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Lactoperoxidase from human colostrum

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The present study has confirmed that human colostrum contains a lactoperoxidase (EC 1.11.1.7) [Langbakk & Flatmark (1984) FEBS Lett. **174**, 300–303], which represents about 0.004% of the total protein in crude colostrum. An apparent 32-fold purification of the enzyme was obtained by a multistep procedure, as modified from that of the bovine enzyme, with a recovery of about 7%. By use of chromatography on an immunoaffinity column (directed against bovine lactoperoxidase B), an apparent 1450-fold purification was obtained in a single step, with a recovery of 21%. The enzyme behaved as a glycoprotein (binding to concanavalin A–Sepharose), and revealed spectral properties (Soret peak at 412 nm) and an M_r (80000) similar to those of the bovine enzyme.

INTRODUCTION

Human breast milk contains many enzymes, some of which may assist in digestion or have other functions (Heitlinger, 1983). Particular interest has been focused on the possible presence of a lactoperoxidase (LPO, EC 1.11.1.7), in view of the proposal that oestrogen-induced peroxidase activity, in the rough endoplasmic reticulum and the secretory granules of the acinar cells, may be used as a criterion for distinguishing between hormone-dependent and hormone-independent mammary cancer (Anderson *et al.*, 1975; De Sombre *et al.*, 1975). However, only fairly recently has an LPO been demonstrated in human colostrum (Langbakk & Flatmark, 1984). In order to characterize this peroxidase further, we have looked for alternative approaches to purify it from pooled colostrum.

MATERIALS AND METHODS

Chemicals

Phenyl-Sepharose CL-4B, concanavalin A–Sepharose, Protein A–Sepharose and Sephadex G-150 were obtained from Pharmacia (Uppsala, Sweden), Bio-Gel P-10 and CNBr-activated Sepharose 4B were from Bio-Rad Laboratories (Richmond, CA, U.S.A.), and CM-cellulose 23 was obtained from Whatman Biochemicals (Maidstone, Kent, U.K.). Phenylmethanesulphonyl fluoride, benzamidine and soya-bean trypsin inhibitor was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Leupeptin was from the Peptide Institute (Osaka, Japan). Other reagents used were of analytical-grade purity. Rabbit anti-(human IgA) antibody, specific for heavy chain and secretory component, was obtained from Dakopatts (Glostrup, Denmark).

Human colostrum

Samples of human colostrum, obtained from healthy donors 2–4 days after delivery, were pooled and kept frozen (maximum 2 weeks) at −25 °C until used.

Isolation of bovine LPO

Bovine LPO B was isolated in a highly purified form as described by Carlström (1969*a*); the specific activity was 9.4 units/mg of protein.

Extraction of peroxidases from human leucocytes

White blood cells were obtained from buffy coats of fresh blood of a patient with allergy (10% eosinophils) as described by Bassøe *et al.* (1983). Myeloperoxidase and eosinophil peroxidase were extracted from the packed cells with 50 mM-potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X-100 and four proteinase inhibitors (see the legend to Fig. 5).

Polyclonal antibodies

Polyclonal antibodies were raised in rabbits against h.p.l.c.-purified bovine LPO B (Fig. 3*c*) by a standard immunization protocol. Specific IgGs were purified as described by DeMey (1983).

Size-exclusion h.p.l.c.

Size-exclusion h.p.l.c. was performed with a TSK-G 3000 SW and a TSK-G 4000 SW (Toyo Soda, Tokyo, Japan) h.p.l.c. columns (both 0.75 cm × 60 cm) coupled in series. The effluent was monitored by a Hewlett–Packard model 1040 A photodiode array detector.

Immunoaffinity adsorption and chromatography

Protein A–Sepharose was swollen in 100 mM-potassium phosphate buffer, pH 8.0. For each adsorption experiment approx. 30 μl of swollen gel (equivalent to 10 mg dry wt.) was preincubated with affinity-purified or partly purified IgGs (directed against bovine LPO B) diluted in the equilibration buffer. The suspension was gently stirred for 1 h at 0 °C. After washing with the equilibration buffer (20 bed volumes × 5), the Sepharose beads were suspended in 200 μl of 100 mM-potassium phosphate buffer, pH 7.4, to which 30–100 μl of the material containing peroxidase activity was added. The suspension was gently stirred for 1 h at 0 °C, and the supernatant (after sedimentation of the Sepharose beads) was assayed for peroxidase activity. After extensive washing of the beads, the adsorbed protein (including IgGs) was eluted with 2% (w/v) SDS in 0.1 M-sodium

Abbreviation used: LPO, lactoperoxidase.
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phosphate buffer, pH 6.8, and the eluate was subjected to SDS/polyacrylamide-gel electrophoresis (see below).

In some experiments partly purified human LPO was pre-adsorbed on a matrix of anti-(human IgA/secretory component) antibody–Protein A–Sepharose, by using essentially the same protocol.

An immunoadsorbent column was prepared by coupling 7.4 mg of affinity-purified anti-(bovine LPO B) antibody covalently to 0.6 g of CNBr-activated Sepharose 4B (DeMey, 1983).

Affinity chromatography on concanavalin A–Sepharose

After removal of fat and casein (see the Results section), colostrum was passed through a concanavalin A–Sepharose column (2.0 cm × 5.0 cm) equilibrated with 0.2 M-NaCl/50 mM-potassium phosphate buffer, pH 6.5. The column was washed with 8 bed volumes of equilibration buffer, and protein was eluted (0.05 ml/min) with 0.5 M-methyl α-D-mannopyranoside in 50 mM-potassium phosphate buffer, pH 7.0.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out by the procedure of Laemmli (1970), with the use of silver staining (Heukeshoven & Dernick, 1985) and quantification by scanning densitometry (Ultroscan XL from LKB, Stockholm, Sweden).

Radioimmunoassay

The radioimmunoassay of human LPO was based on a standard protocol for competitive radioimmune binding assay (Lea, 1985). Highly purified bovine LPO, labelled with ^{125}I, was used as a tracer, and the antibody was polyclonal rabbit anti-(bovine LPO B) antibody (see above).

Peroxidase activity

Peroxidase activity was measured as described previously (Langbakk & Flatmark, 1984).

Other analytical procedures

Protein was measured by dye binding (Bradford, 1976), with bovine serum albumin as a standard. Purified bovine LPO B was assayed spectrophotometrically by using ϵ_{412} 112.2 $mM^{-1} \cdot cm^{-1}$ (Carlström, 1969*c*).

RESULTS

Purification of human LPO

Fat was largely removed from 435 ml of pooled colostrum by centrifugation (27000 $g_{av.}$ for 30 min). A 43% fraction of the peroxidase activity was removed by immunoaffinity adsorption (Table 1). After partial removal of casein (Langbakk & Flatmark, 1984), the whey was precipitated by 50% saturation with $(NH_4)_2SO_4$ for 1 h at pH 7.0. Insoluble protein was dissolved in 10 mM-sodium acetate buffer, pH 5.1 (1 ml of buffer to 12 ml of original colostrum), and desalted on a column (5.0 cm × 17.0 cm) of Bio-Gel P-10, equilibrated with the same buffer. In the last two steps the specific activity of immunoreactive LPO increased about 2-fold (Table 1).

The desalted material was adsorbed on and eluted from a column of CM-cellulose (Table 1), and fractions containing immunoreactive peroxidase activity (270 munits) were precipitated at 60% saturation with

Table 1. Purification of LPO from human colostrum

Specific activities were determined as units (μmol of tetraguaiacol formed/min at 25 °C)/mg of protein. The activities are expressed for the total peroxidase activity and the activity adsorbed on the immunoaffinity matrix of anti-(bovine LPO B) antibody–Protein A–Sepharose (in parentheses). One unit of activity is defined as 1 μmol of oxidation product(s) formed/min at 25 °C.

Step	Volume (ml)	Protein (mg)	Activity (munits)	Specific activity (munits/mg)	Yield of activity (%)
Centrifuged crude colostrum	410	4551	8876.5 (3816.9)	2.0 (0.84)	100 (100)
Acid precipitation	340	3298	6545.0 (2814.4)	2.0 (0.85)	73.7 (73.7)
1st $(NH_4)SO_4$ fractionation	65	1625	3127.8 (2033.1)	2.0 (1.25)	34.1 (53.3)
Bio-Gel P-10 chromatography (desalting)	100	1200	2400.0 (1800.0)	2.0 (1.50)	27.0 (47.2)
CM-cellulose chromatography*	80	152	317.6 (270.0)	2.1 (1.78)	3.6 (7.1)
2nd $(NH_4)_2SO_4$ fractionation	4	44.4	296.9	6.7	3.3
Sephadex G-150 chromatography†	29	17.4	216.4 (216.4)	12.4 (12.4)	2.4 (5.7)
Phenyl-Sepharose CL-4B chromatography‡	12	10.4	274.9 (274.9)	26.4 (26.4)	3.1 (7.2)

* The column (2.5 cm × 18.0 cm) was equilibrated with 10 mM-sodium acetate buffer, pH 5.1. Isocratic elution was with 50 mM-NaCl/30 mM-sodium acetate buffer, pH 5.1, at 0.5 ml/min. Fraction nos. 42–59 (80 ml) were collected.

† The column (1.2 cm × 7.0 cm) was equilibrated with 2 M-sodium acetate, pH 7.0, and eluted isocratically with 50 mM-sodium acetate, pH 7.0, at 0.11 ml/min. Fraction nos. 19–30 (29 ml) were collected.

‡ See Fig. 1. Fraction nos. 16–26 (12 ml) were collected.

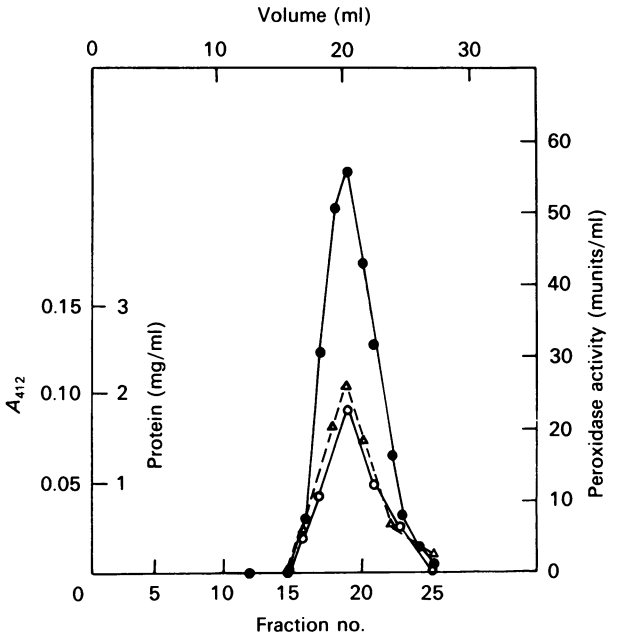

Fig. 1. Elution profile of peroxidase activity from human colostrum on phenyl-Sepharose CL-4B

The phenyl-Sepharose CL-4B column (1.2 cm × 7.0 cm) was equilibrated with 2 M-sodium acetate, pH 7.0, and eluted isocratically with 50 mM-sodium acetate, pH 7.0, at a flow rate of 0.11 ml/min. In each collected fraction (1.1 ml) peroxidase activity (●), A_{412} (○) and protein (△) were measured. For further details see the main text and Table 1.

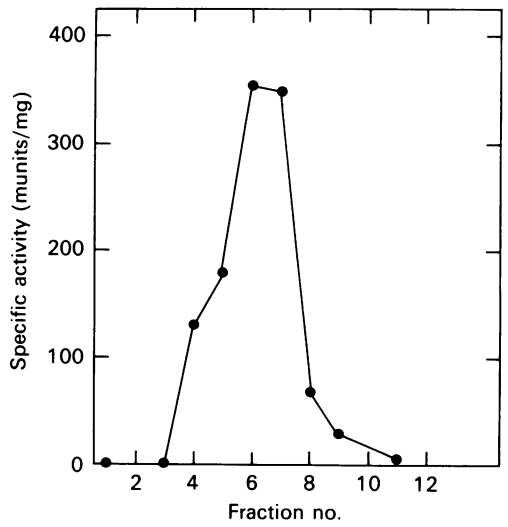

Fig. 2. Elution profile of peroxidase activity from human colostrum on immunoaffinity chromatography

The immunoadsorbent column (see the Materials and methods section) was equilibrated with 100 mM-potassium phosphate buffer, pH 8.0. A 34 ml volume of colostrum (after removal of fat and casein as well as addition of proteinase inhibitors) was passed through the column. After thorough washing ($A_{280} < 0.02$), the enzyme was eluted with 0.1 M-glycine/HCl buffer, pH 2.8, and each collected fraction (0.5 ml) was immediately neutralized. The specific activity of the material applied to the column was 0.25 munit/mg of protein. The concentrations of proteinase inhibitors were 0.1 mM-leupeptin, 0.1 mM-phenylmethanesulphonyl fluoride, 1 mM-benzamidine and 0.1 mM soya-bean trypsin inhibitor.

$(NH_4)_2SO_4$ for 1 h at pH 7.0. The pellet was dissolved in about 3 ml of 2 M-sodium acetate, pH 7.0, and the soluble material was subjected to gel-permeation chromatography on Sephadex G-150 (Table 1). The fractions containing peroxidase activity (100 % of the activity was found to be removed by immunoaffinity adsorption) were combined and subjected to hydrophobic adsorption chromatography on phenyl-Sepharose CL-4B. A single peak of absorption at 412 nm and peroxidase activity was obtained (Fig. 1).

Human LPO was alternatively purified by a single step of immunoaffinity chromatography (Fig. 2). Of the peroxidase activity in the delipidated and decaseinated colostrum 65 % was adsorbed on the column, and 21 % of this activity was recovered in the eluted fractions (Fig. 2). An apparent 1450-fold purification was estimated for the peak fractions.

Size-exclusion h.p.l.c.

Human LPO was subjected to size-exclusion h.p.l.c. Only a single, but slightly asymmetric, peak of protein, absorbing at 412 nm, was detected in the M_r region 20000–670000 (Fig. 3*b*), and the absorption spectrum at the Soret region revealed a maximum at about 412 nm (Fig. 4). The component was eluted with a slightly shorter retention time (96 min) than that of bovine LPO B (Fig. 3*c*).

H.p.l.c. analysis of the LPO fraction recovered from the immunoaffinity chromatography revealed similar chromatographic and spectral properties; the absorption ratio A_{280}/A_{412} was 20:1.

Immunoadsorption on Protein A–Sepharose and SDS/polyacrylamide-gel electrophoresis

The h.p.l.c.-purified bovine LPO (Fig. 5, lane 2) revealed a single major band of M_r 78000 (LPO B) with a minor component of M_r 76000 (LPO A). On adsorption of crude human colostrum on anti-(bovine LPO B) antibody–Protein A–Sepharose, proteins of M_r about 80000 and 64000 were eluted by the SDS buffer (Fig. 5, lane 3). The purified human LPO preparation obtained by final size-exclusion h.p.l.c. (Fig. 3*b*) revealed a major band of M_r 80000, and only a trace amount of protein of M_r 64 000 (Fig. 5, lane 4). Similar results were obtained with the human LPO preparation purified by the single immunoaffinity-chromatography step (results not shown).

Human colostrum contains substantial amounts of secretory IgA (Blanc, 1981; Brandtzaeg, 1987). In order to remove any IgA present in the LPO preparations recovered from, for example, the phenyl-Sepharose CL-4B column (Fig. 1), this material was adsorbed on a matrix of anti-(IgA/secretory component) antibody–Protein A–Sepharose. No peroxidase activity was removed by this adsorption, but from Fig. 5 (lane 5) it is seen that both IgA (M_r 64000 for the heavy chain) and secretory component (M_r 80000) were adsorbed on and eluted from the gel matrix. The supernatant from the pre-adsorbed preparation was found to contain only a single protein, of M_r about 80000 (Fig. 5, lane 6), which adsorbed on anti-(bovine LPO B) antibody bound to

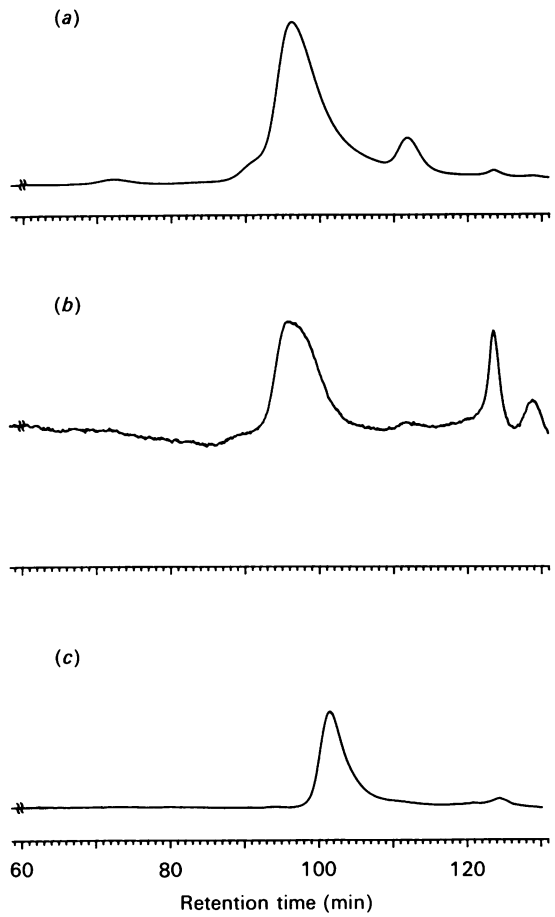

Fig. 3. Size-exclusion h.p.l.c. of partly purified human LPO and bovine LPO

The elution profiles of partly purified human LPO (obtained by affinity chromatography on phenyl-Sepharose CL-4B) at 280 nm (*a*) and 412 nm (*b*) and of bovine LPO at 412 nm (*c*) are shown. The mobile phase was 100 mM-potassium phosphate buffer, pH 7.0, and the flow rate was 0.4 ml/min. The void volume was 51 ml and the total volume 128 ml. The attenuations (full-scale) were $A = 0.2$ (*a*), $A = 0.002$ (*b*) and $A = 0.03$ (*c*).

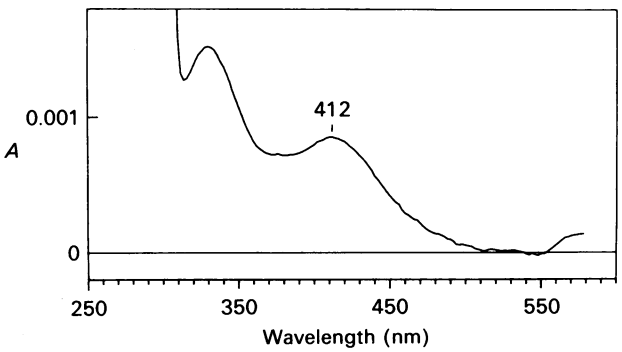

Fig. 4. Soret absorption spectrum of purified human LPO

The spectrum was obtained by a photodiode array detector at the peak (t_R about 96 min) on size-exclusion h.p.l.c. of human LPO as shown in Fig. 3(*b*). Conditions: solvent, 100 mM-potassium phosphate buffer, pH 7.0; light-path, 6 mm.

Protein A–Sepharose, removing all the peroxidase activity. By contrast, Protein A–Sepharose alone did not remove any peroxidase activity, and no protein was eluted from the matrix (Fig. 5, lane 7). When the staining intensity of the 80000-M_r protein (Fig. 5, lane 6) was compared (by densitometric scanning) with known amounts of h.p.l.c.-purified bovine LPO (as in Fig. 5, lane 2), the specific activity of pure human LPO was estimated to be 52.7 ± 3.7 units/mg of protein.

In control experiments human white blood cells were extracted with Triton X-100 (see the Materials and methods section), which gave soluble material with high peroxidase activity (10.6 munits/mg of protein), due to myeloperoxidase and eosinophil peroxidase. However, no activity was adsorbed to the immunoaffinity matrix, and SDS/polyacrylamide-gel electrophoresis of the material eluted from the bead matrix by SDS revealed no protein bands in the 50000–80000-M_r region (Fig. 5, lanes 8 and 9).

Binding of human LPO to concanavalin A–Sepharose and its elution

Affinity chromatography of colostrum proteins on concanavalin A–Sepharose (see the Materials and methods section) revealed a peak of absorption at 412 nm and peroxidase activity with a retention time similar to that of bovine LPO (results not shown).

Radioimmunoassay

That human LPO has immunoreactive properties similar to that of LPO isolated from bovine milk was also confirmed by a competitive radioimmune binding assay. Thus the relative binding of ^{125}I-LPO (bovine) in the presence of increasing amounts of partly purified human LPO revealed a linear curve, which was parallel to the binding curve obtained for bovine LPO. Thus the two enzymes seem to bind with similar affinities to anti-(bovine LPO B) antibody.

DISCUSSION

The demonstration of an LPO in human colostrum (Langbakk & Flatmark, 1984) has been confirmed and extended in the present study. The enzyme accounts for about 0.004% of the total protein in crude colostrum, on the basis of enzyme activity measurements. The potential contribution of non-LPO peroxidase activities to the total activity of human colostrum, notably myeloperoxidase and eosinophil peroxidase from leucocytes (Smith & Goldman, 1968; Moldoveanu *et al.*, 1982; Langbakk & Flatmark, 1984) and haem protein pseudoperoxidases (Blanc, 1981), was eliminated by the selected purification procedure and by LPO-specific immunoaffinity adsorption (Table 1, Fig. 2 and Fig. 5).

LPO was found partly to co-purify with secretory IgA, which constitutes more than one-third of the total protein content of colostrum (Blanc, 1981). In the most purified preparations of human LPO, however, any secretory IgA could be quantitatively removed by specific immunoaffinity adsorption on an anti-(human IgA/secretory component) antibody–Protein A–Sepharose matrix, leaving LPO unadsorbed (Fig. 5). For reasons mentioned above, we have not been able to obtain a high purification factor (based on activity measurements) by conventional procedures (Table 1). However, on immunoaffinity

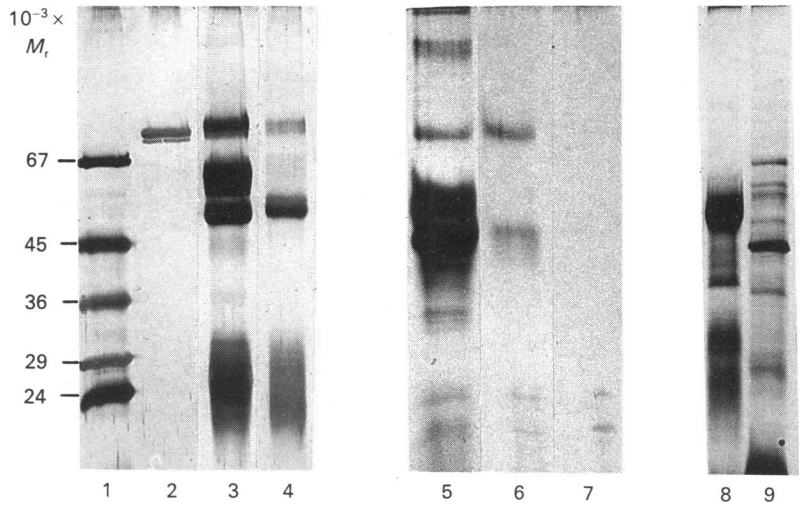

Fig. 5. SDS/polyacrylamide-gel electrophoretograms of bovine LPO and human LPO

SDS/10% (w/v) polyacrylamide gel after electrophoresis was developed by silver staining. Lane 1, protein M_r markers as indicated; lane 2, 1 μg of highly purified bovine LPO B. In lanes 3 and 4 are shown proteins adsorbed on and eluted from the immunoaffinity matrix of anti-(bovine LPO B) antibody–protein A–Sepharose by SDS; the heavy chains and light chains of IgG are also seen in each lane. Lane 3, human colostrum after removal of lipid (100 μl of the fraction was used for adsorption on the immunoaffinity matrix). Lane 4, highly purified human LPO (peak fraction from size-exclution h.p.l.c. shown in Fig. 3). Lane 5, 600 μl of the peak fraction of human LPO obtained by phenyl-Sepharose CL-4B chromatography (Fig. 1) adsorbed on and eluted from the immunoaffinity matrix of anti-(IgA/secretory component) antibody–Protein A–Sepharose. Lanes 6 and 7, 300 μl of the same peak fraction, after pre-adsorption on the immunoaffinity matrix of anti-(IgA/secretory component) antibody–Protein A–Sepharose, followed by adsorption on and elution from the immunoaffinity matrix of anti-(bovine LPO B) antibody–Protein A–Sepharose (lane 6) or by adsorption on and elution from Protein A–Sepharose without IgG (lane 7). Lane 8, Triton X-100 extract of white blood cells (220 μg of protein) adsorbed on and eluted from the immunoaffinity matrix. Lane 9, protein profile of the Triton X-100 extract of white blood cells (41 μg of protein). The M_r markers (lane 1) were bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000) and trypsinogen (M_r 24000).

chromatography an apparent 1450-fold purification was obtained in a single step (Fig. 2).

Although the spectral properties of human LPO, purified by size-exclusion h.p.l.c. (Fig. 3*b*), have been obtained only at the Soret region (Fig. 4), the peak absorption at 412 nm is identical with that of bovine LPO (Carlström, 1969*c*). The estimated M_r value of human LPO (about 80000) is close to that of bovine LPO B (78000) (Carlström, 1969*b*), based on SDS/polyacrylamide-gel electrophoresis (Fig. 5, lanes 4 and 6) and size-exclusion h.p.l.c. (Fig. 3*b*). Both analyses indicate that human LPO is slightly heterogeneous, as also observed for the bovine enzyme (Fig. 5, lane 2), containing about 10% (w/w) of carbohydrate (Carlström, 1969*c*).

The demonstration of a human LPO (Langbakk & Flatmark, 1984; the present study) is of considerable biochemical, physiological and clinical interest. Further studies on its chemical and catalytic properties, as well as its potential use as a criterion to distinguish between hormone-dependent and hormone-independent mammary cancer (Anderson *et al.*, 1975), are desirable.

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REFERENCES

Anderson, W. A., Kang, Y.-H. & DeSombre, E. R. (1975) J. Cell Biol. **64**, 668–681
Bassøe, C. F., Laerum, O. D., Solberg, C. O. & Haneberg, B. (1983) Proc. Soc. Exp. Biol. Med. **174**, 182–186
Blanc, B. (1981) World Rev. Nutr. Diet. **36**, 1–89
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Brandtzaeg, P. (1987) Acta Histochem. Suppl. **34**, 9–32
Carlström, A. (1969*a*) Acta Chem. Scand. **23**, 171–184
Carlström, A. (1969*b*) Acta Chem. Scand. **23**, 185–202
Carlström, A. (1969*c*) Acta Chem. Scand. **23**, 203–213
DeMey, J. R. (1983) in Immunohistochemistry (Cuello, A. C., ed.), pp. 347–372
De Sombre, E. R., Anderson, W. A. & Kang, Y.-H. (1975) Cancer Res. **35**, 172–179
Heitlinger, L. A. (1983) J. Pediatr. Gastroenterol. Nutr. Suppl. **1**, 113–119
Heukeshoven, J. & Dernick, R. (1985) Electrophoresis **6**, 103–112
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Langbakk, B. & Flatmark, T. (1984) FEBS Lett. **174**, 300–303
Lea, O. A. (1985) Clin. Chim. Acta **149**, 197–203
Moldoveanu, Z., Tenovuo, J., Mestecky, J. & Pruitt, K. M. (1982) Biochim. Biophys. Acta **718**, 103–108
Smith, C. W. & Goldman, A. S. (1968) Pediatr. Res. **2**, 103–109

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