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Characterization of Major Radical Scavenger Species in Bovine Milk through Size Exclusion Chromatography and Functional Assays

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Radical scavenging activities of bovine milk components were quantified following size exclusion chromatography (SEC) with postcolumn characterization of fractions using the scavenging of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radicals ($ABTS^{\bullet+}$) in the Trolox equivalent antioxidant capacity (TEAC) assay and peroxyl radicals formed from cleavage of 2,2′-azobis(2-amidinopropane) (AAPH) in the oxygen radical absorbance capacity (ORAC) fluorometric assay. Caseins were quantitatively the major radical scavenger species in both assays, whereas β-lactoglobulin (β-lg) and α-lactalbumin (α-la) were much less active and only in the peroxyl radical assay. The radical scavenging activity of the caseins could be quantitatively accounted for by their constituent amino acids, as there were no effects of denaturing agents or complete digestion with proteases. In contrast, the activities of the whey proteins were dependent on denaturation or partial hydrolysis and dominated by the free thiol in β-lg. A component in milk serum with a molecular mass of ~100 kDa contributed significantly to both $ABTS^{\bullet+}$ and peroxyl radical scavenging but was absent in whey. This radical scavenger was identified as β-casein. The only significant low molecular weight radical scavenger species were identified as ascorbate and urate in both assays.

KEYWORDS: Antioxidants; radical scavengers; milk; TEAC; ORAC; chromatography

INTRODUCTION

Foods are protected against oxidation by the presence of efficient antioxidant systems that combine effects of low molecular weight (LMW) antioxidants, such as α-tocopherol and ascorbate (*1*), and high molecular weight (HMW) proteins. The latter may enzymatically inactivate reactive oxygen species (ROS), for example, superoxide anion, through the successive action of superoxide dismutase and hydrogen peroxide consuming enzymes, such as catalase or peroxidases (*2*). The existence of disulfide reductases and methionine sulfoxide reductases also suggests that both cysteine and methionine serve as an important line of defense of proteins against ROS (*3*). Furthermore, aromatic amino acids are easily oxidized and also contribute as peptides and proteins to the antioxidant effects of proteins (*3*).

The complex chemistry of pro- and antioxidants in foods, such as milk, complicates molecular dissection of early oxidative events that lead to the degradation of lipids and/or proteins (*4*, *5*). Milk caseins and LMW compounds have been recognized as antioxidants during oxidation mediated by transition metals (*6*−*8*), lipoxygenase, or radicals generated by various xenobiotics (*7*). Whey proteins are considered to have less antioxidant activity (*6*), although β-lactoglobulin (β-lg) is an antioxidant in lipid emulsions partly due to its free thiol (*9*) and exposure of amino acids with antioxidant activity in the water−lipid interface (*10*). α-Lactalbumin (α-la) is generally not assigned antioxidant activity (*8*). The antioxidative and radical scavenging activities of crude milk fractions have previously been evaluated according to molecular weight using filtration or dialysis (*9*, *11*, *12*). Although these studies provide some information on the quantitative contributions of milk and whey fractions to milk antioxidative activity, little information on the effects of single components is available. Sample pretreatment may also in previous studies have prevented the interpretation of results and detection of labile antioxidants such as ascorbate (*11*, *12*). Radical scavenging is important for antioxidant activity, and we have therefore investigated and assigned radical scavenging activity of specific milk components. Our approach combines size exclusion chromatography (SEC) with radical scavenging assays and allows us to estimate the contributions of specific milk components, thus overcoming problems of interference between HMW and LMW compounds (*13*). This approach offers a more complete picture of radical scavenging by specific

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milk components and may also be a versatile tool for speciation of radical scavengers in other food matrices.

Antioxidant capacity of foods has been determined with a number of different assays. Two commonly used assays for the assessment of the antioxidant capacity of food components are the oxygen radical absorbance capacity (ORAC) assay and Trolox equivalent antioxidant capacity (TEAC) assay (*12*, *14*). In the former assay, the ability to scavenge peroxyl radicals formed through decomposition of the azo-initiator 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) is evaluated as retardation in bleaching of fluorescein. The antioxidant capacity determined in the ORAC assay measures the ability of an antioxidant to donate a hydrogen atom and is thus closely related to its ability to act as a chain-breaking antioxidant (*15*). The TEAC assay measures the ability of antioxidants to donate an electron to the stable 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical (*13*, *14*). Thus, different properties of the antioxidants are measured with the two assays, and testing antioxidants in both assays provides a more complete picture of their antioxidative activity.

MATERIALS AND METHODS

Chemicals. 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), uricase from *Candida* sp. (EC 1.7.3.3), ascorbate oxidase from *Cucurbita* sp. (EC 1.10.3.3), protease from *Streptomyces griseus* type XIV, ≥3.5 units mg^{-1} solid, and L-amino acids were from Sigma Aldrich (Steinheim, Germany), 2,2′-Azobis(2-methylpropanimidamide) (AAPH) was purchased from Cayman Chemical Co. (Ann Arbor, MI), and fluorescein sodium salt was from Fluka (Steinheim, Germany). Lactic acid, 10% solution, was from Merck (Darmstadt, Germany), *N*-ethylmaleimide (NEM) was from Pierce Biotechnology (Rockford, IL), guanidinium hydrochloride (GndHCl) was from Invitrogen A/S (Taastrup, Denmark), and endoproteinase Lys-C and trypsin were from Boehringer Mannheim (Mannheim, Germany).

Sample Preparation. Fresh bulk milk was collected from the herd of Holstein-Friesian dairy cows at Foulum, Aarhus University. Skim milk was prepared by centrifugation (10000*g*, 5 min at 4 °C). Whey was prepared from skim milk by acidification to pH 4.6 with lactic acid, incubation for 30 min at room temperature, centrifugation (as above), and readjustment of pH of the supernatant with NaOH to 6.7. Milk serum was prepared by ultracentrifugation (320000*g*, 1.5 h at 4 °C).

Experimental Setup. Samples were fractionated using SEC or ion exchange chromatography on an Äkta FPLC system from Amersham Pharmacia Biotech (Uppsala, Sweden). The flow through was collected in 96-well microplates from Bibby Sterilin Ltd. (Stone, Staffordshire, U.K.) as 300 μL fractions. Characterization of fractions was performed with assays designed to reaction volumes of 100−250 μL to allow analysis directly in microplates and together with the appropriate standards in each plate (see later sections).

Size Exclusion Chromatography. Samples were passed through 0.45 μm cellulose filters (Frisenette, Knebel, Denmark) to remove particles, and 0.1−0.5 mL of sample was applied to a Superdex 200 10/300GL column from Amersham Pharmacia Biotech (10 × 300 mm, separation range from 10^4 to 6×10^5 Da). The flow rate was 0.5 mL min^{-1}, and the mobile phase consisted of 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl.

On the basis of a standard curve of diluted samples (milk, milk serum, or whey) in 4.0 M GndHCl, the absorbance at 280 nm of the undiluted sample was estimated. These measurements were performed in UV transparent 96-well micro plates from Corning Inc. (Corning, NY), and all absorbance plate readings were performed on a PowerWave$_X$ microplate scanning spectrophotometer from Bio-Tek Instruments, Inc. (Winooski, VT), with KC4 software for data analysis. Then, the sample amount, expressed as absorbance at 280 nm, was calculated through multiplication of the absorbance of the undiluted sample with the sample volume. For determination of the recovery, this value was compared with the absorbance at 280 nm of all fractions (n) likewise in 4.0 M GndHCl (eq 1)

$$\mathrm{sum}_{A280} = \sum_{i=1}^{n} V_i \times \mathrm{A}_{280i} \quad (1)$$

where V_i and $A_{280,i}$ is the volume and absorbance at 280 nm, respectively, of the ith fraction.

Ion Exchange Chromatography. Selected fractions from SEC were subjected to ion exchange chromatography using the FPLC system described above and using a Resource Q column (1 mL, binding capacity up to 25 mg of protein) from Amersham Pharmacia Biotech. The buffer was 10 mM sodium phosphate, pH 7.4, with 0.15 M NaCl, and elution was carried out with a linear gradient up to 1.0 M NaCl. Fractions of 300 μL were collected.

Preparation of Fractions. All fractions from SEC were diluted 3-fold with buffer (75 mM sodium phosphate, pH 7.4) or the same buffer with 6.0 M GndHCl, resulting in 4.0 M GndHCl. Each sample was divided into two aliquots and treated with either water or the thiol blocking agent NEM (2 mM) for 30 min at room temperature.

Assays for Characterization of All Fractions. *TEAC Assay.* Excess ABTS (18.7 mM) was mixed with ammonium persulfate (8.8 mM) and allowed to react overnight at room temperature for the formation of $ABTS^{\bullet+}$. $ABTS^{\bullet+}$ was diluted to 30 μM using $\varepsilon_{414\ nm} = 3.6 \times 10^4$ L mol^{-1} cm^{-1} (*16*) in a 75 mM aqueous sodium phosphate buffer, pH 7.4, or the same buffer with 4.0 M GndHCl. The TEAC assay was performed by mixing 200 μL of $ABTS^{\bullet+}$ working solution and 50 μL of each fraction from SEC. Absorbance at 414 nm was determined after 60 min along with a Trolox standard, and Trolox equivalents were calculated from a standard curve. Preliminary experiments showed that TEAC values of proteins were highly affected by assay time. Thus, an assay time of 60 min was used for both the ORAC (see below) and TEAC assays.

The total TEAC value and the sum of all fractions was calculated as described in eq 1, substituting A_{280} with the TEAC value. Furthermore, the contribution of individual milk components was estimated as the sum of all fractions containing the component based on the chromatogram.

ORAC Assay. The assay was performed as described in ref *15* with slight modifications. A fluorescein stock solution (4.4×10^{-7} M in water) was diluted to obtain a final concentration of 1.7×10^{-9} M in 75 mM sodium phosphate, pH 7.4, or the same buffer with 4.0 M GndHCl. AAPH (9.2 mM, final concentration, made fresh each day) was dissolved in the same buffers and kept on ice prior to use. The fluorescein working solution (150 μL) was incubated at 37 °C for 20 min, mixed with fractions (25 μL), and incubated for another 10 min. Subsequently the assay was initiated by the addition of 25 μL of AAPH solution, and fluorescence was read every minute for 60 min with excitation at 485 nm and monitoring emission at 515 nm. The areas under the curves were calculated and converted to Trolox equivalents using a standard curve. Fluorescence readings were performed in white 96-well microplates (Costar, Corning, NY) with a Wallac (Wellesley, MA) EnVision 2103 Multilabel reader from Perkin-Elmer.

The same calculations as described for the TEAC assay were carried out for the ORAC assay.

Free Thiols. The content of free thiols was determined using DTNB. A 10 mM solution of DTNB in methanol was diluted to 1.7 mM in 100 mM Tris-HCl, pH 8.0, or 100 mM Tris-HCl in 4.0 M GndHCl, pH 8.0. For determination of free thiols, 50 μL of eluate from SEC was mixed with 50 μL of 100 mM Tris-HCl, pH 8.0, and 10 μL of DTNB solution. After 30 min of incubation at room temperature, the absorbance at 410 nm was read.

Identification of LMW Radical Scavengers. LMW radical scavengers were identified through coelution with pure standards after the addition of urate or ascorbate (60 mg L^{-1}) to milk. Treatment of milk serum with ascorbate oxidase (0.05 unit mL^{-1}) at pH 6.0 for 1 h or treatment of all fractions with uricase (0.2 unit mL^{-1}) at pH 7.4 was used to remove ascorbate or urate, respectively. Complete removal was verified by specific assays for these compounds (*17*). All samples were subjected to the TEAC assay as described above.

Matrix-Assisted Laser Desorption−Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). For analysis of intact proteins, samples from ion exchange chromatography were dialyzed against water, and 0.5 μL was applied to an MTP AnchorChip (Bruker Daltonics, Bremen, Germany) and allowed to dry followed by the addition of 1.0 μL of 10% formic acid and 0.5 μL of matrix (20 mg mL^{-1} 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid in 50% acetonitrile). Ions were detected with a Bruker Ultraflex MALDI TOF Tandem Mass Spectrometer equipped with a 337 nm nitrogen laser in positive mode with an accelerating voltage of 25 kV. Results were analyzed by Flex Analysis software version 2.4 (Bruker Daltonics). Tryptic digests were prepared through a 1 h Lys-C digestion at 37 °C, followed by an overnight digestion with trypsin in 4.0 M urea, pH 8.0, at 37 °C. Digests were desalted on C18 microcolumns prepared in gel-loader tips, and peptides were eluted with 20 mg mL^{-1} α-cyano-4-hydroxycinnamic acid in 70% acetonitrile onto the AnchorChip plate. Peptide fingerprints and fragment sequences were obtained on the instrument described above.

SDS-PAGE. Fractions from SEC or anion exchange were precipitated with 80% ice cold acetone, centrifuged (15000*g*, 5 min), washed three times in acetone, and dissolved in SDS-PAGE sample buffer. After electrophoresis in Tris-Tricine gels (Bio-Rad, Hercules, CA), proteins were stained with Coomassie Brilliant Blue and destained with water.

Protease Digestion. Protease was added to all fractions from SEC of milk and incubated at 37 °C for 2 h. The enzyme/substrate ratio was 1:50 in the peak casein fractions and, thus, significantly higher in other fractions. The pH did not decrease significantly as a result of the digestion, and $ABTS^{\bullet+}$ scavenging was tested in the presence or absence of 4.0 M GndHCl as described above. There was no effect of added protease alone in the TEAC assay.

RESULTS

Milk. Figure 1 shows the radical scavenging profiles of milk (**A**), milk serum (**B**), and whey (**C**) together with the corresponding UV absorption traces. Caseins were the major radical scavengers in both the ORAC and TEAC assays, whereas β-lg and α-la contributed much less. Two LMW peaks with scavenging activity contributed equally to milk radical scavenging and were identified as ascorbate and urate (see below).

Milk Serum. The major $ABTS^{\bullet+}$ scavenging peak in milk serum (apart from ascorbate and urate) was due to one or more components with a molecular mass of ∼100 kDa that were not present in whey (see below), whereas β-lg contributed only slightly to this activity (**Figure 1B**). This result indicates that the major radical scavenger in milk serum may consist of an aggregate of casein molecules. Three major protein fractions with peroxyl radical scavenging activity were detected: the ∼100 kDa fraction, β-lg, and α-la. The identity of the ∼100 kDa component(s) is described below.

Whey. β-Lg and α-la were the major peroxyl radical scavengers in whey, whereas they contributed less to the $ABTS^{\bullet+}$ radical scavenging (**Figure 1C**). The contribution of ascorbate was lower in whey compared to milk and milk serum, which could be due to oxidation during sample preparation, whereas urate appeared to be less affected.

∼100 kDa Radical Scavenger(s) in Milk Serum. The SEC fractions from milk serum with highest $ABTS^{\bullet+}$ scavenging activity (**Figure 1B**) were subjected to anion exchange chromatography coupled with determination of $ABTS^{\bullet+}$ scavenging. One or more components with absorbance at 280 nm eluted in the flow-through, but showed no radical scavenging activity (**Figure 2A**). At least three compounds were eluted with a gradient of NaCl, of which only the major peak showed $ABTS^{\bullet+}$ scavenging. The purity of this component was evaluated through SDS-PAGE (**Figure 2C**). In the fraction from SEC, one dominant band is clearly seen along with a number of components with both higher and lower molecular weight. After ion exchange chromatography, only the dominant band was detected, with an apparent molecular mass of ∼30 kDa. MALDI-TOF MS of the same fraction showed one major peak with *m*/*z* 24.0 × 10^3 and two minor peaks at *m*/*z* 19.0 × 10^3 and 21.7 × 10^3 (**Figure 2B**). Tandem mass spectrometry of a tryptic digest of the peak fraction that eluted between 5.5 and 6.5 mL from the anion exchange column identified a 3.7 kDa fragment as the β-casein peptide Ala_{192}−Val_{224}. The apparent discrepancy between the electrophoretic and mass spectrometric determination of the molecular weight is consistent with the well-known behavior of caseins during SDS-PAGE (*18*), and we conclude that a tetramer of β-casein (with a monomeric molecular mass of 24 kDa) is the major radical scavenger in milk serum.

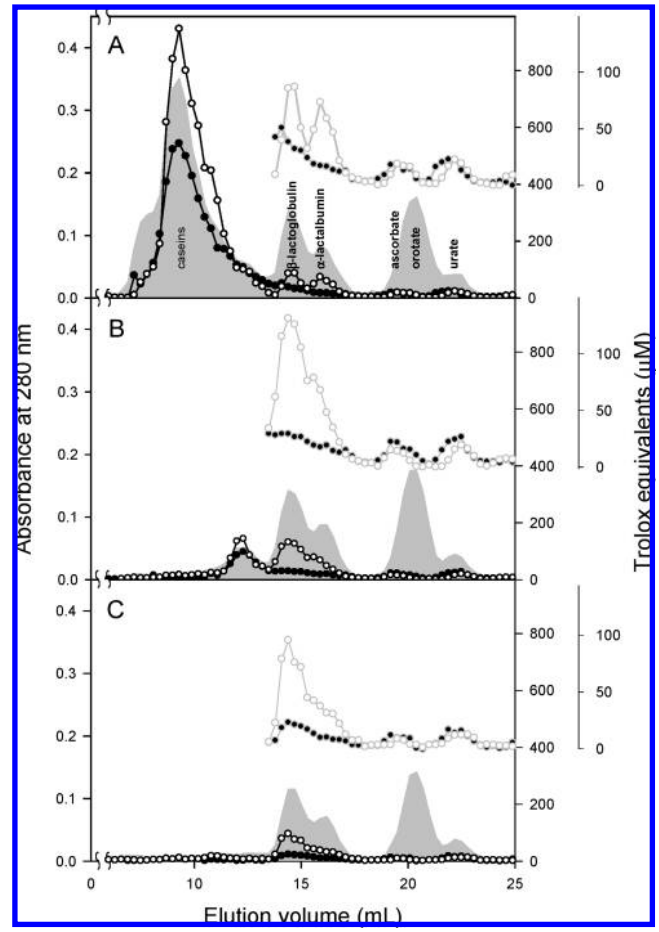

Figure 1. Comparison of radical scavenging activity in the TEAC (●) and ORAC (○) assays after SEC of milk (**A**), milk serum (**B**), and whey (**C**). Absorbance at 280 nm of the SEC fractions is shown as gray shading. Insets with floating axes show expanded views of TEAC and ORAC results for elution volumes between 13 and 25 mL. Representative results of at least five independent experiments are shown.

LMW Radical Scavengers. Two major UV-absorbing LMW compounds were observed in milk, whey, and milk serum and were identified as orotate (1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidinecarboxylic acid, data not shown) and urate (**Figure 3B**) through coelution with pure standards. The radical scavenging profile did not superimpose with the UV profile of orotate, but rather as a shoulder indicating the presence of another compound (**Figure 1**). On the basis of treatment with ascorbate oxidase or uricase, which completely and specifically removed the radical scavenging activity, and coelution with pure standards, these peaks could be unambiguously assigned as ascorbate and urate,

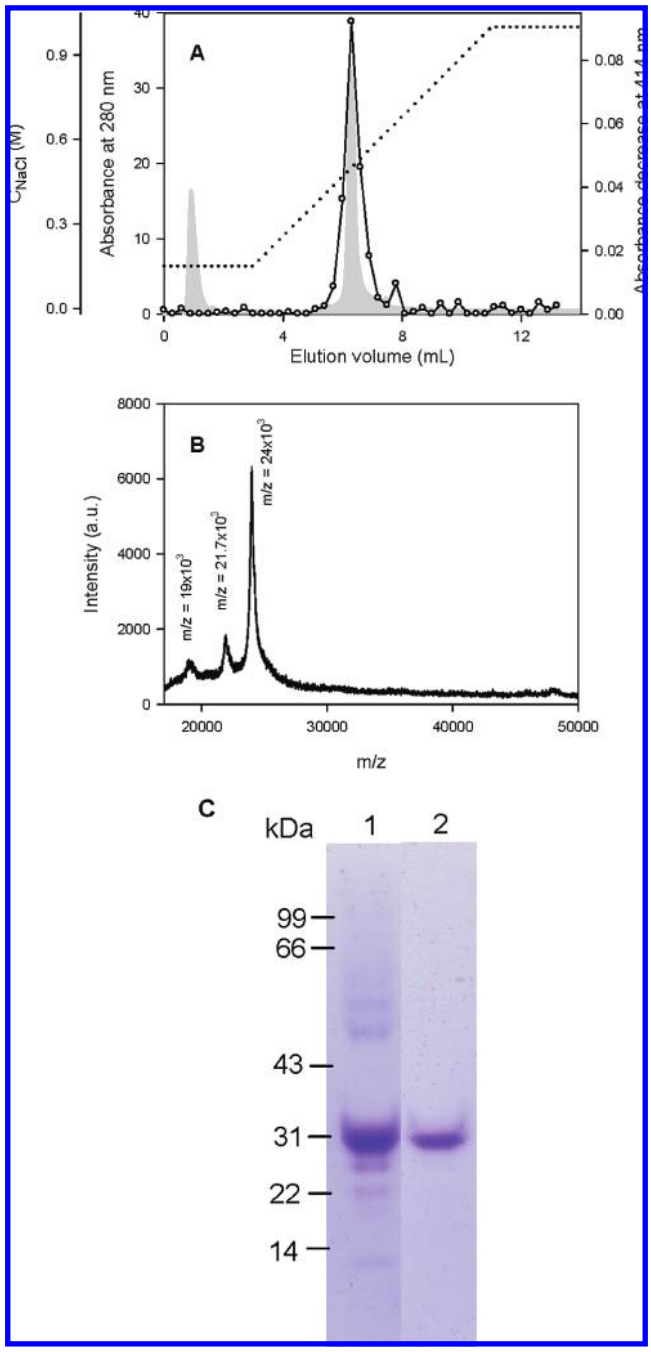

Figure 2. (**A**) Anion exchange chromatography of ~100 kDa fractions (eluted between 12 and 13 mL) pooled from SEC of milk serum coupled with determination of $ABTS^{\bullet+}$ radical scavenging. Absorbance at 280 nm of collected fractions in microplates is shown in gray. Dotted line represents the salt gradient used to elute proteins from the anion exchange column. Means of three independent experiments are shown. (**B**) MALDI-TOF mass spectrum of the peak fraction that eluted between 5.5 and 6.5 mL from the anion exchange column. (**C**) SDS-PAGE of fractions collected from SEC (**Figure 1B**) between 12 and 13 mL (lane 1) and used for anion exchange chromatography and the peak fraction (5.5−6.5 mL) from **A** (lane 2).

which therefore are the only significant LMW radical scavengers in milk (**Figure 3**).

The overall picture of milk radical scavengers provided by our approach relies on the recovery of components loaded onto the column. Summation of absorbance readings at 280 nm of SEC fractions revealed that 97 ± 7% (mean ± SEM) of the loaded absorbance was recovered. Likewise, the recovery of

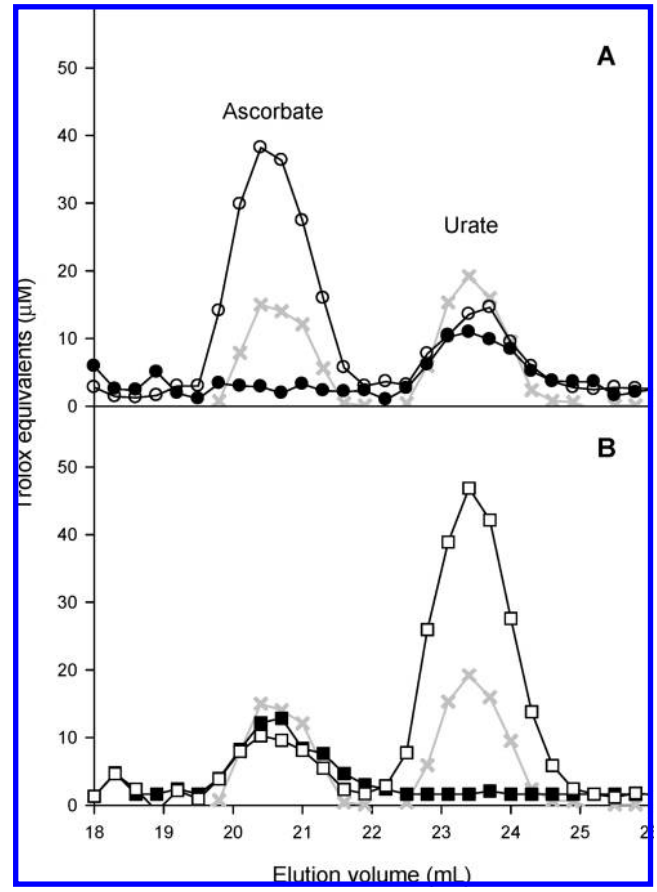

Figure 3. Identification of ascorbate (**A**) and urate (**B**) as the only LMW radical scavengers. $ABTS^{\bullet+}$ radical scavenging (expressed as Trolox equivalents) of samples spiked with ascorbate (○) or urate (□) or after treatment with ascorbate oxidase (●) or uricase (■) is compared to untreated samples (gray ×). Only SEC chromatograms from 18−26 mL are shown.

radical scavenging activity was determined, and as shown in **Table 1** this was ~80−100%. On the basis of these experiments (as summarized in **Table 1**), we conclude that caseins accounted for 89% of the radical scavenging activity in milk, as assessed by the TEAC and ORAC assays. The main radical scavenger in milk serum was β-casein, as opposed to whey, which contained β-lg and α-la as the major radical scavengers. The only LMW radical scavengers were ascorbate and urate, with urate contributing slightly more to both the TEAC and ORAC values of milk than ascorbate.

Activity of Amino Acids in the TEAC and ORAC Assays. The difference in activity of the whey proteins between the TEAC and ORAC assays could be due to the different reactivities of amino acids in the two assays. We therefore determined the radical scavenging of the 20 amino acids (**Figure 4**). Only Trp, Tyr, Cys, Met, Phe, and His were found to be active in the ORAC assay with the relative potencies shown in **Table 2**: Trp was the most potent amino acid, approximately 10 times more potent than Met, which in turn was slightly more active than Cys and Tyr. His and Phe were the least potent amino acids, approximately 170 and 10^4 times less than Trp, respectively. In the TEAC assay Trp, Tyr, and Cys were the most active amino acids, with Phe and His being approximately 4 orders of magnitude less active than Trp. Met was not active at the highest concentrations tested (7 mM). All amino acids (except Cys) were less active in the TEAC assay, and these

Table 1. Total Radical Scavenging of Milk, Milk Serum, and Whey Compared with That of Specific Milk Components after SEC[a]

	milk		milk serum		whey	
	TEAC	ORAC	TEAC	ORAC	TEAC	ORAC
applied sample	2.3 ± 0.5	3.4 ± 0.9	0.37 ± 0.14	0.77 ± 0.09	0.09 ± 0.03	0.28 ± 0.05
caseins	1.6 ± 0.2 (89)	2.58 ± 0.01 (89)				
β-casein			0.18 ± 0.07 (55)	0.33 ± 0.08 (47)		
β-lg	0.078 ± 0.005 (4)	0.20 ± 0.03 (7)	0.052 ± 0.005 (16)	0.22 ± 0.01 (32)	0.031 ± 0.003 (37)	0.14 ± 0.09 (58)
α-la	0.038 ± 0.005 (2)	0.059 ± 0.01 (2)	0.029 ± 0.009 (9)	0.10 ± 0.01 (15)	0.016 ± 0.002 (19)	0.07 ± 0.04 (29)
ascorbate	0.02 ± 0.013 (1)	0.024 ± 0.001 (1)	0.032 ± 0.007 (10)	0.017 ± 0.01 (2)	0.014 ± 0.002 (17)	0.006 ± 0.001 (2)
urate	0.045 ± 0.002 (3)	0.032 ± 0.001 (1)	0.037 ± 0.004 (11)	0.030 ± 0.01 (4)	0.023 ± 0.001 (27)	0.026 ± 0.004 (11)
sum	1.8 ± 0.2 (100)	2.9 ± 0.2 (100)	0.33 ± 0.06 (100)	0.7 ± 0.10 (100)	0.08 ± 0.02 (100)	0.24 ± 0.01 (100)

[a] Mean ± standard deviation of three independent experiments. All values are expressed as Trolox amount (μmol) loaded on column or recovered in fractions. Relative contributions are shown in parentheses.

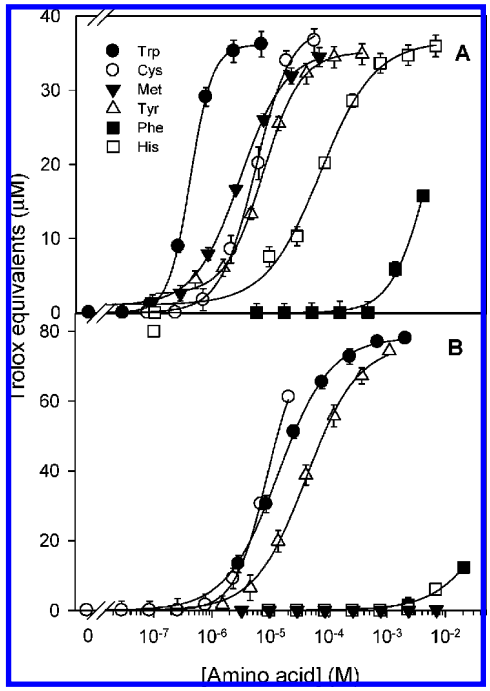

Figure 4. Reactivity of amino acids in the ORAC (**A**) and TEAC assays (**B**). Only amino acids with radical scavenging activity are shown. Results are mean ± SEM from one of two similar experiments.

Table 2. Radical Scavenging of Amino Acids in TEAC and ORAC Assays Expressed as EC_{50} Values Calculated from the Data in **Figure 5**

	EC_{50} (M)	
amino acid	TEAC	ORAC
Cys	8.5×10^{-6}	5.4×10^{-6}
Trp	1.4×10^{-5}	4.1×10^{-7}
Tyr	4.1×10^{-5}	7.3×10^{-6}
Met		3.0×10^{-6}
His	$\sim 1 \times 10^{-1}$	7.0×10^{-5}
Phe	$\sim 1 \times 10^{-1}$	4.7×10^{-3}

results show that the differences between the ORAC and TEAC values of proteins can be related to the higher potencies of the amino acids in the ORAC assay.

Effect of Protein Unfolding. We also expected that radical scavenging activity of buried amino acids would be masked differently as a consequence of the different sized radicals in the two assays. We therefore reasoned that the low radical scavenging activity of the major whey proteins, particularly in the TEAC assay, was due to low accessibility of otherwise reactive amino acids in their globular structure. Unfolding of the proteins in the chaotrope GndHCl was therefore expected to increase radical scavenging, whereas the caseins should be unaffected. Panels **A** and **C** of **Figure 5** show that radical scavenging of the caseins decreased in GndHCl, whereas the scavenging ability of β-lg increased dramatically, but only a small increase for α-la was observed. Furthermore, the β-lg rate appeared to be biphasic with a fast phase followed by a slower reaction, whereas α-la showed only a slow reaction. As shown in **Figure 5B**,**D** these effects can be related to decreased $ABTS^{\bullet+}$ scavenging by Trp and Tyr in GndHCl, whereas the rapid phase observed for β-lg in GndHCl was comparable to that of free Cys.

Denaturation of β-lg in GndHCl also resulted in reaction with DTNB (**Figure 6B**), confirming that a buried thiol was made accessible. Blocking of this thiol (Cys121) with 2 mM NEM completely inhibited the $ABTS^{\bullet+}$ scavenging of β-lg, whereas all other fractions were unaffected (**Figure 6A**).

SEC fractions were also treated with protease before radical scavenging activity was assessed (**Figure 7**). Complete digestion was verified by SEC (data not shown), but this had no effect on radical scavenging by the caseins, indicating that the activity of caseins can be simply accounted for by their constituent amino acids. In contrast, digestion of the globular whey proteins resulted in large increases in their radical scavenging, likely a result of unfolding, release, and solvent exposure of the amino acids. The radical scavenging of β-lg was much less influenced by GndHCl, whereas the radical scavenging of α-la diminished. This is consistent with the moderate effect of GndHCl on Cys $ABTS^{\bullet+}$ scavenging but the significant effect on radical scavenging by Tyr and Trp as shown in **Figures 5** and **6**.

In the ORAC assay, bleaching of fluorescein is decreased in the presence of GndHCl, making direct comparison of results in the presence or absence of GndHCl more difficult. However, after correction for the lower rate and calculation of Trolox equivalents, it was possible, with good approximation, to compare the peroxyl radical scavenging in the presence or absence of GndHCl. Peak fractions of the caseins, β-lg, and α-la were evaluated in the ORAC assay in the absence or presence of GndHCl (**Figure 8**). The radical scavenging of β-lg and α-la increased significantly and to the same extent (~4-fold), whereas that of the caseins did not change significantly. It was furthermore found that treatment of β-lg with 2 mM NEM in GndHCl decreased the ORAC value of this protein by ~10% (not shown). This limited effect of NEM in the ORAC assay as compared to the TEAC assay is consistent with the lower relative activity of Cys in the former assay.

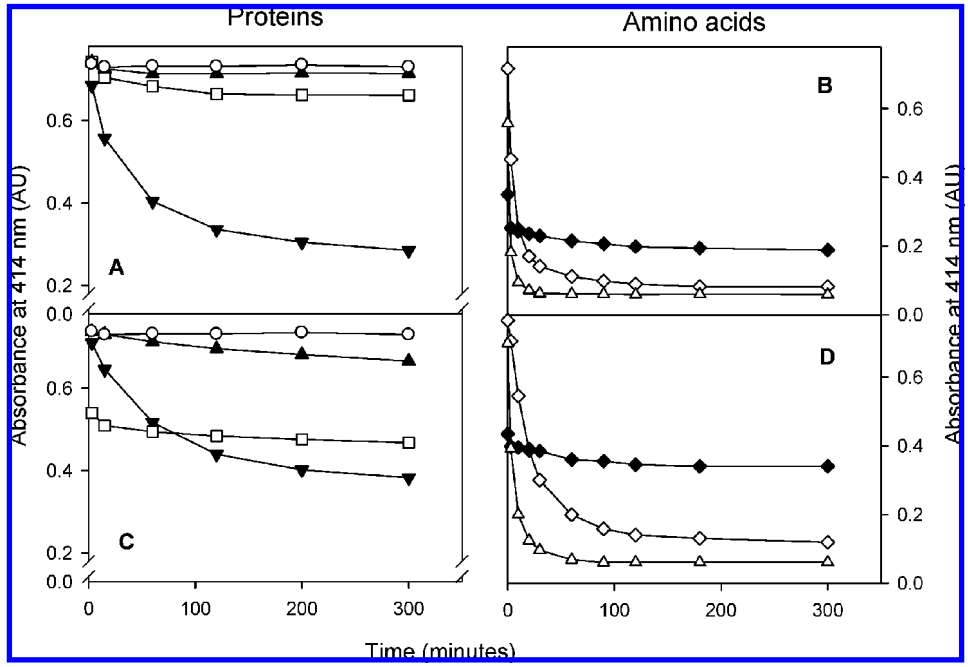

Figure 5. Rate of $ABTS^{\bullet+}$ bleaching in the absence (**A** and **B**) or presence (**C** and **D**) of 4.0 M GndHCl. In displays **A** and **C**, the reactions of caseins (▼), β-lg (□), and α-la (▲) are shown and compared with a blank (○). In displays **B** and **D** the reactions of tyrosine, 120 μM (◇), cysteine, 20 μM (◆), and tryptophan, 220 μM (△) are shown. Amino acid concentrations were chosen to give rates in the same range as the proteins. Results are representative of at least three experiments.

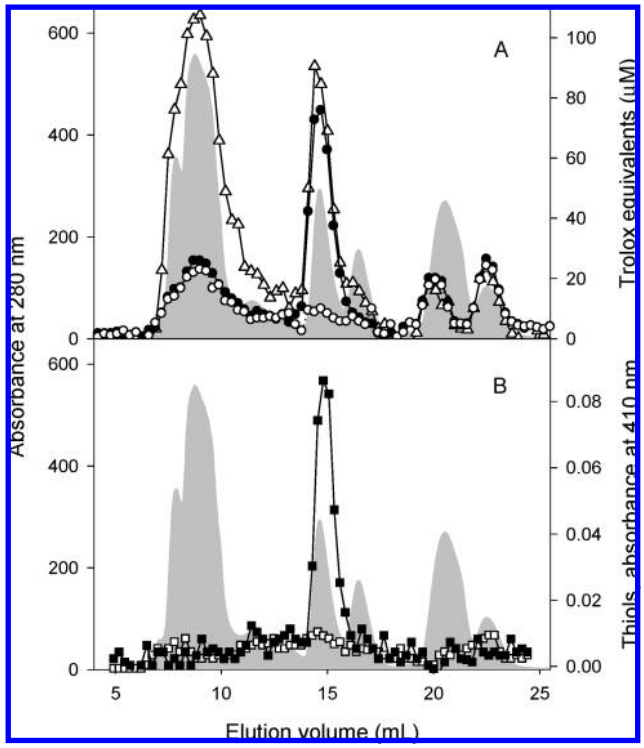

Figure 6. (**A**) Radical scavenging of $ABTS^{\bullet+}$ in the presence of 4.0 M GndHCl after 3 min (●) or 60 min (△). The effect of thiol blocking by NEM is shown after 3 min (○). (**B**) Free thiols determined in the absence (□) or presence (■) of 4.0 M GndHCl. Representative results of two independent experiments are shown.

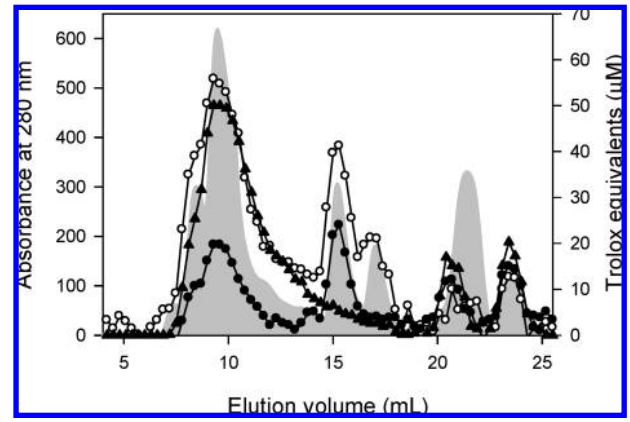

Figure 7. Postcolumn digestion of SEC fractions with protease followed by determination of radical scavenging in the TEAC assay in the absence (○) or presence of 4.0 M GndHCl (●) after 3 min of reaction. For comparison the results before digestion in the absence of GndHCl are included (▲).

DISCUSSION

Antioxidant capacity assays, for example, ORAC and TEAC, are often criticized for neglecting compositional and interfacial effects between aqueous and lipid phases (*19*). In addition, the actual reaction conditions may shift the effects of a compound from antioxidant to pro-oxidant (*20*). Thus, reactivity in these assays cannot be directly correlated with the ability to act as an antioxidant in foods. These problems can probably not be circumvented even by the use of multiple antioxidant assays, as suggested recently (*21*). However, our approach combining SEC with the TEAC and ORAC assays allows us to estimate the relative contributions of specific milk components to the radical scavenging of milk and, therefore, provides some evidence of their significance as antioxidants in milk.

Difference between TEAC and ORAC Assays. The major difference between the TEAC and ORAC assays is the higher sensitivity of the ORAC assay toward whey proteins. We found relatively higher potencies for the free amino acids in ORAC compared to the TEAC assay in accord with earlier papers (*22, 23*). Thus, one explanation for the difference between the assays is the higher number of amino acids with significant radical scavenging activity contributing to the ORAC value. Whereas

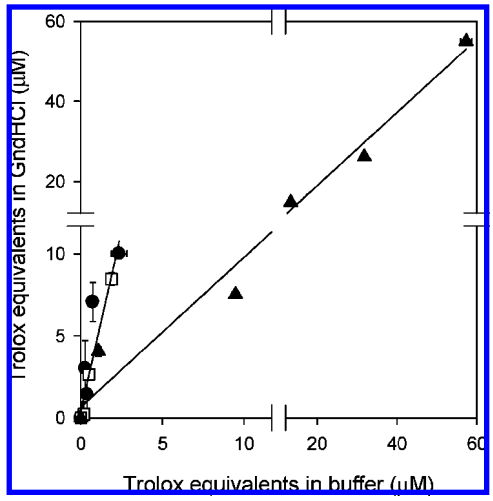

Figure 8. Effect of GndHCl on the radical scavenging activity of caseins (▲), β-lg (□), and α-la (●) from SEC fractions as determined in the ORAC assay. Slope for caseins was 0.91 and that for β-lg and α-la was 4.40. Results are mean ± SEM of triplicates from one or two similar experiments.

the loose structure of the caseins would give good solvent exposure of the amino acids with highest radical scavenging activity (Tyr and Trp), these amino acids are only partly solvent exposed in β-lg: Two tyrosines are buried or partially buried and two are solvent exposed; one Trp is buried and one is partially exposed to solvent, whereas the free Cys is buried in the interior of the protein (*24*). These steric constraints would have a larger effect on the bulky $ABTS^{\bullet+}$ radicals compared with the small peroxyl radicals generated in the ORAC assay (*25*), and our results in fact indicate that Tyr and Trp in β-lg are poorly accessible to $ABTS^{\bullet+}$. Cys and Trp in β-lg have been observed to act as antioxidants during autoxidation in oil-in-water emulsions, whereas Met did not (*10*). Thus, both composition and solvent exposure of amino acids seem to play crucial roles for the antioxidative properties of the native proteins and the results obtained in the two assays.

The slow reaction of Trp and Tyr as compared to Cys was found to further complicate the analysis of proteins in the TEAC assay. Analysis of plasma samples from blood in the TEAC assay reveals both a fast and a slow decay in $ABTS^{\bullet+}$ absorbance, which were related to urate and serum albumin, respectively (*13*). However, the presence of a free cysteine residue, which is also partly masked in albumin, would provide the protein with both a fast-reacting antioxidant along with slow-reacting Trp and Tyr moieties, and thus the reaction time contributes significantly to the TEAC value obtained. This was in fact observed in the case of β-lg when assayed in GndHCl.

Major Protein Radical Scavengers. Our results show that the major $ABTS^{\bullet+}$ and peroxyl radical scavengers in milk are the caseins. This is in accordance with previous results (*12*), where $ABTS^{\bullet+}$ scavenging and ferric reducing ability of different milk components were evaluated. The high radical scavenging activity of the caseins can probably be related to their random coil structure, and as expected our experiments in GndHCl and proteolytic digestion show that activity is neither gained nor lost by caseins under denaturing conditions. The major whey proteins are less potent, with β-lg and α-la being equally active in the ORAC assay. In the TEAC assay, the contribution of β-lg is small unless it is unfolded or digested and that of α-la insignificant. In the presence of GndHCl, the radical scavenging activities of both proteins increase most likely as a result of increased solvent exposure of amino acids previously buried in the interior of the proteins. This is a well-known effect and has been observed also after treatment of β-lg with heat or high hydrostatic pressure (*6*, *9*, *26*).

Simple calculations based on tabulated values for amino acid compositions of the caseins and whey proteins and our data presented in **Table 2** show that if all proteins acted as their constituent amino acids, the ratio between the TEAC value of the caseins and the whey proteins would be 4.2. In **Figure 7** we present the effect of proteolytic digestion, which shows contributions of the proteins after disruption of secondary structure. Here the ratio between caseins and whey proteins appears to be somewhat less (3.3), although more experiments would be required for statistical significance.

The effect of the free thiol group has been estimated to ~20% (*9*) compared with our results of 10% in ORAC. Others found that β-lg accounted for ~50% of the milk antioxidant activity during copper-induced oxidation of low-density lipoproteins (*27*), which further points to the possible importance of structural changes occurring in the lipid−aqueous interface or nature of the radical initiator.

We found that the most prominent radical scavenger in milk serum is β-casein, consistent with the well-known dissociation of β-casein molecules from the casein micelles during the cold storage of milk (*28*). The molecular mass of ~100 kDa corresponds to a tetramer of this protein, which is in good accordance with results from the literature (*29*). Preparation of whey by acidification completely eliminated this scavenging activity, and none of the other proteins in whey with a molecular mass ~100 kDa contributed significantly to radical scavenging. Lactoferrin, which is assigned antioxidant activity due to its ability to bind iron (*30*), is therefore not a significant radical scavenger in milk. Colbert and Decker (*11*) ascribed the antioxidative activity of whey in phosphatidylcholine liposomes to a dialyzable compound with a molecular mass of 500−5000 Da, but we could not detect such a compound with our assays.

LMW Compounds. Oxidation of milk probably begins immediately after secretion from the lactating epithelial cells and is further challenged when it leaves the udder. Our results show that ascorbate and urate are the only significant LMW antioxidants in milk, which is consistent with the results of Østdal et al. (*17*). Whereas some assign urate as the most important LMW antioxidant in milk (*12*), other results show that ascorbate is preferentially oxidized over urate (*17*). Ascorbate will be oxidized preferentially over urate in milk as indicated by the redox potentials of ascorbyl radical/ascorbate (282 mV vs NHE) and urate monoanion radical/urate (590 mV vs NHE) (*31*). Because preparation of whey may lead to accelerated oxidation, this would also explain the failure of others to detect ascorbate in milk (*12*) and why the ascorbate peak in whey is smaller compared to that observed in milk and milk serum.

We conclude that the number of significant contributors to milk radical scavenging activity is relatively low as measured by two complementary assays. Our results indicate that the ORAC assay is probably the most suitable radical scavenging assay for estimation of the antioxidative activity of proteins in food-related systems, such as oil-in-water emulsions, due to the size and nature of the peroxyl radicals generated in this assay. However, we emphasize that our data provide a simplified picture of the major radical scavengers in milk and that more work on early oxidative events in milk and on the interactions between anti- and pro-oxidant compounds is needed.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane); ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GndHCl, guanidinium hydrochloride; HMW, high molecular weight; α-la, α-lactalbumin; β-lg, β-lactoglobulin; LMW, low molecular weight; MALDI-TOF MS, matrix-assisted laser desorption−ionization time-of-flight mass spectrometry; NEM, *N*-ethylmaleimide; ORAC, oxygen radical absorbance capacity; ROS, reactive oxygen species; SEC, size exclusion chromatography; TEAC, Trolox equivalent antioxidant capacity.

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