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Protein Iodination with Solid State Lactoperoxidase†

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ABSTRACT: Bovine lactoperoxidase covalently coupled to Sepharose 4B is demonstrated to be highly versatile in catalyzing the iodination of proteins. Iodinations can be effectively performed even in the presence of urea or sodium dodecyl sulfate and at temperatures as low as 0°. Lactoperoxidase catalyzed iodination of proteins is optimal when the concentration of hydrogen peroxide is essentially equal to that of the total iodide, while enzymatic activity with respect to protein iodinations is reduced with increasing concentrations of enzyme per unit volume of Sepharose beads. Practical applications of the LP-Sepharose iodination procedure are discussed.

A previous report briefly described an improved method for enzymatic iodination of proteins employing solid-state bovine lactoperoxidase (David, 1972). Unlike other methods of iodination, which utilize relatively strong oxidizing agents such as chloramine-T (Hunter and Greenwood, 1962) or iodine monochloride (McFarlane, 1958), enzymatic iodination with lactoperoxidase (Morrison, 1968, 1970; Marchalonis, 1969; Morrison and Bayse, 1970; Phillips and Morrison, 1970; Morrison *et al.*, 1971; Bauer *et al.*, 1971; Miyachi *et al.*, 1972) has been shown to be quite gentle, resulting in little or no detectable denaturation of the protein substrate. The major disadvantage of the soluble lactoperoxidase procedure has been the necessity of introducing contaminating materials, specifically the enzyme and any impurities in the enzyme preparation, into the iodination reaction mixture. By covalently coupling lactoperoxidase to an insoluble matrix, such as Sepharose 4B, it has been possible to circumvent this problem (David, 1972). The resulting solid-state enzyme not only provides a simple method for the gentle iodination of proteins, but is active over a wide range of conditions of pH and concentrations of protein and iodide.

Previously all iodinations were carried out under conditions of a large excess of H_2O_2 (David, 1972). This report presents data which indicate that the iodination reaction is not greatly

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affected by wide variations in the H_2O_2 concentration; in fact, there may be certain advantages to using concentrations somewhat closer to the total concentration of iodide in the mixture. Additional data deal with (1) the activity of the insoluble enzyme with respect to the iodination of macromolecules as a function of the concentration at which the lactoperoxidase is coupled to activated Sepharose, (2) the retention of enzymatic activity at low temperatures or in the presence of denaturing agents, (3) the ability of the enzyme to remain active after several iodinations, and (4) "self-iodination" (David, 1972) by Sepharose-bound lactoperoxidase.

Experimental Section

Preparation of Solid-State Lactoperoxidase. Bovine lactoperoxidase, obtained from Calbiochem, Sigma, or Analytical Aids, was coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the method of Cuatrecasas (1970). The resulting covalent bond (Axén and Ernback, 1971) appears to be quite stable and under the conditions employed here less than 1% of coupled protein, as determined by radiolabel experiments, is eluted from the beads even at pH 2.4 (unpublished data).

The coupling procedure used in this laboratory is outlined in Figure 1. Sepharose 4B was washed thoroughly with distilled water and inspected microscopically for bacterial contamination or bead breakage. The settled beads were then suspended in an equal volume of cold distilled water in an ice bath and were kept in suspension throughout the activation procedure by means of a magnetic stirrer. The pH of the suspension was raised to approximately 11.0 with a drop of 10% NaOH. Excess solid CNBr (4 g/30 ml of suspension) was added, and the reaction was monitored with a Beckman SS-2 pH meter equipped with a combination microelectrode. The pH was held at about 10.8–11.2 by the dropwise addition of 10% NaOH. Upon completion of the reaction, the beads were washed rapidly on a coarse sintered glass funnel with a gentle vacuum with about five volumes of cold coupling buffer (0.01 M $NaPO_4$–0.10 M NaCl (pH 7.5)). The desired quantity of activated beads was transferred to an erlenmeyer flask containing lactoperoxidase in one or two volumes of the cold coupling buffer and agitated on an Eberbach rotary shaker overnight at 4°. Noncoupled lactoperoxidase was determined by optical density measurement of the supernatant material, after removal of the beads, at 412 nm (E_{412}^{1mM} 114) (Morrison, 1970). Coupling efficiency was consistently 90–100%, usually closer to 100%. Bead concentration of lactoperoxidase was determined from the coupled enzyme (total lactoperoxidase added less noncoupled enzyme) and the measured bead volume after having been allowed to settle for 2 days in a graduated conical centrifuge tube.

After thorough washing with cold buffer, the lactoperoxidase-containing beads were suspended in 0.2 M glycine–0.01 M $NaPO_4$ (pH 7.5) for a minimum of 5 hr in the cold, then washed throughly and stored at 4° in phosphate-buffered saline (PBS[1]) (pH 7.0) containing 10^{-5} M merthiolate. In one experiment the glycine was replaced with ethanolamine with no detectable difference in the iodinating properties of the resulting beads. Negligible quantities of lactoperoxidase were found in the washes as determined by optical density measurements at 412 nm.

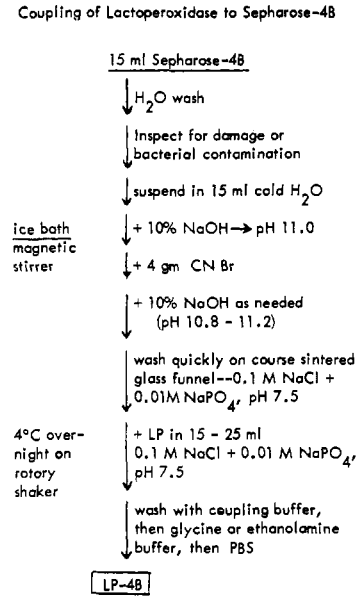

FIGURE 1: Schematic outline of a method for coupling lactoperoxidase (LP) to Sepharose 4B using the CNBr activation method of Cuatrecasas (1970).

Several batches of Sepharose-coupled lactoperoxidase were prepared, containing concentrations of bound lactoperoxidase ranging from 2.3 mg/ml of settled beads to 0.17 mg/ml.

Enzymatic Iodinations. Iodinations catalyzed by insoluble lactoperoxidase were carried out either in 1-ml Fisher centrifuge tubes (Fisher, Cat. 4-978-145) or in 4-ml screw cap vials. When centrifuge tubes were used, agitation was accomplished manually or on a rotator. Disposable magnetic stirring bars were utilized when the reactions were carried out in vials.

The LP-Sepharose beads were washed prior to the iodination reaction to remove the merthiolate. The desired volume of beads, usually 10–20 μl, was added to 0.5 or 1.0 ml of protein solution in pH 7.0 PBS, sometimes containing urea or sodium dodecyl sulfate, followed by 1 μl of KI to give the appropriate final concentration of iodide; 1 μl of $Na^{125}I$ (NEN, high specific activity, protein iodination grade) diluted in PBS was added and a zero time sample was removed and the protein precipitated with Cl_3CCOOH as described below. The iodination was initiated by the addition of 1–10 μl of a hydrogen peroxide solution to give the desired final concentration. The concentration of the stock H_2O_2 solution was determined on a Gilford Model 2400 spectrophotometer using a molar extinction coefficient of 72.4 at 230 nm (Phillips and Morrison, 1970). The enzyme was kept suspended throughout the reaction as described above.

Samples of 20 μl each were withdrawn at various time intervals by means of an Eppendorf pipet and pipetted into 0.2 ml of 2 mg/ml bovine γ-globulin containing 0.025 M NaN_3 and 0.05 M KI. The azide ion inhibits any further lactoperoxidase activity (Morrison, 1970; David, 1972). The lactoperoxidase-containing beads were removed by centrifugation in a Fisher Model 59 centrifuge (7000*g*, 5 min) and 0.1 ml of the supernatant solution was transferred to a 0.4-ml plastic conical microcentrifuge tube (Analytical Aids) and precipitated with an equal volume of cold 10% Cl_3CCOOH. After a minimum of 3 hr at 4° the precipitates were removed by centrifugation in a Beckman Model 152 microfuge (15,000 rpm, 5 min); 0.1 ml of each supernatant was removed with a Kimble automatic pipet and counted along with each precipitate (after 2 washes with cold 5% Cl_3CCOOH) in a Nuclear-Chicago Model 4233 auto-γ

[1] Abbreviations used are: PBS, phosphate buffered saline; CEA, carcinoembryonic antigen; LP-4B, lactoperoxidase coupled to Sepharose 4B.

counter. Per cent Cl_3CCOOH precipitability of the radioiodine remaining after removal of the beads was determined, and the per cent "loss" of ^{125}I counts, presumably due to "self-iodination" (David, 1972) by the enzyme–Sepharose complex, was calculated for each time point based on the total iodine remaining in the supernatant and precipitate as compared to the zero time sample. This "loss" of counts corresponded to the extent of incorporation of counts into the beads. Per cent incorporation of counts into the protein was based on the total zero time counts. Total ^{125}I utilization by the enzyme was considered to be the sum of the counts incorporated into the protein and those "lost" into the beads, since bead iodination appears to be a result of enzymatic activity. This latter calculation may more accurately reflect the effect of various conditions, such as sodium dodecyl sulfate or urea, on the activity of the enzyme than do calculations involving protein iodination alone.

An example of the calculations involved would be as follows: at each time point during the reaction, an aliquot of the iodination mixture was diluted into carrier BGG, carrier iodide, and NaN_3, then a portion precipitated with Cl_3CCOOH.[2] The lactoperoxidase-containing beads were removed prior to Cl_3CCOOH precipitation by centrifugation, along with any bead-bound radioiodide resulting from the "self-iodination" process. The zero time sample ($t = 0$) would represent the point at which neither protein iodination nor bead iodination had yet taken place. All radioiodine would, therefore, be present as free iodide and would consequently appear in the supernatant fraction in the Cl_3CCOOH precipitation step. At each subsequent time point ($t = n$), if "self-iodination" were occurring, that portion of the radioisotope which was "lost" into the beads would not appear in either the precipitate or the supernatant fraction in the Cl_3CCOOH precipitation step, resulting in a corresponding decrease in the total radioiodine (precipitate + supernatant) when compared to the zero-time sample. Thus

$$1 - \frac{(\text{cpm sup} + \text{cpm ppt})_{tn}}{(\text{cpm sup} + \text{cpm ppt})_{t0}} \times 100\% =$$

$$\% \text{ "self-iodination" at } t = n$$

$$\frac{(\text{cpm ppt})_{tn}}{(\text{cpm sup} + \text{cpm ppt})_{tn}} \times 100\% =$$

$$\% \text{ } Cl_3CCOOH \text{ precipitable cpm at } t = n$$

$$\frac{(\text{cpm ppt})_{tn}}{(\text{cpm sup} + \text{cpm ppt})_{t0}} \times 100\% =$$

$$\% \text{ } ^{125}I \text{ incorporation at } t = n$$

$$(\% \text{ "self-iodination" at } t = n) +$$

$$(\% \text{ } ^{125}I \text{ incorporation at } t = n) =$$

$$\text{total } ^{125}I \text{ utilization at } t = n$$

Trace-Labeling of Proteins with Radioiodine. Iodinations of proteins for experimental purposes were carried out as follows: a small volume of buffer, usually PBS, was dispensed into a microcentrifuge tube, followed by the required amount of carrier iodide, if desired. An appropriate volume of radioiodide in 0.1 N NaOH was added, followed by an equal volume of 0.1 N HCl to approximately neutralize the NaOH. The protein to be iodinated and the solid-state lactoperoxidase were added, after which the reaction was initiated with H_2O_2. The LP-Sepharose beads were kept suspended for the duration of the reaction. The reaction was terminated by the addition of about 1/100 volume of 2.5 M NaN_3. A few small crystals of carrier KI were added, followed by carrier protein if necessary. The enzyme and unbound iodide were then removed by passage through a small column of either Sephadex G-25 or Amberlite IR-45 (Hong and Nisonoff, 1965).

Other Materials. The isolation of rabbit IgG has been described (Keckwick, 1940; Levy and Sober, 1960). Urea (ultrapure) was purchased from Mann. In some experiments (LP-4B/2.3 iodinations, see below) the urea was dissolved in pH 7.0 PBS immediately prior to use. Usually a 10 M solution of urea in water was passed through a mixed bed ion exchange resin (Rexyn I-300, Fisher), adjusted to 0.15 M NaCl + 0.01 M sodium phosphate (pH 6.9–7.0) and diluted to the desired concentration with PBS.

Horse heart cytochrome *c* was purchased from Sigma (E_{407}^{1mM} 1.07) (Keilin and Slater, 1953). Carcinoembryonic antigen was kindly donated by Dr. C. W. Todd of the City of Hope National Medical Center or was isolated as previously described (Krupey *et al.*, 1968; Coligan *et al.*, 1972) from the water extract of metastatic gastrointestinal carcinoma supplied by Dr. N. Zamcheck.

Results

Effect of the Concentration at which Lactoperoxidase is Bound to the Sepharose Beads on the Iodination Reaction. In a previous report (David, 1972) data were presented describing the iodination reaction of a sample of Sepharose-bound lactoperoxidase as a function of several variables. The concentration of lactoperoxidase on the Sepharose beads was 2.3 mg/ml of settled beads (henceforth referred to as LP-4B/2.3).

To investigate the effect of varying the LP concentration on the Sepharose beads, samples were prepared ranging from 0.17 mg of coupled enzyme per ml of settled beads (LP-4B/0.17) to 1.3 mg/ml (LP-4B/1.3). As can be seen in Figure 2, the bead concentration of lactoperoxidase greatly affects the rate at which the enzyme is capable of iodinating rabbit IgG. The final enzyme concentration in each reaction was 2.0 μg/ml of reaction mixture. Iodide concentration was 1×10^{-5} M, while H_2O_2 concentration was 2×10^{-5} M.

In various experiments different batches of lactoperoxidase have been employed, ranging in purity from OD_{412}/OD_{280} ratios of 0.66 to 0.80. Within the limits studied, the degree of purity does not appear to affect the iodination rate of the enzyme, at a given bead concentration, with respect to rabbit IgG. There may be some variation in iodination properties, however, depending on the commercial source of the lactoperoxidase.

Effect of Hydrogen Peroxide Concentration on the Iodination Reaction. Since some proteins are sensitive to even mild oxidizing conditions (Miyachi *et al.*, 1972), an experiment was performed to determine whether as large an excess of H_2O_2 as had previously been used was necessary. As can be seen in Figure 3 and Table I, lower H_2O_2 concentrations are not only acceptable, but desirable. "Self-iodination" was found to be lower at low H_2O_2 concentrations and consequently the actual incorporation of ^{125}I into the protein was increased.

Iodinations were carried out using LP-4B/2.3 beads (Figure 3a) and KI concentrations at 1×10^{-5} M while using 9×10^{-5} M H_2O_2, 1.8×10^{-5} M H_2O_2, and 0.9×10^{-5} M H_2O_2, respectively, with the enzyme concentration at 11.4 μg/ml and rabbit

[2] Cl_3CCOOH precipitability was considered to represent protein-bound radioiodide in rabbit IgG experiments. In a separate series of experiments, the Cl_3CCOOH precipitability of radioiodinated cytochrome *c*, under identical conditions of concentration, carrier protein, etc. was determined to be 66% after exposure to PBS or 4 M urea and 72% after exposure to 0.1% sodium dodecyl sulfate. Appropriate corrections were made in the data presented in Table II and Figure 5.

TABLE I: Effect of H_2O_2 Concentration on Iodination Reactions.[a]

LP Source	(LP) (μg/ml)	[KI] (M)	[H_2O_2] (M)	Cl_3CCOOH Precipitability[b] (30 min) (%)	^{125}I Incorporation (30 min) (%)	Total ^{125}I Utilization (30 min) (%)
LP-4B/2.3	11.4	1×10^{-5}	9×10^{-5}	90	55	94
			1.8×10^{-5}	92	59	95
			0.9×10^{-5}	74	59	80
LP-4B/0.17	1.0	1×10^{-5}	9×10^{-5}	79	42	89
			2×10^{-5}	92	50	96
			1×10^{-5}	84	51	91
			0.5×10^{-5}	46	37	57
LP-4B/0.17	1.0	1×10^{-6}	9×10^{-6}	78	49	86
			2×10^{-6}	78	49	86
			1×10^{-6}	79	52	86

[a] Reactions were carried out at room temperature. Rabbit IgG concentration was 1.0 mg/ml in PBS, pH 7.0. [b] Precipitability of radioiodine after removal of LP-4B beads.

IgG at 1.0 mg/ml. In a second experiment LP-4B/0.17 beads were used, with an enzyme concentration of 1.0 μg/ml of reaction mixture, and KI concentrations of 1×10^{-5} M (Figure 3b) or 1×10^{-6} M (Figure 3c), while varying the H_2O_2 concentrations as shown in Table I.

As would be expected, when the molar ratio of H_2O_2:KI was less than unity the total utilization of ^{125}I decreased to a roughly proportional degree. The actual incorporation of ^{125}I counts into the IgG, however, did not diminish to a similar extent. As the data indicate, "self-iodination" was reduced at lower H_2O_2 concentrations, with counts being incorporated preferentially into the protein rather than "losing" much of the iodide into the beads as occurs at higher H_2O_2 concentrations. It would thus appear that a KI:H_2O_2 molar ratio of approximately 1 is usually optimal for iodination reaction conditions. However, in practice it may be preferable to use a slight excess of H_2O_2 to allow for possible problems in manipulation, *e.g.*, H_2O_2 decomposition and pipeting errors. The loss in iodine incorporation under conditions of higher H_2O_2 concentration is relatively insignificant.

It should be pointed out that the concentration of LP-4B/0.17 beads employed in these experiments was less than that which would give maximum iodination rate in order to increase the sensitivity of the assay to H_2O_2 concentration differences. Thus, the lower iodination efficiency of these beads as compared to that of the LP-4B/2.3 beads in these experiments should not be interpreted as an indication that LP-4B/0.17 is inherently less efficient than LP-4B/2.3.

Effect of Denaturing Agents on the Iodination Reaction. The iodination reaction can be used in structural studies of proteins as well as providing a method of trace labeling. For example, the accessibility of a tyrosine residue or the effect of the iodination of a particular tyrosine residue on the biological activity of a molecule is often of interest. It is frequently desirable to carry out an iodination reaction in the presence of a variety of denaturing agents to determine their effect on the tertiary structure of a polypeptide chain, specifically with respect to certain tyrosine residues.

As is shown in Figure 4 and Table II, Sepharose-bound lactoperoxidase is quite active in 0.1% sodium dodecyl sulfate and in concentrations of urea up to about 10 M. Furthermore, since "self-iodination" seemed to decrease in the presence of these denaturing agents, the actual incorporation of ^{125}I into rabbit IgG increase significantly at 4 and 6 M urea and in 0.1% sodium dodecyl sulfate, although the total utilization of the radioiodine had decreased. It is possible that the increase in ^{125}I incorporation may be due to an increase in accessibility of tryosine residues.

As has been reported, cytochrome *c* iodinates poorly, possibly an indication that the tyrosine residues are not readily accessible (Narita *et al.*, 1967; Stellwagen, 1968). As can be seen from Figure 4c and Table II, 4 M urea has little effect on the iodination of horse heart cytochrome *c*. This is in agreement with a report which indicates that the major unfolding of cytochrome *c* takes place at higher urea concentrations (Stellwagen, 1968). On the other hand, 0.1% sodium dodecyl sulfate considerably increases the rate of ^{125}I incorporation under these conditions. This may reflect a significant alteration in the tertiary structure of the cytochrome molecule in the presence of sodium dodecyl sulfate.

Effect of Temperature on the Iodination Reaction. Many isolated proteins are relatively unstable at room temperature with respect to biological activity and physicochemical properties. Thus, it might occasionally be advantageous to carry out an iodination reaction at or near 0° to prevent denaturation of

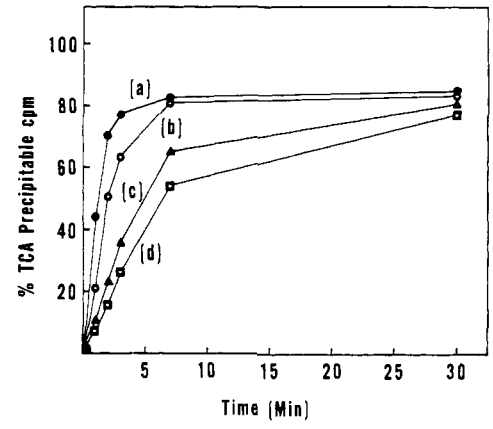

FIGURE 2: Effect of lactoperoxidase concentration on Sepharose 4B on iodination rate. Rabbit IgG concentration = 1.0 mg/ml; [KI] = 1×10^{-5} M; [H_2O_2] = 2×10^{-5} M. The buffer was pH 7.0 PBS. Total lactoperoxidase concentration in each case was 2 μg/ml of reaction mixture. The ratio of lactoperoxidase to Sepharose in mg per ml of settled beads was (a) 0.17, (b) 0.5, (c) 0.99, and (d) 1.3. Results are expressed as per cent precipitability of ^{125}I in 5% Cl_3CCOOH after removal of LP-Sepharose.

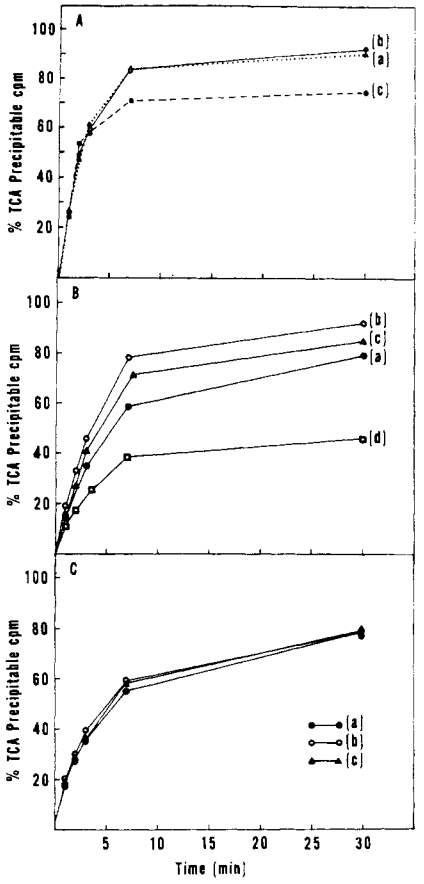

FIGURE 3: Effect of H_2O_2 concentration on iodination catalysis by (A) LP-4B/2.3. Rabbit IgG concentration = 1.0 mg/ml; [KI] = 1×10^{-5} M. Total lactoperoxidase concentration was 11.4 μg/ml of reaction mixture. The reaction was carried out at room temperature in pH 7.0 PBS. Hydrogen peroxide concentrations were (a) 9.0×10^{-5} M, (b) 1.8×10^{-5} M, (c) 0.9×10^{-5} M. Results are expressed as per cent precipitibility of ^{125}I in 5% Cl_3CCOOH after removal of LP-Sepharose; (B) LP-4B/0.17 at [KI] = 1×10^{-5} M. Lactoperoxidase concentration was 1 μg/ml of reaction mixture. Hydrogen peroxide concentrations were (a) 9.0×10^{-5} M, (b) 2.0×10^{-5} M, (c) 1×10^{-5} M, (d) 0.5×10^{-5} M. Other conditions were as in (A); (C) LP-4B/0.17 at [KI] = 1×10^{-6} M. Hydrogen peroxide concentrations were (a) 9.0×10^{-6} M, (b) 2.0×10^{-6} M, (c) 1.0×10^{-6} M. Other conditions were as in (B).

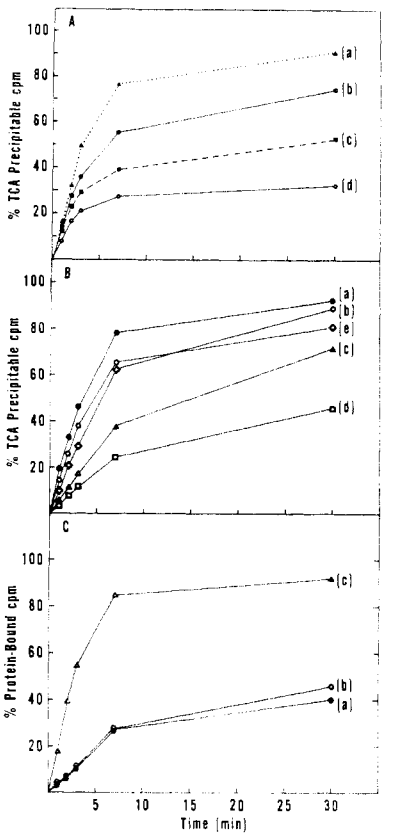

FIGURE 4: Effect of denaturing agents on the iodination of (A) rabbit IgG by LP-4B/2.3. Rabbit IgG concentration = 1.0 mg/ml; [KI] = 1×10^{-5} M; [H_2O_2] = 9×10^{-5} M. Total lactoperoxidase concentration was 11.4 μg/ml of reaction mixture. Reactions were carried out at room temperature in pH 7.0 PBS containing the following concentrations of urea: (a) none, (b) 5.8 M, (c) 7.8 M, (d) 9.7 M. Results are expressed as per cent precipitability of ^{125}I in 5% Cl_3CCOOH after removal of LP-Sepharose. (B) Rabbit IgG by LP-4B/0.17. Lactoperoxidase concentration was 1 μg/ml of reaction mixture; [H_2O_2] = 2×10^{-5} M. Reactions were carried out in pH 7.0 PBS containing (a) no denaturing agents, (b) 4 M urea, (c) 6 M urea, (d) 8 M urea, (e) 0.1% sodium dodecyl sulfate. Other conditions were as in (A). (C) Horse heart cytochrome *c* by LP-4B/0.17. Reactions were carried out in pH 7.0 PBS containing (a) denaturing agents, (b) 4 M urea, (c) 0.1% sodium dodecyl sulfate. Other conditions were as in (B). Results are expressed as per cent protein-bound ^{125}I after removal of LP-Sepharose.

such proteins. Data presented in Table III indicate that insoluble lactoperoxidase is quite capable of carrying out an iodination at low temperatures. The rate of the reaction at 0° is only slightly reduced over that at 22° (data not shown).

Rabbit IgG, 1.0 mg/ml, was iodinated at 1×10^{-5} M KI in pH 7.0 PBS using either LP-4B/2.3 or LP-4B/0.17. With LP-4B/2.3 the H_2O_2 concentration was 9×10^{-5} M and when LP-4B/0.17 was used, the concentration of H_2O_2 was 2×10^{-5} M. Iodinations were performed at room temperature (22°), in a water bath (8°) or in an ice bath (0°). The results show that the iodination reaction is only slightly affected by low temperatures.

Reuse of Insoluble Lactoperoxidase. As reported previously, a significant amount of radioiodine is usually "lost" during an iodination reaction due to the "self-iodination" process alluded to earlier. The enzyme appears to be capable of depositing iodine in the Sepharose bead in some form not yet determined. This iodine is not removed by incubation with an excess of cold iodide and the "self-iodination" process seems to be a function of enzymatic activity; for example, it is inhibited by NaN_3 and is reduced at lower concentrations of enzyme.

While the amount of iodine incorporated into the beads during an iodination reaction would argue against the possibility that self-iodination of the lactoperoxidase molecule itself could account for the total "loss" of radioiodine, it is still conceivable that some enzyme iodination occurs. The inactivation of soluble lactoperoxidase during an iodination reaction (Morrison *et al.*, 1971) might be explained by such a self-destructive mechanism. A similar process might be occurring in the insoluble lactoperoxidase system.

To test the effect of "self-iodination" on the activity of solid-state lactoperoxidase, 11.4-μg samples of Sepharose-bound enzyme (LP-4B/2.3) were pretreated by allowing them to iodinate 1 ml of rabbit IgG at either 10 mg/ml, 1.0 mg/ml, or 0.1 mg/ml, or 1 ml of PBS containing no protein substrate. As has been shown previously (David, 1972), the concentration of protein markedly affects the degree to which the beads will "self-iodinate." After a 30-min reaction, the degree of ^{125}I "loss" was determined and is shown in Table IV. Each sample of enzyme was then washed five times with pH 7.0 PBS and

TABLE II: Effect of Denaturing Agents on Iodination Reactions.[a]

Iodinated Protein	LP Source	LP (μg/ml)	Denaturing Agent	Final Concn	Cl_3CCOOH Precipitability[b] (30 min) (%)	^{125}I Incorporation (30 min) (%)	Total ^{125}I Utilization (30 min) (%)
Rb IgG	LP-4B/2.3	11.4	None		90	54	94
			Urea	5.8 M	74	63	78
				7.8 M	52	47	70
				9.7 M	32	30	36
Rb IgG	LP-4B/0.17	1.0	None		92	50	96
			Urea	4.0 M	89	74	90
				6.0 M	71	60	76
				8.0 M	45	41	51
			SDS	0.1%	81	59	86
Cytochrome *c*	LP-4B/0.17	1.0	None		41[c]	33	51
			Urea	4.0 M	46[c]	41	53
			SDS	0.1%	92[c]	80	93

[a] Reactions were carried out at room temperature. Protein concentration was 1.0 mg/ml; KI concentration was 1×10^{-5} M; H_2O_2 concentration was either 9×10^{-5} M (LP-4B/2.3 iodinations) or 2×10^{-5} M (LP-4B/0.17 iodinations). Urea and SDS (sodium dodecyl sulfate) solutions contained PBS, pH 6.9–7.0. [b] Precipitability of radioiodine after removal of LP-4B beads. [c] Corrected for Cl_3CCOOH precipitability of cytochrome *c* under these conditions after exposure to (a) PBS, 66%; (b) 4 M urea, 66%; (c) 0.1% SDS, 72%. Precipitabilities were determined in separate experiments.

was used a second time to iodinate 1 ml of rabbit IgG at 1.0 mg/ml. Concentrations of KI and H_2O_2 were 1×10^{-5} and 9×10^{-5} M, respectively for all iodinations. As can be seen from Figure 5, "self-iodination" had little detectable affect on the iodination reaction. From these data it would appear that if any iodine is being incorporated into the lactoperoxidase molecule, it does not seem to significantly affect enzymatic activity.

One further experiment was carried out to determine whether LP-4B/0.2 was sufficiently stable to allow the enzyme to be reused several times; 1 ml of a 1.0-mg/ml solution of rabbit IgG in pH 7.0 PBS was iodinated with 2 μg of the LP-4B/0.2 enzyme using 1×10^{-5} M KI and either 9×10^{-5} or 1×10^{-5} M H_2O_2. After 5- and 30-min intervals the degree of iodination was determined as described in the Experimental Section, the enzyme was removed by centrifugation and washed four times with PBS, and the beads were resuspended in 1.0 mg/ml of IgG to their original concentration. A second iodination was then carried out, and the procedure was repeated through a total of five iodinations. The results are presented in Table V. While some loss of activity did occur, it can be seen that the insoluble enzyme retained sufficient activity to participate in several iodination reactions. Furthermore, the lower H_2O_2 concentration seemed to affect enzymatic activity to a lesser extent.

Practical Aspects of the Iodination Procedure. To this point, iodination reactions have been carried out under conditions which were designed to allow one to follow the course of an iodination under a variety of conditions. For practical applications of this technique one might wish to vary the experimental

TABLE III: Effect of Temperature on Iodination Reactions.[a]

LP Source	[LP] (μg/ml)	Temp (°C)	Cl_3C-COOH Precipitability[b] (30 min) (%)	^{125}I Incorporation (30 min) (%)	Total ^{125}I Utilization (30 min) (%)
LP-4B/2.3	11.4	22	90	55	94
		8	83	48	90
		0	76	44	86
LP-4B/0.17	1.0	22	92	50	96
		0	85	55	90

[a] Rabbit IgG concentration was 1.0 mg/ml in PBS, pH 7.0; KI concentration was 1×10^{-5} M; H_2O_2 concentration was either 9×10^{-5} M (LP-4B/2.3 iodinations) or 2×10^{-5} M (LP-4B/0.17 iodinations). [b] Precipitability of radioiodine after removal of LP-4B beads.

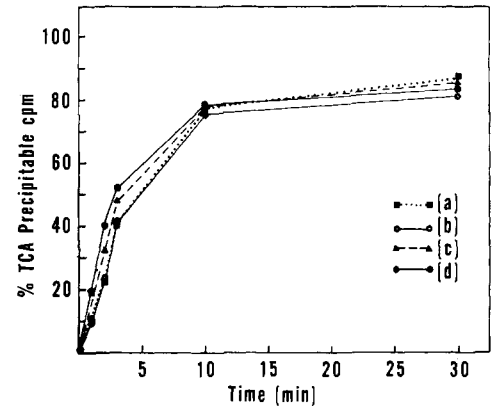

FIGURE 5: Effect of "self-iodination" on enzyme activity. Samples (11.4 μg) of LP-4B/2.3 were allowed to self-iodinate as described in the text, then retested for their enzymatic activity. Extent of iodine uptake by the beads is shown in Table IV. Pretreatment substrate concentrations were (a) 10.0 mg/ml, (b) 1.0 mg/ml, (c) 0.1 mg/ml, (d) no IgG substrate. Concentration of IgG here was 1.0 mg/ml, [KI] = 1×10^{-5} M, [H_2O_2] = 9×10^{-5} M. Reaction volumes were 1.0 ml. Iodinations were carried out at room temperature in pH 7.0 PBS. Results are expressed as per cent ^{125}I precipitability in 5% Cl_3CCOOH after removal of LP-Sepharose.

TABLE IV: ^{125}I Uptake by LP-4B/2.3 During Iodination of IgG.[a]

IgG Concentration (mg/ml)	^{125}I Uptake (%)
10	25
1.0	45
0.1	59
None	41

[a] Sepharose-bound lactoperoxidase (LP-4B/2.3) concentration was 11.4 μg/ml; KI concentration was 1×10^{-5} M; H_2O_2 concentration was 9×10^{-5} M. Reactions were carried out for 30 min at room temperature in PBS, pH 7.0.

TABLE V: Reuse of Solid-State Lactoperoxidase.[a]

	^{125}I Incorporation into IgG (%)			
	9×10^{-5} M H_2O_2		1×10^{-5} M H_2O_2	
Times Used	5 min	30 min	5 min	30 min
1	49	53	54	59
2	42	44	50	56
3	41	44	39	43
4	27	42	46	48
5	16	35	43	48

[a] Sepharose-bound lactoperoxidase (LP-4B/2.3) concentration was 11.4 μg/ml; rabbit IgG concentration was 1.0 mg/ml; KI concentration was 1×10^{-5} M. Reactions were carried out in PBS, pH 7.0 at room temperature.

protocol somewhat. This matter will be explored more extensively in the Discussion. It is worthwhile, however, to describe here specific examples of two slightly different protocols used in this laboratory for the iodination of rabbit IgG at low specific activity and CEA at high specific activity.

Low Specific Activity Rabbit IgG. Two hundred micrograms of rabbit IgG were iodinated as described in the Experimental Section. The total reaction volume was approximately 265 μl, containing 1×10^{-3} μmol of carrier KI, 310 μCi of $Na^{125}I$, 0.5 μg of lactoperoxidase (LP-4B/0.17), and 2×10^{-3} μmol of H_2O_2. The reaction was allowed to proceed for 15 min at room temperature, then stopped as described in the Experimental Section and passed through a 1.5-ml column of Amberlite IR-45 equilibrated in saline. The peak protein tubes were pooled. The ^{125}I label was found to be 97% precipitable in 5% cold Cl_3CCOOH, and the specific activity was 5.5×10^5 cpm/μg (57% counting efficiency).

High Specific Activity CEA. Approximately 4.9 μg of CEA were iodinated in a total volume of about 18 μl containing 270 μCi of $Na^{125}I$, 0.25 μg of lactoperoxidase (LP-4B/0.17), 5×10^{-4} μmol of H_2O_2, and no carrier KI. After 5 min at room temperature the reaction was stopped with NaN_3, and carrier KI and about 180 μg (10 μl) of carrier bovine serum albumin were added. After passage through a 5.4-ml column of Sephadex G-25 equilibrated in PBS containing 0.1% bovine serum albumin and 0.025 M NaN_3, the specific activity of the CEA was found to be about 2.3×10^7 cpm/μg.

The results of these iodinations appear to be quite reproducible under similar reaction conditions.

Discussion

Bovine lactoperoxidase covalently coupled to Sepharose 4B has previously been demonstrated to be an extremely versatile enzyme (David, 1972), catalyzing the iodination of proteins over a wide range of pH conditions and at iodide concentrations varying by several orders of magnitude. While higher protein concentrations favor iodination of the protein substrate rather than "self-iodination" of the enzyme–bead complex, the insoluble enzyme is nevertheless capable of relatively efficient protein iodination even at low protein concentrations. By choosing the appropriate reaction conditions for a given protein, assuming that it has available tyrosine residues, solid-state lactoperoxidase seems to be capable of catalyzing virtually any degree of iodination, with respect to both specific activity and degree of iodine saturation.

It should be stressed, however, that this iodination reaction is enzymatic in nature, and that some data indicate that direct contact is necessary between the enzyme and the residue which ultimately accepts the iodine atom (Morrison, 1968; Morrison and Bayse, 1970). It must, therefore, be expected that any reaction conditions which affect either enzyme activity, such as the presence of trace quantities of NaN_3, or the accessibility of the appropriate residue to the recognition site of the enzyme, such as conformational aspects of the protein or possibly the presence of non-iodine accepting inhibitors, will also affect the iodination reaction. Furthermore, in a peroxide-dependent iodination reaction either reducing agents or extremely sensitive reducing groups on proteins could conceivably compete with the lactoperoxidase for available H_2O_2. In addition, when one uses molar concentration ratios of protein to iodide considerable greater than unity, it is not difficult to imagine a circumstance in which a protein which exhibits an affinity for anions might actually compete with the lactoperoxidase for the iodide ion. In other words, optimal conditions for the enzymatic iodination of rabbit IgG may not necessarily constitute optimal conditions for the iodination of another protein (G. Parsons, I. McIlwaine, R. A. Reisfeld and G. S. David, in preparation). For example, any condition which affects protein conformation, such as pH, might affect the iodination reaction and might show different effects for various proteins.

The data presented here demonstrate the versatility of insoluble lactoperoxidase for protein iodinations, as the enzyme can function in the presence of strong denaturing agents, such as urea and sodium dodecyl sulfate (Table II and Figure 4), as well as at 0° thus permitting the iodination of temperature sensitive proteins. Furthermore, unlike soluble lactoperoxidase the Sepharose-bound enzyme remains active after several protein iodinations (Table V). It should be pointed out that the loss of some iodinating efficiency indicated by data shown here could be due to mechanical problems rather than enzyme inactivation. After several centrifugation steps in the washing procedure, some clumping of the beads was noticed.

The activity of lactoperoxidase as determined by its ability to iodinate proteins decreases as the concentration at which it is coupled to Sepharose is increased. It seems likely that this is a result of steric hindrance. The effects of various reaction conditions upon the enzymatic activity do not appear to be related to the degree of "packing" of lactoperoxidase onto the Sepharose bead. This is demonstrated by the effects of these parameters upon the iodination activities of LP-4B/2.3 and LP-4B/0.17, the two extremes of coupling ratios presented here. As can be seen from the various reaction conditions studied, when the activity of LP-4B/2.3 was increased or decreased that of LP-4B/0.17 was similarly affected.

It appears that the iodination of proteins in the lactoperoxidase system is slightly more efficient at low concentrations of H_2O_2 (Table I). This may reflect the sensitivity of enzyme activity to high H_2O_2 levels reported previously (Morrison, 1968; Morrison *et al.*, 1971). "Self-iodination" occurs to a lesser degree at low H_2O_2 concentration, but it is not clear whether this is the result of increased efficiency of protein iodination or whether the increased incorporation of radioiodide into the protein is a result of the lower degree of incorporation of counts into the beads. Similar arguments can be applied to the shift from bead to protein iodination at low temperatures or in the presence of urea or sodium dodecyl sulfate.

The nature of "self-iodination" of the LP-Sepharose beads is presently not clear. The incorporation of radioiodide into the beads is apparently enzymatic in nature, and counts are not removed from the Sepharose during storage for several days in the presence of 0.05 M KI. Binding, covalent or otherwise, must be to the bead matrix, since too little protein is bound to the Sepharose to account for the extent of the iodination.

For practical purposes, "self-iodination" does not appear to affect the enzymatic activity of lactoperoxidase, although it results in a less efficient incorporation of radioiodide into the protein substrate.

Ideally, to determine optimal conditions for the iodination of a given protein, one should investigate the effects of various parameters, such as pH, protein concentration, and iodide level. However, for practical considerations this is often not feasible. Thus, considering the versatility of Sepharose-bound lactoperoxidase, it would seem logical to attempt an initial iodination under conditions of protein concentration, protein to iodide molar ratios, buffer system, etc., which are most convenient for a particular situation, keeping in mind the effects these parameters have on the iodination of rabbit IgG. If the resulting iodination efficiency is exceedingly low, then various individual parameters may be investigated. If iodination efficiency is not prohibitively low, it is often sufficient to merely increase the amount of radioiodide in the reaction mixture to achieve an acceptable specific activity.

In terms of simplicity, reproducibility, and flexibility, the insoluble lactoperoxidase system may provide an extremely powerful tool for the study of protein structure and function in biological systems. In addition to its usefulness as a radioiodination method, certain aspects of the iodination reaction of the Sepharose-bound enzyme raise some interesting questions. The ability of the enzyme to carry out iodinations at low iodide concentrations in the absence of exogenously added peroxides, although at reduced reaction rates (G. Parsons *et al.*, in preparation), suggests the possible existence of an alternate reaction mechanism. The Sepharose-bound lactoperoxidase system could possibly serve as an interesting model as it may actually mimic a cell membrane-bound enzyme, with its ability to "self-iodinate" suggesting an *in vivo* mechanism for the storage of iodine.

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