Angiotensin-I-converting enzyme-inhibitory peptides in commercial Wisconsin Cheddar cheeses of different ages

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ABSTRACT

Bioactive peptides, including angiotensin-I-converting enzyme-inhibitory (ACEI) peptides, were investigated in commercially produced Wisconsin Cheddar cheeses that ranged in age from ≤ 6 d to more than 2 yr. The ACEI activity of cheese was determined in water-soluble extracts (WSE) that were fractionated for components with molecular weight (MW) $\leq 3,000$ Da, and peptides identified using HPLC and tandem mass spectrometry. The number of types of bioactive peptides increased with an increase in ripening time. Six of the identified ACEI peptides, Ile-Pro-Pro (IPP), Val-Pro-Pro (VPP), Glu-Lys-Asp-Glu-Arg-Phe (EKDERF), Val-Arg-Tyr-Leu (VRYL), Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn (YPFPGPIP), and Phe-Phe-Val-Ala-Pro (FFVAP), with known high ACEI activity (low IC_{50} values, the concentration needed to inhibit ACE to 50% of its original activity) were synthesized and used to quantify the amounts of these peptides in various cheese extracts. The concentrations of these 6 ACEI peptides increased up to a certain stage of ripening. The maximum contents of IPP, VPP, and EKDERF were 2.8, 7.4, and 5.3 mg/100 g of cheese, respectively, and these levels were found in a 1-yr-old Cheddar cheese sample. The maximum content of VRYL (7.5 mg/100 g of cheese) was found in a 2-yr-old Cheddar cheese sample, whereas the maximum content of YPFPGPIP (6.8 mg/100 g of cheese) was found in a 6-mo-old Cheddar cheese sample. Trace amounts of FFVAP were found in these cheeses. Aged Cheddar cheese was found to be a rich source of ACEI peptides even though large differences exist between cheeses from different manufacturers.

Key words: angiotensin-I-converting enzyme-inhibitory peptide, bioactive peptide, cheese ripening, proteolysis

INTRODUCTION

Proteolysis is the major biochemical event during the ripening of Cheddar cheese, starting with preliminary proteolysis, which includes the initial breakdown of the caseins through the action of coagulants, such as rennet, as well as indigenous proteinases, such as plasmin and cathepsin D in milk (Fox, 1989). Secondary proteolysis is performed through the action of proteases and peptidases, such as cell wall-associated proteinases, from starter and nonstarter bacteria. Some of these peptides show biological activity, including hormone- or drug-like properties that eventually modulate physiological functions, and they are called bioactive peptides (Fitzgerald and Murray, 2006). Various types of bioactive peptides have been found in fermented dairy products, with potential properties such as antihypertensive, angiotensin-I-converting enzyme (ACE)-inhibitory, immunomodulatory, antimicrobial, mineral transport, and opiate activities (Fitzgerald and Meisel, 2003; Silva and Malcata, 2005; Korhonen, 2009).

Angiotensin-I-converting enzyme is a peptidyl dipeptide hydrolase that plays an important role in the regulation of hypertension. It increases blood pressure by converting angiotensin I to angiotensin II, a vasoconstrictor, and degrading bradykinin, a vasodilator. By inhibiting these processes, ACE inhibitors have antihypertensive effects (Seppo et al., 2003). Angiotensin-I-converting enzyme-inhibitory (ACEI) peptides have been detected in fermented milk products, including sour milk and cheese (Nakamura et al., 1995a; Hernandez-Ledesma et al., 2005; Bütikofer et al., 2008; Sieber et al., 2010). Sour milk fermented with various strains of *Lactobacillus helveticus* contained the tripeptides IPP and VPP, 2 well-known ACEI peptides, and they exhibited an antihypertensive effect by lowering the systolic blood pressure of mildly hypertensive human subjects (Seppo et al., 2002, 2003; Mizushima et al., 2004; Aihara et al., 2005). The analysis of water-soluble extracts (WSE) of different cheese varieties, such as blue, Camembert, Edam, Emmental, Gouda, Manchego, and Havarti, indicated a possible blood pressure-lowering effect in spontaneously hypertensive

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rats (**SHR**; Saito et al., 2000; Sipola et al., 2001, 2002; Gómez-Ruiz et al., 2002, 2004). Fractions of WSE from Cheddar cheese with high ACEI activity have been collected and a wide range of ACEI peptides, such as α_{S1} -CN (f1–6), α_{S1} -CN (f1–7), α_{S1} -CN (f1–9), α_{S1} -CN (f24–32), α_{S1} -CN (f102–110), β -CN (f47–52), β -CN (f193–209), and κ -CN (f96–102) have been identified (Stepaniak et al., 2001; Ong et al., 2007; Ong and Shah, 2008a). Ong et al. (2007) and Ong and Shah (2008a) reported relative concentrations of ACEI peptides in the WSE of Cheddar cheese but no true quantitative study has been done on the specific ACEI peptides found in Cheddar cheese.

The objective of this study was to determine the types and amounts of ACEI peptides produced during a 2-yr ripening period for commercial Cheddar cheeses. The influence of cheese ripening on the ACEI activity of the WSE of these commercial cheeses was evaluated. This was viewed as a baseline study to understand what types of peptides and amount of ACEI activity is present in existing commercial cheeses, before making any process changes to enhance ACEI activity. Although some types of ACEI peptides have been identified in Cheddar cheese, prior studies specifically enriched these peptides by methods such as use of enzyme-modified cheese or addition of selected probiotic cultures (Cliffe and Law, 1990; Ong et al., 2007; Ong and Shah, 2008a; Pritchard et al., 2010).

MATERIALS AND METHODS

Commercial Cheddar Cheese Produced in Wisconsin

Twelve Cheddar cheeses with different ripening times were kindly provided by 2 Wisconsin Cheddar cheese manufacturers (brand A and brand B). Both manufacturers were large cheese producers located in Wisconsin and they made cheese that is routinely aged for up to 2 yr.

Preparation of WSE

The WSE of Cheddar cheeses were prepared according to the method of Bütikofer et al. (2008) with some modifications. Fifteen grams of each ground cheese sample was homogenized with 30 mL of deionized (**DI**) water at 17,500 rpm for 15 s using an Ultra-Turrax T 25 basic homogenizer (IKA Works Inc., Wilmington, NC). The homogenized sample was incubated at 40°C for 60 min with stirring, before centrifugation at $8,000 \times g$ for 45 min at 4°C. The water-soluble fraction between the upper layer of fat and the lower layer of sediment was collected. The WSE was filtered through Whatman filter paper (Whatman #541) before fractionation by

UF using membranes with nominal molecular weight limits (**NMWL**) of 3,000 and 1,000 Da. The UF was performed using a stirred cell unit (Millipore Corporation, Billerica, MA) under a pressure of ~276 kPa supplied by a compressed nitrogen gas cylinder.

Fractions with molecular weight (**MW**) <3,000 Da were permeates of UF using the 3,000 Da NMWL membrane. These fractions were used for the ACE inhibitory test. Fractions with MW <1,000 Da were permeates of UF using 1,000 Da NMWL membrane. These fractions were used for HPLC-tandem mass spectrometry (MS/MS) analysis. Fractions with MW higher than 1,000 but less than 3,000 Da ($1,000\text{Da} < \text{MW} < 3,000\text{Da}$) were prepared by first collecting permeate from the 3,000 Da NMWL membrane, and then ultrafiltering this permeate using the 1,000 Da NMWL membranes and collecting the retentate. These fractions were also used for HPLC-MS/MS analysis. Fractions with MW <3,000 Da was freeze-dried and stored at –20°C until further analysis (ACE-inhibitory activity test). Fractions with MW <1,000 Da was stored at –20°C until further analysis. Fractions with $1,000\text{Da} < \text{MW} < 3,000\text{Da}$ were washed 2 times using 500 μL of DI water each time and the solutions were collected and stored at –20°C until further analysis (HPLC-MS/MS).

Peptide Identification by HPLC-MS/MS

The HPLC-MS/MS technique has previously been used for identification of cheese peptides (Gagnaire et al., 2001; Hernandez-Ledesma et al., 2004; Dupas et al., 2009). The UF fractions with MW <1,000 Da and $1,000\text{Da} < \text{MW} < 3,000\text{Da}$ from the various aged cheeses were subjected to HPLC-MS/MS to separate and identify the peptides presented in these fractions. Peptide sequences were identified based on the fragments detected from MS/MS. The UF fractions were analyzed for peptides by nano-liquid chromatography-MS/MS using the Agilent 1100 LC/MSD Trap SL spectrometer (Agilent, Palo Alto, CA) with nano electrospray ionization. A 20- μL aliquot of solution of each fraction was loaded onto a trap column (Zorbax 300SB-C₁₈, 5 μm , $5 \times 0.3\text{ mm}$, Agilent) and separation column (Zorbax 300SB-C₁₈, 3.5 μm , $0.075 \times 150\text{ mm}$, Agilent). A 3-step gradient elution was performed at 0.22 $\mu\text{L}/\text{min}$ for the first step: 5% solvent B (95% acetonitrile, 0.1% formic acid) in solvent A (0.1% formic acid in water) to 45% solvent B over 115 min; second step: 45% solvent B to 65% solvent B over 20 min; and third step: 65% solvent B to 100% solvent B over 5 min. The MS/MS spectra were collected from 300 to 2,200 m/z . Peptides were identified using the no-enzyme search of Bovine Taurus NCBI nr amino acid sequence database (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastp&PAGE_

TYPE=BlastSearch&LINK_LOC=blasthome) and Mascot search engine (Matrix Science, London, UK).

Peptide Identification Using HPLC-Electrospray Ionization-Time-of-Flight MS

Because of the detection limit of 500 Da for the MS/MS method, the UF fractions with MW <1,000 Da from different aged cheeses were also subjected to HPLC-electrospray ionization (ESI)-time-of-flight (TOF)-MS to help identify these peptides (Martin et al., 2008). Peptides in the fractions of MW <1,000 Da were identified based on theoretical molar mass of the selected peptides, the detected mass, and elemental composition analysis.

The fractions of MW <1,000 Da were subjected to HPLC-ESI-TOF MS using a Vydac-C₈ column (3 μ m, 2.1 $\times$ 250 mm) on an Agilent 1200 HPLC connected to an Agilent LC/MSD TOF mass spectrometer (Agilent) with electrospray ionization in positive ion mode. Forty microliters of each fraction was injected. A linear gradient elution was performed at a flow rate of 0.25 mL/min with 98% DI water, 2% acetonitrile, and 0.1% formic acid to 90% acetonitrile, 10% DI water, and 0.1% formic acid over 25 min. The lists of peptide masses were generated in Agilent MassHunter Qualitative Analysis using the Find Compound by Molecular Feature. Features were restricted to m/z 200 to 3,000 with no charge state limitation over the retention time of 3.1 to 15.5 min when peaks were eluted by the gradient. Peptides were identified by mass and elemental composition analysis.

Quantitative Analysis of ACE-Inhibitory Peptides

High-performance liquid chromatography MS has been used for quantitative analysis of peptides (Bütikofer et al., 2008). Six ACEI peptides (Table 1) with high ACEI activity (low IC₅₀ value; the concentration needed to inhibit ACE to 50% of its original activity) were synthesized using an automated peptide synthesizer, Symphony Quartet (Protein Technologies

Inc., Tucson, AZ), at the Biotechnology Center of the University of Wisconsin-Madison and were used as standards for the confirmation and quantitative analysis using LC-quadrupole-ion trap MS/MS. These ACEI peptides were Ile-Pro-Pro (IPP), Val-Pro-Pro (VPP), Glu-Lys-Asp-Glu-Arg-Phe (EKDERF), Val-Arg-Tyr-Leu (VRYL), Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn (YPFPGPIPN), and Phe-Phe-Val-Ala-Pro (FFVAP). The synthesized peptides were diluted 10-fold with DI water and the standards were mixed and diluted to concentrations of 10.0, 2.0, 1.0, 0.5, 0.2, and 0.1 μ g/mL with DI water. The MW <1,000 Da fractions and standards were analyzed on a Phenomenex Luna C₁₈ (150 $\times$ 2 mm, 3 μ m) column with an Agilent 1100 LC (Agilent) connected to an Applied Biosystems 3200 Q TRAP LC/MS/MS System (Applied Biosystems, Foster City, CA) with Analyst 1.4.2 software. The column temperature was maintained at 45°C. The mobile phase consisted of 0.1% formic acid in DI water (A) and 0.1% formic acid in acetonitrile (B) with a flow rate of 0.2 mL/min. Gradient elution was performed with 2% solvent B for the first 2 min and then increased linearly to 70% solvent B over 25 min. The column was flushed with 95% solvent B and re-equilibrated for a minimum of 15 min. The samples and standards were maintained at 5°C, and 5 μ L was injected onto the column. The Qtrap parameters were as follows: curtain gas, 207 kPa; CAD medium; ion spray voltage, 5,500 V; temperature, 200°C; ion source gas-1, 138 kPa; ion source gas-2, 138 kPa; declustering potential, 10 V; entrance potential, 10 V; collision energy, 20 V and collision energy spread, 3 V. A 6-point calibration was created with the peptide standards via the following MRM channels [precursor ion (amu)/product ion (amu)]: IPP (326.20/213.20), VPP (312.20/213.20), VRYL (275.70/451.20), EKDERF (412.20/566.20), FFVAP (580.30/295.10), and YPFPGPIPN (501.30/230.20).

ACEI Activity Test

The ACEI activity of WSE was measured according to the method of Cushman and Cheung (1971) with

Table 1. Angiotensin-I-converting enzyme (ACE)-inhibitory peptides selected for synthesis with their reported IC₅₀ values¹

Peptide	Possible source	IC ₅₀ (μ M)	Reference
IPP	β -CN (f74–76)/ κ -CN (f108–110)	5.0	Nakamura et al. (1995b)
FFVAP	α _{SI} -CN (f23–27)	6.0	Maruyama et al. (1987a)
VPP	β -CN (f84–86)	9.0	Nakamura et al. (1995b)
EKDERF	κ -CN (f12–17)	14.3	Schlothauer et al. (1999)
YPFPGPIPN	β -CN (f60–68)	14.8	Saito et al. (2000)
VRYL	α _{S2} -CN (f204–207)	24.1	Gómez-Ruiz et al. (2004)

¹IC₅₀ = concentration needed to inhibit ACE to 50% of its original activity.

modifications as described by Nakamura et al. (1995b), Donkor et al. (2005), and Ong et al. (2007). This method is based on the liberation of hippuric acid from hippuryl-histidyl-leucine (**HHL**) catalyzed by ACE. Fifteen milligrams of freeze-dried UF permeate with MW <3,000 Da was dissolved in 1.0 mL of DI water. Then, HHL (Sigma, St. Louis, MO) was dissolved in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl. Two hundred microliters of 5 mM HHL solution was mixed with 35 μ L of the sample solution and incubated at 37°C for 5 min. The reaction was initiated by addition of 20 μ L of ACE solution (dissolved in DI water, 0.1 U/mL). After 30 min of incubation at 37°C, the reaction was stopped by addition of 250 μ L of 1 M HCl. The hippuric acid liberated by ACE was extracted with 1.7 mL of ethyl acetate using vortex mixing for 15 s. After centrifugation at $729 \times g$ for 10 min at room temperature, 1.4 mL of the organic phase was transferred into a fresh test tube and evaporated to dryness in a water bath for 15 min at 100°C. The hippuric acid was redissolved in 1.0 mL of DI water and the absorbance at 228 nm was measured using a Shimadzu UV-Visible spectrophotometer (Shimadzu UV-1601 PC, Shimadzu Corp., Kyoto, Japan). The percentage of ACE inhibition was calculated according to the equation of Donkor et al. (2005). The ACE activity was measured as the amount catalyzing the formation of 1 μ mol of hippuric acid from HHL in 1 min at 37°C (Cushman and Cheung, 1971). The IC₅₀ values were calculated as a concentration of the cheese WSE required to inhibit 50% of ACE activity (Ong et al., 2007).

Statistical Analysis

Quantitative analysis and ACE-inhibitory experiments were done in triplicate. Statistical analysis was performed using the Statgraphics program, version 5.1 plus (Manugistics, Rockville, MD). One-way ANOVA was performed to determine significant differences between peptide concentrations and IC₅₀ values ($P < 0.05$).

RESULTS AND DISCUSSION

ACEI Peptides in Cheddar Cheese at Different Ripening Times

In this study, we identified many ACEI peptides in commercial Cheddar cheeses manufactured in Wisconsin. The number of ACEI peptides detected changed with ripening time and in cheeses made by different manufacturers. Peptides that have previously been reported to have ACEI activity were identified in the UF

fractions of cheeses. A summary of these ACEI peptides is shown in Table 2. Thirty-nine different types of ACEI peptides were found in these cheese samples, including 10 ACEI peptides derived from α_{S1} -CN, 2 from α_{S2} -CN, 15 from β -CN, 4 from κ -CN, 3 from α -LA, 4 from β -LG, and 1 from BSA. The specific types of these ACEI peptides observed in a cheese sample varied depending on the manufacturer and the age of the sample (Figure 1). Differences in the types of ACEI peptides found in cheeses from the 2 different manufacturers was probably related to specific manufacturing practices, such as starter culture strains used (Fox et al., 1994), and this variability is in agreement with the trend from prior studies on other cheese types (Bütikofer et al., 2008). Their nonstarter lactic acid bacteria (**NSLAB**) flora is also likely to be different. It has been previously reported that the dominant strains of NSLAB in Cheddar cheese can vary between factories (Crow et al., 2002).

Similar numbers of ACEI peptides were found in brand B cheeses over a ripening period of 2 yr, whereas for brand A, the trend was for an increase in the number of different ACEI peptides detected with increasing cheese age. Some of the ACEI peptides such as IPP, VPP, EKDERF, YPFPGPIHN, VRYL, YQEPVLG-PVR, LAHKAL, and IR/RL were present in all the cheeses, including those at 3 to 6 d of age up to 2 yr of age, whereas the other observed ACEI peptides were detected only in cheeses of a certain age.

ACEI Activity of Cheddar Cheese of Different Ages

The overall ACEI activity of WSE fractions of Cheddar cheeses with MW <3,000 Da is shown in Table 3. The IC₅₀ values of WSE from the Cheddar cheeses decreased (i.e., exhibited greater ACEI activity) with an increase in ripening time (Table 2), which in agreement with the results of Ong et al. (2007) and Ong and Shah (2008a,b). Compared with young (3–6 d) cheeses, a significant decrease ($P < 0.05$) in ACE activity was observed in both of the 2-mo-old cheeses (Table 3). We detected no significant differences in ACE activity in cheeses during the ripening time of 60 d to 2 yr, except for the 1- and 2-yr-old cheeses from brand A (Table 3). The 1-yr-old cheese from brand A showed the lowest ACE activity but by 2 yr of age, its ACE activity had increased, possibly due to further breakdown of key ACEI peptides.

Quantitative Analysis of Selected ACEI Peptides

Six ACEI peptides (IPP, VPP, VRYL, FFVAP, EKDERF, and YPFPGPIPN) were chemically synthesized and used as standards for quantitative analysis of

Table 2. Angiotensin-I-converting enzyme (ACE)-inhibitory peptides found in commercial Cheddar cheeses¹

ACE-inhibitory peptide	Age of cheese (d)							IC ₅₀ (μM) ²	Reference for IC ₅₀ values
	3–6	60	180	270	365	>730			
RELEEL	+	+	–	+	+	+	>1,000 ₃	Maeno et al. (1996)	
NVPGEIVE	–	–	–	+	–	+	193.9	Gobbetti et al. (2000)	
DKIHPF	+	–	–	–	+	–	18	Smacchi and Gobbetti (1998)	
LVYPFGPIHNSLPQ	–	–	+	–	+	+	14.8	Saito et al. (2000)	
YFPFGPIHN	–	–	+	+	+	+	749	Abubakar et al. (1998)	
TPVVVPFLQP	–	–	+	+	+	+	9	Nakamura et al. (1995b)	
VPP	+	+	+	+	+	+	5	Nakamura et al. (1995b)	
IPP	–	+	+	+	+	+	430	Kohmura et al. (1989)	
PLP	–	–	–	–	+	–	80	Maryama et al. (1987b)	
AVPYP	–	+	–	–	–	+	209	Yamamoto et al. (1994)	
RDMPIQAF	+	–	–	–	–	–	21	Yamamoto et al. (1994)	
LYEQPVLGPPVRGPPPIIV	–	–	–	–	–	–	300	Meisel and Schlimme (1994)	
YQEPVLGPVR	–	+	+	+	+	+	101	Yamamoto et al. (1994)	
YQEPVLGPVRGPPPIIV	–	–	+	+	+	+	600	Stepaniak et al. (2001)	
QEPVLGPVRGPPPIIV	–	–	+	+	+	+	6	Maryama et al. (1987a)	
FFVAP	–	–	+	+	–	–	–	–	
FVAPFPEVF	–	–	–	–	–	+	>1,000	Gómez-Ruiz et al. (2004)	
PFPE	+	+	+	+	–	–	140	Maryama et al. (1987a)	
FPEVFGK	+	–	–	–	–	–	706.1	Gómez-Ruiz et al. (2004)	
VPSERY	–	–	–	–	–	–	232.8	Gómez-Ruiz et al. (2004)	
VPSERYL	+	–	+	+	–	–	122.1	Mullally et al. (1996)	
YL	+	+	–	–	+	–	716.9	Gómez-Ruiz et al. (2004)	
KKYKVPQ	–	–	–	–	–	–	77.1	Gómez-Ruiz et al. (2004)	
KKYKVPQL	+	+	–	–	–	–	50	Maryama et al. (1987b)	
LW	+	+	–	–	–	–	580	Maeno et al. (1996)	
AMKPW	–	–	–	–	–	–	24.1	Gómez-Ruiz et al. (2004)	
VRYL	+	+	+	+	+	+	14.3	Schlothauer et al. (1999)	
EKDERF	+	+	+	+	+	+	848	Gómez-Ruiz et al. (2007)	
KDERF	–	–	–	–	–	–	720	Yamamoto et al. (1999)	
YP	+	+	–	+	–	–	52.8	Gómez-Ruiz et al. (2007)	
VR	+	+	–	+	+	+	621	Pihlanto-Leppala et al. (1998)	
LAHKAL	+	+	+	+	+	+	409	Mullally et al. (1996)	
YGL	–	+	–	+	–	–	733	Mullally et al. (1996)	
LF	+	–	–	–	–	–	79.8	Hernandez-Ledesma et al. (2002)	
LLF	+	–	–	–	–	–	696	Mullally et al. (1996)	
IR	+	+	+	+	+	+	2,439	Mullally et al. (1996)	
RL	+	+	+	+	+	+	556	Pihlanto-Leppala (2001)	
LAMA	–	+	–	–	–	–	315	Abubakar et al. (1998)	
BSA (f221–222)	+	–	–	–	–	–	–	–	

¹Where + indicates that the peptide was observed in Cheddar cheese, and – indicates that the peptide was not observed in Cheddar cheese.²IC₅₀ = concentration needed to inhibit ACE to 50% of its original activity.³IC₅₀ value was only reported for a collection of peptides (fraction of water-soluble extracts).

Table 3. Experimental IC₅₀ values¹ of water-soluble extract ≤3,000 Da fractions from commercial Cheddar cheeses of different ages

Age (d)	IC ₅₀ (mg/mL)	
	Brand A	Brand B
3–6	11.4 ^{a,A}	11.9 ^{a,A}
60	8.4 ^{b,C}	8.6 ^{b,B}
180	8.8 ^{b,C}	8.5 ^{b,B}
270	9.0 ^{b,C}	8.1 ^{b,B}
365	7.6 ^{c,D}	8.3 ^{b,B}
>730	10.4 ^{d,B}	8.2 ^{b,B}

^{a–d}Means within a column not sharing a common lowercase superscript differ ($P < 0.05$; comparing the effect of ripening time for a single brand).

^{A–D}Means within a row not sharing a common uppercase superscript differ ($P < 0.05$; comparing the effect of brand for a single ripening time).

¹IC₅₀ = concentration needed to inhibit ACE to 50% of its original activity. Values represent the means of triplicates.

these peptides in cheese (Table 1). The concentrations of these selected ACEI peptides in Cheddar cheeses with different ripening times are shown in Figures 2 and 3. Trace amounts of FFVAP were found in Cheddar cheese (Figure 3). High amounts of the other 5 synthesized ACEI peptides, especially the tripeptides IPP and VPP, were found in all Cheddar cheeses.

These results confirmed the presence of considerable amounts of IPP and VPP in Cheddar cheese, with their

concentrations tending to increase with an increase in ripening time (Figure 2). Significantly higher amounts ($P < 0.05$) of IPP and VPP contents were found in brand A Cheddar cheeses compared with brand B cheeses. The maximum concentrations of these 2 tripeptides, IPP (2.8 mg/100 g of cheese) and VPP (7.4 mg/100 g of cheese), were found in the 1-yr-old brand A Cheddar cheese.

The peptides IPP and VPP were first reported by Nakamura et al. (1995b) as key ACEI peptides with high ACEI activities of IC₅₀ = 5.0 and 9.0 μ M, respectively. They were also found to have significant antihypertensive effects in SHR (Nakamura et al., 1995b). The peptides IPP and VPP both have the C-terminal Pro-Pro sequence, which is believed to be resistant to further digestion by peptidases (Kitts and Weiler, 2003). Therefore, IPP and VPP may be effectively absorbed in the small intestine following oral ingestion and thus are available to exert an antihypertensive effect. In vivo studies have shown that oral administration of dairy products containing high levels of IPP (0.3 mg/kg of BW) and VPP (0.6 mg/kg of BW) had antihypertensive effects in SHR (Nakamura et al., 1995a, 1996; Masuda et al., 1996; Sipola et al., 2001, 2002). Clinical studies have shown that administration of fermented milk products containing IPP at a dosage of 1.1 to 4.7 mg/d and VPP at a dosage of 1.15 to 8.3 mg/d can

Figure 1. Number of angiotensin-I-converting enzyme (ACE)-inhibitory peptides in Cheddar cheeses of different ripening times.

Figure 2. Contents of the tripeptides IPP (a, b) and VPP (c, d) in Wisconsin Cheddar cheeses of different ages ($n = 3$, error bars indicate standard deviation).

significantly decrease blood pressure in subjects with mild hypertension or high-normal blood pressure (Hata et al., 1996; Mizushima et al., 2004; Aihara et al., 2005; Foltz et al., 2007).

If IPP and VPP do indeed have *in vivo* antihypertensive effects, then based on the concentrations of these 2 tripeptides present in Cheddar cheeses, we would expect to observe an antihypertensive effect if 100 g of the 1-yr-old brand A Cheddar cheese were consumed by subjects exhibiting mild or high blood pressure.

There is limited quantitative information in other cheese varieties about the content of key ACEI peptides such as IPP and VPP. Bütikofer et al. (2008) studied various cheeses from Switzerland and found that the IPP contents were ~2 to 79 mg/kg (semihard type cheeses) and ~1 to 98 mg/kg (extra hard cheeses), whereas VPP contents were ~5 to 238 mg/kg (semihard type cheeses) and ~1 to 333 mg/kg (extra hard cheese). Our results showed that the maximum IPP and VPP contents in Cheddar cheese samples were 28 and 74 mg/kg, respectively, which were within the ranges observed by Bütikofer et al. (2008). Meyer et al. (2009) suggested that concentrations of VPP and IPP above 100 mg/kg are attainable in Swiss cheeses.

We observed high amounts of VRYL in both Cheddar cheeses and observed a trend for an increasing concentration of VRYL with age in brand B cheeses (Figure 3). A similar trend was found in brand A cheeses but

only during the aging period from 3 to 6 d to 6 mo. The maximum content of VRYL was 7.5 mg/100 g of cheese and it was obtained in the 2-yr-old brand B Cheddar cheese.

The ACEI peptide EKDERF was found in both brands of Cheddar cheeses and its concentration increased with ripening time from 3 to 6 d to 6 mo (Figure 3). For brand A cheese, EKDERF content increased with ripening time up to 6 mo but decreased with longer ripening times, such as 9 mo to 2 yr. For brand B cheeses, an increasing trend was observed in the concentration of EKDERF with aging time. The maximum content of EKDERF was 5.3 mg/100 g of cheese and it was found in the 1-yr-old brand B Cheddar. The peptide EKDERF, κ -CN (f12–17), was first reported as being an ACEI peptide in a milk protein hydrolysate by Gómez-Ruiz et al. (2007). That group estimated its IC_{50} value as 14.3 μM . High concentrations of EKDERF were detected in our Cheddar cheese samples (Figure 3), which might explain, in part, why the WSE of the 1-yr-old brand B cheese showed low ACEI activity ($IC_{50} = 8.3$ mg/mL; Table 3).

The presence of a very small amount of FFVAP was found in brand B Cheddar cheese but was not detected in brand A cheese samples (Figure 3). The peptide FFVAP, α_{S1} -CN (f23–27), was first reported as an ACEI peptide by Maruyama et al. (1987a) in an enzymatic hydrolysate of casein; those authors estimated

Figure 3. Contents of angiotensin-I-converting enzyme (ACE)-inhibitory peptides VRYL, EKDERF, FFVAP, and YPFPGPIP in Wisconsin Cheddar cheeses (brand A, ■, and brand B, □) of different ages ($n = 3$, error bars indicate standard deviation).

that it had high ACEI activity ($IC_{50} = 6.0 \mu M$). In our investigation, this pentapeptide was found in Cheddar cheese, even though its concentration was low.

In this study, high concentrations of YPFPGPIPN were found in both Cheddar cheeses (Figure 3). The maximum content of YPFPGPIPN was 6.8 mg/100 g of cheese and it was detected in the 6-mo-old brand A Cheddar cheese. The peptide YPFPGPIPN, β -CN (f60–68), was first reported by Saito et al. (2000) as an ACEI peptide obtained from an 8-mo-old Gouda cheese; they determined its IC_{50} value as $14.8 \mu M$ and noted that it also showed an antihypertensive effect on SHR (Saito et al., 2000). In this study, we observed high amounts of YPFPGPIPN in both brands of Cheddar cheese (Figure 3).

The highest ACEI activity was observed in 1-yr-old brand A Cheddar cheese (i.e., lowest IC_{50} value; Table 3), which also had the highest concentrations of IPP (2.8 mg/100 g of cheese) and VPP (7.4 mg/100 g of cheese), and some VRYL (0.6 mg/100 g of cheese) and YPFPGPIPN (2.1 mg/100 g of cheese). The 1-yr-old brand B Cheddar also showed high ACEI activity, which was likely caused by its high concentrations of EKDERF (5.3 mg/100 g of cheese), VRYL (5.0 mg/100 g of cheese), and YPFPGPIPN (1.4 mg/100 g of cheese).

The 2-yr-old brand A Cheddar had lower concentrations of IPP (0.2 mg/100 g of cheese), VPP (0.3 mg/100 g of cheese), and YPFPGPIPN (1.7 mg/100 g of cheese) compared with its 9-mo- and 1-yr-old cheeses. The concentrations of VRYL (1.7 mg/100 g of cheese) and EKDERF (0.3 mg/100 g of cheese) were also low. These results might explain why the 2-yr-old brand A cheese sample showed lower ACEI activity compared with the 2-mo-, 6-mo-, 9-mo-, and 1-yr-old brand A cheeses (Table 3). These results were consistent with the findings of Meisel et al. (1997), Ryhnen et al. (2001), Ong et al. (2007), and Ong and Shah (2008a,b), who found that ACEI activity was lower in cheese samples with a low degree of proteolysis (or during the early stage of ripening) and that activity generally increased as proteolysis proceeded up to a certain level but decreased again with more proteolysis, probably due to the further breakdown of ACEI peptides (Meyer et al., 2009).

Possible Mechanisms Responsible for Creating ACEI Peptides

Peptides with ACEI activity are generated by various enzymatic activities during cheese ripening. The possible enzymes responsible for generating the ACEI peptides identified in Cheddar cheeses are listed in Table 4. A large number of peptides, including ACEI peptides, are released as a result of proteolysis during cheese rip-

ening (Rank et al., 1985; Fox, 1989; Sieber et al., 2010). Several enzymes are likely needed to produce a specific ACEI peptide (for both the N- and C-terminal cleavage sites). Most of the ACEI peptides were low in molar mass (i.e., $\leq 3,000$ Da), which would likely have needed the action of bacterial cell wall-associated proteinase and several types of peptidases, which together may have contributed to the production of 18 different types of ACEI peptides during Cheddar cheese ripening. Some of the identified ACEI peptides might have been generated (partly) by the action of indigenous enzymes in milk, such as plasmin, cathepsins B, D, and G, and residual chymosin from cheese making (Table 4).

For the 6 key ACEI peptides that we investigated in detail in this study, the enzymes potentially involved in their formation are known for only some of them. It is still not clear which enzymes contribute to the production of IPP. It has been suggested that VPP might be created partly by the action of the indigenous proteinase in milk, cathepsin G (Upadhyay et al., 2004). The peptide FFVAP, α_{S1} -CN (f23–27), was probably created due to the action of plasmin cleaving at Arg₂₂-Phe₂₃ bond of α_{S1} -CN (Fox et al., 1994) and possibly other enzymes, such as proteinases and peptidases from the lactic acid bacteria in cheese. The peptide YPFPGPIHN could be formed by hydrolysis of the peptide bond Asn₆₈-Ser₆₉ by cell wall-associated proteinase, as it has been reported that *Lactococcus lactis* ssp. *cremoris* strains AC1, SK112, and HP can cleave at Asn₆₈-Ser₆₉ (Fox et al., 1994), or by the action of cathepsin B, which can cleave at Val₅₉-Tyr₆₀ (Upadhyay et al., 2004). The peptide YPFPGPIHN can be hydrolyzed by cell wall-associated peptidases and aminopeptidases into smaller peptides, which might explain the decrease in the concentration of YPFPGPIPN when cheese was aged more than 6 mo (Figure 3). It is not clear which enzymes contributed to the formation of EKDERF.

Some of the ACEI peptides can be further degraded to smaller, inactive fragments through ongoing proteolysis by proteinases and peptidases released by lactic acid bacteria during cheese ripening (Fox, 1989; Meisel et al., 1997). In some cases, an ACEI peptide can be further hydrolyzed to generate another type of bioactive peptide. For example, ACEI peptide YPFPGPIHN, obtained from β -CN (f 60–68), can be degraded by the cell-wall associated peptidase X-prolyl-dipeptidyl aminopeptidase (X-PDAP) from *Lactococcus lactis* ssp. *cremoris* into YP and FPGPIHN (Smid et al., 1991). The peptide FPGPIHN can be degraded by aminopeptidase P (PepP) to remove the N-terminal amino acid and produce Phe and PGPIPN [β -CN (f63–68); Upadhyay et al., 2004], which is an immunostimulating peptide (Migliore-Samour and Jolls, 1988).

Table 4. Enzymes potentially responsible for creating the angiotensin-I-converting enzyme (ACE)-inhibitory peptides during Cheddar cheese ripening

ACE-inhibitory peptide	Possible enzyme	Reference
ACEI peptides possibly created by cell wall-associated proteinases		
LW	α_{S1} -CN (f198–199)	Fox et al. (1994)
FFVAP	α_{S1} -CN (f23–27)	Fox et al. (1994)
FVAPFPEVF	α_{S1} -CN (f24–32)	Fox et al. (1994)
FPEVFGK	α_{S1} -CN (f28–34)	Fox et al. (1994)
YL	α_{S1} -CN (f94–95)/ β -LG (f102–103)	Fox et al. (1994)
VRYL	α_{S2} -CN (f204–207)	Fox et al. (1994)
RELEEL	β -CN (f1–6)	Fox et al. (1994)
AVPYP	β -CN (f177–181)	Upadhyay et al. (2004)
RDMPQAF		
LLYEQPVLGVPVRGBPFIIV	β -CN (f183–190)	Smid et al. (1991); Fox et al. (1994)
YQEPVLGPVRGBPFIIV	β -CN (f191–209)	Fox et al. (1994)
QEPVLGPVRGBPFIIV	β -CN (f193–209)	Reid et al. (1991)
DKIHPF	β -CN (f194–209)	Fox (1989); Fox et al. (1994)
LVYFPFGPIHNSLPQ	β -CN (f47–52)	Visser et al. (1991)
YFPFGPIHN	β -CN (f58–72)	Fox et al. (1994); Upadhyay et al. (2004)
NVPGEIVE	β -CN (f60–68)	Fox et al. (1994); Upadhyay et al. (2004)
KDERF	β -CN (f7–14)	Fox et al. (1994)
YP	κ -CN (f13–17)	Fox et al. (1994)
	κ -CN (f58–59)/ α_{S1} -CN (f146–147)	Smid et al. (1991); Upadhyay et al. (2004)
FP	BSA (f221–222)	Upadhyay et al. (2004)
ACEI peptides possibly created by indigenous proteinases or residual chymosin		
KKYKVPQ	α_{S1} -CN (f102–108)	Fox et al. (1994); Upadhyay et al. (2004)
KKYKVPQL	α_{S1} -CN (f102–109)	Fox et al. (1994); Upadhyay et al. (2004)
PFPE	α_{S1} -CN (f27–30)	Upadhyay et al. (2004)
YQEPVLGPVR	β -CN (f193–202)	Reid et al. (1991); Fox et al. (1994); Gouldsworthy et al. (1996); Upadhyay et al. (2004)
VPP	β -CN (f84–86)	Upadhyay et al. (2004)
LAHKAL	α -LA (f105–110)	Upadhyay et al. (2004)
YGL	α -LA (f50–52)	Upadhyay et al. (2004)

It is notable that some of the ACEI peptides, such as IPP, VPP, EKDERF, YPFPGPIHN, VRYL, YQEPV-LGPVR, LAHKAL, and IR/RL, were present in all cheeses, within the range of ages examined, whereas other observed ACEI peptides were present only in cheeses of a specific age (Table 2).

CONCLUSIONS

Water-soluble extracts from 2 brands of commercial Cheddar cheeses made in Wisconsin showed that ACEI activity increased with an increase in ripening time. Many ACEI peptides were detected and identified in these cheeses and the types of ACEI peptides increased with age. We quantified 6 of the ACEI peptides—IPP, VPP, VRYL, FFVAP, EKDERF, and YPFPGPIPN—and their concentrations increased up to certain level of ripening. We observed large differences in the types and concentrations of ACEI peptides between cheeses produced by the 2 cheese companies; presumably this reflects differences in manufacturing conditions, including type of starter cultures and the NSLAB microflora. Aged Cheddar cheese is a good source of ACEI peptides with high ACEI activity. In vivo studies will be necessary to validate the antihypertensive effects of these ACEI peptides obtained in Cheddar cheese.

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