

**ARTICLE**

# Antioxidant, Antihypertensive, and Anti-inflammatory Activities of Long-Term Ripened Cheddar Cheese Water-Soluble Extract

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 OPEN ACCESS

**Received** July 9, 2024

**Revised** September 9, 2024

**Accepted** September 9, 2024

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**Abstract** During cheese ripening, the proteins in the cheese are broken down, and various functional peptides are produced. This research aimed to investigate the changes in peptides and their physiological activities during the long-term maturation of Cheddar cheese. Young (YC), medium (MC), and 3-year-aged extra-sharp (EC) Cheddar cheeses were extracted with water, and bioactive peptides were identified using ultra performance liquid chromatography-high resolution mass spectrometer. Peptides reported to have antioxidant, angiotensin-converting enzyme (ACE)-inhibitory, and anti-inflammatory effects were identified and evaluated in the extracts. MC and EC showed stronger antioxidant activity than YC. The 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid inhibition rates of MC and EC were similar, but EC exhibited a higher 2,2-diphenyl-1-picrylhydrazyl inhibition rate. The antihypertensive effect was found to increase owing to the appearance of peptides with ACE-inhibitory activity in MC and EC. Quantitative real time polymerase chain reaction and immunoblot were run to evaluate the anti-inflammatory effects. YC did not show anti-inflammatory activity, but MC and EC were shown to effectively inhibit inflammatory mRNA expression. The immunoblot results showed that EC did not inhibit IκBα phosphorylation, but had an inhibitory effect at the mRNA expression level. Overall, the peptides contained in aged Cheddar cheese were shown to have excellent antioxidant, anti-inflammatory, and antihypertensive activities, and long-term ripening appeared to have a positive effect on these activities. This is presumed to have affected not only the already identified peptides but also unknown peptides; therefore, it is expected that the discovery of bioactive peptides will be possible through additional research.

**Keywords** ripened Cheddar cheese, bioactive peptides, antioxidant, antihypertensive effect, anti-inflammatory activity

## Introduction

In bovine milk, functional proteins, such as lactoferrin, immunoglobulins, and growth

factors secreted from the mammary gland exist in an active state and exert biological activity. Milk proteins are superior in nutritional value alone, but they are attracting more attention for their physiological roles and are currently recognized as sources of various biologically active peptides (Pihlanto, 2006).

Bioactive peptides are isolated portions of proteins that have beneficial effects on various physiological functions and physical and mental health. These peptides have positive effects on antioxidant, antithrombotic, antihypertensive, antibacterial, and immunomodulatory properties, and have received considerable attention for their potential bioactivities (Lin et al., 2022). Recent studies have suggested that bioactive peptides may have positive effects on diseases such as obesity and hyperlipidemia (Song et al., 2022).

Cheese is a widely consumed dairy product that is highly regarded for its unique flavor, pleasing texture, and rich nutrients. It is a notable reservoir of proteins, fats, vitamins, and minerals. Cheese is high in protein and is a source of various milk derived peptides, depending on the diversity of proteolytic systems and the intensity of proteolysis during ripening (Park et al., 2007). Enzymes present in milk, derived from rennet or secreted by microorganisms, can break down casein and produce bioactive peptides (Pihlanto, 2006). The degradation of milk proteins is affected by several factors like pH, plasmin, chymosin, enzymes secreted by microorganisms, salt, storage period and temperature, and humidity (Park and Jin, 1998).

The physiologically active peptide content of cheese is enhanced by the specific probiotic strains used in the cheese manufacturing process and ripening. Studies on Cheddar cheese have shown that the use of *Lactobacillus casei* 300 results in peptides having antihypertensive activity (Giosuè et al., 2022). Moreover, it has been demonstrated that the use of specific proteases in cheese production further increases the appearance of bioactive peptides. Therefore, even in the same type of cheese, the type, amount, and function of bioactive peptides are likely to vary depending on the degree of ripening.

Cheddar cheese is a hard, mostly orange-colored, natural cheese. It is known to have originated from the English town of Cheddar in Somerset. Cheddar is the most popular cheese type in the UK, and accounts for 51% of the annual cheese market. In the United States, cheddar is the 2nd most consumed cheese after mozzarella, and the average annual consumption per person is 10 pounds (4.5 kg). Likewise, cheddar is a type of cheese that is easily accessible and loved in many countries.

Therefore, research on Cheddar cheese peptides according to ripening stage is expected to reveal changes in composition and functionality at each stage, and based on this, its value as a fermented food will be reaffirmed. Our study focused on evaluating the anti-inflammatory effect, antioxidant, and antihypertensive functions of Cheddar cheese, a commonly consumed cheese, at different ripening stages.

## Materials and Methods

### Preparation of Cheddar water-soluble extracts

In this study, extra-sharp (3-year-ripened), medium (6-mon-ripened), and young (early stages of ripening, 1-mon-ripened) Cheddar cheeses manufactured by the Animal Resources Research Center of Chungnam National University, Korea, were used as samples and categorized into the extra-sharp Cheddar cheese water-soluble extract (EC), medium Cheddar cheese water-soluble extract (MC), and young Cheddar cheese water-soluble extract (YC) groups, respectively. Each cheese sample was finely ground, and 20 g was placed into 180 mL of distilled water (DW) and extracted with an ultrasonic extractor (1 h, 40°C). Each water-soluble extract (WSE) was cooled and centrifuged (Supra R17, Hanil Science, Ansan, Korea) at 6,000×g (20 min, 4°C) to remove the fat layer, and the WSEs were taken and filtered with Whatman No. 2 (Whatman, Maidstone, UK). Finally, the WSE was lyophilized and kept at -20°C until further analysis.

### **Quantification of water-soluble nitrogen in Cheddar**

To evaluate the ripeness of Cheddar cheese, the nitrogen fraction quantification method (Bütikofer et al., 1993) was applied to measure the change in water-soluble nitrogen (WSN).

Each cheese was finely grinded, mixed in a ratio of 5 g of cheese to 20 mL of sterilized DW, and homogenized at 20,000 rpm for 5 min with a homogenizer (ULTRA TURRAX T25, IKA, Wilmington, NC, USA). It was centrifuged at 4°C, 3,000×g for 30 min to remove the fat, and the supernatant was filtered with by Whatman No. 2 (Whatman).

Subsequently, following Hull's method (Hull, 1947), 2.5 mL of the filtrate was combined with 5.0 mL of reagent A (12% trichloroacetic acid and 0.5 mL of DW). The mixture was left at room temperature for 20 min and then filtered with Whatman No. 42 (Whatman) to obtain filtrate. Then, 2.5 mL of filtrate was taken, 5.0 mL of Reagent B (75 g of sodium carbonate and 10 g of sodium hexametaphosphate dissolved in 500 mL of DW) and 1.5 mL of Reagent C [50 mL of folin & Ciocalteu's phenol reagent (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 100 mL of DW] and reacted for 30 min in a 30°C water bath. After the completion of color development, the WSN content was measured at 570 nm using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan), and their quantification was calculated by a linear regression formula with tyrosine as the standard.

### **Protein content and pH of the Cheddar water-soluble extracts**

Protein quantification and pH measurements were performed to confirm the protein content and characteristics of Cheddar WSEs used in this study. The protein content of Cheddar WSEs were measured using the protein quantification method (Bradford, 1976). Lyophilized WSE diluted in sterilized DW at a concentration of 10 mg/mL was used as a measurement sample. Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA, USA) was diluted with DW in a 1:4 ratio and used as Bradford dye reagent. Measurements were made by placing 10 µL of sample solution and 200 µL of dye reagent in each well of a 96-well plate and measuring absorbance at 595 nm with microplate reader. Afterwards, bovine serum albumin (BSA, Bio-Rad Laboratories) was used as a standard substance to quantify the protein content in the sample.

The pH measurement was performed by mixing YC, MC, and EC lyophilized substances with DW at a concentration of 2% (w/v) and measuring at 25°C with a pH meter (S-20K, Mettler-Toledo, Columbus, OH, USA).

### **Bioactive peptide profiles of Cheddar water-soluble extracts**

Cheddar WSE was dissolved in DW at a concentration of 100 ppm, filtered with a 0.2 µm syringe filter (HM, Anseong, Korea), and used for ultra performance liquid chromatography-high resolution mass spectrometer (UHPLC-HRMS) analysis. HRMS (TripleTOF 5600 plus, SCIEX, Framingham, MA, USA) detector and UHPLC system (1290 Infinity II, SCIEX) were used for analysis. Chromatography was performed using a 2-solvent gradient elution [solvent A: 0.1% formic acid in deionized water (v/v), solvent B: 0.1% formic acid in acetonitrile (v/v)]. The mass spectrometer was operated in (+) ion mode. Data were analyzed using AB SCIEX software. Information on milk-derived peptides was collected from the Milk Bioactive Peptide Database (<https://mbpdb.nws.oregonstate.edu/>) and analyzed.

### **Antioxidant activity evaluation by 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid**

The 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) radical scavenging was measured using a decolorization assay (Re et al., 1999). The ABTS radical solution was prepared by reacting the ABTS (Sigma-Aldrich) solution (7 mM) with potassium persulfate solution (2.4 mM), keeping the mixture in the dark at 25°C for 12–16 h before use. The ABTS radical

solution was diluted with DW to an absorbance of 0.7 ( $\pm 0.02$ ) at 734 nm. A 50  $\mu\text{L}$  sample was mixed with 950  $\mu\text{L}$  of diluted ABTS<sup>•+</sup> solution, and the absorbance (734 nm) was measured after 10 min. L-ascorbic acid was used as a positive control, and the rate of inhibition was calculated as follows:

$$\text{Inhibition rate (\%)} = \{[1 - (\text{Sample} - \text{Sample blank}) / (\text{Control} - \text{Control blank})] \times 100\} \quad (1)$$

### Antioxidant activity evaluation by 2,2-diphenyl-1-picrylhydrazyl

Inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by cheddar cheese was evaluated using a modified free radical method (Brand-Williams et al., 1995). The sample diluted in 20  $\mu\text{L}$  of DW was dispensed into a 96-well plate with 180  $\mu\text{L}$  of freshly prepared 0.2 mM DPPH (Sigma-Aldrich) methanol solution. After reaction in the dark (15 min), the decrease in absorbance at 517 nm was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). In the same way, blank values were determined using DW. Scavenging rate was expressed as follows:

$$\text{Scavenging rate (\%)} = [1 - (A / A_0)] \times 100, \quad (2)$$

Where A is the absorbance of the sample and A<sub>0</sub> is the absorbance of the blank.

### Angiotensin-converting enzyme-inhibitory activity

Angiotensin-converting enzyme (ACE)-inhibitory activity was measured using an *in vitro* spectrophotometric assay (Cushman and Cheung, 1971) with some modifications. Rabbit lung acetone powder (Sigma-Aldrich) was mixed at 1:10 with 0.1 M sodium borate buffer containing 0.3 M NaCl and extracted by shaking at 4°C for 24 h. After centrifugation at 15,000 $\times g$  for 30 min, the supernatant was collected and used as the ACE enzyme. As a substrate, hippuryl-histidyl-leucine (HHL, Sigma-Aldrich) was diluted to a concentration of 5 mM in 0.1 M sodium borate buffer containing 0.3 M NaCl. Captopril (Sigma-Aldrich) was used as the positive control. Then, 100  $\mu\text{L}$  of 0.1 M sodium borate buffer and 50  $\mu\text{L}$  of ACE enzyme were added to 50  $\mu\text{L}$  of the sample, which was preincubated at 37°C for 5 min, after which 50  $\mu\text{L}$  of substrate was added and reacted at 37°C for 30 min. After the reaction, 300  $\mu\text{L}$  of 0.1 N HCl was added to stop the reaction, followed by adding 1 mL of ethyl acetate, stirring for 15 s, and centrifugation at 4°C and 960 $\times g$  for 5 min. The supernatant was completely dried in a vacuum concentrator (Hanil, Daejeon, Korea), 1 mL of DW was added, and the absorbance was measured at 228 nm. A sample blank was prepared by stopping the reaction with 300  $\mu\text{L}$  of 0.1 N HCl before adding the ACE enzyme. The rate of inhibition was calculated as described in the ABTS assay.

### Cell culture

RAW 264.7 cells, purchased from the Korean Cell Line Bank, were used in the experiments. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, WELGENE, Gyeongsan, Korea) supplemented with 10% (v/v) of fetal bovine serum (FBS, WELGENE), and 1% (v/v) of penicillin and streptomycin (WELGENE). The cells were cultured under conditions at 37°C and 5% CO<sub>2</sub>.

### Cell viability assay

To determine the effect of Cheddar WSE on cell activity, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-

(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed. RAW 264.7 cells were seeded in a 96-well plate at 3,000 cells/well and cultured for 24 h. The samples were treated at each concentration in each well and cultured for another 24 h. Subsequently, MTS solution (Promega, Madison, WI, USA) was added to each well, and the absorbance was measured at 490 nm with microplate reader (EMax Plus, Molecular Devices, San Jose, CA, USA). The relative toxicity was measured by expressing the activity of cells treated with the sample as a percentage of the untreated control group.

### Quantitative real time polymerase chain reaction

RAW 264.7 cells were seeded on a 6-well plate at  $1.5 \times 10^5$  cells/well and cultured for 24 h. The cultures were then replaced with culture medium treated with YC, MC, or EC WSE at a concentration of 100  $\mu\text{g}/\text{mL}$ , or with untreated culture medium, and cultured for 24 h. Subsequently, each well was treated with 1  $\mu\text{g}/\text{mL}$  lipopolysaccharide (LPS) and cultured for 6 h. After complete removal of the culture medium, the cells were washed with PBS, lysed with 1 mL of Ribo Ex (Gene All, Seoul, Korea), and RNA was extracted using a Hybrid-R RNA purification kit (Gene All). To synthesize cDNA, RNA was quantified using a Nabi UV/Vis Nano Spectrophotometer (Micro Digital, Seongnam, Korea). Then, 1  $\mu\text{L}$  of random hexamer (100 pmol/ $\mu\text{L}$ ) and 1  $\mu\text{L}$  of dNTP mix (10 mM) were added to 1  $\mu\text{g}$  of total RNA, and the total volume was adjusted to 10  $\mu\text{L}$  using DEPC-treated water. The mixture was reacted at 65°C for 5 min and immediately cooled on ice. Subsequently, 1  $\mu\text{L}$  of M-MLV reverse transcriptase (Promega), 4  $\mu\text{L}$  of 5X M-MLV RT reaction buffer (Promega), 1  $\mu\text{L}$  of RNase inhibitor (Enzymomics, Daejeon, Korea), and 4  $\mu\text{L}$  of DEPC-treated water were added. After incubating at room temperature for 10 min, cDNA was synthesized by reacting at 50°C for 1 h.

The mRNA expression levels of inflammatory cytokines were compared using quantitative real time polymerase chain reaction (qRT-PCR). After diluting 2  $\mu\text{L}$  of cDNA to 1/10, qRT-PCR was performed using 5  $\mu\text{L}$  of nuclease-free water, 10  $\mu\text{L}$  of 2X Prime Q-master Mix (GenET BIO, Nonsan, Korea), 10 pmol/ $\mu\text{L}$  forward primers, and 10 pmol/ $\mu\text{L}$  reverse primers. Using the AriaMx (Agilent, Santa Clara, CA, USA), 40 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 20 s, and elongation at 72°C for 20 s were performed. The nucleotide sequences of the primers used in the experiment were as follows:

IL-1 $\beta$ : (F) 5'-AGG TCA AAG GTT TGG AAG CA-3', (R) 5'-TGA AGC AGC TAT GGC AAC TG-3'; IL-6: (F) 5'- GTC CTT CAG AGA GAT ACA GAA ACT-3', (R) 5'-AGC TTA TCT GTT AGG AGA GCA TTG-3'; TNF- $\alpha$ : (F) 5'-AGG GTC TGG GCC ATA GAA CT-3', (R) 5'-CCA CCA CGC TCT TCT GTC TAC-3'; iNOS: (F) 5'-CAG CTG GGC TGT ACA AAC CTT-3', (R) 5'-CAT TGG AAG TGA AGC GTT TCG-3'; GAPDH: (F) 5'-CCA TGG AGA AGG CTG GGG-3', (R) 5'-CAA AGT TGT CAT GGA TGA CC-3'.

### Immunoblotting

RAW 264.7 cells were seeded on a 6-well plate at  $1.5 \times 10^5$  cells/well and cultured for 24 h. The culture medium was removed, and medium treated with or without YC, MC, and EC WSE at a concentration of 100  $\mu\text{g}/\text{mL}$  was dispensed and cultured for 24 h. LPS was treated at a concentration of 1  $\mu\text{g}/\text{mL}$  for 30 min. Then, each well was then immediately washed with ice-cold PBS, and lysates were extracted using lysis buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, and 1% (v/v) Triton X-100]. Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 1 h and transferred to a nitrocellulose membrane. Next, blocking was performed for 1 h with 5% (w/v) skim milk dissolved in PBST. The membrane was treated with antibodies against p-I $\kappa$ B $\alpha$  (1:1,000; Cell Signaling Technology, Danvers, MA, USA), I $\kappa$ B $\alpha$  (1:1,000; Cell Signaling Technology), and GAPDH (1:2,000; Cell Signaling





**Table 1. Identification of functional peptides in young Cheddar cheese WSE**

Peptide	Protein description	Intervals	Function	Reference
IQP	$\alpha$ s2-CN	209–211	ACE-inhibitory	Jing et al. (2014)
LQP	$\beta$ -CN	103–105	ACE-inhibitory	Tonouchi et al. (2008)

WSE, water-soluble extract; ACE, angiotensin-converting enzyme.

**Table 2. Identification of functional peptides in medium Cheddar cheese WSE**

Peptide	Protein description	Intervals	Function	Reference
IPP	$\kappa$ -CN	129–131	Antioxidant	Chakrabarti et al. (2017)
			Anti-inflammatory	Adams et al. (2020)
			ACE-inhibitory	Jing et al. (2014)
LPP	$\beta$ -CN	166–168	ACE-inhibitory	Norris et al. (2014)
VPP	$\beta$ -CN	99–101	Antioxidant	Chakrabarti et al. (2017)
			ACE-inhibitory	Nakamura et al. (1995)
			Anti-inflammatory	Aihara et al. (2014)

WSE, water-soluble extract; ACE, angiotensin-converting enzyme.

**Table 3. Identification of functional peptides in extra-sharp Cheddar cheese WSE**

Peptide	Protein description	Intervals	Function	Reference
YKVPQL	$\alpha$ s1-CN	119–124	ACE-inhibitory	Maeno et al. (1996)
LPP	$\beta$ -CN	166–168	ACE-inhibitory	Norris et al. (2014)
VP	$\beta$ -CN	23–24, 99–100, 188–189, 193–194	ACE-inhibitory	Norris et al. (2014)
IR	$\beta$ -LG	163–164	ACE-inhibitory	Murakami et al. (2004)
LL	$\beta$ -LG	4–5, 47–48, 73–74, 119–120	ACE-inhibitory	Pan et al. (2012)
IPP	$\kappa$ -CN	129–131	Antioxidant	Chakrabarti et al. (2017)
			Anti-inflammatory	Adams et al. (2020)
			ACE-inhibitory	Jing et al. (2014)
VSP	$\kappa$ -CN Genetic Variant F1	169–171	ACE-inhibitory	Weimann et al. (2009)

WSE, water-soluble extract; ACE, angiotensin-converting enzyme.

(aged for 6 mon), LPP, which has an ACE-inhibiting effect, and IPP and VPP, which are known to have anti-inflammatory, antioxidant, and ACE-inhibiting effects, were discovered. In EC (aged for 3 years), various peptides were identified over the long protein degradation period, and several additional peptides (YKVPQL, LPP, VP, IR, LL, and VSP) were detected. Based on these results, EC, aged for 3 years, contained the most bioactive peptides were found among the three experimental groups. Thus, the results confirm that various bioactive peptides appear during long-term ripening.

### Changes in water-soluble nitrogen content with long-term ripening of Cheddar

The WSN content of Cheddar cheese gradually increased as the ripening period progressed (Table 4). The increase in WSN of cheese during the ripening period is generally caused by casein degradation and varies depending on the rennet used in



**Table 4. Changes in water-soluble nitrogen content during ripening of Cheddar cheese**

Sample	Water-soluble nitrogen content ( $\mu\text{g}$ tyrosine/g)	
	Average	SEM <sup>1)</sup>
Young Cheddar	20.74	0.58
Medium Cheddar	95.18	0.13
Extra-sharp Cheddar	132.13	0.83

<sup>1)</sup> n=3.

cheese production or the protease secreted by the starter (Galán et al., 2008). In addition, it has been confirmed that protease produced from the cheese starter causes continuous protein degradation (Sallami et al., 2004). Therefore, the increase in WSN compounds is used as an indicator of ripening progress. In addition, the results of this study confirmed that WSN increases as the ripening period progresses from YC to MC and from MC to EC and is proportional to the degree of ripening.

### Protein content and pH changes in Cheddar water-soluble extracts

The protein content and pH value of each Cheddar cheese WSE are shown in Table 5. Each sample was compared after completely dissolving the freeze-dried powder in DW at a certain concentration. As a result, the protein content in YC was 20.01 mg BSA/g DM, and MC and EC were slightly reduced to 19.66 mg BSA/g DM and 16.63 mg BSA/g DM, respectively.

The pH value of Cheddar cheese WSE showed no difference from YC (5.69) in MC (5.71), but decreased to 5.57 in EC.

### 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid radical inhibition of Cheddar water-soluble extracts

The ABTS radical inhibition rate (%) was compared by treating each sample at the same concentration, as shown in Fig. 4. YC exhibited a scavenging activity of  $25.99 \pm 0.18\%$ . On the other hand, MC and EC, which underwent longer ripening, demonstrated significantly higher scavenging activities of  $57.90 \pm 0.54\%$  and  $56.08 \pm 0.26\%$ , respectively.

### 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity of Cheddar water-soluble extracts

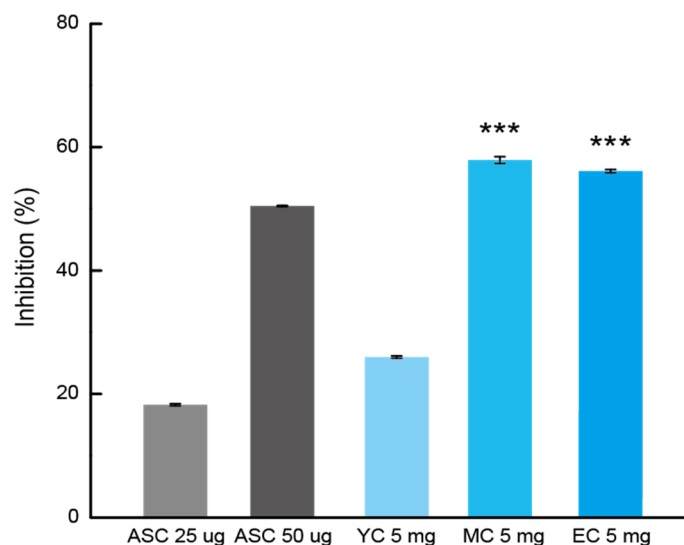
The DPPH radical scavenging ability of Cheddar cheese tended to increase gradually with the maturation period, as shown in Fig. 5. At the beginning of ripening (YC), the activity was  $36.77 \pm 0.14\%$ ; in the middle (MC), it was  $43.73 \pm 0.06\%$ ; and after 3 years (EC), it was  $62.00 \pm 0.10\%$ . Similar to the ABTS assay results, the DPPH radical scavenging ability was higher in MC and EC compared to YC. However, unlike the ABTS assay, EC showed the highest radical scavenging ability, demonstrating excellent DPPH scavenging activity. DPPH inhibition rate, and its activity gradually increased with the ripening period. These results are believed to be related to the presence of peptides (IPP and VPP) with antioxidant activity, as previously

**Table 5. Protein content and pH of Cheddar cheese WSE**

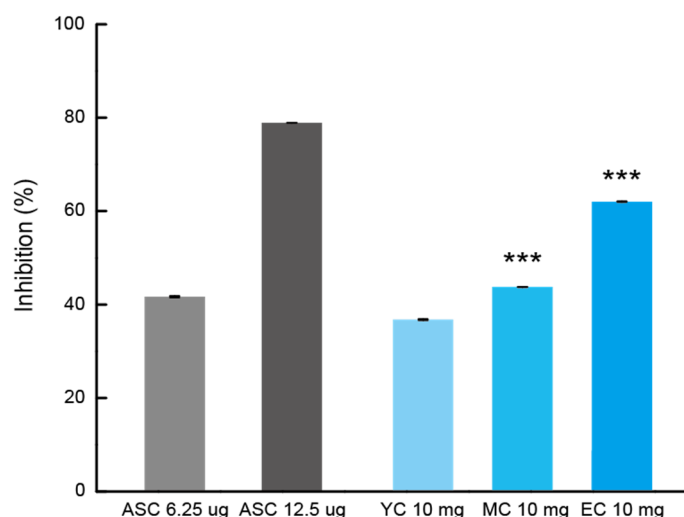
Sample	Protein content (mg BSA/g DM)		pH (2% dissolved in DW)	
	Average	SEM <sup>1)</sup>	Average	SEM <sup>1)</sup>
YC	20.01	0.55	5.69	0.02
MC	19.66	0.02	5.71	0.01
EC	16.63	0.07	5.57	0.01

<sup>1)</sup> n=3.

WSE, water-soluble extract; BSA, bovine serum albumin; DM, dry matter; DW, distilled water; YC, young Cheddar cheese water-soluble extract; MC, medium Cheddar cheese water-soluble extract; EC, extra-sharp Cheddar cheese water-soluble extract.



**Fig. 4. ABTS radical scavenging activity of Cheddar cheese water soluble extract.** The concentration of each sample was the same at 5 mg to compare antioxidant activity on ABTS, and L-ascorbic acid was used as a positive control. Results are expressed as the mean $\pm$ SEM (n=3). \*\*\* p<0.001 (two-sided t-test, versus young Cheddar cheese water soluble extract). ASC, L-ascorbic acid; YC, young Cheddar cheese water-soluble extract; MC, medium Cheddar cheese water-soluble extract; EC, extra-sharp Cheddar cheese water-soluble extract; ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid.



**Fig. 5. DPPH radical scavenging activity of Cheddar cheese water soluble extract.** The concentration of each sample was the same at 10 mg to compare antioxidant activity on DPPH, and L-ascorbic acid was used as a positive control. Results are expressed as the mean $\pm$ SEM (n=3). \*\*\* p<0.001 (two-sided t-test, versus young Cheddar cheese water soluble extract). ASC, L-ascorbic acid; YC, young Cheddar cheese water-soluble extract; MC, medium Cheddar cheese water-soluble extract; EC, extra-sharp Cheddar cheese water-soluble extract; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

confirmed by the Bioactive Peptide Profile. In addition, it was confirmed that Cheddar cheese has excellent antioxidant ability through ripening.

#### Angiotensin-converting enzyme-inhibitory activity of Cheddar water-soluble extracts

The antihypertensive properties of Cheddar WSEs were estimated based on their inhibitory effects on ACE activity. This

enzyme functions to convert angiotensin I to angiotensin II, which produces a powerful vasoconstriction effect and increases blood pressure by inactivating bradykinin, which acts as a vasodilator (Pihlanto-Leppälä et al., 1998). The improved ACE inhibitory activity with ripening suggests that sufficiently mature cheese is a good functional food with antihypertensive activity.

Regardless of the differences in ripening time, the ACE inhibition rate increased in all three experimental groups in this study (Table 6). YC showed relatively low ACE-inhibitory activity (47.02%) because it is a raw cheese in the early stages of ripening after production. In contrast, fully aged MC and EC showed very high ACE-inhibitory activities, which was associated with the appearance of various previously analyzed functional peptides. MC showed a high ACE-inhibitory activity of 80.35%, and while further aging did not increase this activity, EC still exhibited a high ACE-inhibitory activity of 75.04%.

Previous studies on peptides from Cheddar cheese (Ong et al., 2007) reported that the ability to inhibit ACE depends on the degree of protein degradation, with inhibition peaking and then gradually decreasing as a result of extensive protein degradation. The present study showed a similar trend. However, the results revealed that the ACE-inhibitory activity did not significantly decrease after the middle period of ripening (MC) but remained strong even after 3 years (EC).

### Effects of Cheddar water-soluble extracts on RAW 264.7 cells

RAW 264.7 cells were treated with YC, MC, and EC, and the MTS assay was performed to evaluate the survival rate at various concentrations. Concentrations ranged from 500 µg/mL to 50 µg/mL, and each sample was added and incubated for 24 h. Cytotoxicity was assessed by comparing the treated samples with the untreated control cells. Overall, cell activity in the treatment groups was higher than that in the control group, with YC showing the highest cell activity (Fig. 6).

The cell activity under YC treatment was 152.7±3.8% at 500 µg/mL, followed by 138.1±2.2% at 250 µg/mL, 115.3±0.9% at 100 µg/mL, and 102.9±1.5% at 50 µg/mL. However, visual observation of the cells revealed no significant differences from the control cells, indicating that the treatments affected cell activity but not cell number. Therefore, for subsequent experiments, the maximum concentration was set to 100 µg/mL to better assess the effects of YC, MC, and EC on cell activity.

### Effects on inflammatory mRNA expression

Phosphorylation of IκBα promotes NF-κB signaling, which promotes the expression of pro-inflammatory cytokines like IL-6 and IL-1β and inflammatory cytokines like TNF-α and iNOS (Aderem and Ulevitch, 2000). To evaluate the effect of Cheddar WSE treatment on the inflammatory response induced by LPS in RAW 264.7 cells, mRNA expression levels were calculated.

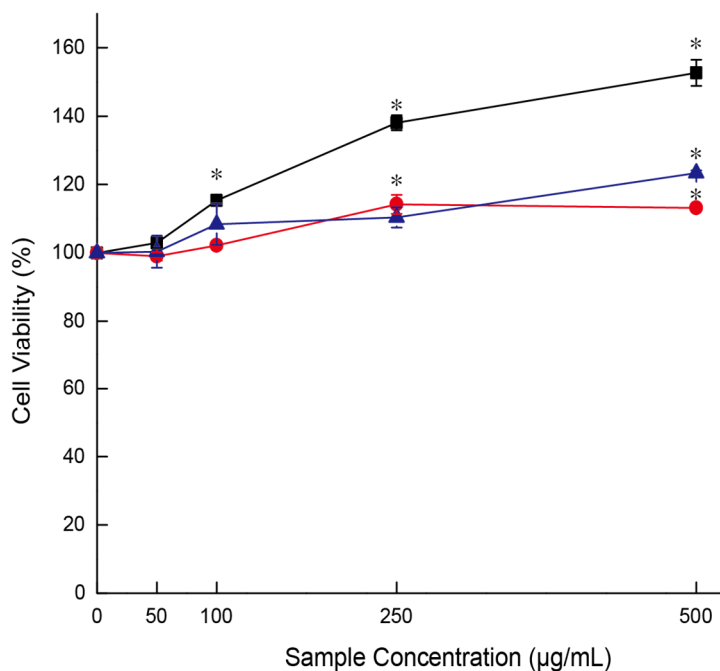
The expression of inflammatory mRNA tended to decrease with the ripening period (Fig. 7). Specifically, the IL-6

**Table 6.** ACE inhibitory effect of Cheddar cheese WSE according to ripening period

Compound (25 mg)	ACE inhibition rate (%)	
	Average	SEM <sup>1)</sup>
Captopril (12.5 mg)	96.01	0.44
YC	47.02	0.02
MC	80.35	0.03
EC	75.04	0.05

<sup>1)</sup> n=3.

ACE, angiotensin-converting enzyme; WSE, water-soluble extract; YC, young Cheddar cheese water-soluble extract; MC, medium Cheddar cheese water-soluble extract; EC, extra-sharp Cheddar cheese water-soluble extract.

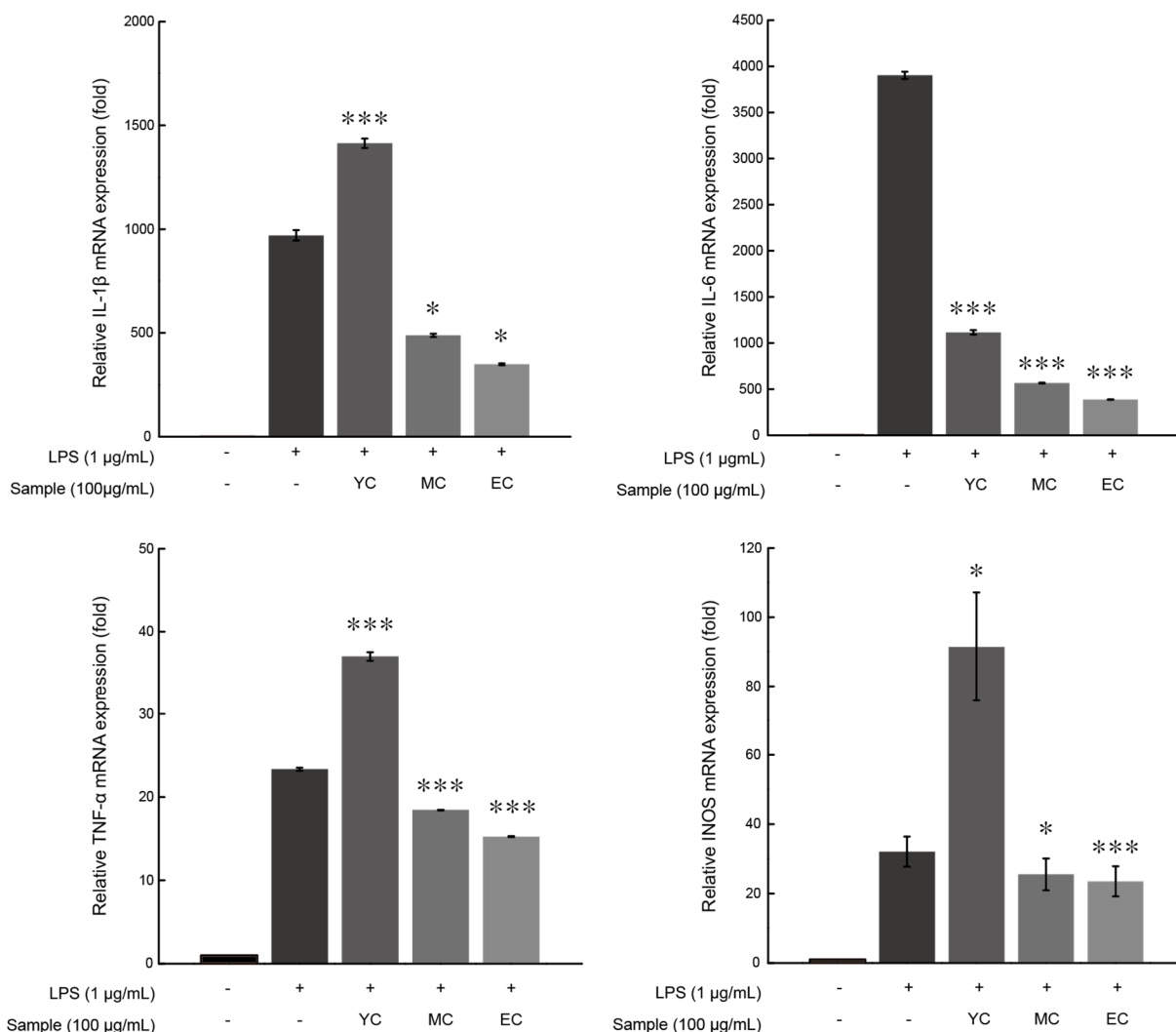


**Fig. 6. Effect of Cheddar cheese water soluble extract on cell viability.** RAW 264.7 cells were treated with indicated concentrations of YC, MC, EC for 24 h. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay. Results are expressed as the mean±SEM (n=3). \* p<0.05 (two-sided t-test, versus control). ■, young Cheddar cheese water-soluble extract (YC); ●, medium Cheddar cheese water-soluble extract (MC); ▲, extra-sharp Cheddar cheese water-soluble extract (EC).

expression level in EC decreased to 9.88%, IL-1 $\beta$  expression to 35.97%, TNF- $\alpha$  expression to 65.29%, and iNOS expression to 72.20%. Consistent with earlier findings in this study, the emergence of anti-inflammatory peptides during Cheddar cheese ripening appears to contribute to the observed anti-inflammatory effects. Accordingly, inflammatory mRNA expression decreased, and the effect became more significant as the ripening period progressed.

### Inhibitory effect on I $\kappa$ B $\alpha$ phosphorylation

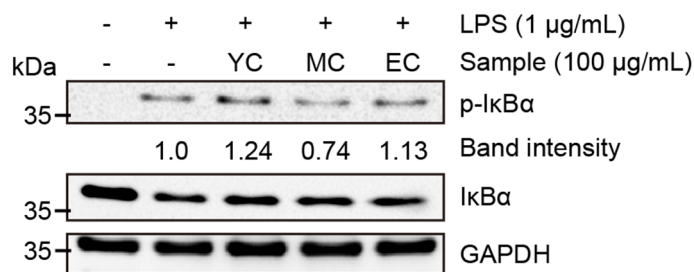
We found that the WSE of sufficiently aged Cheddar effectively inhibited inflammatory signaling in RAW 264.7 cells. Based on these results, it was assumed that Cheddar WSEs would inhibit the phosphorylation levels of I $\kappa$ B $\alpha$ , which induces an inflammatory response. To evaluate the effect of Cheddar WSEs on the inflammatory response, RAW 264.7 cells were treated at a concentration of 100  $\mu$ g/mL and then treated with LPS to induce an inflammatory response. Next, immunoblot analysis was performed to confirm the effect on the signaling action of NF- $\kappa$ B, which mainly regulates the inflammatory response (Fig. 8). The results revealed that YC treatment induced I $\kappa$ B $\alpha$  phosphorylation 24% more strongly than treatment with LPS alone. In contrast, MC inhibited the I $\kappa$ B $\alpha$  phosphorylation induced by LPS treatment by 74%, indicating a strong anti-inflammatory effect. However, under EC treatment, in contrast to MC, I $\kappa$ B $\alpha$  phosphorylation increased by 13%. From these results, it appears that MC suppressed the inflammatory response of macrophages by inhibiting the phosphorylation of I $\kappa$ B $\alpha$ , but no significant inhibitory activity was observed in EC. Evaluation of the anti-inflammatory activity of Cheddar WSEs showed no anti-inflammatory activity at the beginning of ripening (YC); however, the peptides produced during the ripening process effectively suppressed the inflammatory response. Although this inhibition did not inhibit phosphorylation of I $\kappa$ B $\alpha$ , it showed that an inhibitory effect occurred at the level of inflammatory mRNA expression. The underlying mechanism needs to be elucidated in future studies.



**Fig. 7. Effect of Cheddar cheese WSE on inflammatory mRNA expression.** RAW 264.7 cells were pretreated with Cheddar cheese WSE at concentration of 100  $\mu\text{g/mL}$  for 24 h and then incubated with LPS (1  $\mu\text{g/mL}$ ) for 6 h. qRT-PCR was performed to analyze the mRNA expression of the IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , and iNOS. Results are expressed as the mean $\pm$ SEM (n=3). \*  $p < 0.05$ , \*\*\*  $p < 0.001$  (two-sided t-test, versus control). IL-1 $\beta$ , interleukin-1 $\beta$ ; LPS, lipopolysaccharide; YC, young Cheddar cheese water-soluble extract; MC, medium Cheddar cheese water-soluble extract; EC, extra-sharp Cheddar cheese water-soluble extract; IL-6, interleukin-6; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; iNOS, inducible nitric oxide synthase; WSE, water-soluble extract; qRT-PCR, quantitative real time polymerase chain reaction.

## Conclusion

This study was conducted to investigate the changes in peptides and their physiological activities during the long-term aging of cheddar cheese. WSE was prepared for each aging period, and the content of bioactive peptides, antioxidant activity, antihypertensive activity, and anti-inflammatory effects were evaluated. The results show that various peptides present in aged cheddar cheese have excellent antioxidant, anti-inflammatory, and antihypertensive activities, and that long-term aging positively affects these activities. It is presumed that this was influenced not only by previously identified peptides but also by unknown peptides. However, this study did not involve the isolation of peptides or the measurement of their content. Additionally, bioactive peptides that were not discovered could not be confirmed. Therefore, research on the isolation of peptides and their individual activities should be conducted in the future, and biological experiments should be carried out to



**Fig. 8. Inhibitory effect of IκBα phosphorylation of Cheddar cheese WSE.** RAW 264.7 cells were cultured by adding 100 µg/mL of cheddar cheese extracts of different degrees of ripeness for 24 hours, and then stimulated with LPS (1 µg/mL) for 30 minutes. Immunoblotting was performed with proteins extracted from these cells. The numbers at the bottom of the p-IκBα band indicate the relative intensity of the p-IκBα band. Quantitative analysis was performed using densitometry and normalisation to IκBα, with GAPDH serving as the loading control. YC, young Cheddar cheese water-soluble extract; MC, medium Cheddar cheese water-soluble extract; EC, extra-sharp Cheddar cheese water-soluble extract; LPS, lipopolysaccharide; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WSE, water-soluble extract.

provide evidence of their practical activities.

## Conflicts of Interest

The authors declare no potential conflicts of interest.

## Acknowledgements

This study was supported by the Animal Resources Research Center of Chungnam National University for the preparation and ripening of cheese samples.

## Author Contributions

Conceptualization: Nam MS. Data curation: Ki W, Nam MS. Formal analysis: Ki W, Renchinkhand G, Bae H, Nam MS. Methodology: Ki W, Renchinkhand G, Bae H, Nam MS. Software: Ki W. Validation: Nam MS. Investigation: Ki W, Renchinkhand G, Bae H, Nam MS. Writing - original draft: Ki W. Writing - review & editing: Ki W, Renchinkhand G, Bae H, Nam MS.

## Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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