THE ISOLATION AND SOME LIGANDING PROPERTIES OF LACTOPEROXIDASE

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1. Introduction

Lactoperoxidase is an animal protein which participates in antimicrobial mechanisms [1]. Its electron donor profile differs from that of plant peroxidases regarding halide ions [2]. Isolation procedures are available but somewhat tedious [3–5].

Plant isoperoxidases with different affinities for aromatic substrates can be separated on phenyl–Sepharose^R [6]. This observation has now been developed into an isolation procedure for LP. Its essential features are alternations between column materials with ionic and hydrophobic binding forces. The opposite requirements for ionic strength minimize the number of dialyses. The binding of LP to phenyl– and octyl–Sepharose is compared to the binding of horseradish peroxidase to both Sepharoses. Attempts are made to relate these adsorptions to optically operable equilibria between the peroxidases and free, aromatic ligands.

Plant peroxidases can be isolated by means of affinity chromatography on hydroxamic acid–Bio-Gel A^R [7]. An imidazole-carrying polysaccharide binds hemoglobin and myoglobin specifically and unspecifically [8]. Sepharose–concanavalin A binds the glycoprotein HRP [9,10].

2. Materials and methods

Materials for chromatography (CG-50-NH_4, Serva, Heidelberg; Sepharose^R C1-4B, its derivatives, and DEAE–Sephadex A50, Pharmacia, Uppsala; CM-52, Whatman, Maidstone, Kent) were pretreated as prescribed by the manufacturers. Dicarboxidine ($\gamma\gamma'$-(4,4'-diamino-3,3'-bifenylenedioxy)dibutyric acid) was a gift from Dr Å. Jönsson, KABI, Stockholm [11]. The isoperoxidases HRP A2 (pI 3.9) and C2 (pI 8.8) were prepared as in [12]. Rennin was purchased from Sigma (St Louis). Activities were determined with guaiacol and H_2O_2.

Abbreviations and nomenclature: Lactoperoxidase, LP (EC 1.11.1.7, EC 1.11.1.8); horseradish peroxidase, HRP (EC 1.11.1.7)

3. Results

3.1. *Isolation procedure*

To 14 l unpasteurized skim milk were added 56 mg of rennin in a little water, and the milk was stirred at room temperature until coagulation occurred. The whey was passed through several layers of glass wool, moist ion exchanger CG-50-NH_4 was added, 20 g/L whey, and the suspension was stirred for 3 h with cooling in ice-water. After settling, the resin was washed with 2 × 3 l water and 4 × 3 l 50 mM sodium acetate (pH 7.0) bringing A_{280} to <0.02 in the last washing. The resin was transferred to a column, and LP was eluted with 2 M sodium acetate. This step follows essentially a procedure generally applicable to basic proteins [4,13].

The slightly turbid solution was further purified on phenyl–Sepharose^R (fig.1). The resulting/LP solution (160 ml) was concentrated in a Diaflo^R XM-50 cell, dialyzed against 10 mM sodium phosphate (pH 6.0) and centrifuged to remove a little cloudiness. A longer column of phenyl–Sepharose^R would bring about a higher A_{412}/A_{280} ratio, but it is more convenient, and also cheaper, to introduce a third step.

The LP solution was adsorbed on a 25 × 50 mm CM-52 column in 10 mM sodium phosphate (pH 6.0). Some ultraviolet absorbing, peroxidase-negative material left the column during the washing with

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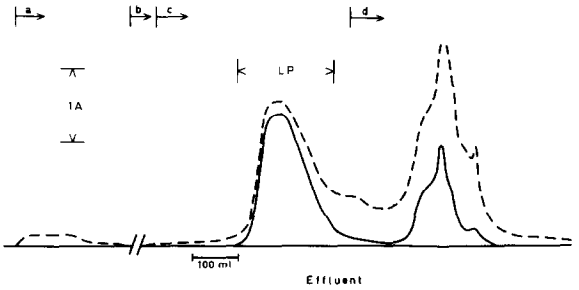

Fig.1. Purification of LP on phenyl–Sepharose^{R} Cl-4B. The column (50 × 120 nm, $d \times l$) was preequilibrated with 2 M sodium acetate. (a) Application (200 ml/h) and washing with 250 ml 2 M sodium acetate; (b) linear gradient from 250 ml 2 M + 250 ml 0.05 M sodium acetate, both at pH 7.0; flow-rate 50 ml/h, (c) 0.05 M sodium acetate (pH 7.0); (d) water. (——) A_{404}; (– – –) A_{280} (Uvicord III, LKB, Stockholm). 4°C.

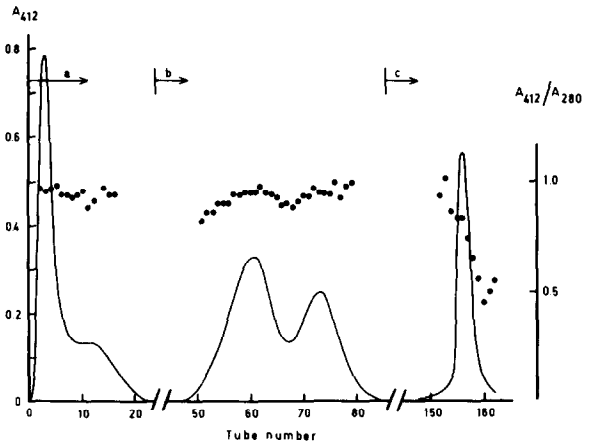

Fig.2. Chromatography of LP on DEAE–Sephadex A-50 medium. The column (30 × 600 mm, $d \times l$) was pre-equilibrated with 10 mM Tris–HCl (pH 9.0). (a) Application and elution with the same buffer; (b) 10 mM Tris–HCl, (pH 8.0); (c) 100 mM Tris–HCl (pH 7.0). Fraction volume: (a) 6.2 ml; (b,c) 3.2 ml 4°C.

10 mM sodium phosphate. A linear gradient from 250 ml 10 mM + 250 ml 130 mM sodium phosphate (pH 6.0) conditioned the column for 130 mM phosphate, which eluted LP as a single fraction with constant A_{412}/A_{280}. It was consistently preceeded by a small fraction, accounting for 2–3% of the total A_{412} and with A_{412}/A_{280} <0.5. A pink pigment [5], lacking a Soret band, appeared on CM-52 only when potassium phosphate (0.9 M) was used to elute LP from CG-50. With sodium acetate (2 M) as eluant no pink pigment appeared in the later step with CM-52. LP was finally concentrated in the Diaflo^{R} XM-50 cell. The ratio A_{412}/A_{280} could not be raised by various chromatographic procedures. Table 1 summarizes the results from 9 preparations.

3.2. *General properties*

When eluted from CG-50 by means of dipotassium phosphate and purified as described, LP always appeared distinctly green when frozen at −70°C. With sodium acetate as eluant from CG-50, the subsequent treatment being identical, the frozen LP appeared reddish-brown. After thawing there was little, if any, difference in colour, both samples being greenish-brown. The spectroscope revealed no difference, and the green form gave only a slightly higher absorbance towards longer wavelengths. The colours reappeared when the solutions were frozen simultaneously at −70°C. The addition of acetate to the phosphate-eluted green form, or vice versa, did not alter the colour. The colour phenomenon may be physical since frozen samples of the red form showed distinct green streaks. The two preparations were enzymatically equally active when compared on the

Table 1
Yield of LP in 9 preparations

Properties after	A_{412}/A_{280}	Yield of LP mg/l milk
Phenyl–Sepharose^{R}	0.72 ± 0.11	10.5 ± 4.0
CM-52	0.90 ± 0.03	7.4 ± 2.3

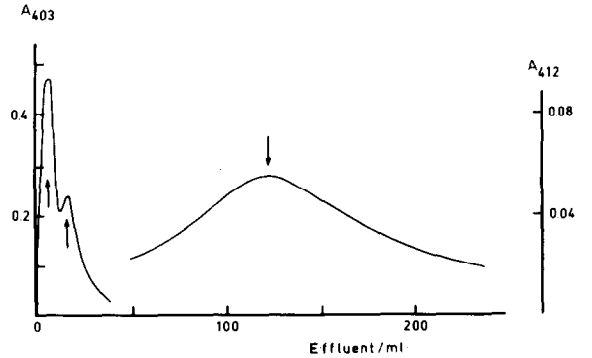

Fig.3. Chromatogram of a mixture of HRP A2 and C2, and LP on phenyl–Sepharose^{R} Cl-4B, 25 × 10 mm, $d \times l$. Elution volumes for HRP A2 and C2, and LP were 6.6, 16.6, and 122.5 ml. The proteins were applied in 0.45 ml 0.5 M sodium sulphate in 10 mM sodium phosphate (pH 6.0) and the chromatogram was also developed with this solution. A_{403}, HRP; A_{412}, LP.

basis of A_{412}. When LP was stored for a few months at −70°C in 10 mM sodium phosphate the A_{412}/A_{280} invariably decreased to ~0.8.

LP is known to migrate inhomogeneously in electric fields [14] and on anion exchangers [5] because of secondary alterations [15]. DEAE–Sephadex^R separated also our LP into 5 fractions but with an A_{412}/A_{280} nearly constant except for the tail fraction (fig.2).

3.3. *A chromatographic comparison of HRP and LP*

A mixture of HRP A2, HRP C2, and LP in a buffer of high ionic strength was resolved by phenyl–Sepharose^R C1-4B into 3 fractions (fig.3). A slightly higher ionic strength separated HRP A2 and C2 completely without moving LP, and a decreasing ionic strength gradient efficiently separated all 3 fractions. The comparison of the affinities of the 3 peroxidases for phenyl–Sepharose C1-4B is, however, simplified if an eluant of constant composition is used.

The exclusion limit for proteins from Sepharose C1-4B^R and its derivatives is 20×10^6 mol. wt [16]. For the 3 peroxidases the sum of the inner and outer solvent volumes (V_s) will therefore be equal to the volume of regained water, giving V_s = 80% of the bed volume [16]. The void volume V_o is 26%, and [ligand] is 40 μmol/ml of the bed volume [16]. For a given column the expression:

$$V_e = V_o + V_s \times K_d^{-1} \times \text{ligand}$$

gives the K_d of the complex between protein and Sepharose-bound ligand. Table 2 summarizes the K_d values, compares them to equilibria with free aromates, and shows the effect of benzene.

When the experiment in fig.3 was repeated with octyl–Sepharose under identical conditions the two plant isoperoxidases were eluted together as a single fraction with K_d 31 mM. LP, on the other hand, was very firmly bound, and could not be eluted by 10 mM sodium phosphate (pH 6.0) alone or containing 20% (v/v) ethanol, 30 mM benzene, 6 M urea, 0.1% (w/v) SDS or 3 mM sodium octanoate. The addition of 3 mM octanoate to LP and the column prior to the application of LP did not affect the adsorption. This concentration of sodium octanoate did not change the spectrum of LP or the activity of LP towards guaiacol.

Table 2
K_d values of ligand–peroxidase complexes, and K_m for the overall oxidation of guaiacol

Ligand	Na_2SO_4 (M)	K_d (mM)		
		HRP A2	HRP C2	LP
Phenyl–Sepharose Cl-4B[e]	0.5	20.8	7.1	1.4
Phenyl–Sepharose Cl-4B[e]	0.5[a]	23.3	9.6	2.1
Phenyl–Sepharose Cl-4B	0.7	[b]	[b]	[b]
Phenyl–Sepharose Cl-4B	0.7[a]	7.0	[b]	[b]
Phenyl–Sepharose Cl-4B	0.8[a]	[b]	[b]	[b]
Octyl–Sepharose Cl-4B	0.5	31	31	[b]
Sepharose Cl-4B	0.5	40	40	40
Guaiacol (n=5)	0	27 ± 7	7.4 ± 1.5	[c]
p-Cresol (n=5)	0	8.4 ± 0.9	2.9 ± 0.3	[c]
			K_m (mM)	
Guaiacol (n=4)[d]	0	1.0 ± 0.1	1.2 ± 0.3	1.5 ± 0.5

Sodium phosphate, 10 mM (pH 6.9) at 25°C

[a] Saturated with benzene
[b] No migration
[c] Optically inoperable
[d] K_m = 0.24 mM with monomeric, free deuterohemin
[e] Same column and temperature

When HRP A2, HRP C2, and LP were applied together onto a column of unsubstituted Sepharose they appeared as one fraction in an elution volume corresponding to K_d ~40 mM (table 2).

3.4. *Equilibria between peroxidases and free, aromatic compounds*

The interaction between peroxidases and phenyl–Sepharose may be related to equilibria between Fe(III) peroxidases and free aromates. HRP A2 or C2 and aromatic substances form optically operable equilibria ([6] and references therein]. It has now been found, however, that interactions of aromates with peroxidases may give rise to a variety of optical effects. Some aromates have no effect on the spectrum of Fe(III) HRP A2 although they are good substrates, e.g., dicarboxidine. In the case of Fe(III)LP most aromates, including guaiacol and dicarboxidine, fail to bring about any spectral change. Some derivatives of benzene, naphthalene, pyridine, dipyridyl and isoquinoline only caused a diffuse and weak bathochromic shift of the Soret band. Very few aromates gave well profiled difference spectra with Fe(III)LP, and so far no aromate has been found that gives an optically operable, reversible 1:1 complex with Fe(III)LP.

3.5. *Other peroxidases*

Cytochrome *c* peroxidase from yeast [17] and myeloperoxidase [18] were very firmly adsorbed onto phenyl–Sepharose Cl-4B. They were not eluted by the solutions listed in the above experiment with octyl–Sepharose.

4. Discussion

Two questions will be discussed: whether phenyl– and octyl–Sepharose^R Cl-4B, and free aromatic compounds interact with the same structure in a peroxidase molecule, and whether the 3 peroxidases carry functionally equivalent binding sites. Information about the interactions between the peroxidases and the 3 Sepharose types could only be obtained from chromatographic procedures. The interactions with free aromates were approached by means of kinetic determinations of K_m and optical determinations of K_d of the peroxidase–aromate complex.

Aromatic compounds cause a variety of changes in the optical spectrum of peroxidases, but only those signalling the reversible formation of a 1:1 complex can be used for a comparison to the chromatographic results. It is irrelevant to the present discussion whether such a complex is catalytically productive or not.

Optically determined K_d values for complexes between an aromatic substrance and the HRP isoenzymes give the K_d A2:K_d C2 $\cong$ 3 [6] (table 2). The ratio between the chromatographically determined dissociation constants of the complexes with phenyl-Sepharose is 2.9 (table 2). This agreement, and the effect of benzene on the apparent affinity of HRP for phenyl–Sepharose suggest that free and Sepharose-bound aromates ligate to the same site in HRP. The comparison can not be extended to LP because of the optical unaccessibility of Fe(III)LP–aromate complexes. The K_d values of the 3 peroxidases with phenyl–Sepharose in table 2 respond, however, in a similar manner to the addition of benzene, what indicates related binding mechanisms.

Octyl–Sepharose holds the 2 HRP isoenzymes equally loosely. Thus the exposed [6], aromate-binding site in HRP interacts, if at all, only weakly and unspecifically with the octyl groups. Alkanes are not substrates to peroxidases, and a very firm binding of the octyl chain to a site in LP designed for aromatic substrates seems unlikely, also by analogy with HRP. HRP and LP were separated much better on octyl– than on phenyl–Sepharose. The retention of LP on octyl–Sepharose therefore suggests the existence of another functional site, able to bind an alkyl group. Topologically, it may consist of a confined area or several disseminated, exposed hydrophobic groups.

LP is secreted into saliva, where it participates in antibacterial mechanisms. It can be adsorbed onto surfaces to be protected against bacterial attacks, e.g., enamel [20], or onto bacteria to be killed [21]. The octyl–Sepharose binding site might mediate the attachment.

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