SOLID STATE LACTOPEROXIDASE: A HIGHLY STABLE ENZYME FOR SIMPLE, GENTLE IODINATION OF PROTEINS

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Summary. A simple, gentle method for radioiodination of proteins has been developed by coupling bovine lactoperoxidase to cyanogen bromide-activated Sepharose-4B. Following iodination the enzyme could be readily removed by centrifugation. Sepharose-coupled lactoperoxidase was capable of iodinating all proteins studied and was active over wide ranges of protein and KI concentrations as well as pH conditions. There has been no detectable loss of activity of the solid state enzyme after storage at 4°C in the presence of 10^{-5} M merthiolate for at least 4 months.

Radioiodine labelling has been used to detect or quantitate biological materials as well as in structural studies of many proteins and peptides. Picogram quantities of iodine labelled proteins can readily be detected, and the incorporation of a single iodine atom per molecule usually has little or no effect on the structure or the biological activity of a protein or peptide. However, until recently chemical iodination procedures have employed relatively high concentrations of strong oxidizing agents such as chloramine-T (1) or iodine monochloride (2). Under such conditions, non-specific side reactions can cause drastic alterations in protein structure and activity (3-5) or can lead to low efficiency of iodine incorporation, especially at low protein concentrations (6). Electrolytic iodination(7,8) can be gentle with little or no denaturation, but specific activities are low. Moreover, if this procedure is carried out over a period of time sufficient to yield a high specific activity, denaturation begins to occur.

Recently developed enzymatic methods of iodination, in particular the lactoperoxidase method (9-15), appear to be quite gentle, employ low levels of relatively weak oxidizing agents, and can yield products with high specific activities. The disadvantage of enzyme catalyzed iodination procedures, when applied to soluble materials,

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has been the necessity of introducing contaminants into the iodination reaction mixture. This problem is compounded by the fact that commercially available enzyme preparations usually contain significant quantities of impurities. An additional problem which may exist involves the incorporation of radioiodine into materials other than those of specific interest, in particular self-iodination by lactoperoxidase. It has generally been assumed, but not well documented, that the low level of enzyme used in an iodination reaction results in the almost exclusive labelling of the major components without any significant iodine incorporation into the enzyme (14, 15). Evidence presented below may indicate a necessity to re-evaluate this problem.

By covalently coupling a lactoperoxidase preparation to cyanogen bromide-activated Sepharose-4B, it has been possible to develop a technique for the iodination of proteins which does not require the introduction of contaminating materials. Sepharose-bound lactoperoxidase appears to be stable over long periods of time when stored at 4°C in phosphate buffered saline (PBS) containing 10^{-5}M merthiolate. Iodination reactions may be carried out over a broad range of conditions of pH, temperature and iodide concentrations. An additional advantage of this newly developed procedure is that following the iodination procedure, the Sepharose-bound enzyme can readily be removed by low speed centrifugation.

MATERIALS AND METHODS

Lactoperoxidase was purchased from Calbiochem. Enzyme concentrations were determined from their optical density at 412 nm (E^{mM}_{412nm} = 114) (11) using a Gilford model 2400 spectrophotometer.

Sepharose-4B (Pharmacia, Uppsala, Sweden) was activated with CNBr according to the method of Cuatrecasas (16). Lactoperoxidase was coupled to the activated Sepharose to a final concentration of 2.3 mg enzyme per ml of settled beads. Following the coupling procedure, any remaining reactive groups on the beads were exposed to 0.2M glycine, 0.01M phosphate, pH 7.5 at 4°C for 5 hrs. After thorough washing the Sepharose-bound

enzyme (LP-4B) was stored in PBS, pH 7.4 containing 10^{-5}M merthiolate.

Iodinations were carried out at room temperature as follows: an appropriate quantity of LP-4B suspension was pipetted into a Fisher centrifuge tube containing approximately 1 ml of the desired buffer. The beads were centrifuged at 2,000 x g in a Fisher Model 59 centrifuge and were washed twice with 1 ml of buffer. Protein solution, usually 0.5 or 1.0 ml, was pipetted into the tube after the final wash had been removed by aspiration. One µl of KI solution was added, followed by 1 µl of $Na^{125}I$ (ICN, 2200 Ci/mmole) containing about 1×10^7 cpm. A zero time sample was removed (20µl) and precipitated with trichloroacetic acid (CCl_3COOH) as described below. The iodination reaction was initiated by the addition of 10 µl of 0.03% H_2O_2 solution per ml of protein solution (when a final concentration 1×10^{-4}M KI was employed, 10 µl of 0.3% H_2O_2 was used). The LP-4B was kept suspended by manual agitation throughout the course of the experiment. Twenty-µl samples were withdrawn at various intervals by means of an Eppendorf pipet and diluted into 0.2 ml of a 2 mg/ml solution of bovine γ-globulin in PBS containing 0.025M NaN_3 and 0.05M KI. The azide ion inhibits any further lactoperoxidase activity (11). Traces of LP-4B and any LP-4B-bound iodine were removed by centrifugation and 0.1 ml of each supernatant was transferred to a Beckman Microfuge tube and precipitated with an equal volume of cold 10% CCl_3COOH. Precipitates were allowed to stand at 0 - 4°C for a minimum of 4 hrs. before removal by centrifugation in a Beckman Microfuge. Precipitates were washed twice with cold 5% CCl_3COOH and radioactivity was determined in a Nuclear Chicago Model 4233 Auto -γ-counter. One-tenth ml of each supernatant was counted and the per cent precipitability of the ^{125}I was determined. Per cent loss of ^{125}I, presumably due to self-iodination by the LP-4B, was calculated from the zero-time sample.

Rabbit IgG was isolated from serum as described by Kekwick (17) followed by passage through DEAE cellulose in 0.0175M phosphate buffer, pH 6.9 (18). Concentrations

were determined by optical density at 280 nm ($E^{1\%}_{280nm}$ = 15) (19). Ribonuclease was purchased from Sigma ($E^{1\%}_{280nm}$ = 7.2) (20). Pepsin was purchased from Worthington ($E^{1\%}_{280nm}$ = 14.3) (21) and horse heart Cytochrome C from Mann (E^{mM}_{407nm} = 1.07) (22). Ovalbumin (5x cryst) was obtained from Calbiochem ($E^{1\%}_{280nm}$ = 7.35) (23). Bovine serum albumin (2x cryst) was purchased from Pentex ($E^{1\%}_{280nm}$ = 6.67) (24).

RESULTS AND DISCUSSION

Figure 1 shows the effect of LP-4B concentration on the iodination of a 1 mg/ml solution of rabbit IgG in PBS, pH 7.4. As can be seen, 0.025M NaN_3 completely inhibits the iodination reaction. Significant iodination occurs at concentrations of about 1 µg of Sepharose-bound lactoperoxidase per ml of solution.

As shown in Fig. 2, LP-4B is active over a wide range of pH values, the optimum being around pH 6.5 - 7.0. In subsequent experiments all reactions were carried out in PBS, pH 7.0.

The effect of KI concentration on the final (30 min.) incorporation of ^{125}I into rabbit IgG (1mg/ml) is shown in Table 1. The optimum concentration is about 1×10^{-5}M KI, but as can be seen significant incorporation can be obtained even when the $Na^{125}I$ is the only source of iodide. In other words, virtually any degree of iodination can be achieved by choosing the appropriate iodination conditions.

As seen in Fig. 3 and Table 2, a wide range of protein concentrations may also be employed. It should be pointed out that the degree of "self-iodination" is markedly affected by substrate concentration. For example, at 10 mg/ml IgG only 18% of the ^{125}I is lost to the LP-4B, while at 0.10 mg/ml IgG 64% of the ^{125}I is lost (Table 2).

Table 2 shows that LP-4B is quite effective with other proteins as well as rabbit IgG. Of the proteins iodinated, only ovalbumin was resistant to iodination under normal conditions (10^{-5}M KI, 9×10^{-5}M H_2O_2). It was necessary to increase both KI and H_2O_2 to obtain significant iodination of this protein. Again, the degree of "self-

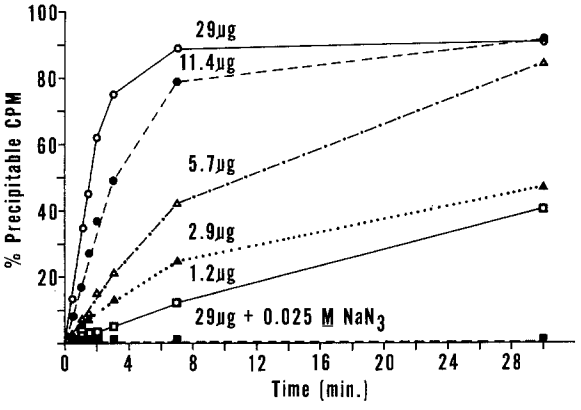

Fig. 1.

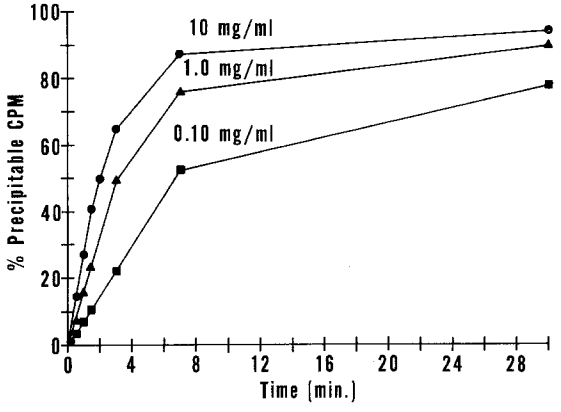

Fig. 3.

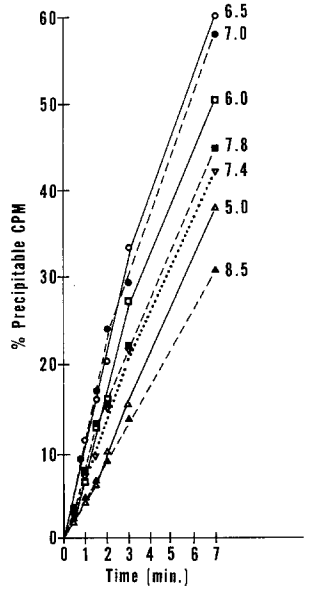

Fig. 2.

Figure 1. Incorporation of ^{125}I into rabbit IgG as a function of Sepharose-bound lactoperoxidase concentration. Each reaction volume was 1 ml, (KI) = 1×10^{-5}M, (H_2O_2) = 9×10^{-5}M, (IgG) = 1.0 mg/ml. Buffer was phosphate-buffered saline, pH 7.4. Experiments were carried out as described in Materials and Methods.

Figure 2. Effect of pH on iodination by Sepharose-bound lactoperoxidase. Each reaction volume was 1 ml, (KI) = 1×10^{-5}M, (H_2O_2) = 9×10^{-5}M, (IgG) = 1.0 mg/ml. Buffers: pH 5.0, 0.1M NaAc; pH 6.0, 6.5, 7.0, 7.4 and 7.8, phosphate-buffered saline; pH 8.5, 0.2M glycine. Experiments were carried out as described in Materials and Methods.

Figure 3. Effect of protein concentration on ^{125}I incorporation into rabbit IgG by Sepharose-bound Lactoperoxidase. (KI) = 1×10^{-5}M, (H_2O_2) = 9×10^{-5}M, buffer was phosphate-buffered saline, pH 7.0. Experiments were carried out as described in Materials and Methods.

TABLE I

EFFECT OF KI CONCENTRATION ON IODINATION

Prot.	Conc.	(KI)	^{125}I Incorporation
Rabbit IgG	1.0 mg/ml	10^{-4}M	40%
"	"	10^{-5}M	53%
"	"	10^{-6}M	36%
"	"	10^{-7}M	42%
"	"	0	26%

Experiments were carried out as described in Materials and Methods. Each reaction volume was 1 ml and 11.4 µg of Sepharose-bound lactoperoxidase was used. Radioiodine incorporation is expressed as the per cent of the initial ^{125}I incorporated into the protein after 30 min.

TABLE II

IODINATION OF DIFFERENT PROTEINS BY SEPHAROSE-BOUND LACTOPEROXIDASE

Protein	conc.	^{125}I Incorporation	^{125}I "loss"
Rabbit IgG	10 mg/ml	78%	18%
"	1.0	54%	40%
"	0.1	29%	64%
BSA	1.0 mg/ml	75%	19%
Pepsin	"	60%	18%
RNase	"	46%	28%
Cyt. C.	"	26%	26%
oval	"	0.9%	9%
oval[a]	"	41%	11%

[a] (KI) = 1×10^{-4}M; (H_2O_2) = 9×10^{-4}M

Iodination conditions are described in Materials and Methods. Reaction volumes were 0.5 ml containing 5.7 µg Sepharose-bound lactoperoxidase. Radioiodine incorporation is expressed as the per cent of the initial ^{125}I incorporated into the protein after 30 min. Radioiodine "loss" represents the per cent of the initial ^{125}I incorporated into the lactoperoxidase-containing Sepharose beads after 30 min.

iodination" varies with the protein substrate (Table 2).

With the exception of the ovalbumin experiments, in all instances in which the lactoperoxidase represented about 1% of the total protein radioiodine loss varied between 19% and 40%. More strikingly, when the rabbit IgG concentration was 10 mg/ml and the lactoperoxidase was only about 0.1% of the total protein, 18% of the ^{125}I was still lost. If such a loss was due in part to self-iodination of the enzyme, there could be cause for concern among investigators using soluble lactoperoxidase for iodinations. Of course, it must be remembered that the properties of solid-state enzymes may be different than those of soluble enzymes.

Data to be presented elsewhere indicate that iodination proceeds virtually as well at 0°C as at room temperature, and that the enzyme retains activity in concentrations of urea as high as 9.6M (25). Preliminary results further indicate that LP-Sepharose may be used more than once.

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