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Production of Lactoferricin and Other Cationic Peptides from Food Grade Bovine Lactoferrin with Various Iron Saturation Levels

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Purification of lactoferricin (Lfcin), a cationic antimicrobial peptide, was achieved by peptic digestion of food grade bovine lactoferrin (LF) followed by fractionation on an industrial grade cation exchange resin with stepwise salt gradient elution. The digest and eluted fractions were partially characterized by MALDI-ToF MS and N-terminal sequencing. A fraction eluted using phosphate buffer with 2.0 M NaCl contained predominantly two peptides with masses of 3196 and 3124 Da, which corresponded to the 26- and 25-amino acid peptides FKCRR WQWRM KKLGA PSITC VRRAF (A), containing the Lfcin sequence. Putative sequences of cationic peptides in other eluted fractions included FKNKS RSFQ, WRMKK LGAPS ITCVR RA, and GAPSI TCVRR AFALE CIRAI AEKKA. The iron saturation level of LF had no effect on the production of Lfcin. Nevertheless, the digestion of LF containing lower iron content led to the production of a higher quantity of low molecular weight cationic peptides. A two-step process using industrial grade cation exchange resin led to 35% recovery of Lfcin and also produced other cationic peptides with potential bioactive properties.

KEYWORDS: **Lactoferrin; lactoferricin; cationic peptides; cation exchange chromatography; iron content**

INTRODUCTION

The presence of an antimicrobial peptide sequence in bovine lactoferrin (LF) was first noted when heat treatment of LF increased its antibacterial activity (*1*). It was suggested that at least one bactericidal domain would be released by heating LF at pH 2.0 at 120 °C for 15 min. Heat-treated LF was later fractionated by reverse-phase HPLC, and a peptide fraction with strong activity, namely, lactoferricin (Lfcin), was identified. Peptic digestion of LF at 37 °C also led to similar results (*2*). Since then, a number of production and purification strategies have been conducted for the isolation of Lfcin, including the use of a cation exchange column followed by reverse-phase HPLC (*3*), bead-based cation exchange chromatography on SP-Sepharose Fast Flow resin (*4*), affinity chromatography with an immobilized heparin column (*5*), and chymosin digestion of LF followed by ion exchange membrane filtration and reverse-phase HPLC (*6*). All of these studies were conducted in laboratory scale using laboratory-grade reagents and conditions, producing a small quantity of Lfcin for scientific research purposes.

Pepsin is a nonspecific protease and could cleave at multiple locations on LF. Because LF is a basic protein with a p*I* of 8.69, it is very likely that cationic peptides other than Lfcin are concurrently produced during the peptic digestion. Furthermore, LF is an iron-binding protein that is 20−25% saturated with iron under normal and healthy physiological conditions. The binding of iron to lactoferrin is an important factor in the thermal stability of its structure. Hadden et al. (*7*) observed that the removal of iron from human LF led to increases in the extent of $^{1}H-^{2}H$ exchange and postulated a “loosening” of human LF upon iron removal. Likewise, Paulsson et al. (*8*) reported that iron-saturated (holo-) bovine lactoferrin was more resistant to heat-induced changes than was the iron-depleted (apo-) LF. However, the potential effect of various iron levels on the profile of peptides produced upon peptic digestion has not been previously reported.

The objectives of this study were to develop an isolation strategy with potential for industrial scale production of Lfcin, to identify other cationic peptides generated during the peptic digestion of LF, and to investigate the effects of initial iron saturation levels of LF on the composition of the resultant peptide mixture produced by peptic digestion.

MATERIALS AND METHODS

Preparation of Lactoferrin with Various Iron Saturation Levels. Food-grade bovine lactoferrin (LF, a gift from DMV International Nutritional, Fraser, NY) was used to prepare samples with different levels of iron saturation on the basis of previously described methods (*9*−*11*). Apo-lactoferrin (apo-LF, iron-depleted) was prepared by dialyzing LF against 0.1 M citric acid at pH 2.0. Holo-lactoferrin (holo-LF, iron-saturated) was prepared by adding a 10-fold molar excess of

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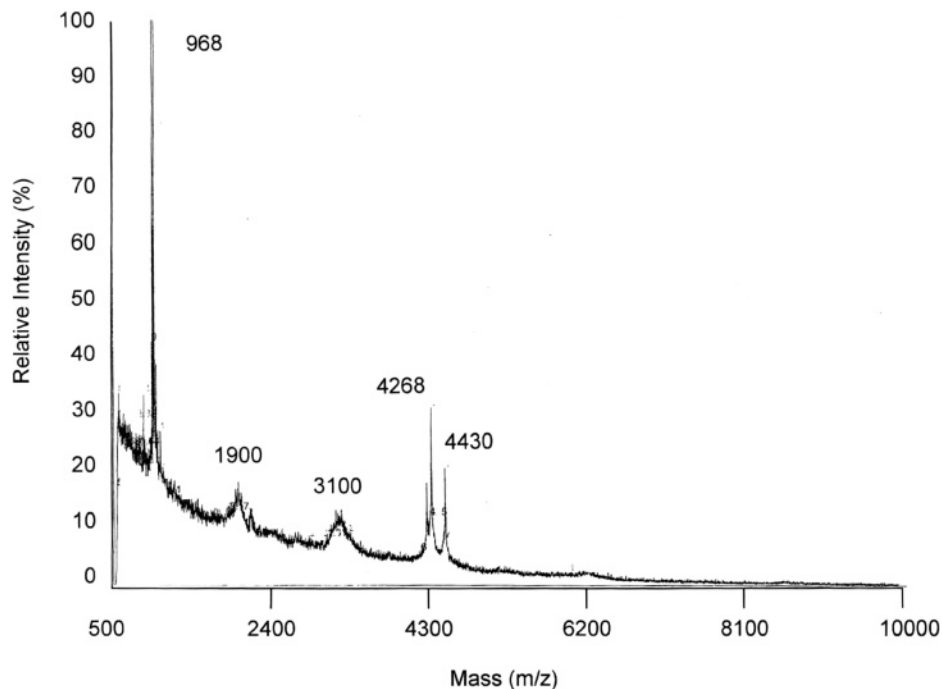


Figure 1. MALDI-ToF mass spectrum of LF digest produced by peptic digestion for 4 h at 37 °C at pH 2.0.

$FeCl_3$ to LF. Excess citric acid or iron was then removed by exhaustive dialysis against double-distilled water. All dialyses were conducted overnight at 4 °C until there was no change in the conductivity of the external buffer solution.

Digestion of Lactoferrin. Peptic digestion of LF was based on methods previously described (*3*, *12*). The LF (5%, w/v) was digested with porcine pepsin (3% of LF, 2500−3500 units mg^{-1} of protein, Sigma Product P7012, Sigma-Aldrich, St. Louis, MO) at pH 2.0 using 2.0 (adjusted using 2.0 M HCl) and 37 °C for 4 h. The digestion was terminated by adjusting the pH to 7.0 using 2.0 M NaOH and heating at 80 °C for 15 min. The resultant digest was centrifuged at 15000*g* for 20 min at 4 °C, and the supernatant was stored at −25 °C until further analysis.

Purification of Lactoferricin and Other Peptides. The supernatant from the peptic hydrolysate was thawed at 4 °C and applied at a flow rate of 0.25 mL min^{-1} to a column packed with the industrial grade, carboxylic acid cation exchanger Purolite C-106 EP (Purolite, Bala Cynwyd, PA), which was pre-equilibrated with 50 mM sodium phosphate buffer (PB) at pH 6.5. A total of 0.50 g of LF digest was applied to 10 mL of the cation exchange resin. After unbound peptides had been washed off with PB at 1.0 mL min^{-1}, bound (cationic) peptides were eluted with PB containing a stepwise gradient of 0.2, 0.5, 0.8, 1.0, and 2.0 M NaCl, at 1.0 mL min^{-1}. The fractionation process was monitored by measuring the conductivity and UV absorbance at 280 nm of the eluant. Fractions were lyophilized and stored at −25 °C.

Peptide Mass Analysis. The whole peptic hydrolysate as well as fractions separated by cation exchange chromatography were analyzed after desalting ($ZipTip_{C18}$, Millipore, Bellerica, MA) by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) using an Applied Biosystems Voyager System (Voyager-DE STR Biospectrometry, AME Bioscience Ltd., London, U.K.) under the following conditions: voltage, 20000 V; laser intensity, 2500; laser rep rate, 20.0 Hz; calibration matrix, α-cyano-4-hydroxycinnamic acid. Post source decay following MALDI-ToF was performed to obtain peptide sequence information. Peptide mass analyses were performed at the LMB-BL Proteomic Core Facility, The University of British Columbia.

Identification of Peptide Sequences. Peptide sequences were identified using the FindPept software tool (http://ca.expasy.org/tools/findpept.html) (*13*).

Iron Content in Lactoferrins. Iron content of lactoferrin solutions containing different iron saturation levels was determined using inductively coupled plasma-mass spectroscopy (ICP-MS) conducted by Elemental Research Inc., North Vancouver, Canada.

Protein Structure by Raman Spectroscopy. Raman spectra of each LF sample (10% w/v, adjusted to pH 2.0) were measured using a laser Raman spectrometer (model NR-1100, JASCO Inc., Tokyo, Japan) with excitation from the 488 nm line of an argon ion laser (Coherent Innova 70C series, Coherent Laser Group, Santa Clara, CA, cooled with the Coherent Laser Pure heat exchanger system). The Raman scattering of samples placed in hematocrit capillary tubes in a transverse arrangement (capillary held horizontally and incident laser beam perpendicular to the capillary axis) was measured under the following conditions: laser power, 200 mW; slit height, 4 mm; spectral resolution, 5 cm^{-1} at 19000 cm^{-1}; sample speed, 120 cm^{-1} min^{-1} with data collected at every cm^{-1}. Spectra from at least six scans were averaged for each duplicate sample. Secondary structure composition of LFs was estimated by applying the algorithm of Williams (*14*) to analyze the Raman spectra in the amide I region, using the RSAP program (version 2.1) of Przybycien and Bailey (*15*).

RESULTS

Production of Lactoferricin and Other Cationic Peptides from Lactoferrin. Pepsin digestion of food-grade LF resulted in a mixture of peptides with a wide range of masses (**Figure 1**). A peptide with a mass of 968 Da was the most dominant product, followed by peptides with masses of 4430 and 4268 Da. Peptides with masses around 3100 and 1900 Da were also detected as broad peaks by MALDI-ToF analysis.

The cation exchange chromatogram of pepsin-digested LF (**Figure 2**) shows that most of the LF digestion products passed through without binding to the industrial-grade cation exchange resin and were recovered in the flow-through fraction eluted with phosphate buffer at pH 6.5. The majority of the bound LF digestion products were eluted using buffers containing 0.2, 0.5, and 0.8 M NaCl. Elutions with 1.0 and 2.0 M NaCl in PB released more tightly bound peptides from the column.

A 965 Da peptide appeared to be the most dominant peptide found in the flow-through fraction as revealed on a MALDI-ToF mass spectrum (**Figure 3a**). Peptides with masses of 4275 and 4433 Da were also detected in the flow-through fraction eluted with phosphate buffer at pH 6.5. The MALDI-ToF mass

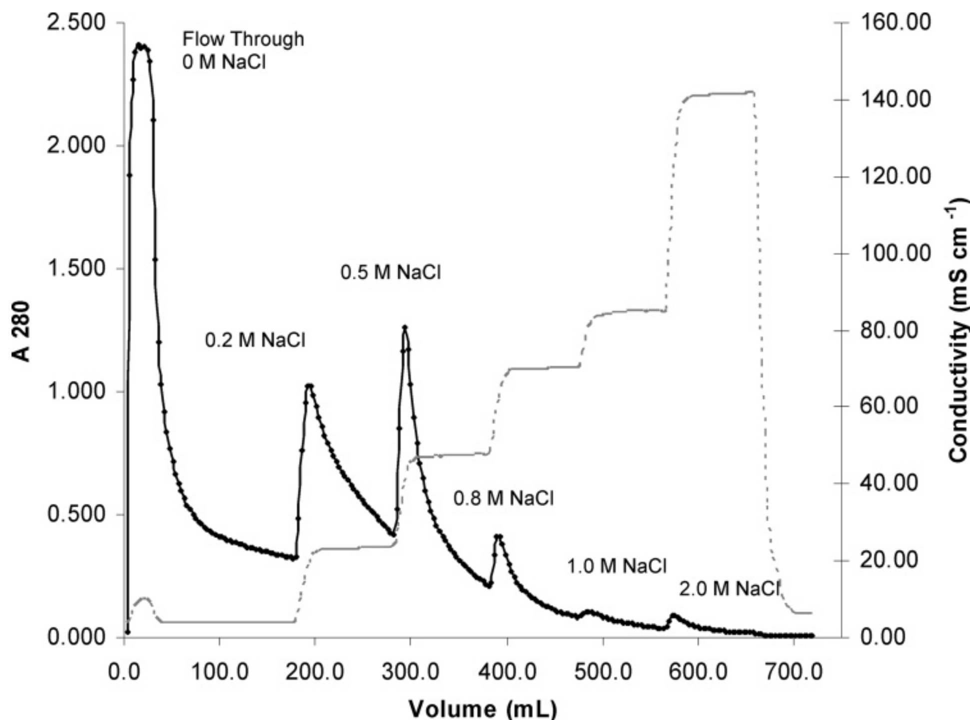


Figure 2. Purolite C-106 cation exchange chromatography of peptic LF digest (solid line, absorbance at 280 nm; dotted line, conductivity). The supernatant of a 0.5 g of peptic LF hydrolysate was loaded onto a 10 mL column and eluted with 50 mM sodium phosphate buffer (pH 6.5) containing 0, 0.2, 0.5, 0.8, 1.0, or 2.0 M of NaCl at a flow rate of 1.0 mL/min. Fraction peaks were collected and further analyzed.

spectrum of the 0.2 M NaCl fraction showed components similar to those of the flow-through fraction (**Figure 3b**); however, the peptides with masses of 4268 and 4430 Da were more dominant constituents in the 0.2 M NaCl fraction, compared to the peptide at 965 Da, and peptides with masses near 2700 Da also became noticeable in this fraction. The addition of 0.5 M NaCl to PB eluted a peptide mixture with a very different mass profile (**Figure 3c**). In addition to the 4268 and 4430 Da peptides that were previously detected in other fractions, broad peaks near 1971 and 3196 Da were observed, indicating that many peptides with masses near 1971 and 3196 Da were present in the fraction. The 0.8 M NaCl eluate contained peptides with masses close to 3196 Da. In addition, peptides with lower masses (1971 and 2675 Da) were also noted (**Figure 3d**). Peptides with masses of 1052, 1971, 3196, 4275, and 4430 Da were found in the 1.0 M NaCl fraction (**Figure 3e**). Peptides with masses of 3124 and 3196 Da dominated the 2.0 M NaCl fraction (**Figure 3f**). These masses correspond to the 25- and 26-mer of Lfcin, residues 17−41 or 42 of LF, respectively (*4*).

Two-Step Process for Lactoferricin Isolation. The aforementioned results indicated that the peptides of interest, Lfcin with a mass of 3124 or 3196 Da, were eluted by PB containing high NaCl levels, suggesting that Lfcin was highly cationic and bound strongly with the cation exchange resin. Hence, a large amount of LF hydrolysate was loaded to exceed the column capacity to allow a competitive displacement of the highly cationic Lfcin against other peptides in the LF digest. LF peptic hydrolysate from 5.0 g of LF was applied to 5.0 mL of cation exchange resin and fractionated using conditions described in the previous section, except that only PBs containing 1.0 and 2.0 M NaCl were used to elute Lfcin. **Figure 4a** shows the elution profile of the fractionation. A large amount of peptides passed through the column as LF hydrolysate was being loaded onto the column. These peptides include those that passed through the column without binding to the cation exchange resin, as well as some that might have formed weak bonds with the resin and were later displaced by other stronger cationic peptides in the digest. Peptides that formed stronger bonding with the cation exchange resin were later eluted off the column by 1.0 and 2.0 M NaCl in PB.

Panels **b−d** of **Figure 4** illustrate the MALDI-ToF mass spectra of fractions eluted at various points in time. Toward the end of the sample loading, the flow-through fraction eluted with phosphate buffer at pH 6.5 contained primarily peptides with masses around 3200 and 4200 Da (**Figure 4b**). Subsequent elution using PB buffer containing 1.0 M NaCl produced a mixture of peptides with masses of 3196 and 3124 Da, along with lower levels of peptides with masses around 1970 and 1053 Da (**Figure 4c**). The MALDI-ToF mass spectrum of the 2.0 M NaCl fraction (**Figure 4d**) shows that the fraction was predominantly composed of peptides of masses of 3196 and 3124 Da. The signal intensity observed in **Figure 4d** indicates that the purity of Lfcin recovered in the 2.0 M fraction is comparable to the purity (>99%) of the standard used for the calibration of the MALDI-ToF mass spectrometer (data not shown). Assuming that the peptides with masses of 3196 and 3124 Da corresponded to the 26- and 25-mers of Lfcin, a total of 70 mg (35% of the theoretical yield from 5 g of LF) of highly purified Lfcin was recovered in the 2.0 M NaCl fraction using this purification strategy.

Identification of Lfcin and Other Cationic Peptides. Identification of the fractionated peptides using FindPept software was based on the mass data obtained, cleavage properties of pepsin, digestion conditions, and known sequence information of LF. The results indicated that peptides with masses of 3196 and 3124 Da could represent the 26- and 25-mer peptide segments of residues 17−42 and 17−41, respectively, in LF, with the following sequences: ^{17}FKCRR WQWRM KKLGA PSITC VRRAF^{41} A^{42}. These findings were verified by post source decay performed on the 3196 and 3124 Da peptides detected in the 2.0 M NaCl fraction, yielding many smaller peptides with masses of 122, 445, 485, 488, 2319, 2372,

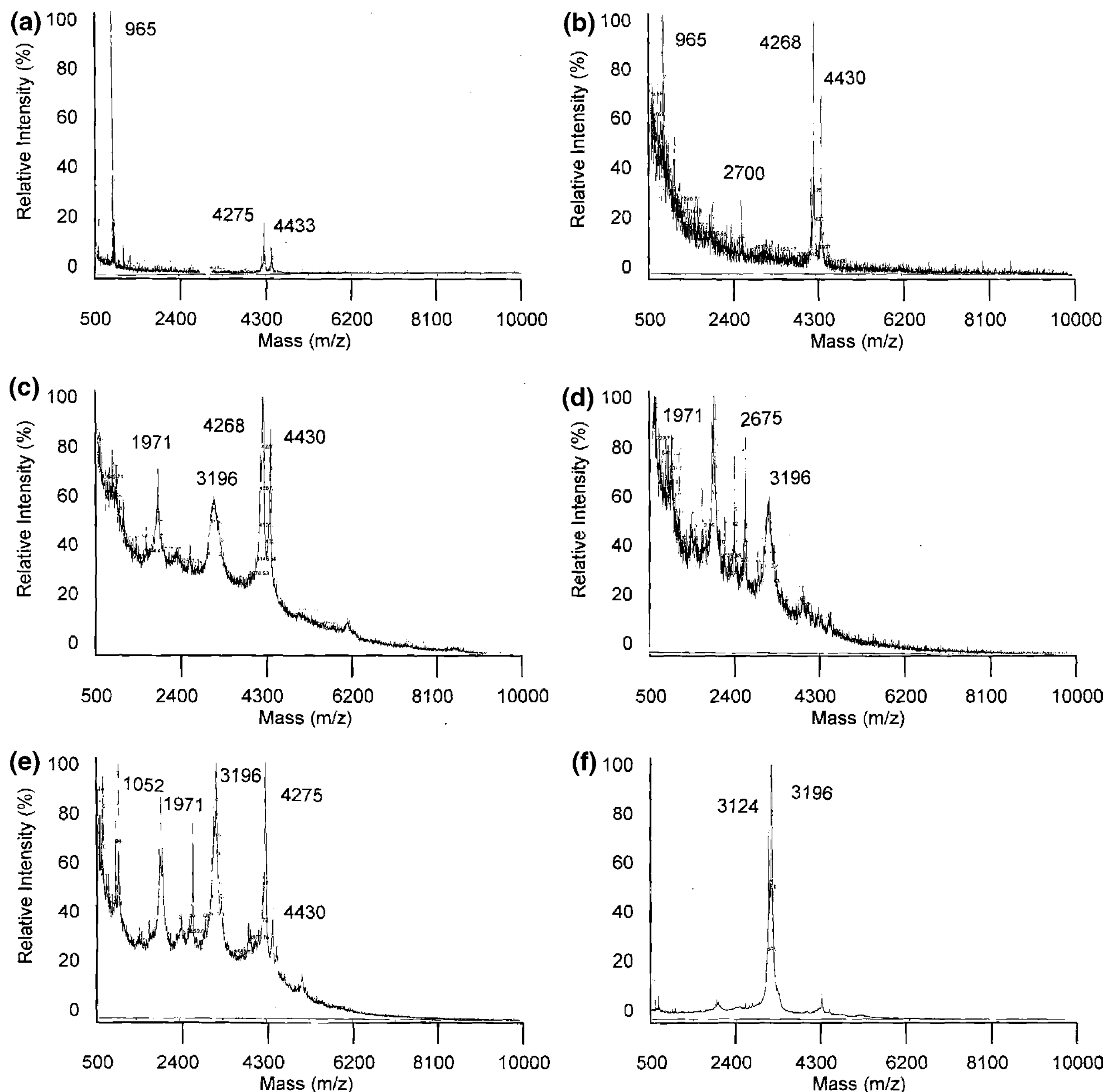


Figure 3. MALDI-ToF mass spectra of LF digest fractions eluted from a 10 mL cation exchange column with Purolite C-106 resin using 50 mL of phosphate buffer at pH 6.5 containing no NaCl (**a**), 0.2 M NaCl (**b**), 0.5 M NaCl (**c**), 0.8 M NaCl (**d**), 1.0 M NaCl (**e**), or 2.0 M NaCl (**f**).

3124, and 3196 Da (**Figure 5a**). These masses correspond to peptide fragments isolated from FKCRR WQWRM KKLGA PSITC VRRAF A (**Figure 5b**).

Table 1 summarizes the sequence information of other possible cationic peptides produced during peptic hydrolysis of LF. Although many of these potentially bioactive peptides are located near the current peptide of interest (lactoferricin), a number of them are at different locations on the lactoferrin sequence. For instance, the MALDI-ToF mass spectrum of 1.0 M NaCl eluant indicated that several peptides with masses of 1052, 1971, 3196, and 4275 Da were present (**Figure 3e**). The PeptIden tool was used to identify potential peptide sequences in LF containing cationic amino acids with high p*I* values and having calculated mass values within ±2 Da of the mass measured by MALDI-ToF MS (**Table 1**).

Effects of Iron Saturation Levels of Lactoferrin. ICP-MS analyses showed the iron saturation level of the LF solution dialyzed against 0.1 M citric acid (apo-LF) was decreased to 16%, compared to 20% in the original untreated LF. When excess Fe^{3+} was added to the LF (holo-LF), iron saturation was increased to 350%. Structural analyses of the LFs determined by Raman spectrometry show that the Raman amide I band near 1500−1800 cm^{-1} of apo-LF was less intense than the spectra of LF and holo-LF (data not shown). In addition, as determined using the RSAP program, holo-LF contained little α-helical structure accompanied with more β-sheet and overall random content at pH 2.0 (**Table 2**). The Raman spectra and estimated secondary structural content of LF and apo-LF appeared to be similar. Removing some of the Fe from LF decreased the α-helix content from 42 to 34%, increased the β-sheet content from 32 to 47%, and lowered the overall random content from 27 to 19% (**Table 2**).

Results from the MALDI-ToF analyses show that digests of LF (**Figure 3**) and apo-LF contained more peptide fragments with lower masses (<1000 Da) than digest of holo-LF (**Figure 6**). In addition, large quantities of peptides with masses of <1000 Da appeared on the mass spectrum of the digest of apo-LF (**Figure 6**). Similarly, peptides eluted by 1.0 M NaCl buffer

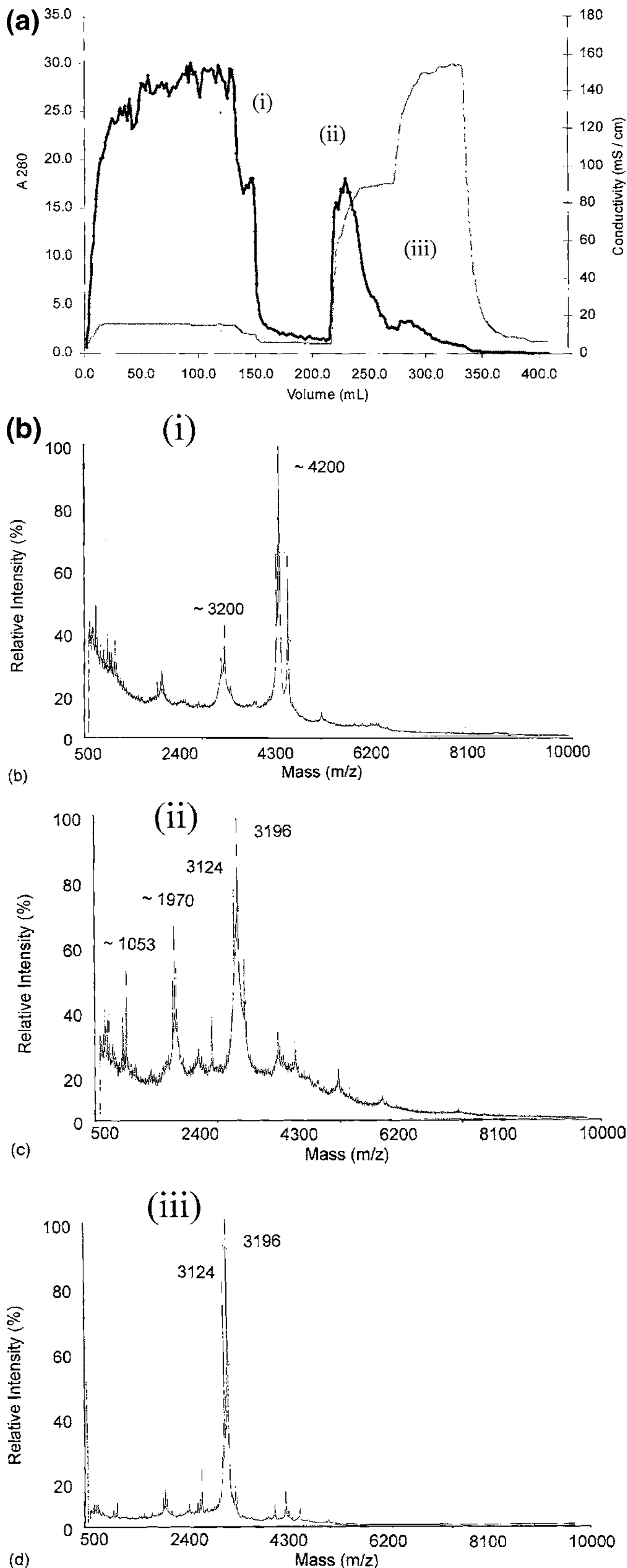
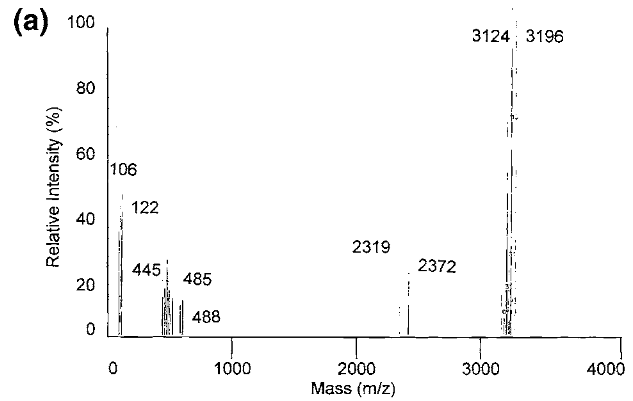


Figure 4. Purolite C-106 cation exchange chromatography in competitive displacement mode of peptic LF digest (**a**, solid line, absorbance at 280 nm; dotted line, conductivity). The supernatant of a 5.0 g of peptic LF digest was loaded onto a 5.0 mL column and eluted with 50 mM sodium phosphate buffer (pH 6.5) containing 0, 1.0, or 2.0 M NaCl (**a**). Fraction peaks were collected and further analyzed by MALDI-ToF [**b**, (i) fraction collected after sample loadingwas completed; (ii) 1.0 M NaCl fraction; (iii) 2.0 M NaCl fraction].



(b)

Mass (Da)	Sequences and Locations
3196	17 - FKCRR WQWRM KKLGA PSITC VRRAF A - 42
106.05	S
122.03	C
122.03	C
444.25	GA PSI
444.25	LGA PS
445.41	KKLG
485.31	KLGA P
488.27	A PSIT
2319.22	CRR WQWRM KKLGA PSITC V
2319.22	QWRM KKLGA PSITC VRRAF A
2372.31	RR WQWRM KKLGA PSITC VR
2372.31	R WQWRM KKLGA PSITC VRR
3124.69	FKCRR WQWRM KKLGA PSITC VRRAF

Figure 5. MALDI-ToF post source decay mass spectrum of peak with mass of 3196 Da (**a**) and possible peptides that resulted from the post source decay (**b**).

from the LF and apo-LF digest also contained more peptide fragments with lower masses (<1000 Da) than those of holo-LF (**Figure 7**). However, the compositions of the 2.0 M fractions produced from LFs with different levels of iron saturation were not very different (**Figure 8**). The 3196 Da peptide remained the most dominant peptide in all three preparations, whereas the 3124 Da peptide was the second most abundant peptide.

DISCUSSION

Production of Lactoferricin and Other Cationic Peptides from Lactoferrin. Peptic digestion of food-grade bovine lactoferrin (LF) produced multiple peptides (**Figure 1**) with various masses because pepsin is an enzyme with low substrate specificity (*18*). Pepsin could cleave at the P1 or P1′ position of Phe, Tyr, Trp, and Leu, corresponding to over 200 possible cleavage sites on LF. Furthermore, its cleavage specificity is lost at pH ≥2.0 (*18*). Slight changes of the pH during the digestion process would result in varying peptic cleavage patterns. Hence, the resulting profile of peptides would vary greatly depending on the digestion conditions used. Under the digestion conditions used in the present study, 5% (w/v) LF solution with 3% (w/w) pepsin at pH 2.0 maintained by 2.0 M HCl and 37 °C for 4 h, an array of peptides with wide ranges of sizes and charge properties were produced. Due to differences in the amino acid composition of these peptides, they carried different charges and were bound to the cation exchange column with different strengths. The use of buffer containing various levels of salt allowed the fractionation of peptides produced

Table 1. Possible Cationic Peptides That Were Produced from the Peptic Digestion of Bovine Lactoferrin at 37 °C for 4 h at pH 2.0[a]

buffer NaCl concn[b]	obsd mass (Da)	calcd mass (Da)	putative peptide sequence[c]	peptide position	p*I*[d]
0.2 and 0.5 M NaCl	4268	4267.94	QG**RK**S CHTGL G**R**SAG WIIPM GIL**R**P YLSWT ESLEP LQGA	110–148	9.31
	4268	4269.83	G**R**DPY **K**L**R**PV AAEIY GT**K**ES PQTHY YAVAV V**KK**GS NFQ	68–105	9.52
	4268	4269.95	QLDQL QG**RK**S CHTGL G**R**SAG WIIPM GIL**R**P YLSWT ESL	105–142	9.31
	4430	4427.95	EEV**K**A **R**YT**R**V VWCAV GPEEQ **KK**CQQ WSQQS GQNVT CATA	336–374	8.02
	4430	4427.95	TAEEV **K**A**R**YT **R**VVWC AVGPE EQ**KK**C QQWSQ QSGQN VTCA	334–372	7.62
0.8 M NaCl	1971	1971.27	AP**RK**N V**R**WCT ISQPE W	1–16	9.51
	1971	1973.43	WRM**KK** LGAPS ITCV**R** **R**A	24–40	11.72
	2675	2675.21	GAPSI TCV**RR** AFALE CI**R**AI AE**KK**A	30–54	9.50
	3162	3162.69	AP**RK**N V**R**WCT ISQPE WF**KCR** **R**WQW	1–24	10.86
	3162	3162.88	KC**RR**W QW**R**M**K** **K**LGAP SITCV **RR**AFA L	18–33	11.84
	3162	3163.64	AVAPN HAVVS **R**SD**R**A AHV**K**Q VLLHQ QALF	590–618	10.84
1.0 M NaCl	1053	1051.17	G**K**N**K**S **R**SFQ	279–287	11.17
	1973	1973.43	WRM**KK** LGAPS ITCV**R** **R**A	24–40	11.72
	2675	2675.21	GAPSI TCV**RR** AFALE CI**R**AI AE**KK**A	30–54	9.50
	3196	3196.90	F**KCRR** WQW**R**M **KK**LGA PSITC V**RR**AF A	17–42	11.84
	3196	3197.68	NSL**K**D **KK**SCH TAVD**R** TAGWN IPMGL IVNQ	447–477	9.20
	3196	3198.63	G**K**N**K**S **R**SFQL FGSPP GQ**R**DL LF**K**DS ALGF	279–307	10.28
	4275	4276.80	SPQTH YYAVA VV**KK**G SNFQL DQLQG **RK**SCH TGLG**R** SAGW	97–125	9.86
2.0 M NaCl	3124	3125.82	F**KCRR** WQW**R**M **KK**LGA PSITC V**RR**AF	17–41	11.84
	3196	3196.90	F**KCRR** WQW**R**M **KK**LGA PSITC V**RR**AF A	17–42	11.84
	3196	3197.68	NSL**K**D **KK**SCH TAVD**R** TAGWN IPMGL IVNQ	447–477	9.20
	3196	3198.63	G**K**N**K**S **R**SFQL FGSPP GQ**R**DL LF**K**DS ALGF	279–307	10.28

[a] Cationic amino acids are indicated in boldface type. [b] In 50 mM phosphate buffer at pH 6.5. [c] As determined using PeptideCutter tool available at http://ca.expasy.org/tools/peptidecutter/ (*16*). [d] As determined using Compute pI/Mw tool available at http://ca.expasy.org/tools/pi_tool.html (*17*).

Table 2. Secondary Structure of Bovine Lactoferrin Preparations (LF) at pH 2.0 As Determined Using Raman Spectroscopy in This Study and Human Lactoferrin (LFH) As Reported in the Literature

	Raman			X-ray[a]		FTIR[b]		
	α-helix	β-sheet	coil	α-helix	β-sheet	α-helix	β-sheet	coil
untreated LF	42	32	27					
apo-LF	34	47	19					
holo-LF	8	57	35					
apo-LFH						43	27	22
holo-LFH				41	24	44	28	21

[a] X-ray data from ref *26*. [b] FTIR data from ref *7*.

from pepsin-digested LF as was shown in **Figure 2**. MALDI-ToF analyses showed that fractions eluted with buffers containing different levels of salt were composed of peptides with differing masses (**Figure 3**). Peptides with a mass of 3196 Da could be fractionated with a PB buffer containing 0.8 M NaCl along with many other peptides with lower molecular masses (**Figure 3d**). The use of PB containing 1.0 M NaCl allowed the purification of the 3196 Da components together with a few other peptides (**Figure 3e**). Subsequently, eluting the cation exchange column with PB with 2.0 M NaCl at pH 6.5 led to the production of a peptide mixture containing two predominant peptides with masses of 3196 and 3124 Da from the digest of food-grade LF (**Figure 3f**); these peptides were verified to be Lfcin using post source decay analysis (**Figure 5**). Lfcin was thus successfully isolated from a peptide mixture using a stepwise salt gradient on an industrial cation exchange resin.

Although the digestion and isolation of Lfcin have been reported in the past, many of these purifications were performed using laboratory-grade reagents resulting in only small quantities of recovered Lfcin. For example, both Dionysius and Milne (*3*) and Hoek et al. (*6*) reported the isolation of Lfcin from peptic digest of bovine LF followed by cation exchange chromatography on a column of S-Sepharose Fast Flow, HPLC using a preparative C18 reverse-phase column, and HPLC on an analytical C18 column. Lfcin was produced at the microgram

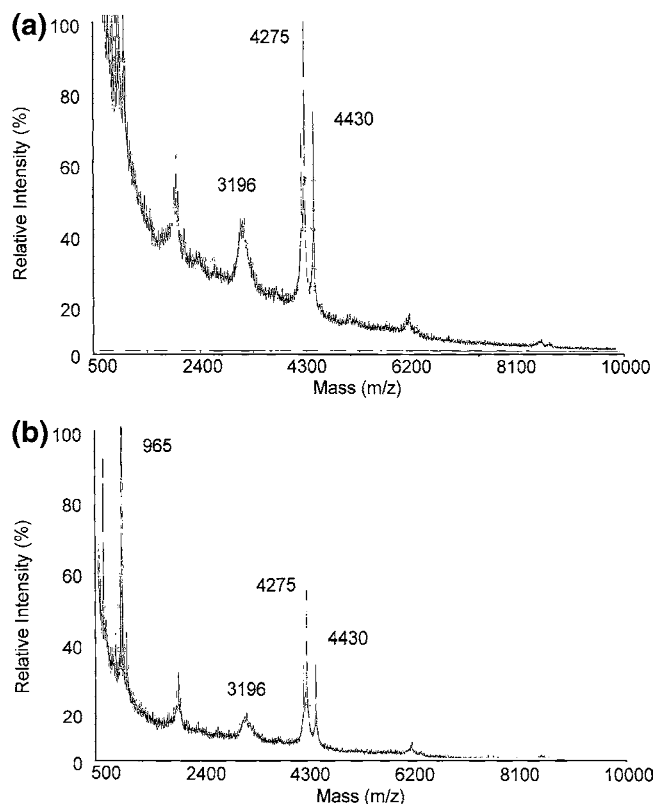


Figure 6. MALDI-ToF mass spectra of lactoferrin digest resulting from peptic digestion of apo-LF (**a**) and holo-LF (**b**).

level following the method described by Recio and Visser (*4*). Affinity chromatography (*5*) was also attempted, but again, only microgram levels of Lfcin were produced.

A patented method (*19*) used 3000 mL of butyl moiety-containing hydrophobic or carboxymethyl moiety-containing cation exchange chromatography medium to purify Lfcin from 600 g of pepsin-digested LF. Lfcin was desorbed from the

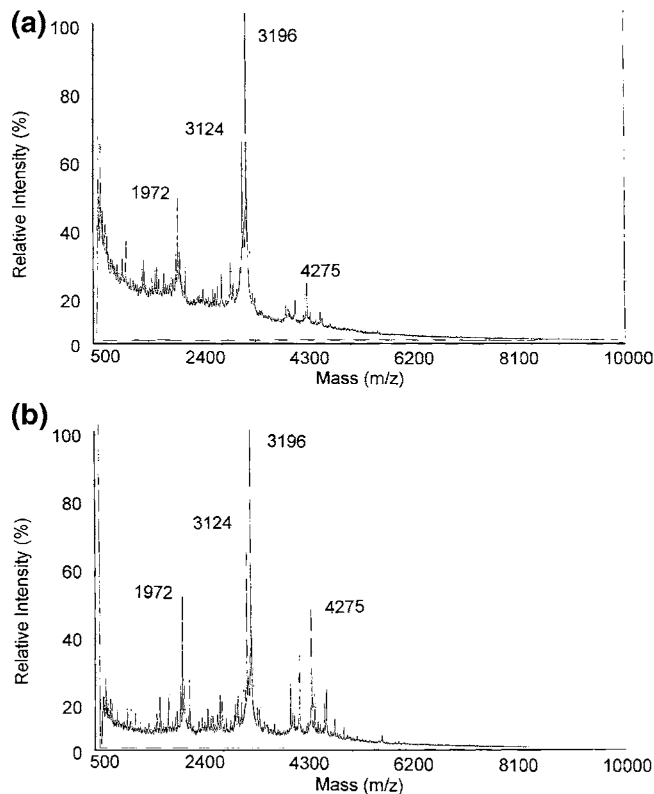


Figure 7. MALDI-ToF mass spectra of 1.0 M NaCl fractions resulting from peptic digestion of apo-LF (**a**) and holo-LF (**b**).

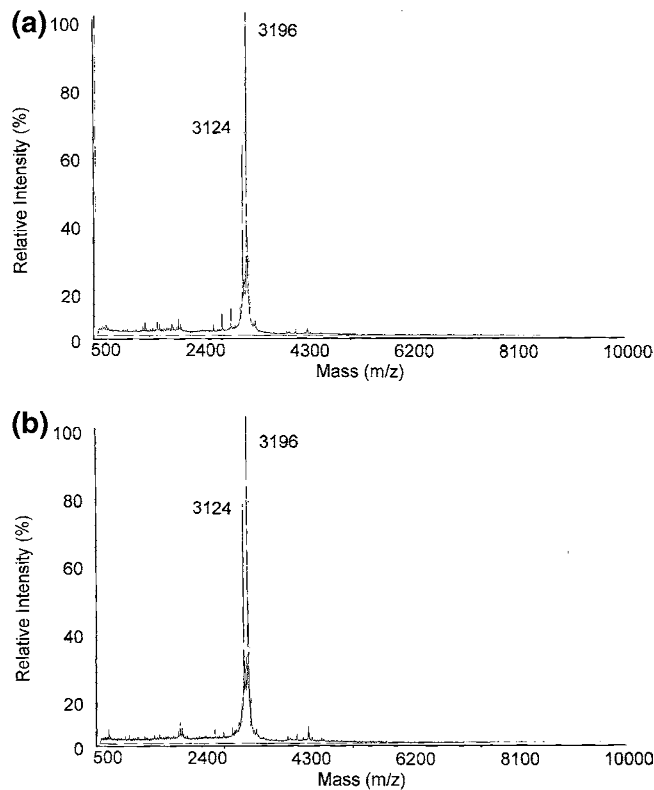


Figure 8. MALDI-ToF mass spectra of 2.0 M NaCl fractions resulting from peptic digestion of apo-LF (**a**) and holo-LF (**b**).

hydrophobic medium using 10 mM HCl along with a mixture of 0.1 M citric acid and 0.2 M Na_2HPO_4 at pH 5.0. When cation exchange chromatography was used, 1−4 M salts were used to elute Lfcin off the medium. Following the methods described, 10.5 g of highly pure (>90%) Lfcin was produced from 600 g of LF, corresponding to approximately 40% of the theoretical yield. In the present study, the concept of competitive displacement chromatography (*20*) was applied to isolate the highly positively charged Lfcin from other cationic peptides in a two-step process. First, pepsin-digested LF was loaded beyond the capacity of the cation exchange column. Because of the high p*I* of Lfcin, other less positively charged peptides were continuously displaced by Lfcin during the loading of the pepsin digested LF, eventually resulting in a cation exchange column loaded primarily with highly purified Lfcin. It was therefore possible to produce highly purified Lfcin in the subsequent elution step. Results from the present study showed that peptic digestion of food-grade LF followed by cation exchange chromatography on industrial-grade resin allowed the production of highly pure Lfcin in milligrams quantity, a 35% recovery of its theoretical yield.

Identification of Other Cationic Bioactive Peptides. LF itself is a cationic protein with antimicrobial activity, and a previous study has shown that the digestion of LF could enhance its antimicrobial functions (*12*). Although it is commonly believed that the enhanced antimicrobial property is mainly due to the release of Lfcin during the digestion, it is possible that other functional peptides may also be released during peptic treatment. In the present study, MALDI-ToF analyses of the peptic digest of food-grade LF showed that a wide range of cationic peptides were indeed released during the digestion.

On the basis of the molecular masses detected by MALDI-ToF and knowledge of the sequence of LF, the potential candidate sequences and positions of these cationic LF peptides were identified. For instance, the mass of the 4268 Da fragment found in the 0.2 and 0.5 M NaCl fractions could be one or more of the following peptides: ^{110}QGRKS CHTGL GRSAG WIIPM GILRP YLSWT ESLEP LQGA^{148} with a p*I* of 9.31, ^{68}GRDPY LKRPV AAEIY GTKES PQTHY YAVAV VKKGS NFQ^{105} with a p*I* of 9.52, or ^{105}QLDQL QGRKS CHTGL GRSAG WIIPM GILRP YLSWT ESL^{142} with a p*I* of 9.31. In fact, all or some of these peptides with similar masses and p*I* values could have been eluted at the same time, as suggested by the broad bases on the MALDI-ToF mass spectra (**Figure 3b**,**c**). The broad 3196 bands appearing in the 0.5, 0.8, and 1.0 M NaCl fractions (**Figure 3c**,**d**,**e**, respectively) could consist of more than one peptide. As demonstrated using the proteomic tools on www.expasy.com, peptic digestion of LF could result in multiple peptides with a similar mass of 3196 Da, but with different p*I* values. Results presented in this study indicated that some of the peptides, with a mass of 3196 Da but lower p*I* than Lfcin, could be eluted by NaCl PB containing lower ionic strengths. Among all of the possible cationic peptides that could be produced from the peptic digestion of bovine LF, many of the putative peptides contain either arginine (R) or lysine (K) and have an overall p*I* >7.0. The presence of these positively charged amino acids, R and K, is a common feature among many bioactive peptides and is responsible for their bioactive functions (*21*). The presence of R and K near the N terminus of some of the peptides is especially important for the antimicrobial properties (*22*). Future research should be conducted to confirm the biological activities of these cationic peptides produced during peptic hydrolysis of food-grade LF.

Effects of Iron Saturation Levels of Lactoferrin. Under normal physiological conditions, bovine LF contains approximately 20−25% iron (*23*). The food-grade LF used in this study was consistent with the reported level and had an iron level of 20% as determined by ICP-MS analysis. At this iron saturation level and at pH 2.0, Raman spectroscopic analyses

showed that approximately 32% of LF folded into β-sheet structures, 41% of LF had α-helical structures, and the rest of the molecule was present as random coil structures (**Table 2**).

After some iron was removed from LF by dialyzing LF against citric acid, apo-LF (16% as determined by ICP-MS) contained less α-helical structure and random coil. Consequently, more β-sheet conformation was found in apo-LF. When LF was saturated with iron, a dramatic drop in the α-helical content with a concurrent increase in the β-sheet structure was detected. The Raman spectroscopic results showed that the presence of iron was important for the overall structural integrity of LF.

Previous data from the literature supported results observed in the present study. The differential scanning calorimetry (DSC) thermogram of native LF revealed two denaturation temperatures at 65 and 92 °C (*8*). However, apo-LF and holo-LF showed prominent thermal denaturation peaks at 71 and 93 °C, respectively. The transition at high temperature (92 °C) indicated the unfolding of the iron-binding domains of LF (*8*). Apo-LF containing 30% Fe also denatured more quickly than holo-LF that was 100% saturated with Fe (*24*).

The binding of iron to human LF has been reported to be an important factor in the stabilization of its structure. Iron-depleted human LF demonstrated an increase in the extent of $^1H-^2H$ exchange, suggesting a "loosening" of human LF upon iron removal (*7*). Using DSC, Mata et al. (*25*) showed significant increases in maximum peak temperature, transition enthalpy, and activation energy of human milk LF that was fully saturated with iron (90.6 °C, 3209 kJ mol^{-1}, and 387.6 kJ mol^{-1}, respectively), compared to the corresponding values for LF that was <15% saturated with iron (67.0 °C, 2276 kJ mol^{-1}, and 275.5 kJ mol^{-1}, respectively). Crystallographic analysis of human LF also showed that the binding of iron to LF caused conformational changes and the molecule became more compact. Iron entered into the open interdomain cleft in each lobe, and then the domains closed over the iron atoms (*26*). This change in lactoferrin structure explained why iron-saturated LF was more resistant to denaturation and proteolysis than the apo form (*27*).

On the basis of results reported in this section, it can be speculated that removal of iron from LF decreased its overall stability and increased accessibility of pepsin to potential cleavage sites on LF. Hence, more cleavages led to the production of a larger amount of low-mass peptide fragments in the digest of apo-LF. In contrast, the addition of iron increased the β-sheet content in holo-LF, making it more resistant to structural changes under the thermal and acidic conditions for peptic digestion. Decreased accessibility of peptic cleavage sites on holo-LF thus may have led to more larger-sized peptide fragments in the digest of holo-LF. These effects of iron saturation levels are deemed to be important when specific peptides are needed. For instance, when peptides of higher masses (e.g., 4065 and 4275 Da) are desired, the addition of Fe^{3+} to the LF solution would allow a higher yield of larger peptides than untreated LF or iron-depleted solutions (**Figure 7**).

Nevertheless, the level of iron saturation of LF did not affect the production and purity of Lfcin. This is probably due to the fact that the Lfcin sequence, residues 17−41 or residues 17−42, is located far from the iron-binding domains of LF (D60−Y92−Y192−H254 and D395−Y433−Y526−H595). Hence, changes in the microenvironment of these two iron-binding sites exert little effect on the structure and stability around residues 17−42. The disulfide bond between C19 and C36 on Lfcin further enhances the stability of this peptide fragment, making it resistant to further peptic digestion regardless of the iron status in the environment.

Furthermore, the 2.0 M NaCl fractions recovered by cation exchange chromatography from the peptic digests of all three LF preparations (untreated LF, apo-LF, and holo-LF) demonstrated similar peptide mass profiles. All other peptides produced during the digestion processes, regardless of the initial iron saturation levels, were eluted by buffers with lower ionic strengths, leaving only the highly cationic Lfcin on the cation exchange resin. When eluted with phosphate buffer containing 2.0 M NaCl, the 26-mer and 25-mer peptides corresponding to Lfcin were the most dominant peptides, resulting in a highly pure Lfcin fraction.

In summary, the production of Lfcin was not affected by the iron status of LF. However, the removal of iron from LF increased the production of small-size peptides, from LF and the addition of iron to LF increased the production of large-size peptides. We also presented an economically feasible isolation and purification strategy for the production of Lfcin from food-grade LF using an industrial-grade cation exchange resin. At the time of the present study, it cost approximately U.S. $450 for each kilogram of LF and U.S. $2000 for each gram of Lfcin. Using the purification strategy described in the present study, 14 g of Lfcin can be produced from each kilogram of LF.

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LITERATURE CITED

(1) Saito, H.; Miyakawa, H.; Tamura, Y.; Shimamura, S.; Tomita, M. Potent bactericidal activity of bovine lactoferrin hydrolysate produced by heat treatment at acidic pH. *J. Dairy Sci.* **1991**, *74*, 3724−3730.

(2) Bellamy, W.; Takase, M.; Wakabayashi, H.; Kawase, K.; Tomita, M. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *J. Appl. Bacteriol.* **1992**, *73*, 472−479.

(3) Dionysius, D. A.; Milne, J. M. Antibacterial peptides of bovine lactoferrin: purification and characterization. *J. Dairy Sci.* **1997**, *80*, 667−674.

(4) Recio, I.; Visser, S. Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin in situ enzymatic hydrolysis on an ion-exchange membrane. *J. Chromatogr. A* **1999**, *831*, 191−201.

(5) Shimazaki, K.; Tazume, T.; Uji, K.; Tanake, M.; Kumura, H.; Mikawa, K.; Shimo-Oka, T. Properties of a heparin-binding peptide derived from bovine lactoferrin. *J. Dairy Sci.* **1998**, *81*, 2841−2849.

(6) Hoek, K. S.; Milne, J. M.; Grieve, P. A.; Dionysius, D. A.; Smith, R. Antibacterial activity of bovine lactoferrin-derived peptides. *Antimicrob. Agents Chemother.* **1997**, *41*, 54−59.

(7) Hadden, J. M.; Bloemendal, M.; Haris, P. I.; Srai, S. K. S.; Chapman, D. Fourier transform infrared spectroscopy and differential scanning calorimetry of transferrrin: human serum transferrin, rabbit serum transferrin and human lactoferrin. *Biochim. Biophys. Acta* **1994**, *1205*, 59−67.

(8) Paulsson, M. A.; Svensson, U.; Kishore, A. R.; Naidu, S. Thermal behavior of bovine lactoferrin in water and its relation to bacterial interaction and antibacterial activity. *J. Dairy Sci.* **1993**, *76*, 3711−3720.

(9) Nagasako, Y.; Saito, H.; Tamura, Y.; Chimamura, S.; Tomita, M. Iron-binding properties of bovine lactoferrin in iron-rich solution. *J. Dairy Sci.* **1993**, *76*, 1876−1881.

(10) Marchetti, M.; Superti, F.; Ammendolia, M.; Rossi, P.; Valenti, P.; Seganti, L. Inhibition of poliovirus type 1 infection by iron-, manganese- and zinc-saturated lactoferrin. *Med. Microbiol. Immunol.* **1999**, *187*, 199−204.
(11) Moore, S. A.; Anderson, B. F.; Groom, C. R.; Maridas, M.; Baker, E. N. Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution. *J. Mol. Biol.* **1997**, *274*, 222−236.
(12) Tomita, M.; Bellamy, W.; Takase, M.; Yamauchi, K.; Wakabayashi, H.; Kawase, K. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J. Dairy Sci.* **1991**, *74*, 4137−4142.
(13) Gattiker, A.; Bienvenut, W. V.; Bairoch, A.; Gasteiger, E. *FindPept,* a tool to identify unmatched masses in peptide mass fingerprinting protein identification. *Proteomics* **2002**, *2*, 1435−1444.
(14) Williams, R. W. Estimation of protein secondary structure from the laser amide I spectrum. *J. Mol. Biol.* **1983**, *152*, 783−813.
(15) Przybycien, T. M.; Bailey, J. C. Structure-function relations in the inorganic salt-induced precipitation of α-chymotrypsin. *Biochim. Biophys. Acta* **1989**, *995*, 231−245.
(16) Gasteiger, E.; Hoogland, C.; Gattiker, A.; Duvaud, S.; Wilkins, M. R.; Appel, R. D.; Bairoch, A. Protein identification and analysis tools on the ExPASy server. In *The Proteomics Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 2005; pp 571−607.
(17) Bjellqvist, B.; Hughes, G. J.; Pasquali, C.; Paquet, N.; Ravier, F.; Sanchez, J.-C.; Frutiger, S.; Hochstrasser, D. F. The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* **1993**, *14*, 1023−1031.
(18) Keil, B. *Specificity of Proteolysis*; Springer-Verlag: Berlin, Germany, 1992.
(19) Tomita, M.; Shimamura, S.; Kawase, K.; Fukuwatari, Y.; Takase, M.; Bellamy, W.; Hagiwara, T.; Matukuma, H. A process for large-scale production of antimicrobial peptide in high purity. Canadian Intellectual Property Office, CA 2070882, 1999.
(20) Li-Chan, E.; Kwan, L.; Nakai, S. Isolation of immunoglobulins by competitive displacement of cheese whey proteins during metal chelate interaction chromatography. *J. Dairy Sci.* **1990**, *73*, 2075−2086.
(21) Lehrer, R. I.; Ganz, T. Cathelicidins: a family of endogenous antimicrobial peptides. *Curr. Opin. Hematol.* **2002**, *9*, 18−22.
(22) Nakai, S.; Chan, J.; Li-Chan, E.; Dou, J.; Ogawa, M. Homology similarity analysis of sequences of latoferricin and its derivatives. *J. Agric. Food Chem.* **2003**, *51*, 1215−1223.
(23) Shimazaki, K.-I. Lactoferrin: a marvelous protein in milk? *Anim. Sci. J.* **2000**, *71*, 329−347.
(24) Sánchez, L.; Peiró, J. M.; Castillo, H.; Pérez, M. D.; Ena, J. M.; Calvo, M. Kinetic parameters for denaturation of bovine milk lactoferrin. *J. Food Sci.* **1992**, *57*, 873−879.
(25) Mata, L.; Sánchez, D.; Headon, D. R.; Clavo, M. Thermal denaturation of human lactoferrin and its effect on the ability to bind iron. *J. Agric. Food Chem.* **1998**, *46*, 3964−3970.
(26) Anderson, B. F.; Baker, H. M.; Norris, G. E.; Rice, D. W.; Baker, E. N. Structure of human lactoferrin: crystallographic structure analysis and refinement at 2.8 Å resolution. *J. Mol. Biol.* **1989**, *209*, 711−734.
(27) Anderson, B. F. Apolactoferrin structure demonstrates ligand-induced conformational change in transferrins. *Nature* **1990**, *344*, 784.

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