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Solid Lactoperoxidase in the Iodination of L-Tyrosine and Albumin

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Abstract

Solid lactoperoxidase (LP-sorbent) was prepared from lactoperoxidase and a carrier copolymer of maleic anhydride and butanediol divinylether. The properties of LP-sorbent in the iodination of tyrosine and albumin were examined. For optimizing the pH of the iodination mixture, buffers in the pH range 4.5–8.0 were used. Albumin was iodinated using $Na^{125}I$ and tyrosine using KI. The effect of substrate concentration and the sequential addition of reagents was examined in the iodination of tyrosine. The optimum pH for iodination of albumin was 6.5 and that for the iodination of tyrosine 6.0–6.5. The iodination reactions were effective over a broad pH range around 6.5 resulting in almost equal iodinations. The optimum concentrations expressed as mmol/L/ μmol lactoperoxidase/mg sorbent at pH 6.5 were: H_2O_2, 70; KI, 64; and tyrosine, 128. The maximum catalytic activity of the LP-sorbent at pH 6.5 was 4.22 μkat/mg LP-sorbent or 28.8 kat/mol (mol of L-3-ITyr/s/mol lactoperoxidase). After the primary reaction of the LP-sorbent with hydrogen peroxide, both the supernatant and the washed solid phase exhibited iodinative activity. The iodination of tyrosine by LP-sorbent was also found to be possible in water.

Index Entries: Lactoperoxidase, in iodination; solid-phase iodination; L-tyrosine, iodination with lactoperoxidase; albumin, iodination with lactoperoxidase; iodination, of L-tyrosine and albumin.

Introduction

The use of immobilized lactoperoxidase for trace iodination has previously been described by several groups (*1–4*). David (*1, 4*) used a polydextran carrier, Thorell et al. (*2*) a polyacrylamide carrier, whereas Karonen et al. (*3*) utilized a

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polyvinyl butanediol derivative carrier. Recently, lactoperoxidase (LP) has been covalently coupled to the surface of polystyrene vessels (*5*) and to a ferrisepharose carrier (*6*).

Iodination by use of immobilized lactoperoxidase has proven superior to iodination with soluble lactoperoxidase for several reasons. Solid lactoperoxidase allows iodination at a pH where the protein best maintains its tertiary structure resulting in a controllable efficiency of iodination. Further, solid lactoperoxidase is easily separated from the iodination mixture and the reaction is easily stopped by centrifuging without any need for the addition of reducing agents. The easiness of separating out the solid lactoperoxidase is a noteworthy advantage if the size of the peptide to be iodinated is close to that of lactoperoxidase that can potentially be self-iodinated (*2, 4*).

In the present study, the coupling of LP to a carrier copolymer of maleic anhydride and butanediol divinylether is described. Further, the iodination of tyrosine was studied and the incorporation of iodine into albumin was used to monitor the effect of various experimental parameters on the reaction.

Experimental

Lactoperoxidase, B grade (EC 1.11.1.8) was obtained from Calbiochem, La Jolla, USA. The copolymer of maleic anhydride and butanediol divinylether was purchased from E. Merck, Darmstadt, West Germany; $Na^{125}I$ (IMS 30) from Amersham, Buckinghamshire, England; L-tyrosine from Sigma Chemical Co., St. Louis, Missouri, USA; and bovine serum albumin from the Armour Pharmaceutical Co., Eastbourne, England. The ion-exchangers used were DEAE-Sephacel®, Pharmacia, Uppsala, Sweden, and CM 22 Cellulose®, Whatman, H. Reeve Angel Inc., Clifton, New Jersey, USA.

The buffers used were sodium acetate at 0.01–0.1 mol/L and sodium phosphate buffer at 0.05 mol/L. Measurements were carried out with a Beckman DB and a Cary 118 spectrometer. Tyrosine iodinating activity was measured according to Morrison and Bayse (*7*) at 290 nm and by counting the radioactivity of the albumin fraction in an LKB-Wallace GTL gamma counter when albumin was the reagent.

Preparation of Lactoperoxidase Sorbent

After the simultaneous addition of 7 mL of 0.5 mol/L sodium phosphate buffer containing 5, 15, 30, or 50 mg of LP and 100 mg carrier (dry weight), titration of the mixture was carried out on ice with 0.1 mol/L NaOH, keeping the pH at 7.0–8.5 using a pH-Stat® (Radiometer). After 60 min the suspension was transferred to a beaker and homogenized with an Ultra-Turrax® as described earlier (*3*).

The Tyrosine Iodinating Activity of the LP-Sorbent

For the study of the activity of LP-sorbent, tyrosine was iodinated in the pH range of 4.5–8.0 and the optimum substrate concentration was examined at

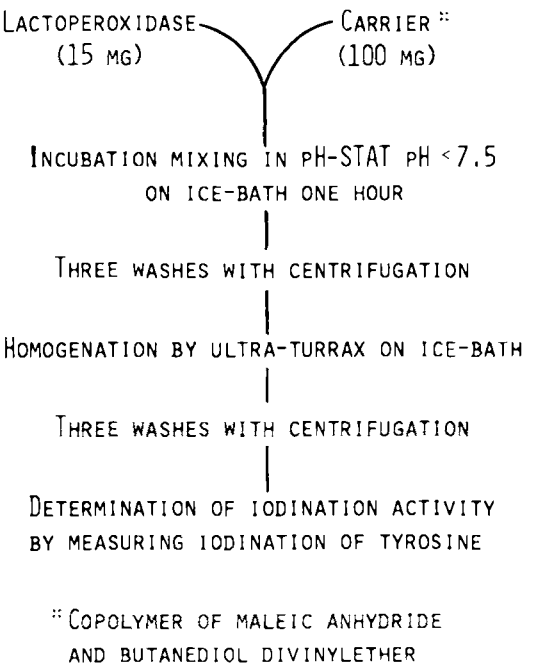

FIG. 1. Preparation of solid lactoperoxidase. Outline of the coupling of lactoperoxidase to copolymer of maleic anhydride and butanediol divinylether.

pHs 5.0, 5.5, 6.0, and 6.5, respectively. The concentrations used were H_2O_2 88–560 μmol/L, KI 10–500 μmol/L, tyrosine 100–200 μmol/L. The LP-sorbent was diluted from stock solution with sodium acetate buffer whereafter 50 μL of a 1:20 dilution was added to the reaction mixture, the total volume of which was 1 mL. The reactions were initiated by the addition of H_2O_2.

The Effect of Temperature

The reaction temperatures examined were 0 (ice-bath), 25, 37, and 56°C (thermal block) at pHs 5.0, 5.5, 6.0, and 6.5. In these experiments the tyrosine concentration was 400 μmol/L, that of KI 100 and 200 μmol/L, and that of H_2O_2 175 μmol/L, while the LP-sorbent was diluted as above.

The Effect of the Amount of LP-Sorbent

The LP-sorbent dilutions used were from 1:10 to 1:200 using tyrosine at 400 μmol/L, KI at 100 μmol/L, and H_2O_2 at 88 μmol/L in the pH range given above.

The Iodination of Albumin

Albumin was iodinated at 22°C (*3*). For kinetic studies several 1 μg batches of albumin were iodinated at pHs 5.0, 5.5, 6.0, 6.5, and 7.0, respectively. The reactions were initiated by addition of 1 μL H_2O_2 88 μmol/L, followed by new additions at 5-min intervals, and then stopped by centrifugation.

The Examination of the Different Reaction Steps

To investigate the different reaction steps, all combinations of reactants were tested by measuring the L-3-ITyr formed. The difference in absorbance at 290 nm was measured after the addition of H_2O_2 and KI and tyrosine without LP-sorbent to evaluate possible blank reactions. The other combinations of reactants and their additions to the reaction mixtures are listed in Table 3.

Results

Effect of the Lactoperoxidase/Carrier Ratio

Maximal tyrosine iodination activity was obtained at an enzyme/carrier ratio of 15 mg enzyme/100 mg carrier (Table 1). The peroxidase activity of the LP-sorbent correlated linearly to its iodinating capacity.

The effect of pH measured at 22°C is shown in Fig. 2. LP-sorbent also catalyzed iodination of Tyr whether suspended in diluted acetic acid or in distilled water. The solidifying of LP seemed to broaden the optimum pH range.

Optimum substrate concentrations between pH 5.0 and 6.5 are shown in Table 2. Reaction rates were altered by various substrate concentrations.

The effect of temperature is shown in Fig. 3. The LP-sorbent was effective over a broad temperature range; LP-sorbent was active at 0°C when sufficient amounts of substrate were added. Best results were, however, achieved at 37°C and at pH 6.0 with 175 μmol/L H_2O_2 and 200 μmol/L KI. The effect of pH was clearcut at 56°C.

The effect of the amount of LP-sorbent is shown in Table 1 with dilutions ranging from 1:10 to 1:50. Solid lactoperoxidase was still active at very low enzyme concentrations maintaining good catalytic activity.

TABLE 1
Various Preparations and Decrease of Iodinating Activity in Relation to Dilution of LP-Sorbent[a]

Amount of LP, mg	Preparation, LP/carrier, mg	Peroxidative activity, U/mg LP	Iodinating activity, nkat/mg LP	Iodinating activity in relation to dilution of LP-sorbent, %		
				(0.2)	(0.1)	(0.05)
5	4/100	326	1200	25	—	—
15	10.5/100	445	2560	76	45	20
30	16/100	580	2350	50	—	—
50	18/100	310	1200	—	—	—

[a]Peroxidative activity was measured according to Maehly (12) (U ≙ 0.100/min/3 mL) and tyrosine iodinating activity according to Morrison and Bayse (7).

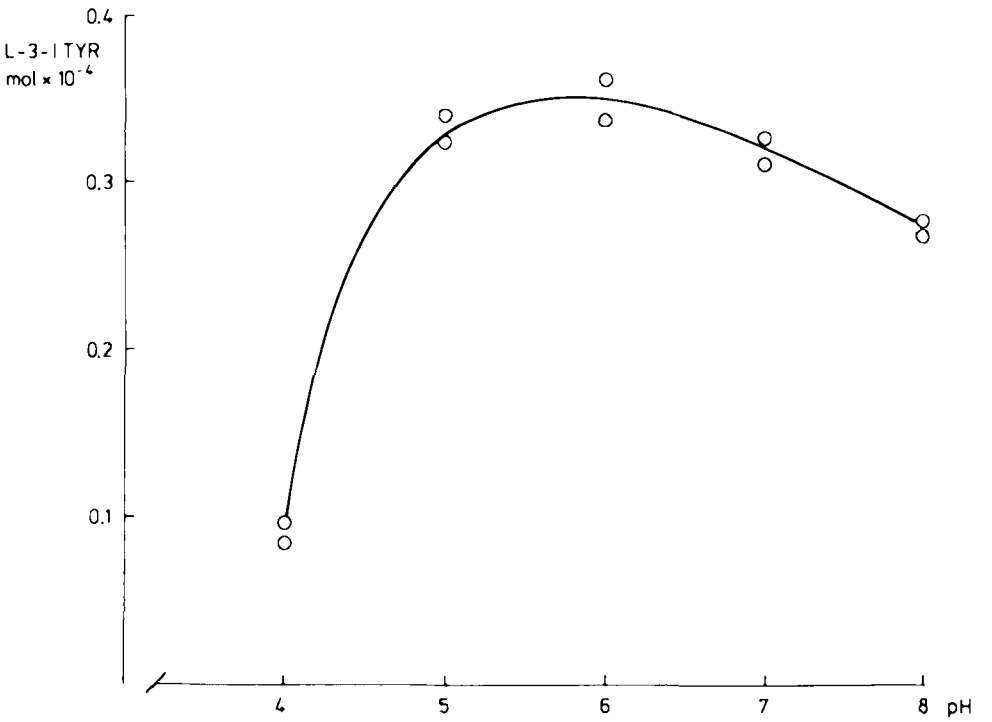

FIG. 2. The effect of pH on the iodination of tyrosine. Iodination activity was determined according to Morrison and Bayse (*7*) by measuring L-3-ITyr at 290 nm. Substrate concentrations were as follows: 88 nmol H_2O_2, 100 nmol KI, and 400 nmol L-Tyr in 1 mL of the reaction mixture.

The apparent K_m for iodination of tyrosine was assessed according to the method of Eisenthal and Cornish-Bowden (*8*), as suggested by Sundaram and Pye (*9*). The apparent K_m was 350 μmol/L tyrosine and the V_{max} was 43 μmol/L/s iodotyrosine as shown in Fig. 4.

For the iodination of albumin a pH of 6.5 was found to be optimal (Fig. 5).

TABLE 2
Optimal Conditions for the Iodination of Tyrosine as a Function of pH[a]

pH	Substrate concentration			Specific activity, kat/mol LP
	H_2O_2, μmol/L	I^-, μmol/L	Tyr, μmol/L	
5.0	0.26	0.40	0.40	18.7
5.5	0.26	0.40	0.40	22.0
6.0	0.26	0.40	0.80	25.9
6.5	0.44	0.40	0.80	28.8

[a]Lactoperoxidase concentration was 125 μmol LPS/mL 0.08 mol/L buffer of sodium acetate. Rates were measured as L-3-I-tyrosine/L-tyrosine at 290 nm (7).

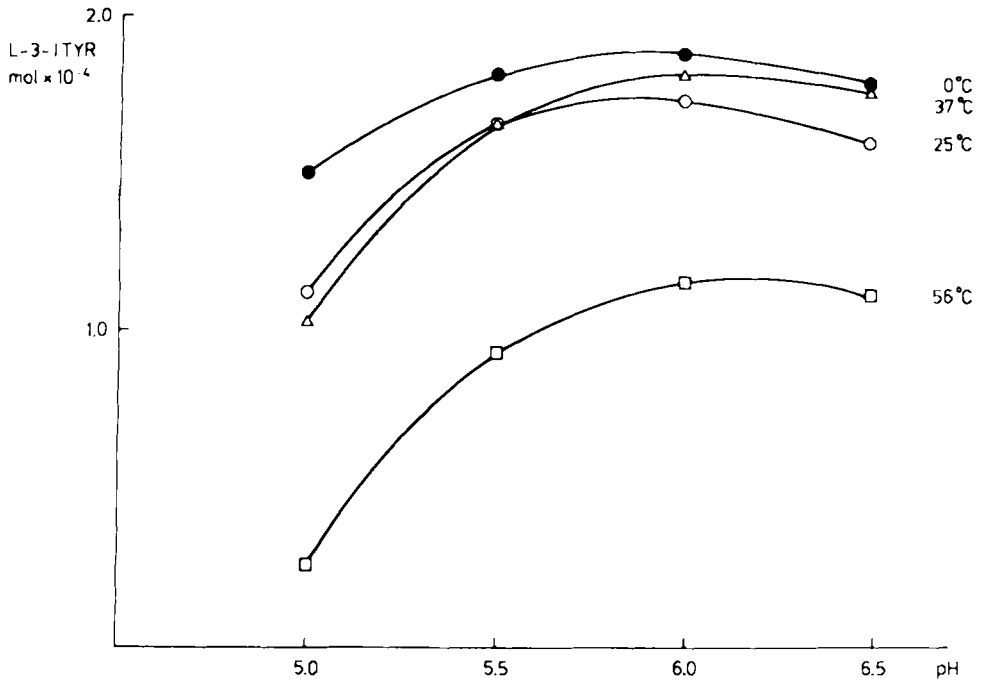

FIG. 3. The effect of temperature on the iodination reaction. Effect of temperature was tested at various temperatures, between 0 and 56°C. Substrate concentrations used were: 132 nmol H_2O_2, 200 nmol KI, and 400 nmol Tyr in 1 mL of the reaction mixture.

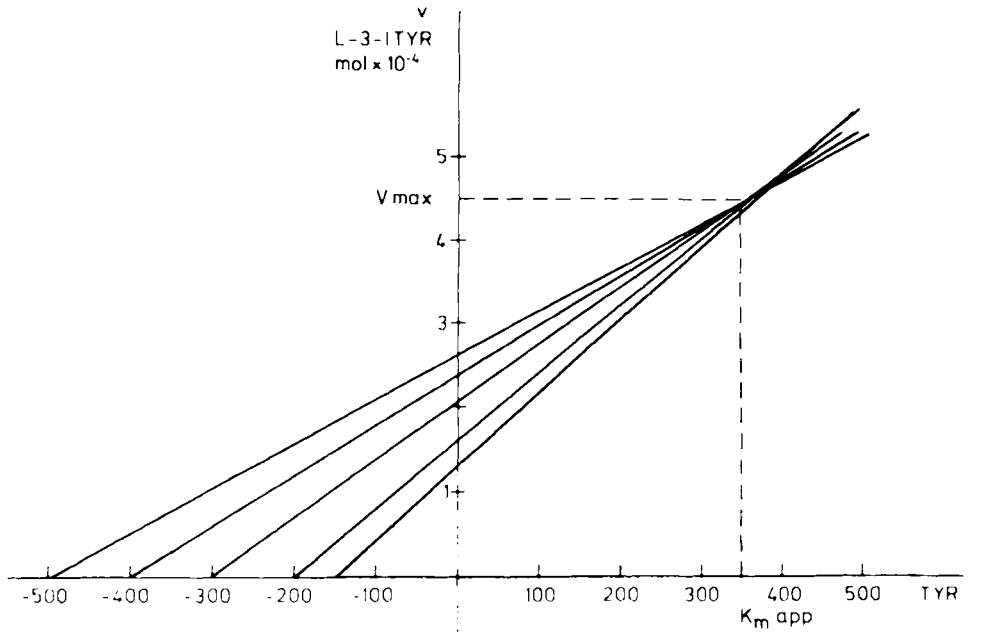

FIG. 4. Iodination at various tyrosine concentrations, presented as a direct linear plot according to Eisenthal and Cornish-Bowden (*8*). Substrate concentration is plotted as −S on the abscissa against the observed velocity as the ordinate. V_{max} is 43 nmol/s iodotyrosine and K_m app. is 350 nmol tyrosine in 1 mL of the reaction mixture.

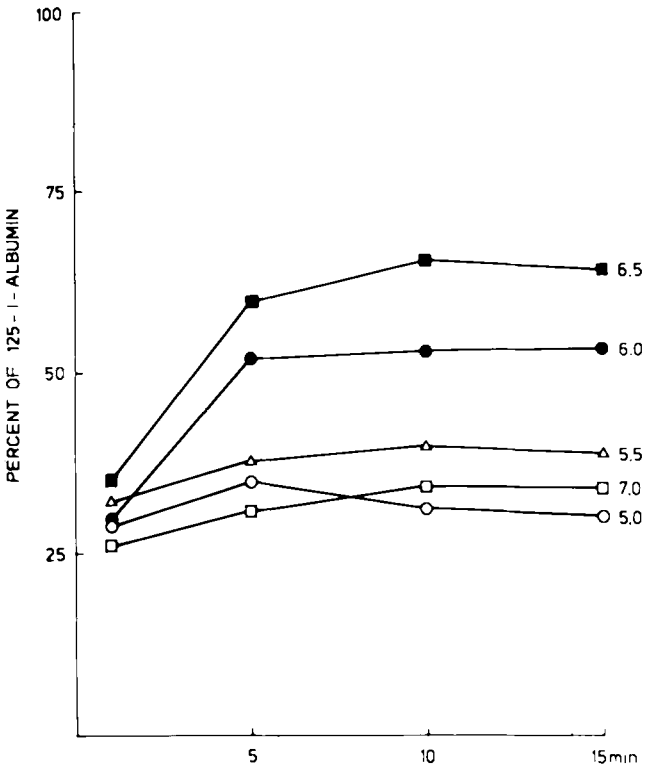

FIG. 5. Iodination of albumin. Effect of pH on the iodination of albumin was tested at different pHs: 5.0, 5.5, 6.0, and 7.0. The optimum pH was 6.5.

When 1 μg of albumin was iodinated by the addition of peroxide (1, 0.5, 0.5 μL of 88 μmol/L solution) at pH 6.5, the maximal response was achieved with 250 μCi of $Na^{125}I$ and 1:60 dilution of LP-sorbent.

Iodination reaction. The blank reaction tested caused no detectable rise at 290 nm, neither did the LP-sorbent react with iodide nor with iodide and tyrosine without added peroxide [Table 3, reactions (1)-(5)]. When the LP-sorbent was allowed first to react with peroxide, tyrosine was iodinated [reactions (6) and (9) in Table 3] although the solid phase was washed after the first reaction with peroxide and no further addition of peroxide was made. On the other hand, when the LP-sorbent was allowed first to react with potassium iodide and thereafter with peroxide and tyrosine, a small amount of iodinated tyrosine was generated. When the LP-sorbent was washed after the first reaction with KI, no detectable amount of iodinated tyrosine was found (*4*), although when the solid phase was washed after the primary reaction with peroxide it still maintained activity to iodinate (*6*), not only as solid phase, but also as supernatant when transferred to another reaction vessel [Table 3, (9)-(11)]. This iodinating activity was also transferable from the supernatant; when the supernatant was treated with an anion exchanger after separation of the solid phase it lacked capacity to iodinate while the anion exchanger exhibited activity even after one wash. Iodination was effective for at least 10 min after becoming detectable and all components of its activity were preserved in a similar way.

TABLE 3
Iodination Reactions[a]

	First incubation			Second incubation or treatment	Result
(1)		H_2O_2	+ KI		Neg
(2)		H_2O_2	+ KI	+ tyrosine	Neg
(3)			KI	+ tyrosine	Neg
(4)	LPS		+ KI	+ tyrosine	Neg
(5)	LPS		+ KI	+ tyrosine	Neg
(6)	LPS	+ H_2O_2		+ KI + tyrosine	Pos
(7)	LPS		+ KI	+ H_2O_2 + tyrosine	Low pos
(8)	LPS		+ KI	Washed solid + H_2O_2 + tyrosine	Neg
(9)	LPS	+ H_2O_2		Washed solid + KI + tyrosine	Pos
(10)	LPS	+ H_2O_2		Supernatant + KI + tyrosine	Pos
(11)	LPS	+ H_2O_2		Anion exch. + wash + KI + tyrosine	Pos

[a]LP-sorbent was allowed to react with various combinations of the reagents involved the iodination of tyrosine. The three first reactions (1)–(3) were carried out without LP-sorbent (in the table LPS). In reactions (4) and (5) the capacity of LP-sorbent for reacting without H_2O_2 were tested. In the reaction (6) LP-sorbent was allowed to prereact with H_2O_2 and in (7) and (8) prereact with KI. In the reactions (8)–(11) LP-sorbent was treated by washing and after that allowed to react. In (9) and (10) the solid phase and liquid phase were separated from each other and continued by addition of reagents into both the supernatant and solid LP-sorbent. In the reaction (11) the supernatant was treated with anion exchanger, solid anion exchanger washed and allowed to react with reagents.

In order to exclude the possibility that the activity of the supernatant owed to "broken" sorbent, e.g., that in the supernatant there was free lactoperoxidase, dissociated from the LP-sorbent, the reaction was performed *de novo* and the reaction mixture dialyzed. The dialyzed supernatant still exhibited iodinating activity. Experiments done with ESR failed to give evidence of any free radicals in the supernatant or in the solid phase.

Discussion

From the present results, we can say clearly that the use of solid lactoperoxidase for the iodination of tyrosine and albumin offers the following advantages:

(a) The buffering capacity of the electrically charged carrier eliminates hazardous pH effects by offering the possibility of choosing the pH to be within a broad range and allowing the use of diluted buffers in which the iodination takes place efficiently.
(b) The easiness of separating the reaction product is advantageous not only in the purification of the iodinated product, but also allows the generation of the iodination reagents beforehand.

It should be pointed out that the choice of carrier seems to be very important because of the low optimum pH of lactoperoxidase. The carrier, a copolymer of maleic acid anhydride and butanediol divinylether, has one great advantage when compared to other carriers used in the coupling of lactoperoxidase. Because the surface of LP-sorbent contains lactoperoxidase molecules and carboxyl groups, the same groups that occur in the common media used in iodination, i.e., sodium acetate buffer, it offers independence from the liquid media in iodination reactions.

According to Katchalski (*9*) covalent binding of enzymes to uncharged carriers via enzymatically nonessential groups does not affect their catalytic behavior. On the other hand, negatively as well as positively charged carriers possibly have a very strong effect on the behavior of the enzyme. The different pH optimum of solid lactoperoxidase compared to that of the soluble form was probably partly caused in this study by the negatively charged carrier and to a lesser extent by the solidifying of the enzyme.

Because the hydrogen ion concentration within a negatively charged polyelectrolyte gel is higher compared to that in the bulk of the solution, the pH dependent activity curve of lactoperoxidase embedded in a negatively charged carrier is displaced more towards an alkaline pH not only when using small substrate molecules such as tyrosine, but also with larger substrate molecules such as albumin.

The electric field owing to the negatively charged carrier also affects the distribution of positively charged low-molecular-weight substrates and products (*10*). It thus seems possible that positively charged compounds formed in the reaction remain on the surface of the carrier and this could

explain why solid lactoperoxidase is still able to iodinate although no free radicals are detectable. In addition, in the supernatant there are anions that are also able to iodinate and can be detected in the supernatant by binding to an anion exchanger. These results fit well to the theory of the two-route mechanism of the action of peroxidase put forward by Chance et al. (*11*). They stated that the mechanism includes only binary complexes of the enzyme and peroxide, in this system compound I is a cation, which after reaction with iodide forms compound II, respectively.

In conclusion, solid lactoperoxidase seems to be an excellent tool for the study of the reaction steps in the iodination of tyrosine and for the evaluation of primary peroxidative reactions with lactoperoxidase.

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