

Research Communication

Identification of novel antibacterial peptides isolated from a commercially available casein hydrolysate by autofocusing technique

Hend A. Elbarbary,^{1,2} Adham M. Abdou,^{2*} Yasushi Nakamura,¹ Eun Young Park,¹ Hamdi A. Mohamed,² and Kenji Sato¹

¹Division of Applied Life Sciences, Graduate School of Life & Environmental Sciences, Kyoto Prefectural University, Shimogamo, Kyoto, Japan

²Faculty of Veterinary Medicine, Department of Food Control, Benha University, Moshtohor, Kaliobiya, Egypt

Abstract.

Autofocusing, as a simple and safe technique, was used to fractionate casein hydrolysate based on the amphoteric nature of its peptides. The antibacterial activity of casein hydrolysate and its autofocusing fractions (A1–10) was examined against *Escherichia coli* and *Bacillus subtilis*. The basic fraction A9 exhibited the highest activity with minimum inhibitory concentration (MIC) of 150 µg/mL, whereas casein hydrolysate showed MIC values ranging from 2000 to 8000 µg/mL. The antibacterial peptides in A9 were purified by using a series of size exclusion and

reversed phase chromatographies. Three peptides exhibited the most potent antibacterial activity with MIC values ranging from 12.5 to 100 µg/mL. These peptides were generated from α_{s2} -casein, α_{s1} -casein, and κ -casein and identified as K₁₆₅KISQRYQKFALPQYLKTVYQH₁₈₈, I₆KHQGLPQEV₁₅, and T₁₃₆EAVESTVATL₁₄₆, respectively. Therefore, the results revealed that casein hydrolysate had potent antibacterial peptides that could be isolated by autofocusing technique.

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E-mail: dradham@yahoo.com

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1. Introduction

The new advances in protein bioengineering help to explore numerous potentials for releasing biologically functional peptides due to degradation of proteins by specific enzymes. From the recent research reports, it has become clear that proteins are sources of biologically active peptides. These peptides are inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing. This aspect has been studied and several peptides, which exhibit various activities, have been found. Nowadays, it has been proved that food proteins can act as antibacterial peptide precursors. The antibacterial peptides can be generated from proteins of different food origins like royal jelly, spinach, egg white, milk, and marine fish [1–3].

In the last two decades, a number of bioactive peptides encrypted within the primary structure of milk proteins with much casein-derived peptides were described [4]. Among these, several peptides with antibacterial activity were found within the amino acid sequence of this group of milk proteins by employing different enzymatic strategies [5–8].

The first antibacterial peptides of casein origin were identified by Hill et al. [9]. Other studies have identified antibacterial peptides derived from α_{s1} -casein [7,10,11], α_{s2} -casein [8,12], κ -casein [5,13], and β -casein [8,14].

Isracidin is a bovine α_{s1} -casein segment 1–23 that has been obtained by chymosin digestion. It was found to inhibit the growth of a variety of Gram-positive bacteria [11]. Other antibacterial peptides were isolated and identified from α_{s1} -casein; fragment 99–109 that has shown a broad spectrum of activity against Gram-positive and Gram-negative bacteria [7]. Moreover, Hayes et al. [10] identified fragments 21–29 and 30–38 that revealed a potent activity against pathogenic strains of *Enterobacter sakazakii* and *Escherichia coli*.

Casocidin-I was isolated from acidified milk and determined to be a segment 165–203 of bovine α_{s2} -casein [15].

*Address for correspondence: Adham M. Abdou, Faculty of Veterinary Medicine, Department of Food Control, Benha University, Moshtohor 13736, Kaliobiya, Egypt.
Tel.: +20 222 873 193; Fax: +20 222 873 199; E-mail: dradham@yahoo.com
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Other two peptides corresponded to fragments 183–207 and 164–179 [12]. Moreover, three antibacterial peptides corresponded to fragments 181–207, 175–207, and 164–207 of α_{s2} -casein exhibited antibacterial activity against a variety of Gram-positive and Gram-negative bacteria [6].

Kappacin is an antibacterial peptide derived from κ -casein. It exhibited growth inhibitory activity against Gram-positive (*Streptococcus mutans*) and Gram-negative (*Porphyromonas gingivitis*) bacteria [5]. Other six antibacterial peptides were identified from peptic digest of κ -casein. These peptides showed antibacterial activity against *Listeria innocua*, *Salmonella carnosus*, and *E. coli* [13].

Caseicin 15 and 17 were found on the C-terminal of bovine β -casein and showed antibacterial activity against *E. coli* [8]. These casein-derived antibacterial peptides were isolated by liquid chromatography that allows high resolution of peptides in short time. However, it is difficult to use such purified peptides for food applications due to high cost and the use of some harmful solvents. Meanwhile, other techniques for peptide separation like ion exchange or membrane filtration are still much complicated and expensive. On the other hand, autofocusing is a simple technique that was used to isolate peptide fractions based on their amphoteric nature. In addition, it has advantages of cost and biocompatibility over other techniques as it does not require chemically synthesized ampholytes or organic solvents [16]. Therefore, autofocusing can be used for separation of antibacterial peptides from protein hydrolysates [17].

Thus, the objectives of this work were to isolate antibacterial peptides from a commercially available casein hydrolysate by autofocusing technique, to purify and identify antibacterial peptides, and to assess the antibacterial activity of the chemically synthesized active peptides.

2. Material and methods

2.1. Fractionation of casein hydrolysate by autofocusing

A commercially available bovine casein hydrolysate (Catalog number: CE90GMM) was purchased from DMV Japan (Tokyo, Japan). The hydrolysate was prepared by a mixture of proteases and had peptides with an average molecular weight of 640 Da with free amino acid up to 15%. Peptides in casein hydrolysate were fractionated by autofocusing according to Hashimoto et al. [16]. The autofocusing apparatus is assembly composed of a tank (20 cm width \times 25 cm height \times 120 cm length) was made from polyacryl plate of 5 mm thickness. Eleven rows of slots (0.5 cm width \times 1 cm depth) were made in the inner surface of the tank every 10 cm. To divide the tank into 12 compartments, 11 polyacryl plates (25 cm \times 20 cm), each with a window (10 cm \times 10 cm), were prepared. Nylon screen (100 mesh) was mounted on the window and fixed by Scotch tape and a frame with four screws. The screen was wetted with 1% hot agarose solution and allowed to stand for a few minutes to form a thin agarose gel layer on the screen. On the agarose gel layer, additional agarose solution was loaded to 1–2 mm in thickness and also allowed to stand for a few minutes. Conse-

quently, an agarose gel layer of 1–2 mm in thickness was prepared on the screen. The polyacryl plate with the agarose gel layer is hereafter referred to as a separator. A separator was inserted into each slot of the tank. The compartment at either end of the tank was filled with 0.1 N phosphoric acid (anode) or 0.1 N NaOH (cathode) and used as, respectively, electrode compartments. The sample compartments were numbered from the anode side (number 1) to the cathode side (number 10). A titanium plate coated with platinum (6.5 cm \times 3.5 cm) was purchased from Tanaka Kikinzoku Kogyo (Tokyo, Japan) and used as an electrode. To cool the sample during focusing, water jackets were attached to both sides of the tank. The water jackets were filled with 80% ethylene glycol cooled to -20°C . In addition, a silicon tube, in which cold 80% ethylene glycol was circulated, put into the sample compartments through the lid on the tank. The polyethylene glycol solution in the silicon tube was cooled and circulated by an Eyela Coolace CCA-1100 (Tokyo Rikakikai, Tokyo, Japan). This assembly of the tank, separators, water jackets, and lid with silicon tube is referred to as a “focusing apparatus.” Autofocusing of peptides was performed at constant voltage of 500 V in which sample compartments 4 and 5 were filled with 5% sample and the others were filled with deionized water. During focusing, all solutions in the compartments were stirred with magnetic stirrers. The autofocusing fractions were collected after 24 h, and then freeze dried for further experiments.

2.2. Amino acid analysis

To identify amino acids composition of the autofocusing fractions (A), amino acid analysis was performed according to the method of Bidlingmeyer et al. [18] with some modifications according to Sato et al. [19].

2.3. Chromatographic techniques

Freeze dried A fractions with the highest antibacterial activity were loaded onto a Superdex Peptide 10/30HR (GE Healthcare UK, Amersham Place, UK) equilibrated with 30% (v/v) acetonitrile containing 0.1% trifluoroacetic acid for size exclusion chromatography (SEC). The absorbance at 230 nm was also monitored. Then the collected SEC fractions (S) were freeze dried and tested for their antibacterial activity.

Then peptides in the S fractions with high antibacterial activity were further fractionated by reversed phase-high performance liquid chromatography (RP-HPLC) using an Inertsil ODS-3 (4.6 mm \times 250 mm, GL Science, Tokyo, Japan). The S fraction was dried under vacuum to remove acetonitrile and then dissolved in 50 μL of 10 mM HCl. Elution was performed with binary gradient elution at 1 mL/min. Solvent A consisted of 10 mM/L HCl and solvent B consisted of 10 mM/L HCl containing 80% acetonitrile. Gradient program was as follows: 0–5 min, B; 0%. 5–35 min, B; 0–50%. 35–40 min, B; 50–100%. 40–45 min, B; 100%. 45–55 min, B; 0%. The column was maintained at 40°C . The absorbance at 214 nm was also monitored. The fractions from RP-HPLC fractionation (R fractions) were collected at programmed time intervals. The fractions were frozen at -20°C until use.

2.4. Amino acid sequencing

An automated pulse-liquid peptide sequencer based on Edman degradation (Model PPSQ-21, Shimadzu, Kyoto, Japan) was used to detect amino acid sequence of most of the highest RP-HPLC peaks, and then checked their antibacterial activity.

2.5. Synthesis of antibacterial peptides

All purified peptides exhibiting antibacterial activity were subsequently chemically synthesized by the Fmoc strategy by using a PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan). Synthetic peptides were purified by SEC and RP-HPLC as described above. Purity of synthetic peptides was checked by amino acid sequence and electron spray ionization mass spectrometry (MS) analyses by using LC-Q (Thermo Fisher Scientific, Waltham, MA). For MS analyses, peptides were dissolved in 30% acetonitrile containing 0.1% formic acid. The synthetic peptides (SR) were examined for their antibacterial activity.

2.6. Antibacterial assay

E. coli NBRC 3301 and *Bacillus subtilis* NBRC 3134 (NITE Biological Resource Center, Chiba, Japan) were used in this study. Both strains were activated and adjusted to about 10^4 CFU/mL as previously described by Elbarbary et al. [17].

Determination of bacterostatic and bactericidal activity of casein hydrolysate, A fractions, S, R, and SR peptides were examined. The antibacterial assay was based on the method used by Branen and Davidson [20] with some modifications as described by Elbarbary et al. [17]. The minimum inhibitory concentrations (MIC) were determined by using a temperature controlled 96-well plate reader (Model 550, Bio-Rad Laboratories, Hercules, CA) and monitoring the absorbance at 660 nm. The MIC was considered as the lowest concentration that completely inhibited the growth. Growth inhibition was defined as the concentration in which absorbance of the test well minus the absorbance of the control well was ≤ 0.05 [20]. To determine the minimum bactericidal concentrations (MBC), 100 μ L was taken from each well which showed growth inhibition and was plated on tryptic soy agar plates. The viability of bacteria was assessed from the forming colonies and scored after incubation at 30°C for 24–48 h. The lowest concentration of the fractions or peptides showing no growth of bacteria was recorded as the MBC [21].

2.7. Statistical analysis

Statistical comparisons were made by using one-way analysis of variance (ANOVA) and Fisher's PLSD method using StatView 4.11 (Abacus Concepts, Berkeley, CA).

3. Results and discussion

3.1. Antibacterial activity of casein hydrolysate and autofocusing fractions

Milk has provided the largest amount of antibacterial peptides and it stands reason that several of them can be

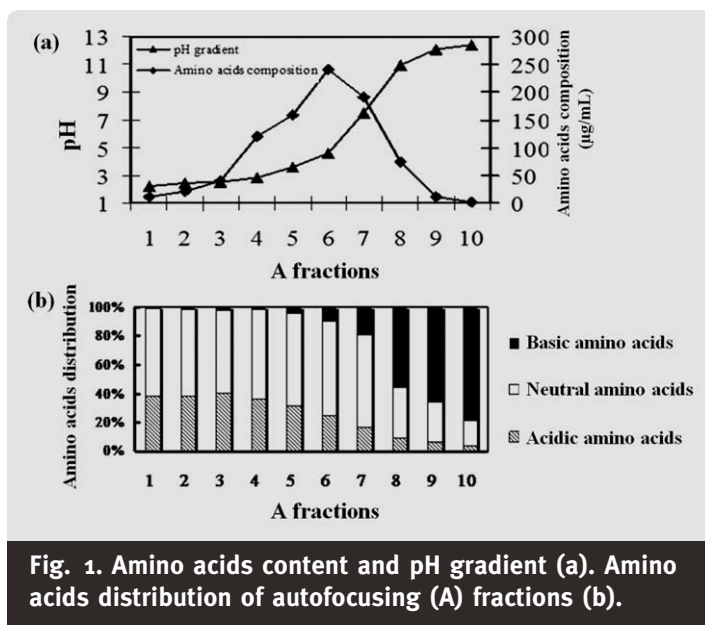


Fig. 1. Amino acids content and pH gradient (a). Amino acids distribution of autofocusing (A) fractions (b).

released from casein as it is the most abundant milk protein [4]. It may be possible to envisage the use of crude casein hydrolysate instead of those highly purified as is required for pharmaceutical uses. The commercially available casein hydrolysate was subjected to fractionation using autofocusing technique that has the advantages of cost and biocompatibility and does not require chemical or organic solvents [16]. Hence the use of casein hydrolysate or its autofocusing fractions (A fractions) may thus be easily approved by health regulatory authorities for use in foods. Therefore, we have examined the antibacterial activity of casein hydrolysate and A fractions.

The A fractions subjected to acid hydrolysis and their amino acid composition was determined. The amino acids composition and pH of A fractions are shown in Fig. 1a. The basic A fractions were characterized by higher content of basic amino acids (>50%) and lower content of acidic ones in comparison to both acidic and neutral A fractions (Fig. 1b). As shown in Table 1, the tested concentrations of casein hydrolysate were ranged from 16,000 to 125 μ g/mL, whereas those of A fractions were ranged from 2,400 to 18.7 μ g/mL. The basic A fractions showed higher antibacterial efficacy than casein hydrolysate and other A fractions. Fractions A 8, 9, 10 had significant antibacterial activity at concentrations ranging from 150 to 600 μ g/mL compared to 2,000 to 8,000 μ g/mL for casein hydrolysate. Fraction A9 was the most active fraction with MIC of 150 μ g/mL and MBC of 300 μ g/mL against both *E. coli* and *B. subtilis* (Table 1).

3.2. Purification and identification of antibacterial peptides

Fraction A9 was subjected to size exclusion chromatography (SEC). The SEC fractions (S1–8) were collected as shown in (Fig. 2a), and their antibacterial activity were examined at concentrations ranging from 100 to 6.25 μ g/mL. As shown in

Table 1
The MIC and MBC of casein hydrolysate and its autofocusing fractions (A) against *E. coli* and *B. subtilis*

µg/mL	<i>E. coli</i>			<i>B. subtilis</i>		
	MIC ^a		MBC ^b	MIC		MBC
	24 h	48 h		24 h	48 h	
Casein hydrolysate	4,000	8,000	8,000	2,000	2,000	2,000
A (1-5)	>2,400	>2,400	>2,400	>2,400	>2,400	>2,400
A 6	>2,400	>2,400	>2,400	1,200	1,200	>2,400
A 7	600	1,200	>2,400	600	1,200	>2,400
A 8	300	600	600	300	300	600
A 9	150	150	300	150	150	300
A 10	600	600	600	300	600	600

^aMinimum inhibitory concentration.

^bMinimum bactericidal concentration.

The mean values indicated were observed in three experiments.

Fig. 2b. Fractions S4, 5, and 6, that eluted between 30 and 40 min, had more potent antibacterial activity against the tested bacterial strains (Fig. 2b). Peptides in S4, 5, and 6 were purified by RP-HPLC as shown in Fig. 3a. The RP-HPLC peaks (R fractions) were collected (R1–14) and examined for their antibacterial activity. R peptides were examined at concentrations ranging from 100 to 6.25 µg/mL. As shown in Fig. 3b, the highest activity results were met with six peptides (R1, 4, 7, 8, 9, 13).

Amino acid sequence and casein origin of the antibacterial R fractions have shown in Table 2. The sequences of the active six peptides R1, 4, 7, 8, 9, 13 showed their corre-

spondence to α_{s2} -casein fragment 194–205, α_{s2} -casein fragment 165–188, α_{s1} -casein fragment 6–15, β -casein fragment 177–183, α_{s2} -casein fragment 188–193, κ -casein fragment 136–146, respectively.

Three peptides (R1, 4, 9) were found within the sequence of the C-terminal part of α_{s2} -casein. Several antibacterial peptides have been previously isolated from α_{s2} -casein including different fragments; 150–188 [15], 164–279, 183–207 [12], and 181–207, 175–207, 164–207 [6]. The α_{s2} -casein fragment 150–188, known as casocidin-1, was the first described antibacterial peptide isolated from α_{s2} -casein; R4

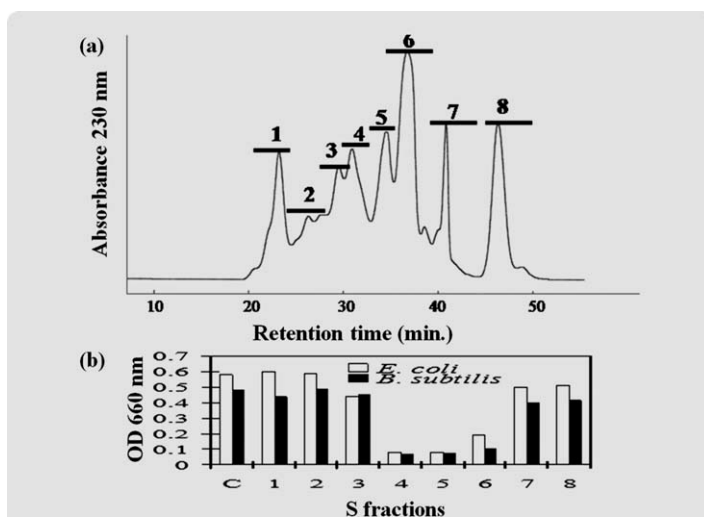


Fig. 2. Fractionation of autofocusing (A) fraction 9 by size exclusion chromatography (a). The antibacterial activity of size exclusion chromatographic (S) fractions against *E. coli* and *B. subtilis* (b).

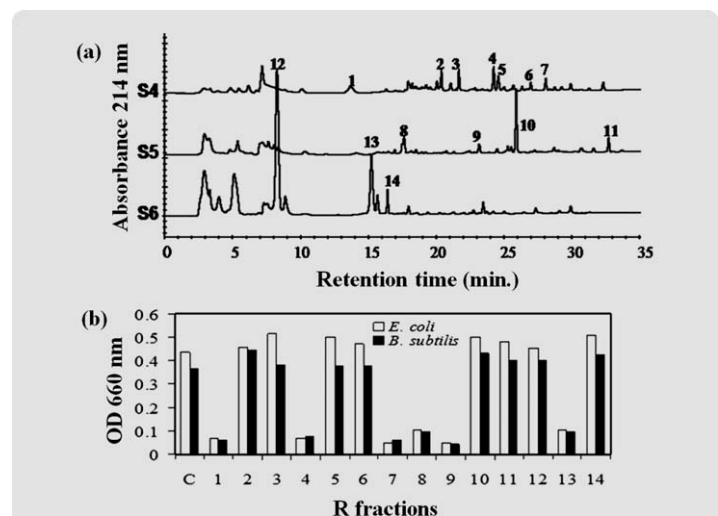


Fig. 3. Reversed phase high performance liquid chromatography (RP-HPLC) for the active size exclusion chromatographic (S) fractions (a). The antibacterial activity of RP-HPLC (R) fractions against *E. coli* and *B. subtilis* (b).

Table 2
Summary of amino acids sequences of most peptides (R) purified by reversed phase high performance chromatography (RP-HPLC)

R fractions	Sequence	Origin
1	I ₁₉₄ QPKTKVIPYVR ₂₀₅	α_{s2} -casein
2	S ₈₀ NTVPAKSCQAQP ₉₂	κ -casein
3	A ₅₃ QTQSLVYPPGPIPNSLPQN ₇₃	β -casein
4	K ₁₆₅ KISQRYQKFPALPQYLKTVYQH ₁₈₈	α_{s2} -casein
5	K ₄₆ PVALINNQFL ₅₆	κ -casein
6	S ₁₆₆ QSKVLPVPQK ₁₇₆	β -casein
7	I ₆ KHQGLPQEV ₁₅	α_{s1} -casein
8	A ₁₇₇ VPYPQR ₁₈₃	β -casein
9	K ₁₈₈ AMKPW ₁₉₃	α_{s2} -casein
10	I ₄₉ HPFAQ ₅₄	β -casein
11	No sequence	–
12	T ₁₉₄ TMPLW ₁₉₉	α_{s1} -casein
13	T ₁₃₆ EAVESTVATL ₁₄₆	κ -casein
14	R ₁₆₀ LNFL ₁₆₄	α_{s2} -casein

(α_{s2} -casein fragment 165–188) is a part from casocidin-1. The three α_{s2} -casein fragments in this study (R1, 4, 9) are novel compared to the previously isolated peptides; however, they are all derived from the C-terminal part of α_{s2} -casein.

Our data showed that peptide R7 (α_{s1} -casein fragment 6–15) is a part of isracidin which is the first active peptide isolated from α_{s1} -casein [9] and corresponding to N-terminal fragment of α_{s1} -casein fragment 1–23 [11]. While peptide R13 (κ -casein fragment 136–146) is recognized as part from the previously isolated kappacin that derived from κ -casein fragment 106–169 [5]. Meanwhile, few studies searched for antibacterial peptides from β -casein. We could identify R8 with

seven amino acid residues corresponding to β -casein fragment 177–183. A different antibacterial peptide corresponding to human β -casein segment 184–210 has been isolated by Minervini et al. [14].

Therefore, the novel six peptides in this study have not been reported previously as possessing antibacterial activity. However, they have several features in common with other reported casein-derived antibacterial peptides.

3.3. Antibacterial activity of the synthetic peptides

The six most active peptides (R1, 4, 7, 8, 9, 13) were chemically synthesized and denoted as SR1, 4, 7, 8, 9, 13. Then, they were subjected to antibacterial assay to ensure their responsibility for the activity rather than other factors. SR peptides were examined at concentrations ranging from 100 to 6.25 $\mu\text{g}/\text{mL}$. As shown in Table 3, three peptides (SR4, 7, 13) revealed the highest antibacterial activity with MIC and MBC values ranging from 50 to 100 $\mu\text{g}/\text{mL}$ against the tested bacteria. The most potent peptide against *E. coli* was SR7 that exhibited MIC of 25–50 $\mu\text{g}/\text{mL}$ and MBC of 50 $\mu\text{g}/\text{mL}$. The highest activity against *B. subtilis* was shown by SR4 that exhibited MIC of 12.5–25 $\mu\text{g}/\text{mL}$ and MBC of 50 $\mu\text{g}/\text{mL}$.

Peptide R4 (α_{s2} -casein fragment 165–188) was recognized as part of casocidin-1 (α_{s2} -casein fragment 150–188); however, its antibacterial activity against *E. coli* was higher than that of casocidin-1, which was reported by Zucht et al. [15]. Moreover, Hayes et al. [10] mentioned that caseicin A, had nine amino acid residues representing amino acids 6–14 of α_{s1} -casein. Caseicin A showed MIC as same as that of SR7 (α_{s1} -casein fragment 6–15). Both caseicin A and SR7 are representing amino acid residues in the amino acid sequence of isracidin (α_{s1} -casein fragment 1–23), however, they showed higher antibacterial activity than that of isracidin. In the

Table 3
The MIC and MBC of the SR of reversed phase high performance chromatographic fractions against *E. coli* and *B. subtilis*

$\mu\text{g}/\text{mL}$	<i>E. coli</i>			<i>B. subtilis</i>		
	MIC ^a		MBC ^b	MIC		MBC
	24 h	48 h		24 h	48 h	
SR 1	50	50	>100	50	100	>100
SR4	50	50	100	12.5	25	50
SR7	25	50	50	50	50	100
SR8	100	100	>100	50	100	>100
SR9	100	100	>100	50	100	>100
SR13	50	100	100	50	50	100

^aMinimum inhibitory concentration.

^bMinimum bactericidal concentration.

The mean values indicated were observed in three experiments.

same manner, peptide SR13 (κ -casein fragment 136–146) is a part of kappacin (κ -casein fragment 106–169) and showed better activity than that reported for kappacin by López-Expósito et al. [13]. Moreover, López-Expósito et al. [13] demonstrated that the isolated peptide fragments showed higher antibacterial activity than their parent peptides. As well as, some antibacterial peptides in the current study showed some biological activities in previous studies such as, Maruyama et al. [22] reported that the peptide corresponding to β -casein fragment 177–183, which is similar to SR8 in the current study, is considered as angiotensin converting enzyme (ACE) inhibitor. Moreover, it would favor bradykinin and, thus, act as immunomodulators that exert a protective action against some pathogens.

Most of the previously investigated casein-derived peptides possess cationic character and a positive charge that are important to exhibit their antibacterial activity [23,24]. However, our results showed that not all peptides that contain basic amino acids exhibited antibacterial activity (Table 2). The active peptides R1, 4, 7, 8, and 9 contain 3, 7, 2, 1, and 2 basic amino acids, respectively. However, R13 contains no basic amino acid, yet, it exhibited antibacterial activity. On the other hand, R2 and 6 had no antibacterial activity while they contain basic amino acids (Table 2). This is in agreement with the findings of López-Expósito et al. [25] who reported that the peptide corresponding to α_{s2} -casein fragment 165–181 with only three positively charged residues showed higher antibacterial activity than the peptide α_{s2} -casein fragment 184–208 with five positively charged residues. As previously demonstrated with model peptides, the positioning of the positive charge within the sequence of peptides has been proven to modulate their antibacterial activity. In addition, hydrophobic residues also have an indirect effect on activity by influencing structure or structure stability [26]. Therefore, positive charge is an important factor for peptide antimicrobial activity; however, it is not the only factor that guarantees antibacterial activity.

The present antibacterial peptides can be used as compounds for elucidating critical sequence and amino acid residue by substituting amino acid residues, which might produce a peptide with stronger antibacterial activity. However, a chemically synthesized peptide cannot be used as a food ingredient. The present autofocusing fraction has a potential for preservation as a food ingredient. Further studies on antibacterial activity and safety of the present autofocusing fraction in food system are now in progress.

4. Conclusions

This study showed that autofocusing technique is suitable for isolation of potent antibacterial peptides from casein hydrolysate which is considered as an inexpensive source of antibacterial peptides. The basic autofocusing fraction that exhibited the highest activity offered three novel and potent antibacterial peptides. Moreover, autofocusing technique is simple, cost-effective, and operated by food-grade solvents.

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