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Identification of lactoperoxidase in mature human milk

Kouichirou Shin,*,† Mamoru Tomita,* and Bo Lönnerdal†

**Nutritional Science Laboratory, Morinaga Milk Industry Co. Ltd., Zama, Kanagawa, Japan; and †Department of Nutrition, University of California, Davis, CA USA*

Myeloperoxidase (MPO) derived from milk leukocytes and lactoperoxidase (LPO) secreted from the mammary gland have been identified previously in human colostrum. These peroxidases are known to play host defensive roles through antimicrobial activity. The goals of this study were to measure the peroxidase activity in mature human milk and to characterize the enzyme responsible for the activity. As determined using 3,3′,5,5′-tetramethylbenzidine as substrate, whey prepared from human milk samples obtained 1 and 5 months postpartum showed levels of peroxidase activity equivalent to 0.13 ± 0.18 and 0.24 ± 0.21 μg/mL bovine LPO (bLPO; n = *13), respectively. Whey from early milk was fractionated into two peaks of peroxidase activity by cation-exchange chromatography; the peroxidase in the first peak was sensitive to dapsone, which is an inhibitor of LPO, whereas the second peroxidase was not. Whey from mature milk showed only the first peak. Purified bLPO and MPO showed chromatographic behaviors that were similar to the first and second peaks, respectively. The dapsone-sensitive peroxidase from mature milk was further purified (952-fold from whey) by hydrophobic interaction chromatography. This preparation showed two bands with molecular masses of 80 and 90 kDa by polyacrylamide gel electrophoresis and immunoblotting using an antibody against bLPO. After deglycosylation, two distinct proteins with lower molecular weights were observed. Amino acid sequencing indicated that both of these proteins are LPO. These results provide evidence that LPO is present in mature human milk and that it is responsible for most of the peroxidase activity in mature milk.* (J. Nutr. Biochem. 11:94–102, 2000)

Keywords: lactoperoxidase; myeloperoxidase; human milk; amino acid sequence

Introduction

In all mammalian species tested thus far, peroxidase activity is present in the milk. Activity is highest in guinea pigs, intermediate in cows, sheep, pigs, llamas, and mice, and lowest in humans.[1–4] Peroxidase activity is also found in saliva, tears, cervical mucus, and other biological secretions.[5] In these secretions, peroxidases play a role in host defense through antimicrobial action and removal of toxic hydrogen peroxide (H_2O_2).[5–7] The peroxidase activity in bovine milk is attributable to lactoperoxidase (LPO) secreted from the mammary gland. This enzyme is a heme-containing glycoprotein with a molecular mass of approximately 78 kDa.[7,8] In the presence of H_2O_2, LPO catalyzes the oxidation of thiocyanate to produce hypothiocyanate, which has broad-spectrum antimicrobial activity.[1,7] Other biological effects of this protein including anti-tumor activity[9] and immunoregulatory effects[10] have been reported.

The origin of peroxidase activity in human milk has been a subject of controversy.[1,7,11,12] Two different heme-containing peroxidases—myeloperoxidase (MPO) and LPO—have been identified in human colostral whey, mainly by means of immunologic techniques using antibodies directed against MPO or bovine LPO (bLPO). MPO is found in blood neutrophils and monocytes and is released from these cells by stimulation.[13,14] This enzyme plays an important role in the oxygen-dependent antimicrobial system of neutrophils. In this system, MPO catalyzes the oxidation of chloride ions (Cl^-) by H_2O_2 to produce hypochlorous acid, which has potent antimicrobial activity.[13] Early reports on the identification of peroxidase in colostrum concluded that the peroxidase activity in human colostral whey is attributable to MPO released from milk leukocytes.[15,16] However, other purification studies concluded that colostrum contains

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Address correspondence to Dr. Bo Lönnerdal, Department of Nutrition, University of California, One Shields Ave., Davis, CA 95616.
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LPO, but only at a very low concentration, representing 0.004% of total milk protein.[17,18] Furthermore, in a comparative kinetic study using several substrates, the ratio of LPO activity to total peroxidase activity in colostrum was estimated at only 2.6%.[19] It has been commonly observed that total peroxidase activity in human milk is highest in colostrum and that the activity declines rapidly during the first several days of lactation.[2,20] This phenomenon appears to be closely associated with the decreasing number of milk leukocytes during this period.[15,21] These previously reported studies on characterization of LPO were all performed using human colostrum.

Although the origin of the activity has not been known, some studies have noted that mature human milk samples have peroxidase activity[2–4,7,20] at levels sufficient to exhibit a bactericidal effect against *Escherichia coli* in the presence of added thiocyanate and H_2O_2.[20] It seems likely that such an enzyme secreted in milk throughout the lactation period would exert some physiologically relevant activity in terms of both the mammary gland and the gut of infants, even if the concentration is relatively low. Therefore, it is of interest to measure the peroxidase activity in mature human milk and to determine the relative contribution of LPO and MPO to the activity.

In the present study, we employed a sensitive colorimetric assay using 3,3′,5,5′-tetramethylbenzidine (TMB)[7,22] as substrate to detect low levels of peroxidase activity in human milk. The peroxidase found in mature milk was partially purified by column chromatography and biochemically characterized as LPO, which is a true secretory enzyme.

Methods and materials

Materials

HiTrapSP 1 mL and 5 mL columns, a Phenyl Superose HR 5/5 column, and Superloop 10 mL were purchased from Pharmacia (Uppsala, Sweden). Nylon membrane filter cartridges with a 0.45 μm pore size were obtained from Gelman Sciences (Ann Arbor, MI USA). The centrifugal membrane concentrator used, Centriplus 30, was from Amicon (Beverly, MA USA). LPO purified from bovine milk (A_{412}/A_{280} = 0.93), MPO purified from human leukocytes, proteases, TMB, dapsone (4,4′-diaminodiphenylsulfone), and Coomassie Brilliant Blue R concentrate were from Sigma Chemical Co. (St. Louis, MO USA). Rabbit anti-MPO antibodies were from Athens Research and Technology (Athens, GA USA). Catalase from beef liver was purchased from Boehringer Mannheim Co. (Indianapolis, IN USA). Enhanced chemiluminescence (ECL) reagent and peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) were from Amersham Life Science (Arlington Heights, IL USA). GlycoShift™ protein de-N-glycosylation kit was from Oxford Glycosystems (Rosedale, NY USA). Cetyltrimethylammonium chloride (CETAC) was from Aldrich Chemical Co. (Milwaukee, WI USA). Other chemicals used were of the highest grade available.

Human milk

Human milk samples were donated by healthy mothers (n = 13) at various times during the lactation period and were kept frozen at −20°C until use. Each sample was centrifuged at 264,000 × g at 4°C for 1 hour. The lipid layer was removed and the supernatant (whey) was filtered through a membrane cartridge. In some experiments, the remaining sediment was washed with phosphate buffered saline (PBS; 138 mmol/L sodium chloride, 2.7 mmol/L potassium chloride, 10 mmol/L phosphate buffer, pH 7.4) and extracted with the same original volume of 0.1% CETAC in 25 mmol/L sodium acetate buffer, pH 5.4. The sediment extract was centrifuged at 26,000 × g at 4°C for 15 minutes to remove particulate material.

Peroxidase activity

Peroxidase activity was assayed by measuring H_2O_2-dependent oxidation of TMB as described by Thomas et al.[7,22] with slight modifications. Assay mixtures containing 50 mmol/L sodium acetate buffer, pH 5.4, 0.3 mmol/L H_2O_2, 1.4 mmol/L TMB, 1.4% (v/v) N,N′-dimethylformamide, 0.01% Triton X-100, and 0.3 M sucrose in 1.75 mL were incubated at 37°C for 3 minutes. The reaction was stopped by the addition of 50 μL of 0.3 mg/mL catalase in PBS and 1.7 mL of cold 0.2 M acetic acid. The mixture was centrifuged to clarify the solution and the absorbance was measured at 655 nm. One unit of activity was defined as the amount producing a change in absorbance of 1 unit per 3 minutes per milliliter of the final assay mixture.

Protein assay

Protein content was determined by the method of Lowry et al.[23] using bovine serum albumin (BSA) as the standard.

Column chromatography

Whey proteins were fractionated by fast protein liquid chromatography (FPLC) using a 1 mL or 5 mL HiTrapSP cation-exchange column in 50 mmol/L sodium phosphate buffer, pH 6.4. The flow rate was 1 or 5 mL/min for the 1 or 5 mL column, respectively. Ten or 50 mL of whey were diluted twice with starting buffer and applied to the column using a Superloop or a peristaltic pump. The column was washed until the absorbance at 280 nm returned to baseline level. Then, a linear gradient of 0 to 1 mol/L sodium chloride was applied and programmed for 60 minutes. Fractions were collected every 1 minute and assayed for peroxidase activity in the presence or absence of 0.3 mmol/L dapsone.

From cation-exchange chromatography of whey, fractions found to display dapsone-sensitive peroxidase activity were pooled and adjusted to contain 1.7 mol/L ammonium sulfate by adding solid ammonium sulfate. The mixture was then fractionated by FPLC using Phenyl Superose HR 5/5 column equilibrated in 50 mmol/L sodium phosphate buffer, pH 7.0, and 1.7 mol/L ammonium sulfate. A linear gradient of 1.7 to 0 mol/L ammonium sulfate at a flow rate of 0.5 mL/min was applied and programmed for 60 minutes. Fractions of 1 mL each were collected and assayed for peroxidase activity. The fractions showing the highest levels of peroxidase activity (total 3 mL) were pooled and concentrated to 0.4 mL by a centrifugal membrane concentrator.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli.[24] The acrylamide concentration of the gels used was 8% throughout the investigation. To detect the proteins, gels were stained with Coomassie Brilliant Blue R solution and destained with 25% ethanol with 10% acetic acid.

Immunoblotting

Antibodies against bLPO were prepared by Antibodies, Inc. (Davis, CA USA). A standard immunization protocol was employed to immunize female New Zealand white rabbits. Antigen

(100 μg of bLPO) was injected five times into each rabbit with an interval of 2 weeks between injections. Antiserum was fractionated by ammonium sulfate precipitation to obtain IgG. Antibody specificity was tested by immunoblotting using bovine acid whey by the following method. From 1 μL of bovine whey, the antibodies gave a single band at a position corresponding to a molecular mass of 78 kDa. The proteins fractionated by SDS-PAGE were electrotransferred to nitrocellulose sheets by the method of Towbin et al.[25] using a Hoefer model TE22 transfer apparatus (Pharmacia). After electrotransfer, the blots were blocked at room temperature for 1 hour with 5% BSA in PBS containing 0.1% Tween 20 (PBS-Tween). Then the blots were washed three times in PBS-Tween for 10 minutes and incubated for 1 hour in a solution of anti-bLPO antibodies at 1:5,000 dilution in PBS-Tween. After three washes with PBS-Tween for 10 minutes, the blots were incubated for 1 hour in a solution of peroxidase-conjugated donkey anti-rabbit IgG at 1:10,000 dilution in PBS-Tween. After three washes with PBS-Tween for 10 minutes, the blots were visualized with ECL reagent and X-ray film according to manufacturer's instructions. For comparison, rabbit anti-MPO antibodies were used as the primary antibody instead of anti-bLPO antibodies at the same dilution.

Deglycosylation

bLPO and human LPO (hLPO) were deglycosylated using the GlycoShift protein de-N-glycosylation kit with peptide-N-glycosidase F according to manufacturer's instructions. Then, samples were analyzed by immunoblotting using anti-bLPO antibody as described above.

Amino acid sequencing

Amino acid sequencing was performed in the Protein Structure Laboratory of the University of California, Davis. For analysis of the amino-terminal sequence, 3.2 μg of hLPO was subjected to SDS-PAGE and electro-transferred to a polyvinylidinedifluoride membrane (ProtBlot, PE Biosystems, Foster, CA USA) by the method described above in Immunoblotting. The membrane was briefly rinsed with distilled water and soaked in 100% methanol for a few seconds. Then the proteins on the membrane were stained with 0.1% Coomassie Brilliant Blue R in 40% methanol and 1% acetic acid for 1 minute with gentle shaking and destained with 50% methanol. The membrane was thoroughly rinsed with distilled water and dried. The two protein bands corresponding to those visualized after immunoblotting with anti-bLPO antibodies were individually excised and their amino-terminal sequences were analyzed using an ABI model 477A protein sequencer (Applied Biosystems, Foster, CA USA). For determination of the internal sequence, the modified in-gel digestion technique described by Shevchenko et al.[26] was used. hLPO (12.8 μg) was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R. The two bands corresponding to those visualized after immunoblotting with anti-bLPO antibodies were individually excised from the gel. These proteins were digested with trypsin (sequencer grade modified porcine trypsin; Promega, Madison, WI USA) and fractionated by reversed-phase high performance liquid chromatography (HPLC) using a C_{18} microbore column (1 × 250 mm; Vydac, Hesperia, CA USA). The solvents for HPLC were as follows: A, 0.1% trifluoroacetic acid in water; B, 70% acetonitrile, 0.1% trifluoroacetic acid, and 30% water. A linear gradient of 10 to 70% solvent B with flow rate of 70 μL/min was applied and programmed for 115 minutes. Peptides were detected by monitoring the absorbance at 210 nm. The peptide constituents of selected peaks were analyzed to determine their amino acid sequences. A search for sequences similar to the determined sequences was conducted through the SWALL protein sequence database using the FASTA program at the European Bioinformatics Institute (http://www2.ebi.ac.uk/fasta3/).[27]

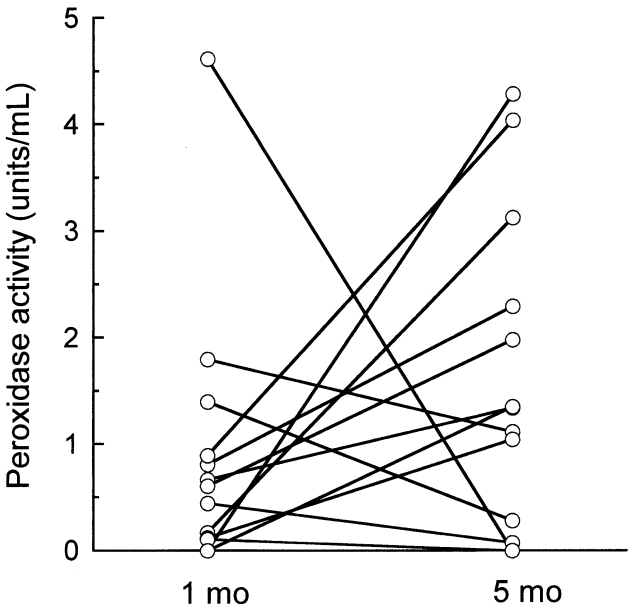

Figure 1 Levels of peroxidase activity in whey prepared from milk samples obtained 1 and 5 months after start of lactation (n = 13). Values represent the mean of duplicate assays.

Results

Peroxidase activity in human whey was effectively measured using TMB as substrate. Although we omitted the final dilution of the reaction mixture from the original method described by Thomas et al.[7,22] so that we could detect relatively low levels of peroxidase activity in human milk, the definition of units of activity in our method was identical to the original one. The levels of peroxidase activity varied greatly in whey samples (*Figure 1*). In whey prepared from milk obtained from 13 different mothers 1 and 5 months after the start of lactation, the levels of peroxidase activity were 0.89 ± 1.20 and 1.61 ± 1.41 units/mL (mean ± SD; 35 ± 3 and 150 ± 8 days postpartum), respectively. These values are equivalent to 0.13 ± 0.18 and 0.24 ± 0.21 μg/mL of bLPO as calculated by the standard equation: peroxidase activity [units] = 6.77 × bLPO [μg] − 0.01 (r = 1.00). Thus, overall the mean activity of peroxidase in human milk increased from 1 to 5 months of lactation; however, there was considerable variation among women.

Figure 2 shows typical chromatograms for peroxidases from different sources. No peroxidase activity was detected in the flow-through fraction. Whey prepared from early milk showed two distinctive peaks of peroxidase activity (*Figure 2A*; 10 days postpartum); the peroxidase in the first peak (0.2 mol/L NaCl) was sensitive to dapsone, which is a specific inhibitor of LPO activity,[7,22] whereas the peroxidase in the second peak (0.5 mol/L NaCl) was not. The sediment extract from this milk sample showed a few peaks of dapsone-insensitive peroxidase (*Figure 2B*). In contrast,

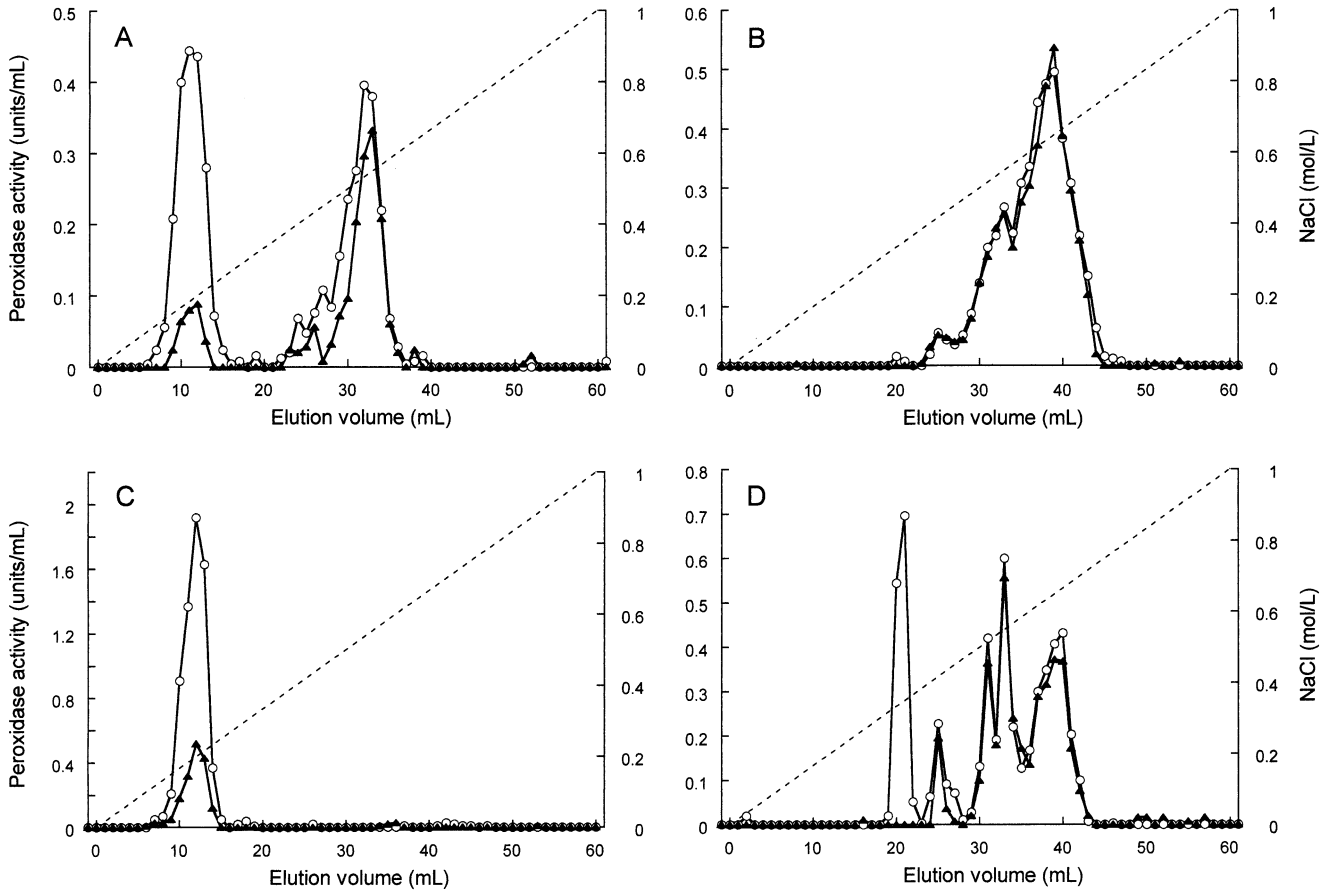

Figure 2 Cation-exchange chromatography of peroxidases. Proteins were eluted with a gradient of 0 to 1.0 M NaCl in 50 mmol/L sodium phosphate buffer, pH 6.4. Each fraction was assayed for peroxidase activity in the presence (closed triangle) or absence (open circle) of 0.3 mmol/L dapsone. (A) 10 mL of whey prepared from early milk (0.62 units/mL, 10 days postpartum). (B) 30 mL of sediment extract from early milk (0.45 units/mL, obtained from the same milk sample as panel A). (C) 10 mL of whey prepared from mature milk (4.48 units/mL, 59 days postpartum). (D) Bovine lactoperoxidase 5 μg and myeloperoxidase 5 μg.

whey prepared from mature milk showed only one peak of dapsone-sensitive peroxidase, which eluted at 0.2 mol/L NaCl (*Figure 2C*; 59 days postpartum). Because only a trace level of peroxidase activity was detected in the sediment extract from this milk sample, fractionation was not performed. However, the chromatograms for whey prepared from some samples of mature milk showed peaks of dapsone-insensitive peroxidase activity. In this case, the ratio of dapsone-insensitive peroxidase activity to the total activity recovered from the column was much lower compared with early milk (data not shown). Chromatography of purified bLPO and MPO resulted in a single dapsone-sensitive peak and several dapsone-insensitive peaks of peroxidase activity, which eluted at 0.3 mol/L and 0.4 to 0.7 mol/L NaCl, respectively (*Figure 2D*). These purified peroxidases also showed their characteristic peaks when chromatographed individually (data not shown). Although the ionic strength required for elution from the column is slightly different, the elution behavior of the dapsone-sensitive peroxidase in the whey resembles that of bLPO. Similarly, the elution behavior of the dapsone-insensitive peroxidase in the whey and sediment extract resembles that of MPO. These results demonstrate the presence of LPO in mature milk as well as in early milk. It is also apparent that LPO is responsible for most of the peroxidase activity in mature milk, whereas MPO contributes only a small portion of the total activity.

We tested the stability of peroxidase activity in whey from mature milk against low pH and proteases. No significant inactivation of peroxidase activity was observed at pH 4.0 (*Figure 3A*). At pH 3.0, a mean of 47% of the activity remained after 2 hours at 37°C. Peroxidase was completely inactivated below pH 2.5. Interestingly, compared with the result from incubation of whey at pH 3.0 or 4.0, no further inactivation was observed by addition of pepsin at a concentration of 1.0 mg/mL at the same pH (*Figures 3A and 3B*). Treatment of whey with trypsin and pancreatin (both at 1.0 mg/mL) for 2 hours at 37°C reduces the activity to 70 and 83% of control, respectively. Chymotrypsin did not show any significant effect on peroxidase activity. Further studies are needed to elucidate the reasons for the high stability of the LPO activity against proteases.

For further purification of hLPO, 50 mL of a whey sample with a high level of peroxidase activity (10.45

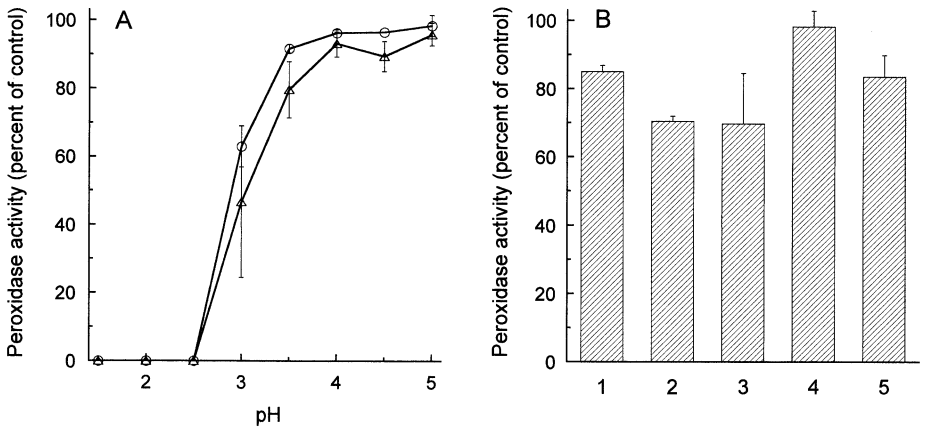

Figure 3 Effect of pH and proteases on peroxidase activity in whey from mature milk. (A) Two whey samples from mature milk (3.23 and 2.61 units/mL; 30 and 53 days postpartum, respectively) were adjusted to pH from 1.5 to 5.0 by addition of 1 mol/L HCl and incubated at 37°C for 1 (open circle) or 2 (open triangle) hours. Samples were neutralized by addition of 1 mol/L sodium bicarbonate. (B) Samples were treated with proteases at a final concentration of 1.0 mg/mL for 2 hours. Bar 1, pepsin at pH 4.0; bar 2, pepsin at pH 3.0; bar 3, trypsin; bar 4, chymotrypsin; bar 5, pancreatin. Remaining peroxidase activity is expressed as a percentage relative to the activity in original whey. Values represent the mean ± SD of two samples.

units/mL; 121 days postpartum) was fractionated by cation exchange chromatography using a 5 mL HiTrapSP column. The fractions found to contain dapsone-sensitive peroxidase activity were pooled and subjected to hydrophobic interaction chromatography using Phenyl Superose. *Table 1* summarizes the purification of hLPO. In a typical round of purification, 94-fold purification was achieved in the first cation-exchange chromatography step with a yield of 25%. In the second step, hydrophobic interaction chromatography, 952-fold purification was achieved with a yield of 16%.

The specific activity of the hLPO (857.4 units/mg; *Table 1*) was lower than that of bLPO (6,770 units/mg). Although this preparation of hLPO was not completely purified, as shown in the SDS-PAGE profile, each of the protein bands was nicely resolved (*Figure 4A*, lane 3). We used this preparation for further characterization of the hLPO. The absorption spectrum of the hLPO showed two main peaks at 280 and 412 nm as commonly observed for peroxidases containing the "heme 1" structure as a prosthetic group.[28] The A_{412}/A_{280} ratio in the case of hLPO was 0.36, whereas the ratio was 0.93 for bLPO.

Upon immunoblotting using anti-bLPO antibodies, hLPO was visualized as two distinct bands at positions corresponding to 80 and 90 kDa, whereas bLPO appeared as a single band at 78 kDa (*Figure 4B*). MPO was not detected with the same antibodies, suggesting that the antibodies were specific for LPO. The anti-MPO antibodies employed detected the heavy chain of MPO at 59 kDa but did not react with bLPO or partially purified hLPO (*Figure 4C*). Examination of a Coomassie-stained gel revealed that this sample of MPO was contaminated with a protein with a molecular mass of approximately 80 kDa (*Figure 4A*). After N-deglycosylation, a single band for bLPO was detected by immunoblotting with an intensity similar to that of the band of untreated bLPO, but it showed a smaller molecular mass of 70 kDa (*Figure 4D*). In the same experiment, two distinct bands of hLPO were observed at positions corresponding to 72 and 82 kDa (*Figure 4D*). These results indicate that hLPO is glycosylated to the same extent as bLPO and that the oligosaccharide chains have a molecular mass of approximately 8 kDa. These results also indicate that the anti-bLPO antibodies mainly recognize the polypeptide portion of these enzyme molecules. Taking these results into consideration, it seems more likely that hLPO displays molecular heterogeneity in its polypeptide chain rather than different glycosylation patterns.

We determined the amino-terminal and internal amino acid sequences of these two proteins visualized by immunoblotting using anti-bLPO antibodies. By searching a database, we detected a complete match between our amino acid sequences and the deduced sequence of salivary peroxidase (SPO),[29] which is another true secretory peroxidase found in human saliva.[6,30] The deduced amino acid sequence of SPO[29] matches that of hLPO[31,32] except for residue 244 (A for SPO, T for hLPO). This might suggest that SPO and hLPO are products of the same gene. The 80 kDa protein gave the amino-terminal sequence XRTAIRN corresponding to residues 81 to 87 of hLPO.[32] This region is upstream of the possible starting sites (position 101 or 129) for bLPO.[8,31] This result is in agreement with the observation that the molecular mass of this protein is slightly greater than that of bLPO, in both native and

Table 1 Purification of human lactoperoxidase

Step	Volume (mL)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Whey	50	580.2	522	0.9		100
HiTrapSP	20	1.53	129	84.3	94	25
Phenyl Superose	3	0.10	85	857.4	952	16

Fifty milliliters of whey from mature milk (10.45 units/mL; 121 days postpartum) was fractionated by cation exchange chromatography using a 5 mL HiTrapSP column. The fractions found to contain dapsone-sensitive activity were pooled and subjected to hydrophobic interaction chromatography using a Phenyl Superose HR5/5 column.

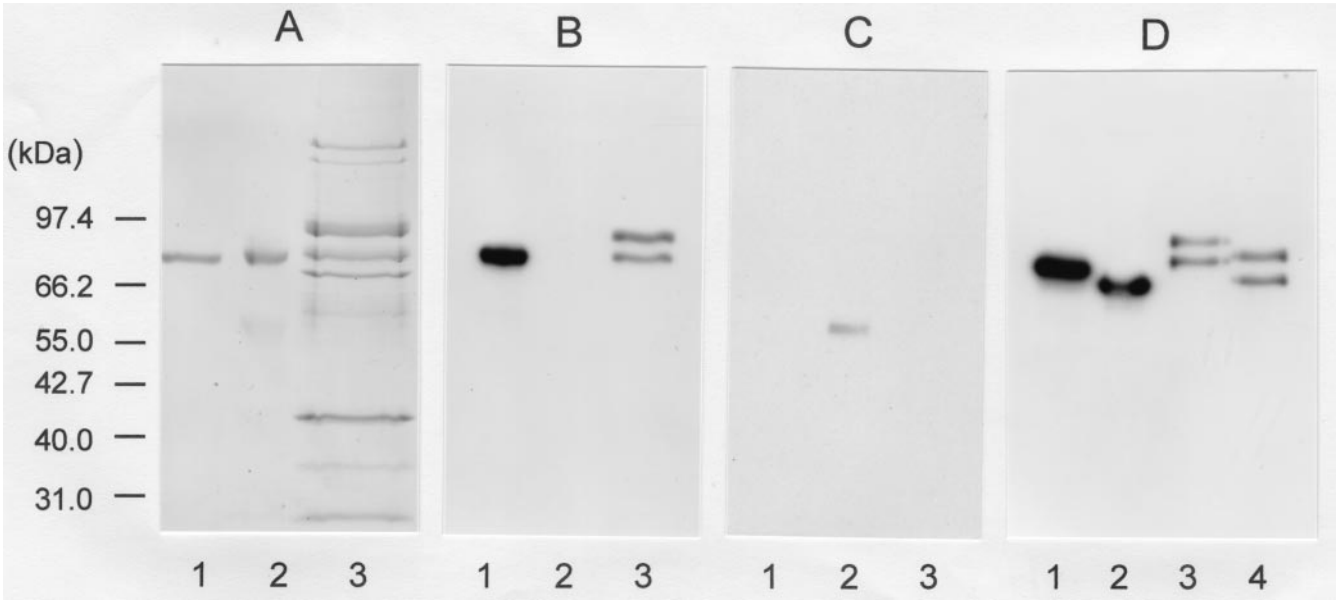

Figure 4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of peroxidases. (A) SDS-PAGE. Lane 1, 0.4 μg of bovine lactoperoxidase (bLPO); lane 2, 0.4 μg of myeloperoxidase (MPO); lane 3, 3.2 μg of partially purified human lactoperoxidase (hLPO) containing peroxidase activity equal to 0.4 μg of bLPO. (B) Immunoblotting using anti-bLPO antibodies. (C) Immunoblotting using anti-MPO antibodies. For the panels (B) and (C), the lanes are as follows: lane 1, 0.1 μg of bLPO; lane 2, 0.1 μg of MPO; lane 3, 0.8 μg of partially purified hLPO containing peroxidase activity equal to 0.1 μg of bLPO. (D) Deglycosylation of bLPO and hLPO. Peroxidases in samples treated with peptide-N-glycosidase F or untreated were detected by immunoblotting using anti-bLPO antibodies. Lane 1, 0.1 μg of control bLPO; lane 2, 0.1 μg of deglycosylated bLPO; lane 3, 0.8 μg of control hLPO; lane 4, 0.8 μg of deglycosylated hLPO. The blots were visualized by treatment with enhanced chemiluminescence reagent.

deglycosylated forms, as shown in *Figure 4*. The amino-terminal sequence could not be obtained from the 90 kDa protein due to poor recovery of amino acids in the analysis. This protein might be amino-terminally blocked as reported for bLPO.[31] Therefore, the amino-terminal structure of the 90 kDa protein could not be compared with that of 80 kDa protein. The elution profiles observed in reverse-phase HPLC analysis of peptides generated from the 80 and 90 kDa proteins were similar (data not shown). Thus, for analysis of the internal sequence of each of these two proteins, we selected a peptide peak derived from each protein that showed the same retention time by HPLC separation. The sequences of these homologous peptides were determined to be WLPAEYED in the 80 kDa protein (positions 163–170) and XLPAEYE in the 90 kDa protein (positions 163–169). This sequence is found in hLPO, MPO, and eosinophil peroxidase, but not thyroid peroxidase.[32–35] We determined another internal sequence of the 90 kDa protein and the sequence EQINALXSFLDASFVY (positions 295–310), which is unique to hLPO, was found. These results clearly indicate that both the 80 and 90 kDa proteins are LPO.

Figure 5 compares the sensitivity of hLPO, bLPO, and MPO to inhibitors and Cl^-. The sensitivity of hLPO to dapsone was similar to that of bLPO, whereas MPO was much less sensitive to this reagent (*Figure 5A*). Sodium azide, a classical peroxidase inhibitor, inhibited hLPO but to lesser extent than bLPO (*Figure 5B*). It is well known that bLPO and MPO show similar substrate specificity in terms of reactions with halide, but only MPO can catalyze the oxidation of Cl^-. This feature of the substrate specificity of peroxidases has been applied to study the origin of peroxidases in secretions.[7,19,36] The observed activity of MPO was inhibited by addition of Cl^- (*Figure 5C*), suggesting that competition may occur between TMB and Cl^- as substrates. In contrast, Cl^- slightly increased the observed activity of both bLPO and hLPO.

Discussion

Several techniques differing in sensitivity and specificity have been used to assay peroxidase activity in human milk.[1–4,15–21] Therefore, values in previous reports cannot be directly compared with our data. It is apparent from our data that mature milk contains low but detectable peroxidase activity. The levels of peroxidase activity in human whey at 1 and 5 months of lactation were 0.89 ± 1.20 and 1.61 ± 1.41 units/mL (mean ± SD, $n = 13$), respectively. These average values are more than 100 times lower than that of peroxidase activity in acid whey from pooled bovine milk (179.6 ± 63.2 units/mL, mean of three experiments). The difference in the levels of peroxidase activity in human and bovine milk is consistent with other reports.[1–4,7,20]

Our report provides the first evidence of the presence of LPO in mature human milk. The cation-exchange chromatogram of whey from mature milk showed a single major peak of dapsone-sensitive peroxidase. Our method for purification of hLPO consists of a cation exchange chromatography step and a subsequent hydrophobic interaction chromatography step. This method is principally the same as that applied in the case of bovine milk[37] or human colostrum.[18,19] The preparation of bLPO with a purity index $A_{412}/A_{280} = 0.90$ was obtained from bovine acid whey by our method (details not shown). The purification index of

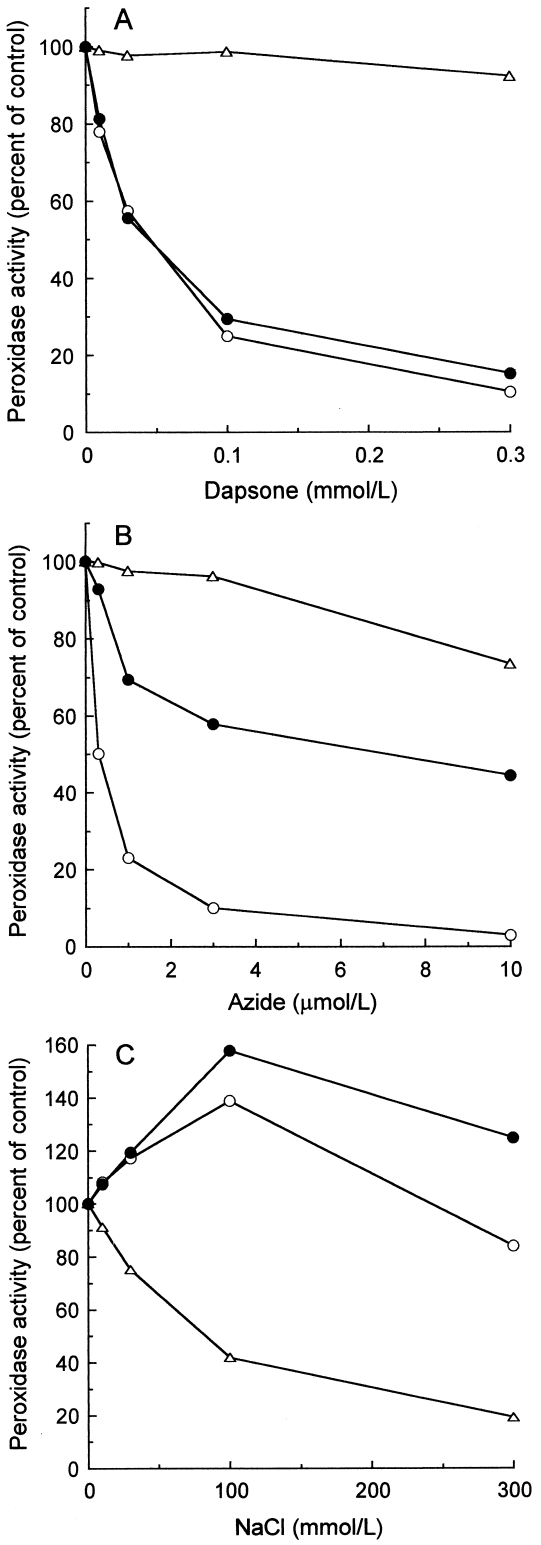

Figure 5 Influence of inhibitors and chloride ions on the peroxidase reaction. The peroxidase activity of 0.1 μg of bovine lipoperoxidase (open circle), 0.8 μg of human lactoperoxidase (closed circle), or 0.1 μg of myeloperoxidase (open triangle) was assayed in the presence of various concentrations of the following inhibitors: (A) dapsone, (B) sodium azide, and (C) sodium chloride. Activity is expressed as a percentage relative to the activity in the absence of the inhibitor or Cl^-. Values represent the mean of duplicate assays.

952-fold for hLPO in this study was comparable to that of 1,450-fold purification reported for this protein from colostral whey by immunoaffinity chromatography.[18] Partially purified hLPO obtained by hydrophobic interaction chromatography showed properties similar to bLPO in terms of absorption spectrum, recognition by anti-bLPO antibodies in immunoblotting, molecular mass, glycosylation, and sensitivity to inhibitors. We further identified hLPO by amino acid sequencing of an electrophoretically purified sample.

The low abundance and large variability in levels of peroxidase activity in human milk may have made the characterization of this enzyme difficult. Our results obtained from the cation exchange chromatography and characterization of the peroxidase suggest that LPO contributes most of the peroxidase activity in mature milk, whereas MPO contributes only a small part of the total activity. The average levels of peroxidase activity detected in mature milk samples at 1 and 5 months after the start of lactation are equivalent to 0.13 and 0.24 μg/mL bLPO, respectively. If it is assumed that bLPO and hLPO have the same specific activity, these concentrations represent 0.0025 and 0.0046% of the total whey protein in a typical sample of mature milk (5.17 mg/mL).[38] Such a content is similar to that (0.004%) estimated for hLPO in colostrum.[18] Therefore, LPO appears to be present at appreciable concentrations throughout lactation, whereas MPO levels decrease drastically together with the decline in the number of leukocytes in the early period of lactation.

The peroxidase system is one of the important host defense systems in the oral cavity.[6] The average concentrations of SPO and MPO in the supernatant of whole saliva have been estimated as 1.7 and 0.6 μg/mL, respectively, using a colorimetric assay similar to the one we used.[22] In another study using an immunometric assay, slightly lower levels of these enzymes were reported; the average concentrations of SPO and MPO were 0.34 and 0.24 μg/mL, respectively.[39] SPO is true secretory enzyme derived from the salivary gland and it cross-reacts with antibodies against bLPO.[22,30,39] Purified SPO differs slightly from bLPO in terms of its substrate specificity, azide sensitivity, glycosylation, and molecular weight.[6,30,36] However, a recent genetic study has suggested that an interrelationship exists between SPO and LPO. The cDNA for SPO cloned from the salivary gland was found to be homologous to a partial cDNA encoding the carboxy-terminal 324 residues of hLPO cloned from a mammary gland library and the genomic LPO gene, which is on chromosome 17.[29,31,32] Our amino acid sequencing further revealed the primary structure of the amino-terminal region of hLPO, which had not been determined in the cDNA cloning study on this enzyme.[31] Our sequencing results completely match the amino acid sequence deduced from the cDNA for SPO[29] and that deduced from the chromosomal gene for hLPO.[32] Therefore, it is likely that hLPO and SPO are products of the same gene and have the same biochemical and biological characteristics. In our study, two bands with molecular masses of 80 and 90 kDa were visualized upon immunoblotting using anti-bLPO, suggesting heterogeneity of the hLPO molecule. After deglycosylation, two bands with molecular masses of 72 and 82 kDa were observed. One possible mechanism for

the heterogeneity of hLPO is that the 80 kDa protein is secreted from the mammary gland into milk after complete proteolytic processing, whereas the 90 kDa protein is secreted with less processing. Similar structural heterogeneity has been also reported for SPO.[22,30,40] The average activity of SPO (6 units/mL) reported for the enzyme in saliva using TMB as a substrate[22] is somewhat higher than the level of peroxidase activity in mature human milk, which is mainly attributable to LPO. Further study is needed to elucidate the regulatory mechanisms controlling secretion of this enzyme.

LPO may exert some protective effects such as antimicrobial activity and removal of toxic H_2O_2 both in the mammary gland and in the gut of infants throughout the lactation period. In our study, LPO in mature human milk was stable at pH 4.0. This value is lower than pH 5.2, which has been reported for gastric contents in infants 2 hours after feeding.[41,42] In addition, LPO in human milk was not susceptible to pepsin and other proteases. Therefore, it is likely that some activity of ingested LPO will survive during digestion in the stomach and intestine of infant. Human milk contains many other protective factors including secretory IgA, lactoferrin, and lysozyme.[11,12,21] Some additive or synergistic antimicrobial effects between LPO and these protective factors have already been reported.[43–45] A detailed study focusing on the antimicrobial activity of LPO at physiologic concentrations in the presence or absence of these other protective factors is now in progress.

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