble peptide extract of milk fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium lactis* (IC₅₀ of 27.79 µg/mL; Donkor et al., 2007). However, comparisons of these studies with the present work assumed equivalent peptide mass calculated in relation to the BSA standard by Bradford's method as it was carried out in this study. Therefore, wild ADP

strains Q1 and Q2 presented good potential for use in the manufacture of antihypertensive dairy products.

It is important to note that WSE obtained from milk fermented with most CSC *L. lactis* strains also presented low IC₅₀ (20–50 µg/mL). Thus, the ACEI activities of these CSC strains could be exploited commercially in addition to their other technological properties.

CONCLUSIONS

Our results suggest that when screening for useful strains in the production of potentially antihypertensive dairy foods, it is important to consider the ecological niche of wild *L. lactis*. Artisanal dairy products were the preferred isolation source because the WSE from ADP presented superior ACEI activity and low IC₅₀ values. In addition, *L. lactis* strains isolated from CSC presented good possibilities for use in the manufacture of dairy products with ACEI properties.

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