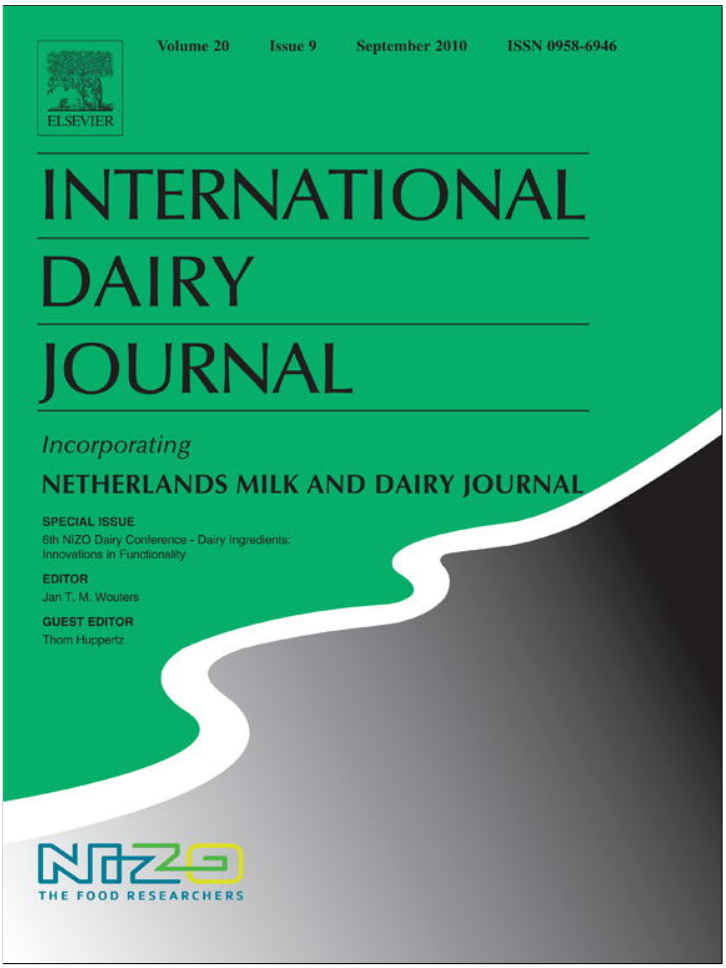

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Novel antibacterial lactoferrin peptides generated by rennet digestion and autofocusing technique

Hend A. Elbarbary [a,b], Adham M. Abdou [b,*], Eun Young Park [a], Yasushi Nakamura [a], Hamdi A. Mohamed [b], Kenji Sato [a]

[a] Division of Applied Life Sciences, Graduate School of Life & Environmental Sciences, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan
[b] Department of Food Control, Faculty of Veterinary Medicine, Benha University, Moshtohor, Kaliobiya 13736, Egypt

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ABSTRACT

Bovine lactoferrin was hydrolysed with a range of proteolytic enzymes including calf rennet, fungal rennin, and porcine pepsin. Lactoferrin hydrolysates were assessed for their antibacterial activities against *Escherichia coli* and *Bacillus subtilis*. At pH 3, calf rennet lactoferrin hydrolysate before (LFH) showed the highest antimicrobial activity, then pepsin LFH, while fungal rennin LFH was the least active. The calf rennet and pepsin LFH were fractionated using autofocusing and chromatographic techniques. The activity-guided fractionation of calf rennet LFH identified a potent antimicrobial peptide of 11-residues, lactoferricin B (Lf-cin B), and three other novel antibacterial peptides. The 11-residues Lf-cin B was the most potent antibacterial peptide and was isolated from both rennet and pepsin LFH. Pepsin LFH had a main antimicrobial peptide of 25-residues, which was not detected in calf rennet LFH. It could be concluded that calf rennet LFH had stronger antibacterial properties than porcine pepsin LFH. Besides, autofocusing could be used for scaling up the isolation of the potent rennet LFH peptides that would have a widespread commercial use as a natural food preservative substituting porcine pepsin digest, especially in Islamic communities.

1. Introduction

Food preservation procedures such as pasteurization, refrigeration, canning, modified atmosphere packaging, or the incorporation of chemical preservatives in food are usually employed to prevent the growth of microorganisms that may cause human disease or food spoilage. Chemical preservatives such as nitrites, benzoates, sorbates, and sulfites have been widely and effectively used in food practices (Deng, 2006; Seman, Quickert, Borger, & Meyer, 2008). Recently, natural antimicrobials have received considerable interest from the food industry due to consumers' preferences. Consequently, manufacturers are urged to develop a preserving system based on natural compounds.

Lactoferrin (LF) is a well-known natural antimicrobial protein that has attracted considerable interest as a natural food preservative. As a member of the transferrin family, LF is a cationic iron-binding protein that is present in many secretions including milk, tears, saliva, and serum (Bellamy et al., 1993; Chantaysakorn & Richter, 2000). Lactoferrin is a single chained molecule of approximately 78 KDa, composed of two lobes with four domains that facilitate the reversible binding of two iron ions. Therefore, the antibacterial effect of LF is exerted by limiting iron availability for microorganisms (Dionysius, Grieve, & Milne, 1993; Ye, Wang, Liu, & Ng, 2000). However, its antibacterial activity is limited through its bacteriostatic activity even at high concentration (Murdock & Matthews, 2002). In addition, it is not convenient to use native protein for food preservation as protein could be denatured or precipitated frequently and consequently lose its activity at low pH, high temperature, and high salt conditions.

It has been demonstrated that peptides derived from LF by porcine pepsin digestion showed higher antimicrobial activity than that of intact LF (Tomita et al., 1991). Depending on digestion condition, antimicrobial peptides with different lengths varying from 11 to 47 and from 6 to 25 amino acid residues are obtained from human and bovine LF, respectively. These antimicrobial peptides are known as lactoferricin H (human; Lf-cin H) or lactoferricin B (bovine; Lf-cin B) (Chapple et al., 1998a,1998b; Nakai, Chan, Li-Chan, Dou, & Ogawa, 2003). The antimicrobial effect of Lf-cin has been recognized to be through the destabilization of the plasma membrane of Gram-positive and Gram-negative bacteria, resulting in disrupting membrane permeability (Ulvatne, Haukland, Olsvik, & Vorland, 2001; Vorland, Ulvatne, Rekdal, &

* Corresponding author. Tel.: +20 222 873 193; fax: +20 222 873 199.
E-mail address: dradham@yahoo.com (A.M. Abdou).

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Svendsen, 1999). In addition, Lf-cin B has shown to exhibit some biological activities as immunomodulatory and anticancer effects (Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002; Wakabayashi, Takase, & Tomita, 2003).

Porcine pepsin, which has been used for generation of Lf-cin, can not be used in Islamic communities because of religious rules. Furthermore, there is no commercial product of food-grade pepsin from non-porcine origin. Meanwhile, other proteases such as trypsin, chymotrypsin, plant and microbial proteases had been studied, but significant antimicrobial peptides could not be generated from LF (Tomita et al., 1991).

Rennin enzyme, present in calf rennet, shares some characteristics with pepsin; however, it does not elaborate antimicrobial peptides at pH 6 from LF (Shimazaki, Nagata, & Yoo, 1991), but other digestion conditions were not tested. In addition, food-grade rennin and/or calf rennet is commercially available and accepted by consumers worldwide. Therefore, the aim of the current study was to investigate whether any antibacterial peptides could be released from LF upon digestion with calf rennet or rennin. The results obtained were compared with those from porcine pepsin and microbial rennin digestion of LF. Autofocusing and chromatographic techniques were used to isolate and identify the active peptides.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Escherichia coli NBRC 3301 and *Bacillus subtilis* NBRC 3134 strains were obtained from NITE (NITE Biological Resources Centre, Chiba, Japan). Bacterial strains were streaked on tryptic soy agar (TSA) and then inoculated in tryptic soy broth (TSB) with incubation at 30 °C overnight. The cultures were centrifuged at 3000 × *g* for 3 min, the precipitates were suspended in 1:20 dimethyl sulfoxide: phosphate buffer saline (PBS) and maintained as stocks at −80 °C. *E. coli* and *B. subtilis* counts in this stock were 10^9 and 10^8 cfu mL^{-1}, respectively.

Cultures were obtained by transferring a loopful stock culture on TSA, incubating the plate at 30 °C overnight and then transferring a single colony to 10 mL of TSB. Inoculated TSB was incubated at 30 °C for 16–20 h and then 0.5 mL of the pre-cultured TSB was inoculated into 10 mL fresh TSB, which was subsequently incubated for a further 6 h. The cultures obtained were added to 5 mL 0.07 mol L^{-1} PBS at pH 7.4. Bacteria were harvested by centrifugation at 3000 × *g* for 10 min, washed and re-suspended in TSB. Before using in assays, the latter cultures were diluted 1:10 in fresh TSB.

2.2. Preparation of lactoferrin hydrolysates

Bovine lactoferrin (LF) was obtained from Wako Pure Chemicals (Osaka, Japan). Pepsin (EC 3.4.23.1), calf rennet (EC 3.4.23.4), and microbial rennin from *Mucor miehei* (EC 3.4.23.6) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Lactoferrin hydrolysates was prepared by the method of Bellamy, Takase, Wakabayashi, Kawase, and Tomita (1992). In brief, bovine LF was dissolved in sterile distilled water to obtain a concentration of 5% (w/v) and then adjusted to pH 3 or 6 using 1 M HCl or 1 M NaOH. Enzymes were added to a final concentration of 3% (w/w of substrate). The mixtures were incubated at 37 °C for 4 h. The reaction was terminated by heating at 80 °C in a water bath for 20 min. The pH of the solutions was readjusted to 7 using 1 M NaOH. Insoluble solids were removed by centrifugation at 6000 × *g* for 10 min. Supernatants were freeze-dried and used in the following experiments.

2.3. Purification of antibacterial peptides

2.3.1. Autofocusing of peptides

Peptides in LFH were first fractionated by autofocusing, which is a preparative isoelectric focusing based on amphoteric nature of sample peptides. Autofocusing was carried out using a Rotofor (Bio-Rad Laboratories, Inc., Hercules, CA, USA) by the method of Yata, Sato, Ohtsuki, and Kawabata (1996). Autofocusing fractions were collected on the basis of pH gradient, neutralized, freeze-dried, and then tested for antibacterial activity in TSB.

2.3.2. Size exclusion chromatography (SEC)

Peptides in the autofocusing fraction with the highest antimicrobial activity were dissolved in 30% (v/v) acetonitrile containing 0.1% trifluoroacetic acid (TFA) to obtain a concentration of 5%. They were loaded onto a Superdex Peptide 10/30HR column (GE Healthcare, Amersham Place, UK) equilibrated with 30% (v/v) acetonitrile containing 0.1% TFA and eluted at 0.5 mL min^{-1}. For antibacterial assay in broth, 600 μL of SEC fractions (SEC-Fr) were dried under vacuum, and then 50 μL of 10 mM HCl was added to each tube and their contents were re-dried to remove TFA and subsequently dissolved in 500 μL of TSB.

2.3.3. Reversed phase-high performance liquid chromatography

Peptides in the SEC-Fr with high antibacterial activity were further resolved by reversed phase-high performance liquid chromatography (RP-HPLC) using an Inertsil ODS-3 (4.6 mm × 250 mm, GL Science, Tokyo, Japan). Solvent A consisted of 10 mmol L^{-1} HCl and solvent B consisted of 10 mmol L^{-1} HCl containing 80% acetonitrile (v/v). Peptides were eluted at a flow of 1 mL min^{-1}. Gradient program was as follows: 0–5 min, 0% B; 5–35 min, 0–50% B; 35–40 min, 50–100% B; 40–45 min, 100–0% B. The column was maintained at 40 °C. The RP-HPLC fractions (RP-Fr) were frozen at −20 °C until use.

All active peptides were finally re-chromatographed using 0.01% TFA (solvent C) and 0.01% TFA containing 60% acetonitrile (v/v) (solvent D) before subjection to mass spectrometry (MS) analysis. In some cases, samples were reduced and alkylated with 1 mol L^{-1} dithiothreitol and 2.5 μL of 4-vinylpyridine in presence of 8 mol L^{-1} urea, 1 mol L^{-1} Tris–HCl, pH 8.5, and 4 mmol L^{-1} ethylendiamine tetraacetate for 2 h at 37 °C. The reduced sample was then re-chromatographed by RP-HPLC using Cosmosil 5C18-300 column (Nacalai Tesuque, Kyoto, Japan). The gradient program with elution rate of 1 mL min^{-1} was as follows: 0–30 min, 0–100% D; 30–35 min, 100–0% D.

2.3.4. Amino acid analysis

Amino acid analysis was performed according to method of Bidlingmeyer, Cohen, and Tarvin (1984) with a slight modification according to Sato et al. (1992).

2.3.5. Amino acid sequencing

Amino acid sequence of the peptides was determined with an automated pulse-liquid peptide sequencer based on Edman degradation (Model PPSQ-21, Shimadzu, Kyoto, Japan). All operations were carried out according to instructions of the supplier.

2.3.6. Mass spectrometry

All purified peptides were analysed by MS in electron spray ionization (ESI) mode by using LCQ (Thermo Electron, San Jose, CA, USA) in an auto-tune model.

2.3.7. Peptide synthesis

All purified peptide exhibiting antibacterial activity were subsequently chemically synthesized by the FMOC strategy by

Table 1
Antibacterial activity of lactoferrin hydrolysates (LFH) prepared at pH 3 by different enzymes[a].

Bacterial culture	Pepsin LFH			Calf rennet LFH			Fungal rennin LFH		
	MIC		MBC	MIC		MBC	MIC		MBC
	24 h	48 h		24 h	48 h		24 h	48 h	
E. coli	4000	4000	8000	2000	2000	4000	8000	8000	>16,000
B. subtilis	1000	1000	1000	250	250	1000	4000	8000	>16,000

[a] Abbreviations are: MIC, minimum inhibitory concentration (μg mL^{-1}); MBC, minimum bactericidal concentration (μg mL^{-1}); The values indicated were the mean of three experiments.

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 and MS analyses.

2.4. Assay for antibacterial activity in TSB

The antibacterial assay was carried out by the method of Branen and Davidson (2000). Stock solution of protein hydrolysate was prepared in sterile distilled water at a concentration of 32,000 μg mL^{-1}. The resulting mixture was filter sterilized by 0.45 μm pore size cellulose acetate and stored at −20 °C until used. Further dilutions were made in sterile TSB (two-fold serial dilutions). The concentrations of the antibacterials tested ranged from 16,000 to 125 μg mL^{-1}. The concentration of autofocusing fractions ranged from 2400 to 18.7 μg mL^{-1}. The minimum inhibitory concentration (MIC) was determined by using a temperature controlled 96-well plate reader (Model 550, Bio-Rad Laboratories, Inc.). In each well, 120 μL of diluted antimicrobial agents and 120 μL of diluted inoculum to achieve 10^4 colony forming units (cfu) mL^{-1} were added. The micro-titre plate was incubated at 30 °C and the absorbance at 655 nm was determined after 24 and 48 h. The MIC was considered the lowest concentration that completely inhibited microbial 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 (Branen & Davidson, 2000). To determine the minimum bactericidal concentration (MBC), 100 μL was taken from all wells after 48 h incubation, spread on TSA and incubated at 30 °C for 48 h. The MBC was designated as there was 99.9% loss of viable cells (Barry, 1976). All assays were performed in triplicates and the results are expressed as the means of three independent experiments. Similarly, the synthetic peptides were checked for their antibacterial activity at concentrations ranging from 100 to 6.25 μg mL^{-1}.

2.5. 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, USA). The results were considered significantly different with $p < 0.05$.

3. Results and discussion

3.1. Antibacterial activity of lactoferrin hydrolysates

Experimental conditions for antibacterial assays differ per researchers. In some cases, MIC and MBC activities have been evaluated after relatively short periods such as 2 or 14 h (Tomita et al., 1991). However, after initial suppression of growth by a potential antibacterial agent, bacteria frequently restart to grow. Therefore, to evaluate the antibacterial activity, the MIC and MBC should be examined after incubation for a few days. In the present study, antibacterial activity was considered to be present if suppression of growth or bactericidal effect were observed after 48 h, as described by Branen and Davidson (2000).

In an attempt to generate antibacterial peptides, LF was hydrolysed with porcine pepsin, calf rennet and fungal rennin at pH 3 and 6. Hydrolysates produced at pH 6 by all these enzymes showed no antibacterial activity. Shimazaki et al. (1991) demonstrated that calf rennet could not generate antibacterial peptides from LF at pH 6. On the other hand, all hydrolysates produced at pH 3 in the current study showed antibacterial activity against the strains tested. Calf rennet LFH showed the lowest MIC after 48 h incubation and showed a smaller MBC against *E. coli* than pepsin LFH, while fungal rennin LFH showed no bactericidal activity (Table 1).

3.2. Identification of antibacterial peptides

To isolate and identify the antibacterial peptides in calf rennet and pepsin LFH prepared at pH 3, the hydrolysates were fractionated by autofocusing. The peptide content of the 20 fractions obtained is shown in Fig. 1a. These fractions were combined and divided into four autofocusing fractions Fr I –IV according to their pH profile as shown in Fig. 1b. The basic autofocusing fractions Fr III and IV exhibited potent antibacterial activity against *E. coli* and *B. subtilis* contrary to the acidic ones (Fig. 2a and b). Fr IV had higher

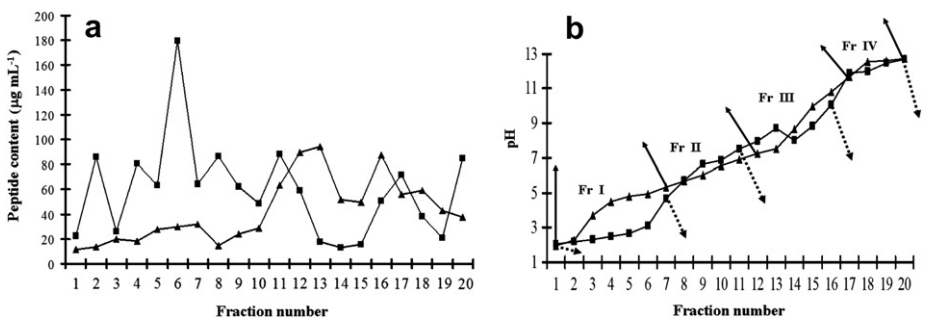

Fig. 1. Fractionation by autofocusing: (a) Peptide content (μg mL^{-1}) and (b) pH gradient of rennet (■) and pepsin (▲) lactoferrin hydrolysates. Fractions collected were indicated with arrows and referred to autofocusing fraction (Fr) I–IV.

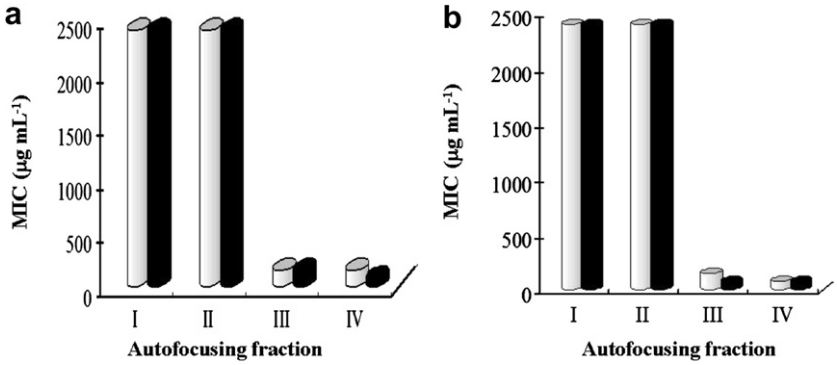

Fig. 2. Minimum inhibitory concentration (MIC) of autofocusing fractions obtained from rennet (■) and pepsin (□) lactoferrin hydrolysates against (a) *E. coli* and (b) *B. subtilis*.

activity than Fr III. The MIC after 48 h for Fr IV against *E. coli* was 75 and 150 μg mL^{-1} for rennet and pepsin LFH, respectively. It was 75 μg mL^{-1} for both rennet and pepsin LFH against *B. subtilis*.

Fr IV was subjected to SEC. The SEC-Fr 1–7 were obtained as shown in Figs. 3a and 4a. In both rennet and pepsin LFH, the SEC-Fr 4 and 5 had the most potent antibacterial activity against both *E. coli* and *B. subtilis* as compared with other fractions (Figs. 3b and 4b). In rennet LFH, an additional fraction (SEC-Fr 2) exhibited antibacterial activity (Fig. 3b).

The active peptides in the SEC-Fr were purified by RP-HPLC. The SEC-Fr 2 obtained from rennet digestion gave a broad peak (Fig. 5a), which was divided into 3 fractions (A, B & C) and their antibacterial activity was examined. Antibacterial activity was observed only in fraction C. After reduction, alkylation and rechromatography, four major sharp peaks (C1, C2, C3 & C4) were observed as shown in Fig. 5b. The RP-peak C4 was the only peak with antibacterial activity. The sequence analysis revealed that the RP-peak C4 yielded one peptide with a sequence of TRVVWCAVG (Table 2).

For SEC-Fr 4 and 5, the results of the RP-HPLC are shown in Fig. 6 together with that of the authentic 11-residues lactoferricin B (Lf-cin B) fragment. All peaks were collected, combined, and divided into 4 fractions (RP-Fr 1–4). The RP-Fr 3 showed potent antibacterial activity in both rennet and pepsin LFH, while only RP-Fr 4 in rennet LFH showed antibacterial activity. All peaks in the active fractions (RP-Fr 3 and 4) were subjected to antibacterial assay. The peaks with antibacterial activity were marked with letters (A–F) in Fig. 6 and were re-chromatographed for further purification. The peptide in RP-peak A showed antibacterial activity against *B. subtilis* only, while the other purified peptides had antibacterial activity against both *E. coli* and *B. subtilis*. In Table 2, the sequence and MS analyses revealed that pepsin LFH contained the 25-residues- (peak B) and 11-residues Lf-cin B (peak C) fragments, which both had the same antibacterial potency. These results are in agreement with the previous studies carried on porcine pepsin LFH by Nakai et al. (2003). Rennet LFH contained also the same 11-residues Lf-cin B (peak F). Rennin is an endogenous calf enzyme active on cows' milk LF, which may suggest that the 11-residues Lf-cin B fragment is a naturally occurring endogenous antibacterial peptide for the calf.

The full sequences of antibacterial peptides are summarized in Table 2. It is clear that rennet LHF had an 11-residue Lf-cin B identical to that in pepsin LFH, besides some other novel antibacterial peptides that were also identified in RP-peaks C4, D and E. It could be concluded that rennin has a substrate specificity partially different from that of porcine pepsin, and, consequently, generates different antibacterial peptides from LF.

3.3. Antibacterial activity of the identified peptides

The antibacterial activities of the peptides identified were confirmed by using chemically synthesized peptides against *E. coli* and *B. subtilis*. The growth of both bacteria in TSB was inhibited by the four synthetic peptides in a dose-dependent manner with different potency. As shown in Table 3, peptide C/F, the 11-residues of Lf-cin B fragment, showed the highest activity, followed by peptide D. In contrast to the peptides C/F and D, the peptides C4 and E had no bactericidal activity against the bacterial strains tested at concentrations less than 100 μg mL^{-1}.

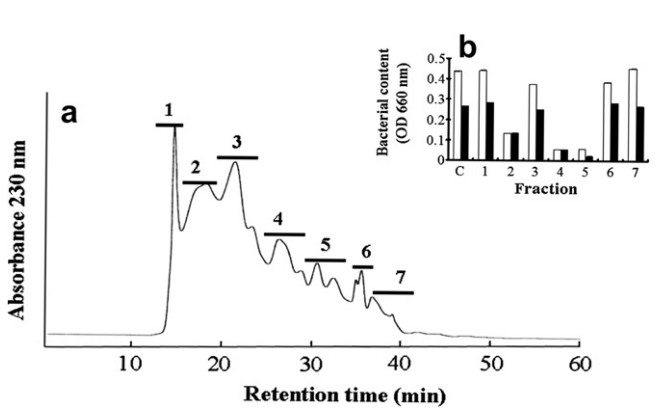

Fig. 3. Size exclusion chromatography (a) of autofocusing Fraction IV of rennet lactoferrin hydrolysates and (b) the antibacterial activity of the resulting fractions against both *E. coli* (□) and *B. subtilis* (■).

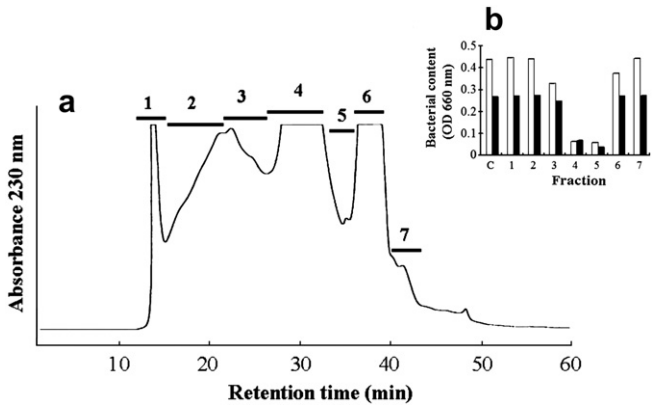

Fig. 4. Size exclusion chromatography (a) of autofocusing Fraction IV of pepsin lactoferrin hydrolysates and (b) the antibacterial activity of resulting fractions against both *E. coli* (□) and *B. subtilis* (■).

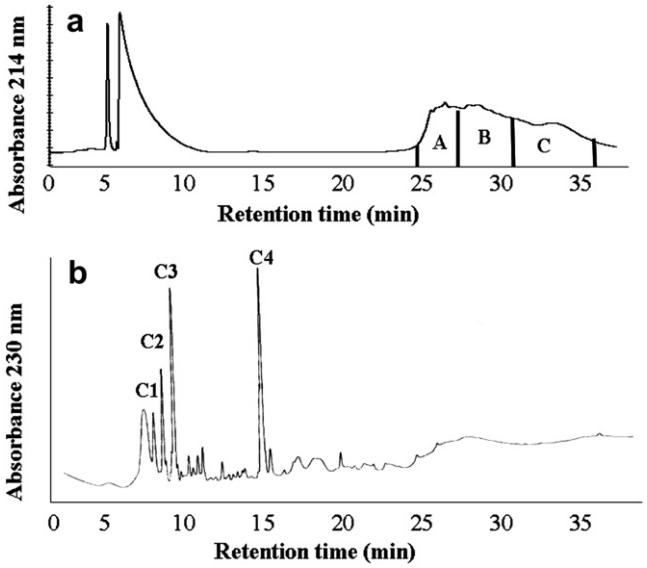

Fig. 5. Separation of (a) the antibacterial peptides from size exclusion chromatography fraction 2 of rennet lactoferrin hydrolysate by reversed phase-high performance liquid chromatography RP-HPLC using an Inertsil ODS-3 (4.6 mm × 250 mm) column (fractions marked A, B and C were collected) and rechromatography (b) of fraction C after reduction of disulphide bond by using Cosmosil 5C18-300 column (peaks marked C1 – C4 were collected).

Table 2
Summary of sequences of antibacterial peptides isolated from autofocusing fraction IV of rennet and pepsin lactoferrin hydrolysates (LFH).

SEC-Fr[a]	RP-Peak[b]	Pepsin LFH	RP-Peak	Rennet LFH
2			C4	TRVVWCAVG
4	A	GRDPYKLRPV	D	KLLSKAQEKFGKNKSRSFQL
	B	FKCRRWQWRMKKLGAP–SITCVRRAF	E	APRKNVRWCTISQPEWFKCR
5	C	RRWQWRMKKLG	F	RRWQWRMKKLG

[a] SEC-Fr: size exclusion chromatography fractions.
[b] RP-Peak: reversed phase-high performance liquid chromatography fractions.

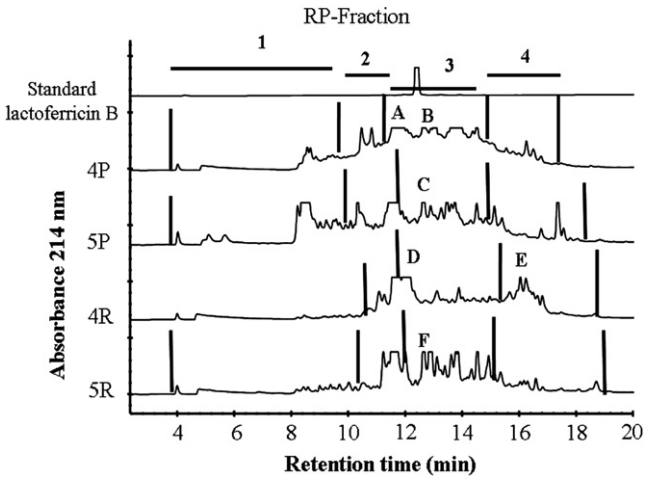

Fig. 6. Reversed phase-high performance liquid chromatography of size exclusion chromatography fractions 4 and 5 of rennet (4R, 5R) and pepsin (4P, 5P) lactoferrin hydrolysates. The fractions collected were termed RP-Fr 1 to RP-Fr 4. The letters A to F refer to the active peaks. Standard lactoferricin B is included for comparison.

Table 3
Antibacterial activity of synthetic peptides against growth of *E. coli* and *B. subtilis* in tryptic soy broth[a].

Peptide[a]	*E. coli*			*B. subtilis*		
	MIC[b]		MBC[c]	MIC		MBC
	24 h	48 h		24 h	48 h	
C/F	25	25	25	6.25	12.5	12.5
C4	100	>100	>100	50	100	>100
D	50	50	100	50	50	100
E	50	100	>100	50	100	>100

[a] Abbreviations are: Peptide C/F, RRWQWRMKKLG (lactoferricin B fragment); peptide C4, TRVVWCAVG; peptide D, KLLSKAQEKFGKNKSRSFQL; peptide E, PRKNVRWCTISQPEWFKCR; MIC, minimum inhibitory concentration (μg mL^{-1} of each peptide); MBC, minimum bactericidal concentration (μg mL^{-1} of each peptide); The values indicated were the mean of three experiments.

The detailed mechanism of actions of antimicrobial LF-derived peptides is unknown, however, it has been suggested that the positive charge of peptides plays a major role in antibacterial activity as most of antibacterial peptides have a basic nature (Epand & Vogel, 1999; Ulvatne et al., 2001). This is in an agreement with the result in the present study as only basic autofocusing fractions showed antibacterial activity (Fig. 2a and b). Moreover, Stark, Liu, and Deber (2002) reported that the hydrophobic core portion of the peptides would be able to interact with a lipid bilayer, causing displacement of lipids and alteration of the membrane structure, resulting in disruption of the bacterial membrane. In the present study, the active peptides were absorbed to RP-HPLC column and eluted with an increasing concentration of acetonitrile, which indicated that these peptides had also a hydrophobic core. As shown in Fig. 6, peptides eluted near the antibacterial peptide did not show antibacterial activity, although these basic and hydrophobic peptides were of similar molecular size. The antibacterial activity presumably depends on primary structure of the peptide. Further study on the relation between antibacterial activity and structure of the peptide is under investigation.

4. Conclusions

The present study demonstrated that the 11-residues Lf-cin B fragment had significant activity against *E. coli* and *B. subtilis*. Rennin also generated an additional peptide with the sequence KLLSKAQEKFGKNKSRSFQL (peptide D) that had significant antibacterial activity (Table 3), while it was not detected in the pepsin digest (Table 2). This peptide may also contribute to the higher antibacterial activity of the rennet LFH.

Rennet digest of lactoferrin was shown to have potential for use as a natural antimicrobial food preservative against both Gram-negative and Gram-positive bacteria. Natural preservatives, like nisin and lysozyme, are known to be active against Gram-positive bacteria only. Also, rennet LFH would be applicable as a substitute for porcine pepsin digest, especially in Islamic communities to fulfill the specific consumer's demand. In addition, basic autofocusing fractions exhibited higher antibacterial activity than the crude digest. Autofocusing does not use harmful chemicals and solvents (Hashimoto, Sato, Nakamura, & Ohtsuki, 2005), therefore, it would be useful to scale-up the potent antibacterial fractions in rennin LFH, even in an industrial setting.

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