ABSTRACT

The ability of specific wild *Lactococcus lactis* strains to hydrolyze milk proteins to release angiotensin I-converting enzyme (ACE) inhibitory peptides was evaluated. The peptide profiles were obtained from the <3 kDa water-soluble extract and subsequently fractionated by reversed-phase HPLC. The fractions with the lowest half-maximal inhibitory concentration estimated values (peptide concentration necessary to inhibit ACE activity by 50%) were *Lc. lactis* NRRL B-50571 fraction (F)1 (0.034 ± 0.002 μg/mL; mean ± SD) and *Lc. lactis* NRRL B-50572B F1 (0.041 ± 0.003 μg/mL; mean ± SD). All peptide fractions were analyzed by reversed-phase HPLC tandem mass spectrometry. Twenty-one novel peptide sequences associated with ACE inhibitory (ACEI) activity were identified. Several novel ACEI peptides presented peptides encrypted with proven hypotensive activity. In conclusion, specific wild *Lc. lactis* strains were able to hydrolyze milk proteins to generate potent ACEI peptides. However, further studies are necessary to find out the relationship between *Lc. lactis* strain proteolytic systems and their ability to biogenerate hypotensive peptides.

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

INTRODUCTION

Dairy proteins may act as precursors of biologically active peptides with different physiological effects. Among these biological activities, the inhibition of the angiotensin-converting enzyme (ACE) is one important target of antihypertensive drugs to prevent hypertension (Hernández-Ledesma et al., 2005). One way to liberate bioactive peptides from proteins in fermented dairy foods is by the use of highly proteolytic strains of lactic acid bacteria (López-Fandiño et al., 2006). During growth, lactic acid bacteria not only generate peptides by the action of cell wall proteinases but also produce free AA by the action of intracellular proteinases (Hugenholtz, 2008).

Lactic acid bacteria strains have been selected for their ability to ferment milk and hydrolyze milk proteins into peptides with ACE inhibitory (ACEI) activity (Nielsen et al., 2009). Lactic acid bacteria genera used to generate ACEI peptides mainly include lactobacilli and lactococci (Gobbetti et al., 2000; FitzGerald and Murray, 2006; Nielsen et al., 2009; Otte et al., 2011). For example, the commercial drink Calpis, made by milk fermentation with *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, was shown to contain peptides with ACEI activity (Nakamura et al., 1995).

Torres-Llanez et al. (2011) recently reported that a wild *Lactococcus lactis* strain presented ACE activity in Mexican Fresco cheese. Also, specific wild *Lc. lactis* strains were explored for their ability to produce ACEI activity in fermented milk (Rodríguez-Figueroa et al., 2010; Otte et al., 2011). Therefore, native *Lc. lactis* strains could not only be excellent aroma producers (Gutiérrez-Méndez et al., 2008) but also be able to generate ACEI peptides in fermented dairy products. Thus, the objective of this study was to identify and compare the ACEI peptides released from milk proteins through lactic acid fermentation by specific wild *Lc. lactis* strains.

MATERIALS AND METHODS

**Materials**

Sodium borate; SDS; 2-mercaptoethanol; ACE (EC 3.4.15.1; 5 U), which was from rabbit lung powder; hippuryl-l-histidyl-l-leucine (Hip-His-Leu); and trifluoroacetic acid (TFA) were obtained from Sigma.
Chemical Co. (St. Louis, MO). Acetonitrile was from J. T. Baker Chemical Co. (Phillipsburg, NJ). Lactose, M17 broth, and agar were obtained from Difco (Sparks, MD). United States Department of Agriculture organic grade A nonfat dry milk was from Organic Valley Family of Farms (La Farge, WI).

**Strains and Growth Conditions**

Two wild *Lc. lactis* strains (NRRL B-50571 and NRRL B-50572) obtained from the dairy laboratory collection at Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD, Hermosillo, Sonora, Mexico) were deposited in the Agricultural Research Service Culture Collection (NRRL, Peoria, IL) of the US Department of Agriculture. The strains were propagated in 10 mL of sterile lactose (5 g/L) M17 broth and incubated at 30°C for 24 h. Fresh cultures were obtained by repeating the same procedure. Initial starter cultures were prepared by allowing *Lc. lactis* strains to reach 10^6 to 10^7 cfu/mL, as enumerated on M17 agar containing lactose (5 g/L).

**Manufacture of Fermented Milk**

Reconstituted nonfat dry milk (10%, wt/vol) was sterilized at 100°C for 20 min. A loop of *Lc. lactis* single preculture (7–8 log cfu/mL) was inoculated into sterilized milk. The inoculated milk was incubated for 12 h at 30°C. Then, cultures were added (3% vol/vol) to reconstituted nonfat dry sterilized milk to get the different fermented milk batches. Incubation was carried out at 30°C and stopped at 48 h by pasteurization at 75°C for 1 min.

**Preparation of the Water-Soluble Extracts from Fermented Milk**

Fermented milk was centrifuged at 20,000 × g (J2–21 rotor; Beckman Coulter Inc., Brea, CA) for 10 min at 0°C. Then, supernatants were collected and ultrafiltered through 3-kDa-cutoff membranes (Pall Life Sciences, Port Washington, NY) at 9,800 × g for 6 min (J2–21 rotor; Beckman Coulter Inc.). Permeates were collected, filtered through a 0.45-μm disposable hydrophilic filter, and frozen at −80°C until analyses were done.

**Isolation of ACEI Peptide Fractions by Reversed-Phase HPLC**

Peptide profiles from water-soluble extracts (WSE) were obtained by reversed-phase HPLC (1100 series; Agilent Technologies Japan Ltd., Tokyo, Japan). Separation was carried out with a Discovery-C18 (250 × 4.6 mm, 5-μm particle size, 180-Å pore size) column from Supelco Inc. (Bellefonte, PA) with a solvent flow rate of 0.25 mL/min. Once the column was equilibrated with solvent A (0.04% TFA in water), 20 μL of the WSE were injected. Peptides were eluted with an increasing gradient of solvent B (0.03% TFA in acetonitrile) from 0 to 45% in solvent A for 60 min. Peptide profiles monitored at 214 and 280 nm were collected from 5 chromatographic runs and freeze dried to be subjected to ACEI activity analysis and half-maximal inhibitory concentration (IC_{50}) determination. Peptide fractions (214 nm) with the lowest IC_{50} were eluted once more to achieve better separation. This second elution was carried out by using a linear gradient of solvent B (0–15%) in A for 85 min, with a flow rate of 0.5 mL/min. Peptide fractions from this second elution were collected from 5 chromatographic runs, freeze dried, and stored for further analysis.

**Analysis of Peptides by Tandem Mass Spectrometry**

Mass spectrometry analysis was performed using a 1100 Series LC/MSD Trap (Agilent Technologies Inc., Waldbronn, Germany) equipped with an electrospray ionization source (LC-ESI-MS). The nanocolumn was a C18-300 (150 mm × 0.75 μm, 3.5 μm; Agilent Technologies Inc.) The sample injection volume was 1 μL. Solvent A was a mixture of water-acetonitrile-formic acid (10:90:0.1, vol/vol/vol) and solvent B contained water-acetonitrile-formic acid (97:3:0.1, vol/vol/vol). The gradient was based on the increment of solvent B, which was initially set at 3% for 10 min and it took 23 more min to reach 65%. The 0.7 μL/min flow rate was directed into the mass spectrometer via an electrospray interface. Nitrogen (99.99%) was used as the nebulizing and drying gas and operated with an estimated helium pressure of 5 × 10^5 Pa. The needle voltage was set at 4 kV. Mass spectra were acquired over a range of 300 to 2,500 mass/charge (m/z). The signal threshold to perform auto MS analyses was 30,000. The precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp from 0.35 to 1.1 V. Peptide sequences were obtained from mass spectrometry data using the Mascot server (Perkins et al., 1999) through the UniProtKB/Swiss-Prot database (http://www.matrixscience.com/help/seq_db_setup_Sprot.html) sequences.

**Assay of ACEI Activity**

Peptide fractions were dissolved in 1 mL of water and the pH was adjusted to 8.3 using 10 N NaOH. For ACEI
activity analysis of the peptide fractions, the method of Cushman and Cheung (1971) was applied with some modifications as reported by Rodríguez-Figueroa et al. (2010) using 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, and ACE solution prepared from rabbit lung (Sigma Chemical Co.).

The IC50, which is the peptide content (μg/mL) required to inhibit the original ACE activity by 50%, was also measured. Peptide content (μg/mL) was determined using the method of Bradford (1976) using a Bio-Rad protein assay (Bio-Rad Laboratories Inc., Hercules, CA) and bovine serum albumin as a standard. Due to some limitation of the Bradford (1976) method used for determining the peptide content, IC50 are considered IC50 estimate values. The IC50 was calculated by plotting ACE inhibition as a function of peptide content as reported by Donkor et al. (2007). Thus, each sample was adjusted to at least 3 levels of known peptide concentration (μg/mL). Measurements were taken in duplicate.

**Statistical Analysis**

Experiments were carried out in triplicate and the normality of data was evaluated as a prerequisite before one-way ANOVA analysis was performed. Differences between means were assessed using the Tukey-Kramer multiple-comparison test and were considered significant when \( P < 0.01 \). Statistical analysis was performed using NCSS 2007 software (NCSS LLC, Kaysville, UT).

**RESULTS AND DISCUSSION**

**Peptide Fraction Profiles from Milk Fermented by Specific Wild Lc. lactis Strains**

Figure 1A shows WSE peptide fraction profiles produced by specific wild *Lc. lactis* strains monitored at 214 nm absorbance. Unfermented milk was used as a control. The area under the curve of each peptide profile was evaluated as an indirect measure of proteolysis. Results showed significant differences \( (P < 0.01) \) between fermented milk peptide profiles and the control. However, the peptide profiles obtained from milk fermented with different strains of *Lc. lactis* were similar. The first peak eluted after 12 min in all samples. The largest concentration of peptides eluted between 12 and 25 min when the concentration of acetonitrile was between 9 and 13.5%, which may be related to the relatively hydrophobic nature of the eluted peptide. It has been suggested that a close relationship exists between hydrophobicity and positively charged AA in the C-terminal position and ACEI peptides derived from milk proteins (Pripp et al., 2004). Thus, it is very likely...
that peptides eluting in the first 25 min present ACEI activity.

On the other hand, when WSE were monitored at 280 nm, only 3 peaks eluted between 16 and 20 min (Figure 1B). These peptides may have ACEI activity, as they were of aromatic nature. Wu et al. (2006) reported the relevant presence of aromatic AA in the ACEI peptides structure.

A comparison of *Lc.* lactis strains ability for hydrolyzing milk proteins was shown by recording area counts from the peptide chromatographic profiles (Figure 2). Even though no significant difference (*P* > 0.01) was observed, milk fermented by *Lc.* lactis NRRL B-50571 presented lower proteolysis than NRRL B-50572. In general, *Lc.* lactis strains have a complex proteolytic system that is able to hydrolyze milk proteins to AA or peptides essential for strain growth (Savijoki et al., 2006). Thus, milk proteolysis should be a prerequisite to find out peptides with bioactivity. On the other hand, Pripp et al. (2004) established a relationship between milk-derived peptides and their ACEI activity through a quantitative structure-activity relationship model. The objective of that study was to relate peptide structure (AA sequence) and ACE inhibition. Therefore, in the current study, it was necessary to identify the AA sequence of peptides associated with ACE inhibition.

**Peptide Fractions with Angiotensin I-Converting Enzyme Inhibitory Activity**

Peptide chromatographic profiles were divided into 6 fractions and collected for further evaluation. Peptide profiles obtained at 214 nm were divided into F1 to F5 fractions (Figure 1A); meanwhile, peptide profiles obtained at 280 nm corresponded to F6 (Figure 1B). Peptide fractions F1 to F6 showed remarkable IC$_{50}$ estimate values (means ± SD), ranging from 0.034 ± 0.002 to 0.61 ± 0.19 μg/mL (Figure 3). Results did not show significant differences (*P* > 0.01) among all peptide fraction IC$_{50}$. However, the peptide fraction IC$_{50}$ estimate values (means ± SD) obtained from milk fermented by *Lc.* lactis strains NRRL B-50571 (0.076 ± 0.004 and 0.034 ± 0.002 μg/mL for F1 and F6, respectively) and milk fermented by *Lc.* lactis NRRL B-50572 (0.041 ± 0.003 and 0.084 ± 0.003 μg/mL for F1 and F2, respectively) showed the lowest values (Figure 3). Quirós et al. (2007) reported a <3 kDa subfractionated WSE with an IC$_{50}$ of 0.8 μg/mL released by the hydrolytic action of *Enterococcus faecalis* on skim milk during a 48-h fermentation. Moreover, *Lactobacillus helveticus* and *Saccharomyces cerevisiae* were able to hydrolyze skim milk obtaining 2 different peptides with the minimum IC$_{50}$ (2.8 and 1.6 μg/mL; Nakamura et al., 1995). Therefore, the results suggest that the specific wild *Lc.* lactis strains of this study have remarkable ACEI activity. Both strains did not present a
significant difference ($P > 0.01$) in IC$_{50}$ estimate values and proteolysis, which are related to ACEI activity.

**Identification of Peptides with ACEI Activity Derived from Lc. lactis Fermented Milk**

Peptides associated with every fraction ($<$3 kDa) were identified by tandem mass spectrometry. Twenty-one new peptides associated with ACEI activity were identified and their molecular weight was calculated (Table 1). The only peptide already reported was LHL-PLPL, which was found in milk fermented by *Enterococcus faecalis* (Quirós et al., 2007). The presence of the peptide sequence TVQVTSTAV in milk fermented by specific wild *Lc. lactis* strains suggested that *Lc. lactis* NRRL B-50571 and NRRL B-50572 strains may present similar proteolytic systems. On the other hand, only 1 peptide ($<$3 kDa) sequence presented Pro in the C-terminal position; therefore, these results may suggest the absence of proline-specific peptidases such as Pep I, Pep R, and Pep X.

The peptide sequence with the lowest molecular weight (505 Da) was AESIS, derived from αS1-CN (f62–65). On the other hand, the longest sequence obtained presented 21 amino acids with a molecular weight of 2,048 Da, derived from serotransferrin (f506–526) according to the UniProtKB/Swiss-Prot database. It has been suggested that ACEI peptides usually include 2 to 12 AA; however, it has been reported that peptides of up to 27 AA may present ACEI. Another key point is the ability of C-terminal hydrophobic AA, such as Pro, to bind ACE (López-Fandiño et al., 2006). In fact, peptide fraction 1 (F1) obtained from milk fermented with *Lc. lactis* NRRL B-50571 presented the peptide sequence HPHPHLSFMAIPP with Pro in the C-terminal position, which has a hydrophobic nature with increased ACEI activity. Pripp et al. (2004), also specified a relationship between hydrophobicity and ACEI activity.

**Table 1.** Identification of peptides obtained from milk fermented by specific wild *Lactococcus lactis* strains associated with angiotensin-converting enzyme inhibitory (ACEI) activity

<table>
<thead>
<tr>
<th>Sample$^1$</th>
<th>Molecular ion (m/z) (charge)</th>
<th>Protein fragment</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>362.9 (+2)</td>
<td>α-LA (f63–68)</td>
<td>DDQNPH</td>
</tr>
<tr>
<td></td>
<td>517.4 (+2)</td>
<td>α-LA (f82–89)</td>
<td>LDDDLTDDI</td>
</tr>
<tr>
<td></td>
<td>350.3 (+2)</td>
<td>κ-CN (f35–40)</td>
<td>YPSYGL</td>
</tr>
<tr>
<td></td>
<td>740.5 (+2)</td>
<td>κ-CN (f98–110)</td>
<td>HPPHILSMAIPP</td>
</tr>
<tr>
<td></td>
<td>518.8 (+2)</td>
<td>α-La (f55–62)</td>
<td>YDTQAIQV</td>
</tr>
<tr>
<td></td>
<td>462.3 (+3)</td>
<td>κ-CN (f100–111)</td>
<td>DDDLTDDIMCV</td>
</tr>
<tr>
<td></td>
<td>586.7 (+1)</td>
<td>αs-CN (f63–39)</td>
<td>YPSYG</td>
</tr>
<tr>
<td>F2</td>
<td>506.9 (+1)</td>
<td>αs-CN (f62–66)</td>
<td>AESIS</td>
</tr>
<tr>
<td>F3</td>
<td>416.1 (+2)</td>
<td>β-CN (f22–28)</td>
<td>SITRINK</td>
</tr>
<tr>
<td></td>
<td>526.7 (+2)</td>
<td>αs-CN (f80–88)</td>
<td>HIQKEDVPS</td>
</tr>
<tr>
<td></td>
<td>453.0 (+2)</td>
<td>κ-CN (f116–169)</td>
<td>TVQVTSTAV</td>
</tr>
<tr>
<td>F4</td>
<td>453.2 (+2)</td>
<td>κ-CN (f116–169)</td>
<td>TVQVTSTAV</td>
</tr>
<tr>
<td>F5</td>
<td>859.4 (+2)</td>
<td>β-CN (f194–209)</td>
<td>QEPVLPGRPGFFPIIV</td>
</tr>
<tr>
<td></td>
<td>576.2 (+2)</td>
<td>β-CN (f199–209)</td>
<td>GPVGRRPGFPPIIV</td>
</tr>
<tr>
<td></td>
<td>489.6 (+2)</td>
<td>β-CN (f69–77)</td>
<td>SLPNQIPPL</td>
</tr>
<tr>
<td>F6</td>
<td>548.2 (+2)</td>
<td>κ-CN (f125–33)</td>
<td>YIPQQVLS</td>
</tr>
<tr>
<td></td>
<td>453.5 (+2)</td>
<td>κ-CN (f161–169)</td>
<td>TVQVTSTAV</td>
</tr>
<tr>
<td></td>
<td>198.3 (+3)</td>
<td>κ-CN (f157–169)</td>
<td>PEINTVQVTSTAV</td>
</tr>
<tr>
<td></td>
<td>592.3</td>
<td>Serotransferrin (f448–453)</td>
<td>GYALAV</td>
</tr>
</tbody>
</table>

$^1$Fractions collected from milk fermented by *Lc. lactis* NRRL B-50571 and NRRL B-50572. NRRL = US Department of Agriculture Agricultural Research Service Culture Collection (Peoria, IL).

$^2$MS/MS = tandem mass spectrometry.
Figure 4. Typical mass spectrum corresponding to a peptide sequence (DDQNPtH) collected from the water-soluble extract peptide fraction 1 (WSE F1) obtained from milk fermented by Lactococcus lactis NRRL B-50571. (A) Double-charged ion 362.9 m/z; (B) tandem mass spectrometry (MS/MS) spectrum for the specified ion in (A). After interpretation and comparison in the database, the fragment AA sequence matched α-LA (563-68). NRRL = US Department of Agriculture Agricultural Research Service Culture Collection (Peoria, IL).
positively charged AA in the C-terminal position and ACEI activity. Indeed, the peptide sequence DDQNPH, which also was present in F1, presented the positively charged residue histidine in the C-terminal position (Figure 4). Both of these peptide sequences presented in F1 produced by Lc. lactis NRRL B-50571 fermented milk had the lowest IC_{50} estimate values.

It has been reported that α-, β-, and κ-CN are precursors of bioactive peptides (Mills et al., 2011). However, in the current work, casein proteins as well as whey proteins, such as β-LG and α-LA, were found to be important sources of peptides with ACEI activity.

Milk fermented by Lc. lactis NRRL B-50571 showed HPHPHLSFMAIPP derived from κ-CN (98–110) and SLPQNIPL derived from β-CN (69–77), which have encrypted the hypotensive tripeptide (IPP) reported by Nakamura et al. (1995). Additionally, milk fermented by Lc. lactis NRRL B-50572 showed QEPVLGPVRGFPIIV, derived from β-CN (f194–209). Thus, this AA sequence included the peptide VLGPVRGPFP, which was reported by Quiros et al. (2007). Finally, the peptide fragment YPSYGL, obtained from κ-CN (335–40) found in milk fermented by Lc. lactis NRRL B-50571 and NRRL B-50572 strains, showed the dipeptide YP reported before (Yamamoto et al., 1999).}

CONCLUSIONS

The peptide profiles of the distinct fractions obtained from the hydrolysis of milk proteins by specific wild Lc. lactis strains were similar. Nevertheless, differences were observed in the degree of proteolysis, which may be related to the action of specific peptidases and proteinases of each Lc. lactis strain. Moreover, this research suggests that proteolysis may be a prerequisite to ACEI activity; however, it seems that differences in Lc. lactis strain proteolytic systems determine the peptide sequences associated with ACE inhibition. Therefore, studies are underway to determine enzymatic activity differences among specific wild Lc. lactis strains. Specific wild Lc. lactis strains were able to release 21 new encrypted milk peptides with potent ACEI activity through a fermentation process not only from caseins but also from whey proteins.

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REFERENCES


