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Antioxidant Activities of Buttermilk Proteins, Whey Proteins, and Their Enzymatic Hydrolysates

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ABSTRACT: The oxygen radical absorbance capacities (ORAC) and metal chelating capacities (MCC) of protein concentrates prepared from buttermilk and cheese whey by ultrafiltration were compared with those of skim milk protein. Samples were also heat-denatured and hydrolyzed by pepsin for 2 h followed by trypsin for 3 h. The highest MCC was obtained for hydrolyzed skim milk protein. ORAC values ranged from 554.4 to 1319.6 μmol Trolox equivalents/g protein, with the highest value obtained for hydrolyzed buttermilk protein. Liquid-phase isoelectric focusing (IEF) of this hydrolysate yielded peptide fractions with lower ORAC values. LC-MS analysis of the hydrolyzed skim milk and buttermilk proteins and IEF fractions of the latter showed that peptides derived from milk fat globule membrane proteins, primarily butyrophilin, could be responsible for the superior antioxidant activity of buttermilk. These results suggest overall that hydrolyzed buttermilk protein could be used as a source of natural antioxidants.

KEYWORDS: *buttermilk, cheese whey, enzymatic hydrolysates, antioxidant activity, free radical scavenging activity, metal chelating capacity, bioactive peptides, mass spectrometry*

INTRODUCTION

Buttermilk, the byproduct of the butter-making process, has an overall composition and appearance very similar to that of skim milk. Its principal difference from skim milk is that of being richer in milk fat globule membrane (MFGM) components. For this reason, buttermilk solids have attracted attention as a source of high added-value ingredients over the past decade.[1−4] Despite this growing interest in buttermilk components such as MFGM proteins, their actual potential as ingredients with health benefits remains uncertain.

The antioxidant properties of peptides released during enzymatic hydrolysis of major milk proteins are well recognized.[5] Consisting generally of 3−20 amino acid residues, these antioxidant peptides can inhibit lipid or protein oxidation by any of several mechanisms,[6] of which the best known are scavenging of free radicals and chelation of pro-oxidative metal ions.[7]

The aim of the present study was to evaluate the antioxidant activity of buttermilk and cheese whey proteins hydrolyzed sequentially with pepsin and then trypsin and of peptides obtained from such hydrolysates using liquid-phase isoelectric focusing (IEF). Skim milk was used as a control. The proteins were first concentrated using a pilot-scale ultrafiltration unit, and the impact of thermal denaturing on the antioxidant activity was also studied. Antioxidant activity was determined using two different chemical assays, namely, the oxygen radical absorbance capacity−fluorescein ($ORAC_{FL}$) method, used to evaluate free radical scavenging activity, and ferrous ion binding measurements to assess the transition metal ion chelating capacity (MCC). Finally, potentially antioxidant peptides were identified using liquid chromatography coupled with mass spectrometry (LC-MS).

MATERIALS AND METHODS

Dairy Products and Reagents. Fresh buttermilk and fresh whey (the latter from mozzarella cheese production) were obtained from a local dairy (L'Ancêtre, Bécancour, Canada). Skim milk powder was purchased at a local retail outlet. Porcine pepsin (EC 3.4.23.1, 2500−3500 U/mg protein), bovine trypsin (EC 3.4.21.4, type I, 10000 BAEE U/mg protein), *o*-phthaldialdehyde (OPA), 2,2′-azobis(2-methylpropionamide) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ferrous chloride, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,3-triazine (Ferozine), and Na_2EDTA were purchased from Sigma-Aldrich (Oakville, Canada). The Lactose/D-Galactose test kit was obtained from R-Biopharm (Darmstadt, Germany). All SDS-PAGE reagents were purchased from Bio-Rad (Mississauga, Canada).

Preparation of Buttermilk and Cheese Whey Concentrates. Fresh buttermilk and cheese whey were skimmed at 37 °C using a milk separator (Alpha-Laval, Lund, Sweden). Cheese whey was then microfiltered (MF) with cocurrent recirculation of permeate using a Bactocatch module (TetraPak, Lund, Sweden) containing a 1.4 μm ceramic membrane (Membralox, Mississauga, Canada). Microfiltered cheese whey and skim buttermilk were then concentrated to 9.4% (w/v) protein using a spiral wound UF membrane (MWCO 5 kDa, Koch Filtration Systems, Wilmington, MA, USA). Part of the UF retentate was freeze-dried, and the resulting powders were stored frozen (−35 °C) until further analysis, whereas the other part was frozen (−35 °C) until the denaturing step. The above processing procedure was repeated on two separate batches of buttermilk and whey, and the replicates were pooled to minimize the batch effect.

Denaturing of the Dairy Proteins. The pH of UF-concentrated buttermilk and whey (both dissolved at 9.4% w/v in 2.0 L of distilled water) was adjusted to 4.6 with 6 N HCl, and the solutions were then heated to 90 °C with constant stirring for 30 min. The heat-treated

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Table 1. Sample Composition and Degree of Hydrolysis (DH) during Treatment with Pepsin (2 h) followed by Trypsin (5 h)

	dry matter[a] (%)				DH[a,b] (%)	
sample[c]	protein	lactose	lipid	ash	2 h	5 h
BC	54.6 ± 0.2	28.0 ± 0.3	14.9 ± 0.3	6.0 ± 0.0	1.7 ± 0.1 d	7.5 ± 0.1 c
DBC	54.4 ± 0.1	19.9 ± 0.3	13.4 ± 0.4	6.0 ± 0.0	2.5 ± 0.1 c	6.4 ± 0.3 d
WC	74.5 ± 0.2	10.2 ± 0.3	7.8 ± 0.1	1.7 ± 0.0	2.0 ± 0.3 cd	8.2 ± 0.5 c
DWC	75.9 ± 0.1	8.6 ± 0.0	6.9 ± 1.5	1.7 ± 0.0	10.1 ± 0.5 a	17.1 ± 0.6 a
SM	37.2 ± 0.0	54.2 ± 0.6	1.0 ± 0.0	8.4 ± 0.1	3.2 ± 0.3 b	10.1 ± 0.2 b

[a]Data are the mean ± SD of three independent experiments ($n = 3$). [b]Different letters within a column indicate significant difference ($p < 0.05$). [c]BC, buttermilk concentrate; DBC, denatured buttermilk concentrate; WC, whey concentrate; DWC, denatured whey concentrate; SM, skim milk.

suspensions were then passed five times at 65.5 MPa through an Emulsiflex-C50 device (Avestin, Canada) to homogenize the denatured proteins and then freeze-dried. The powders thus obtained were stored frozen (−35 °C). The denaturing procedure was performed three times, and the replicates were pooled to minimize the batch effect.

In Vitro Enzymatic Hydrolysis. The procedure described in Conway et al.[3] was used. Briefly, 1 kg batches of protein solution (5% w/w in gastric salts solution) containing pepsin (enzyme to substrate mass ratio of 1:100) were stirred at constant speed for 2 h at 37 °C and pH 2.0 (adjusted with 1 N HCl). The pH was then adjusted to 8.0 with 1 N NaOH, and trypsin (enzyme to substrate mass ratio of 1:250) was added for an additional 3 h of hydrolysis. The enzymes and nonhydrolyzed proteins in the final hydrolysates (5 h) were removed by UF (30 kDa MWCO) followed by diafiltration. All hydrolysates were freeze-dried and kept at −35 °C for further analysis.

Fractionation of the Buttermilk Hydrolysate by Isoelectric Focusing. UF-processed hydrolyzed buttermilk protein (49.9% w/w protein) was further fractionated using a Rotofor liquid-phase IEF cell (Bio-Rad, Hercules, CA, USA) as described by Groleau et al.,[8] with some modifications. The protein was dissolved in distilled water (1.25% w/v) at room temperature with constant stirring for 30 min. Each run (3.5 h) was carried out at constant power (12 W) and temperature (4 °C). In the course of each run, 20 peptide fractions were collected. These were pooled into three pH ranges, namely, F1 (pH <4.0), F2 (pH 4.0−8.0), and F3 (pH >8.0). The pooled fractions were freeze-dried and stored frozen (−35 °C). The procedure was repeated until sufficient powder was obtained to perform further analysis. The protein contents of F1, F2, and F3 were found to be 51.6, 65.3, and 38.5% (w/w), respectively, using the Dumas combustion method as described below.

Chemical Analyses. Moisture and ash contents were determined gravimetrically by drying at 100 °C for 5 h in a drying oven, followed by incineration in an ashing oven at 550 °C for 18 h. Nitrogen content based on the Dumas combustion method[9] was determined using an FP-528 Leco apparatus (Leco Corp., St. Joseph, MI, USA) with an EDTA sulfate (99.99% pure, w/w) standard curve. Nitrogen was expressed as protein using a conversion factor of 6.38. Lipids were extracted by using the Mojonnier gravimetric extraction method,[10] and the lactose content was measured using an enzymatic method.[11] The degree of protein hydrolysis (DH) was based on α-amino group titration using the *o*-phthaldialdehyde spectrophotometric method.[12] Samples were removed before enzyme was added (0 h) and at the end of the peptic (2 h) and tryptic (5 h) treatments. These analyses were performed in triplicate ($n = 3$).

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles before and during digestion were obtained using 12.5% polyacrylamide gel under reducing conditions as described elsewhere.[13] Individual proteins were identified using a prestained SDS-PAGE MW broad-range standard from Bio-Rad. The protein bands were assigned according to the method of Mather.[14]

Free Radical Scavenging Activity ($ORAC_{FL}$). The ORAC assay with fluorescein was performed according to a method described elsewhere.[15] Briefly, the assay was performed in duplicate on a BMG Fluostar Galaxy microplate reader (Durham, NC, USA) equipped with an incubator and an injection pump, at excitation and emission wavelengths of 485 and 520 nm, respectively. The procedure was conducted at 37 °C and pH 7.0 using Trolox as the standard. Microplate wells each contained 200 μL of fluorescein solution (0.036 mg/L), 20 μL of diluted sample (13 μg/μL), and AAPH (8.6 mg/L) as peroxyl generator. Fluorescence was recorded for 35 cycles of 210 s each. Results are expressed as micromole Trolox equivalents (TE) per gram of protein. Three independent assays were performed for each sample ($n = 3$).

Metal Ion Chelating Capacity (MCC). MCC was measured using ferrous ion binding capacity of our samples based on the method described by Dinis et al.[16] Briefly, 20 μL of 1.2 mM ferrous chloride ($FeCl_2$) was added to 200 μL of sample at different protein concentrations (1, 2, and 4 mg/mL), and the reaction was initiated by adding 20 μL of 2.4 mM ferrozine (i.e., a reducing agent). All solutions were prepared in Milli-Q water, and Na_2EDTA was used as the standard. The mixture was first shaken vigorously for 10 min and then left to stand at room temperature for 10 min. As ferrozine−ferrous ion complexes form a stable magenta color that can be measured at 562 nm, the absorbance decrease was measured at 562 nm with a Multiskan model 1500 microplate reader (Thermolabsystem, Waltham, MA, USA). The MCC (percent), more precisely the inhibition of ferrozine−ferrous ion complex formation (percent), was calculated using the equation

$$[(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the negative control and A_1 is the absorbance of the sample (or standard positive control). The negative control did not contain complex-forming molecules.

LC-MS Analysis. The peptide contents of the hydrolyzed proteins and their IEF fractions were analyzed using a Quad Agilent Series 1100 LC-MS (Agilent Technologies, Palo Alto, CA, USA) consisting of a vacuum degasser (G1322A), a binary pump (G1312A), an autosampler (G1329A), a thermostat-controlled column compartment (G1315A), and a diode array detector (G1315A) monitoring at 214 and 280 nm. The C18 column used was a Luna 5 μm (2 mm i.d. × 250 mm, Phenomenex, Torrance, CA, USA) maintained at 40 °C. Data processing was carried out using Chem Station software (version B.01.03 SR1, Agilent Technologies, Santa Clara, CA, USA).

Elution was performed under the following conditions: injection volume, 5 μL; flow rate, 0.2 mL/min; solvent A, water/acetonitrile/trifluoroacetic acid (TFA) (99:1:0.1, v/v); solvent B, acetonitrile/water/TFA (90:10:0.11, v/v). Samples were rehydrated in solvent A (20 mg/mL) and eluted with a linear gradient from 0 to 40% of solvent B over 90 min, followed by a 35 min linear gradient of 40−100% of solvent B.

The flow was split prior to the detector, and electrospray mass spectrometry was performed in the positive ion mode using a direct infusion method at a rate of 0.5 mL/min. Peptides eluted from the HPLC were mixed with propionic acid (2%). Nitrogen was used as the nebulizer and drying gas at a rate of 9.0 L/min, 350 °C, and 20 psi. The capillary voltage was kept at 4000 V. Mass spectra were acquired over the mass/charge (m/z) range 200−1600. Peptide tools software (version 8.03, Agilent Technologies, Santa Clara, CA, USA) was used to interpret MS spectra.

Statistical Analysis. Statistical differences were revealed on the basis of two-way factorial ANOVA using the MIXED procedure of SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Different variances were

modeled on each level of "SAMPLE" to meet the model assumptions for "DH−SAMPLES" and "MCC−SAMPLES". When a significant effect was found, post hoc multiple comparisons were made using the step-down Bonferroni correction. A p value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Protein Concentrate Composition and Hydrolysate Characterization. The protein, lactose, lipid, and ash contents of the protein concentrates and of skim milk are expressed in Table 1 as percent dry matter, along with degree of hydrolysis (DH) by pepsin followed by trypsin. Buttermilk concentrates (BC) differed from cheese whey concentrates (WC) and skim milk (SM) in lipid content (13−15%). Buttermilk protein (native and denatured) showed low final (5 h) degrees of hydrolysis compared to all other samples. It is known that most MFGM proteins are glycosylated. This particularity has been reported to hinder hydrolysis by pepsin.[17,18] Even though denaturing a protein usually increases its susceptibility to proteolysis, the heat-denatured buttermilk protein concentrate (DBC) gave the lowest final DH (6.4%). This may be associated with increased milk fat globule size following heat treatment[19] resulting from interactions of whey proteins and caseins with denatured MFGM proteins.[20] Indeed, it is a known fact that larger fat particles are digested more slowly.[21] We can presume that skim milk proteins aggregated at the MFGM surface have increased the hindrance of enzymatic hydrolysis of MFGM proteins.

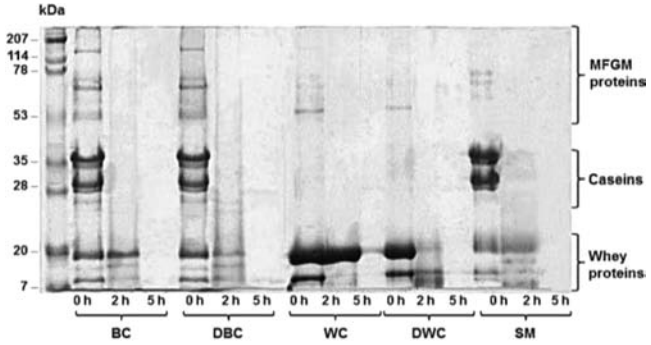

Figure 1. SDS-PAGE analysis of dairy protein concentrates before (0 h) and during hydrolysis with pepsin (2 h) followed by trypsin (5 h). MFGM, milk fat globule membrane; BC, buttermilk concentrate; DBC, denatured buttermilk concentrate; WC, whey concentrate; DWC, denatured whey concentrate; SM, skim milk.

Table 2. Free Radical Scavenging Activity[a] before (0 h) and during Treatment with Pepsin (2 h) followed by Trypsin (5 h)

	$ORAC_{FL}$ (μmol TE/g protein)		
sample[b]	0 h	2 h[c]	5 h[c]
BC	4.6 ± 4.3	900.5 ± 34.5 a	1319.6 ± 46.7 a
DBC	6.1 ± 0.8	476.5 ± 48.7 b	1052.0 ± 42.6 b
WC	<LD[d]	336.8 ± 24.3 cd	782.5 ± 34.8 c
DWC	3.7 ± 0.2	356.9 ± 30.5 bcd	554.4 ± 14.5 d
SM	<LD	458.6 ± 35.7 b	811.7 ± 8.0 c

[a]Radical scavenging activity data ($ORAC_{FL}$ values) are the mean ± SD of three independent experiments (n = 3). [b]BC, buttermilk concentrate; DBC, denatured buttermilk concentrate; WC, whey concentrate; DWC, denatured whey concentrate; SM, skim milk. [c]Means with different letters within a column indicate significant difference (p < 0.05). [d]$ORAC_{FL}$ values below the limit of detection.

Table 3. Metal-Chelating Capacity (MCC) of Dairy Proteins Hydrolyzed by Pepsin and Trypsin Tested at Different Concentrations and Compared to a Standard (Na_2EDTA)

	$MCC^{a,b}$ (%)		
sample[c]	1 mg/mL	2 mg/mL	4 mg/mL
HBC	16.4 ± 3.2 b	30.4 ± 4.1 c	46.7 ± 4.7 c
HDBC	12.5 ± 9.0 bc	24.7 ± 10.0 c	41.9 ± 7.5 cd
HWC	1.0 ± 1.9 c	5.4 ± 1.6 d	27.8 ± 3.6 d
HDWC	1.5 ± 1.0 c	3.0 ± 2.1 d	18.7 ± 6.2 e
HSM	25.5 ± 4.4 b	42.3 ± 6.2 b	63.6 ± 4.1 b
EDTA	100.0 ± 0.1 a	99.9 ± 0.2 a	99.9 ± 0.1 a

[a]Data are the mean ± SD of three independent experiments (n = 4). [b]Different letters within a column indicate significant difference (p < 0.05). MCC was measured by the ferrous ion binding capacity of samples. [c]HBC, hydrolyzed buttermilk concentrate; HDBC, hydrolyzed denatured buttermilk concentrate; HWC, hydrolyzed whey concentrate; HDWC, hydrolyzed denatured whey concentrate; HSM, hydrolyzed skim milk.

Table 4. Free Radical Scavenging Activity of Hydrolyzed Buttermilk Concentrate (HBC) and Its Liquid-Phase IEF Fractions (F1−F3)

sample	$ORAC_{FL}^{a,b}$ (μmol TE/g protein)
HBC[c]	1319.6 ± 46.7 a
F1	555.1 ± 38.0 c
F2	752.1 ± 23.4 b
F3	476.6 ± 52.4 c

[a]Radical scavenging activity ($ORAC_{FL}$ values) are the mean ± SD of three independent experiments (n = 3). [b]Means with different letters within the column indicate significant difference (p < 0.05). [c]Value reported in Table 2.

The SDS-PAGE profiles of the concentrates before (0 h) and during treatment with pepsin followed by trypsin show that buttermilk protein (heat-denatured or not) contained the most MFGM protein, whereas whey concentrate (WC) contained the most β-LG and α-LA but no caseins (Figure 1). After peptic digestion, MFGM-derived proteins and caseins were hydrolyzed in all samples, whereas β-LG appeared to resist the action of pepsin, except in the denatured concentrates (BDC and DWC), in which the β-LG band (~18 kDa) disappeared. It is known that following heat treatment, whey proteins lose their globular conformation, which increases their susceptibility to hydrolysis.[22] At the end of tryptic digestion, all proteins were hydrolyzed to peptides smaller than 7 kDa, except in WC, in which a faint β-LG band remained visible.

Effect of Enzymatic Hydrolysis on Free Radical Scavenging Ability. Table 2 presents the free radical scavenging activity ($ORAC_{FL}$) of the dairy protein concentrates and skim milk proteins before and after hydrolysis with pepsin followed by trypsin. Hydrolysis increased the free radical scavenging activity in all cases. The highest $ORAC_{FL}$ values were obtained after the 5 h treatment, with values ranging from 554.4 to 1319.6 μmol TE/g protein. It is plausible that unfolding of the protein structure exposed amino acids previously unavailable within the native protein structure, resulting in an overall increase of the antioxidant activity.[7] The observed increase in free radical scavenging activity was significant (p < 0.05).

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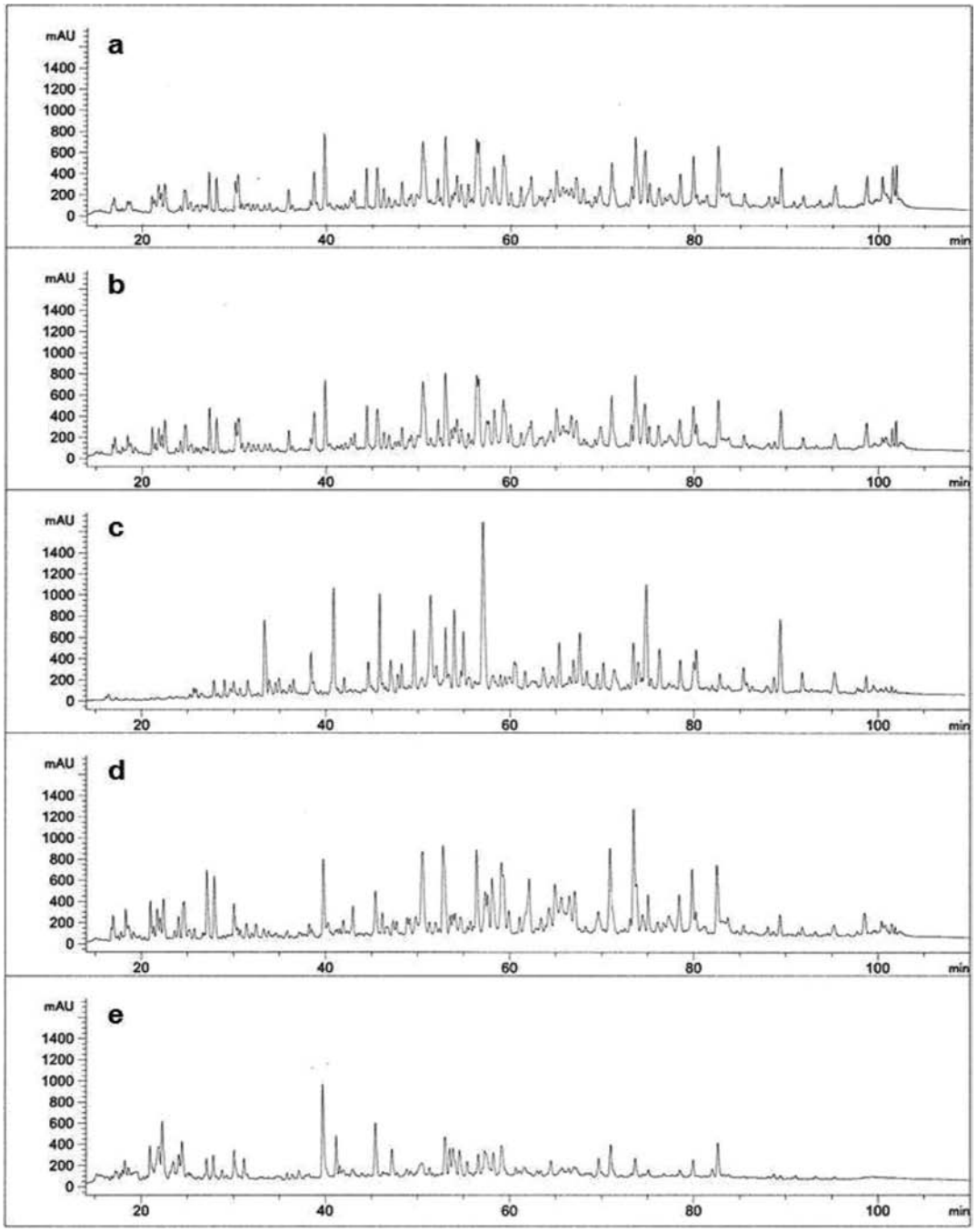

Figure 2. RP-HPLC elution profiles (absorbance measured at 214 nm) of hydrolyzed (2 h pepsin followed by 3 h trypsin treatment) skim milk proteins (a), hydrolyzed buttermilk protein concentrate (b), and peptide fractions obtained by liquid-phase IEF of hydrolyzed buttermilk protein concentrate (c = F1; d = F2; e = F3). F1 (pH <4.0), F2 (pH 4.0−8.0), and F3 (pH >8.0).

Buttermilk concentrates gave the highest $ORAC_{FL}$ scores following the 5 h treatment, with values of 1319.6 and 1052.0 μmol TE/g protein, respectively, for native (BC) and heat-denatured protein (DBC). Although denaturing had a negative impact on radical scavenging activity, the final hydrolysate was still more active than all other samples. The MFGM proteins occurring as minor components of buttermilk are most likely responsible for this characteristic. As confirmed by SDS-PAGE (Figure 1), buttermilk contained larger amounts of MFGM proteins than did the other dairy products. Britten et al. have reported that MFGM proteins account for up to 19% of the total protein in buttermilk,[23] which suggests that "minor" may be an inappropriate qualifier. Chen et al. have suggested the contribution of these proteins to the antioxidant capacity of milk.[24] These authors reported a significant increase in the free radical scavenging activity of milk in proportion with the milk fat and thus MFGM substance content. Furthermore, buttermilk is well-known for its high content of polar lipids compared to skim milk (2.03 vs 0.28%, on a dry matter basis).[2] These polar lipids may also play a role in the superior antioxidant activity found in buttermilk concentrates. Gavella et al.[25] have reported the protective action of gangliosides against reactive oxygen species. The authors attributed this phenomenon mainly to the ability of gangliosides to chelate iron. In a

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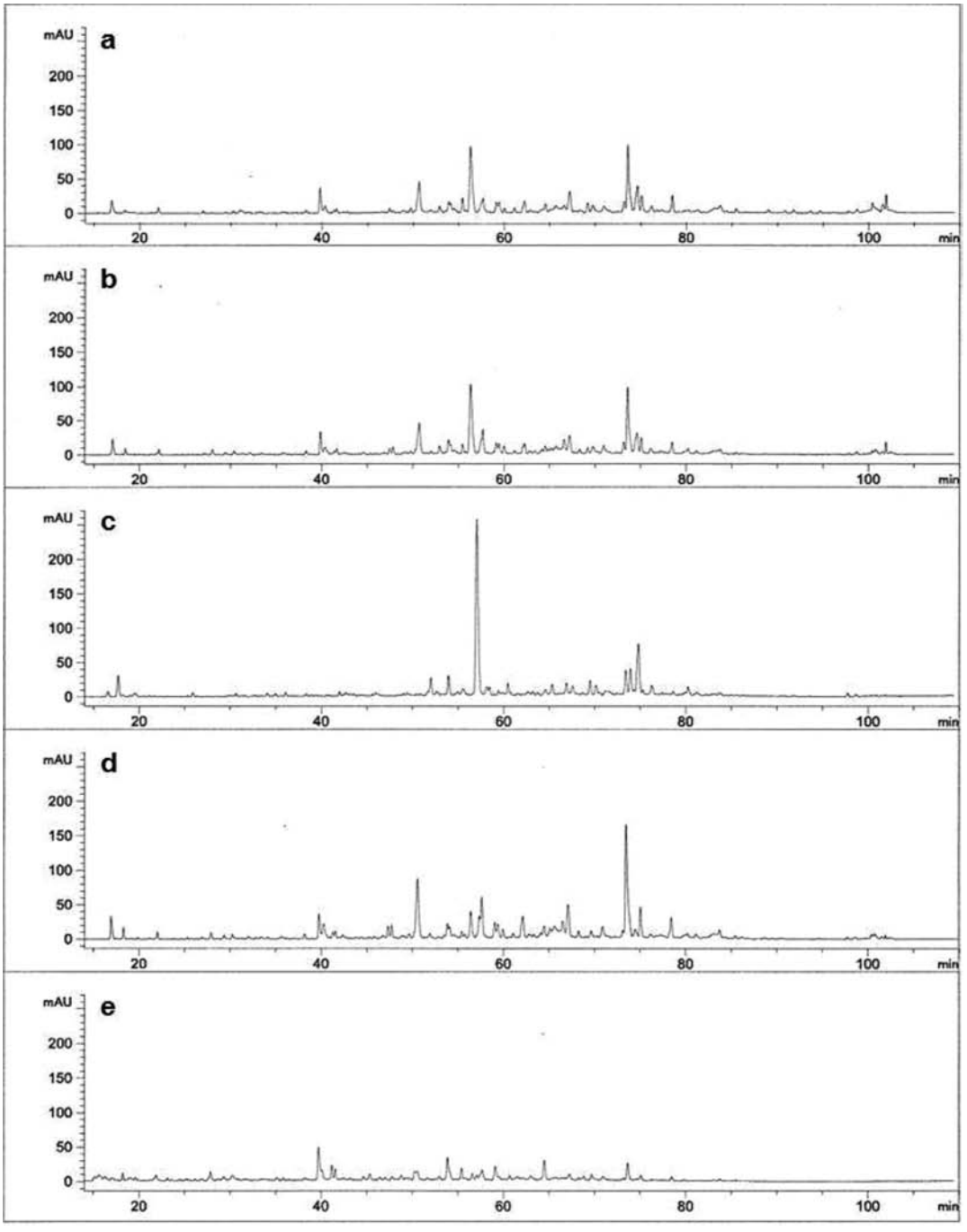

Figure 3. RP-HPLC elution profiles (absorbance measured at 280 nm) of hydrolyzed (2 h pepsin followed by 3 h trypsin treatment) skim milk proteins (a), hydrolyzed buttermilk protein concentrate (b), and peptide fractions obtained by liquid-phase IEF of hydrolyzed buttermilk protein concentrate (c = F1; d = F2; e = F3). F1 (pH <4.0), F2 (pH 4.0−8.0), and F3 (pH >8.0).

similar manner, MFGM phospholipids with long polyunsaturated fatty acids are known for their cation-binding capacity.[26]

No significant difference between the scavenging activities of enzyme-hydrolyzed whey concentrate and skim milk was observed in this experiment, based on the $ORAC_{FL}$ assay. This suggests that peptides originating from major whey proteins (α-lactalbumin and β-lactoglobulin) were most likely responsible for the antioxidant activity of these samples, because the casein content of skim milk (about 80%) did not improve its scavenging activity to any significant degree. The antioxidant activity of bovine whey protein peptides has been reported previously.[27]

Our results also revealed a greater impact of denaturing on the free radical scavenging activity of cheese whey protein concentrate, which yielded the lowest $ORAC_{FL}$ values (554.4 μmol TE/g protein) following the 5 h sequential hydrolysis. This decrease may result from thermally induced polymerization. It is well-known that heated whey proteins form homopolymers or heteropolymers through hydrophobic interactions and/or thiol/disulfide exchanges.[28] Interactions of this type likely affected the nature of the peptides released upon digestion and the ability of denatured whey protein to scavenge free radicals. In fact, protein hydrolysates display different antioxidant activities depending on peptide size, amino acid

Table 5. Scale Used To Attribute Antioxidant Scores to Peptides Based on the Presence and Position of Certain Amino Acid Residues[a] within the Amino Acid Sequence

peptide characteristic	points[b] added to the antioxidant score
W within the amino acid sequence	5
Y within the amino acid sequence	2
short sequence length (2−10 residues)	2
H, K, P, F, V, or I within the sequence	1
Y, W, V, or L residues at N-terminus	0.5
W, Y, and M residues at C-terminus	0.5

[a]The single-letter amino acid code is used. [b]Relative value is based on the literature.[7,29,33−35,37,39]

sequence, and the presence of amino acids that are capable of involvement in oxidative reactions.[27] We also note that denatured whey protein (DWC) gave the highest DH (Table 1). As reported elsewhere, extensive proteolysis is known to decrease antioxidant activity.[29]

Metal Chelating Capacity of Enzymatic Hydrolysates. As shown in Table 3, all of the dairy proteins subjected to the 5 h hydrolytic treatment had ferrous ion reducing capacity as expressed in MCC. At both 4 and 2 mg/mL, hydrolyzed skim milk protein (HSM) showed the highest chelating capacity. At 1 mg/mL, no significant difference was noted between the ferrous ion binding capacities of hydrolyzed skim milk protein (HSM), buttermilk protein concentrate (HBC), and denatured buttermilk protein concentrate (HDBC). The lowest iron-binding capacity was observed for hydrolyzed denatured whey protein concentrate (HDWC). Our results suggest that casein peptides were largely responsible for the iron-chelating capacity of our samples. Casein-derived peptides, more specifically phosphopeptides, have been reported to possess a capacity for binding minerals such as ferric (Fe^{3+}) ion.[30] Indeed, the polar side chains of certain amino acid residues (C, S, W, or Y) have been associated with the ability of phosphopeptides to interact with metals.[30,31]

Denaturing the protein using heat did not have a significant effect on the iron-binding ability of the final hydrolysate in the case of buttermilk proteins or whey proteins at any of the concentrations tested, except for the decrease at the highest concentration tested in the case of whey protein (Table 3). The ferrous ion binding capacity of our hydrolysates seems to be associated with casein and hence the presence of phosphopeptides. Because the strong negative charge borne by phosphopeptides interferes with their hydrolysis,[30] we reason that it also interferes with the aggregation of peptides and thus the loss of chelating capacity.

Overall, our results suggest that peptides released specifically from buttermilk proteins by pepsin and trypsin provide antioxidation capacity mainly by acting as free radical scavengers (Table 2) rather than by chelating metals such as iron (Table 3).

Fractionation and Identification of Potential Antioxidant Peptides. Because the greatest free radical scavenging activity was observed in the case of hydrolyzed buttermilk protein concentrate (Table 2), this material was selected for further separation into three peptide fractions by liquid-phase IEF. Table 4 presents the $ORAC_{FL}$ values for these fractions compared to the hydrolyzed protein concentrate. Our results suggest that the components responsible for the free radical scavenging activity are found mainly in the neutral fraction (F2, pH 4.0−8.0). These results are consistent with published studies, in which amino acid residues with hydrophobic or uncharged side chains are associated frequently with antioxidant activity of peptides. However, fractionation reduces the overall ability to scavenge free radicals. This observation suggests the presence of a wide range of antioxidant peptides contained in hydrolyzed buttermilk protein and, possibly, the presence of synergistic interactions between peptides.

On the basis of the $ORAC_{FL}$ value obtained for the neutral fraction (F2) and on the RP-HPLC profiles obtained at 214 nm (Figure 2) and 280 nm (Figure 3) for hydrolyzed skim milk protein and for buttermilk protein and its IEF fractions, we selected the peaks that were largest in both F2 and hydrolyzed buttermilk protein or found only in the latter. For subsequent identification of potentially antioxidant buttermilk protein-derived peptides, a scale was established for scoring peptide sequences based on the presence and position of certain amino acid residues, based on a literature review and summarized in Table 5. Peptide peaks scoring >5.0 were selected and are shown in Table 6 as potentially antioxidant sequences in buttermilk protein hydrolysate.

Table 6 shows that several of the potentially antioxidant peptides identified by LC-MS were derived from MFGM proteins. In fact, these minor dairy proteins are composed of a very diverse group of proteins represented predominatey by butyrophilin (BTN), xanthine dehydrogenase/oxidase (XO), cluster of differentiation (CD36), periodic acid Shiff III (PAS III), adipophilin (ADPH), fatty-acid binding protein (FABP), and periodic acid Shiff 6/7 (PAS 6/7). BTN represents 20−43% of bovine MFGM proteins[14] and is known as the most abundant MFGM protein, followed by XO, which accounts for up to 20%.[32] BTN is the main source of the peptides identified by LC-MS in hydrolyzed buttermilk proteins (Table 6), accounting for 20 of 48. Moreover, antioxidant scores of these peptides range from 5.5 to 16.5, suggesting that this MFGM protein is likely the principal source of the antioxidant peptides in our buttermilk hydrolysate. BTN fragments f396−412 (VGVFLDYESGDIFFYNM), f316−334 (DSWPCVMGREAFTSGRHYW), f231−249 (LGLLTIGSIFFTWRLYKER), and f413−433 (TDGSHIYTFSKASFSGPLRPF) all have antioxidant scores >10.0 (10.0−16.5). Sequences of such length (17−21 residues) have been associated previously with antioxidant activity.[6] Meanwhile, CD36 f200−212, PAS 6/7 f259−278, PAS 6/7 f297−319, and XO f1060−1098 have antioxidant scores in the range of 10.0−16.0 (Table 5). All of these peptides are rich in amino acid residues associated with antioxidant activity (W, Y, H, K, P, F, V, I, L, or M), sometimes bearing them (W, V, L F, K, M, or I) at the N- or C-terminal position. It has been suggested that large peptides may play the role of a physical barrier against oxidation initiators by forming a membrane protecting more oxidation labile compounds such as unsaturated fatty acids.[29] Of course, it is likely that these peptides are hydrolyzed by digestive enzymes in the gastrointestinal tract, thus releasing shorter potentially antioxidant peptides.

Among the other antioxidant peptides identified by LC-MS (Table 6), five were derived from α_{S2}-CN, four each from α_{S1}-CN and β-LG, and two from β-CN. Of the 48 peptide sequences listed in Table 6, 40 contain one or more W and/or Y residues in their sequence, which explains the high antioxidant scores attributed to them (Table 5). Twelve peptides possess a terminal W residue, 11 peptides have a C-terminal Y residue, 5 have an N-terminal V residue, and 5 have

Table 6. Potentially Antioxidant Peptides Derived from Buttermilk Proteins, As Identified by LC-MS

observed mass	calculated mass[a]	protein fragment[b]	amino acid sequence[c]	antioxidant score[d]
305.13	305.14	BTN f242−243	TW	7.5
351.11	351.16	BTN f40−41	WF	8.5
	351.16	BTN f367−368	FW	8.5
351.16	351.16	BTN f40−41	WF	8.5
	351.16	BTN f367−368	FW	8.5
	351.18	PAS 6/7 f181−183	VAY	6
422.23	422.22	MUC1 f408−410	QIY	5.5
	422.22	PAS 6/7 f72−74	IQY	5.5
	422.22	PAS 6/7 f338−341	VAAY	6
	422.25	BTN f245−247	LYK	5.5
437.21	437.26	α_{s1}-CN f102−104	KKY	6.5
	437.23	α_{s2}-CN f78−80	YQK	5.5
		α_{s2}-CN f89−91	YQK	5.5
		α_{s2}-CN f171−173	YKQ	5.5
503.21	503.24	BTN f368−371	WAVE	8.5
514.22	514.24	α_{s1}-CN f91−94	YLGY	7
545.31	545.3	CD36 f52−55	NWVK	9
	545.32	β-LG f78−82	IPAVF	6
572.32	572.22	BTN f373−377	YGNGY	7
607.26	607.31	XO f335−339	WFAGK	9.5
616.28	616.32	BTN f368−372	WAVEL	8.5
620.96	620.34	BTN f38−41	LRWF	8.5
673.37	673.34	β-CN f157−162	FPPQSV	6
	673.38	XO f998−1003	CIIPTK	6
695.29	695.33	β-LG f15−20	VAGTWY	11
747.32	747.36	α_{s1}-CN f194−199	TTMPLW	8.5
842.43	842.36	BTN f119−126	GSDPHISM	5.5
865.3	865.38	α_{s1}-CN f157−164	DAYPSGAW	10.5
902.73	902.46	α_{s2}-CN f182−188	TVYQHQK	7
	902.56	β-LG f76−83	TKIPAVFK	8
919.67	919.59	BTN f486−493	LHSKLIPL	6.5
978.79	978.57	CD36 f285−292	KGIPVYRF	9
1064.86	1064.58	β-LG f 92−100	VLVLDTDYK	7.5
1099.54	1102.61	XO f290−292	TLTLSFFFK	6
	1100.63	BTN f264−271	LEELKWKR	9.5
1156.95	1156.59	α_{s2}-CN f171−179	YQKFALPQY	10
	1156.62	β-CN f193−202	YQEPVLGPVR	8.5
1199.52	1199.49	BTN f410−419	YNMTDGSHIY	9
1395.21	1395.76	BTN f269−280	WKRATLHAVDVT	9.5
1490.13	1490.91	BTN f228−241	LVVLGLLTIGSIFF	6.5
1564.6	1564.72	CD36 f200−212	FYPYNNTADGIYK	10
2015.66	2014.9	BTN f396−412	VGVFLDYESGDIFFYNM	11
2285.49	2283.99	BTN f316−334	DSWPCVMGREAFTSGRHYW	16.5
2313.14	2312.3	BTN f231−249	LGLLTIGSIFFTWRLYKER	12.5
	2314.14	BTN f413−433	TDGSHIYTFSKASFSGPLRPF	10
	2314.16	PAS 6/7 f259−278	KDNTIPNKQITASSYYKTWG	15
2594.87	2594.24	PAS 6/7 f297−319	FNAWTAQTNSASEWLQIDLGSQK	13
4123.17	4122	XO f1060−1098	IYISETSTNTVPNSSPTAASVSTDIYGQAVYEACQTILK	16

[a]Monoisotopic mass (Da). [b]α_{s1}-CN, alpha$_{s1}$-casein; α_{s2}-CN, alpha$_{s2}$-casein; β-CN, beta-casein; β-LG, beta-lactoglobulin; BTN, butyrophilin; PAS III, periodic acid Shiff III; XO, xanthine dehydrogenase/oxidase; MUC1, mucin 1; CD36, cluster of differentiation; PAS 6/7, periodic acid Shiff 6/7; ADPH, adipophilin. [c]The single-letter amino acid codes are used. [d]The antioxidant score was attributed as described in Table 4.

a M residue either in their sequence or at the C-terminal position. The presence of terminal Y and W residues has been associated with superior free radical scavenging ability,[33] as has a C-terminal M residue.[34] In addition, peptide XO f998−1003 (CIIPTK) contains amino acid residues (K and I) that are highly correlated (i.e., $p < 0.001$) or (in the case of T) correlated (i.e., $p < 0.05$) with antioxidant activity, as reported previously.[35] Furthermore, this peptide contains a P residue, which has been described previously as one of the most important residues (along with H) in the lipoprotein peroxidation inhibitory activity of peptides derived from soybean protein.[36] Finally, three of the peptide fragments listed in Table 6 have already been associated with biological activity relevant to cardiovascular health. Indeed, fragments α_{S1}-CN f194−199 (TTMPLW), β-LG f15−20 (VAGTWY), and β-

LG f91−100 (VLVLDTDYK) are recognized as antihypertensive peptides.[33,37]

Although assays such as ORAC or metal chelating capacity can provide information on the antioxidant potential of peptides, those in vitro methods do not necessarily reflect physiological conditions or mechanistic actions. Among the methods available in the literature, the ORAC assay, by using free peroxyl radical as well as physiological conditions, seems to be the preferred choice for in vitro antioxidant analysis, and it represents the assay with the broader application possibilities.[38] Furthermore, because antioxidants have different mechanisms of action (i.e., radical chain inhibition, metal chelation, oxidative enzyme inhibition, or antioxidant enzyme cofactors), it is preferable to use a combination of methods in the assessment of the antioxidant's capacity.[38] For this reason, we have chosen to study the free radical scavenging ability (i.e., a radical chain inhibition assay) as well as the metal chelating capacity of our samples. Because ferrous ions are the most effective prooxidants in food systems, we used the ferrous ion chelating ability of our sample to assess the metal chelating capacity. Moreover, due to the extreme diversity of buttermilk proteins, the peptides identified in the present study are not likely the only ones responsible for the antioxidant activity of the hydrolysates. However, peptides such as those derived from BTN very likely make a substantial contribution to this activity.

To the best of our knowledge, this is the first study to highlight the strong free radical scavenging ability of hydrolyzed buttermilk proteins and the likely contribution of MFGM protein fragments to the antioxidant capacity of buttermilk. This study also shows the impact of thermal and enzymatic processing on the antioxidant activity of proteins obtained from buttermilk and whey and suggests future industrial treatments for these products.

In conclusion, this study confirms the superior potential of buttermilk proteins for free radical scavenging, compared to whey proteins and skim milk proteins. It also shows the limited impact of processing on the free radical scavenging capacity of buttermilk proteins in comparison with whey and skim milk, and no impact on its ferrous ion sequestering capacity. This study thus provides useful information for increasing the commercial value of buttermilk as a multifunctional ingredient and suggests new ways to increase its recognition as a natural source of antioxidants capable of preventing oxidation processes in food systems.

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Notes

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