Characterization of Bromine-77-Labeled Proteins Prepared Using Bromoperoxidase

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The halogenating enzyme bromoperoxidase, isolated from the red algae *Bonnemaisonia hamifera* and *Penicillus capitatus*, was used to catalyze the radiohalogenation of proteins at neutral pH. Human serum albumin and canine fibrinogen were halogenated as model compounds; the proteins were labeled with Br-77, produced by the $^{75}As(\alpha,2n)^{77}Br$ reaction. For each enzyme, the essential reaction parameters (including the concentrations of hydrogen peroxide or of protein, the amount of enzyme used to catalyze the reaction, the pH of the reaction mixture, and the reaction time) were varied to obtain conditions that resulted in the highest yield of radiolabeled protein.

The labeled proteins prepared with bromoperoxidase are stable with respect to loss of the radiolabel by hydrolysis and retain their biologic activity. The extension of this method to radiobromination of other types of compounds for imaging and receptor studies seems promising. Also, proteins and other compounds may be labeled with shorter-lived, positron-emitting isotopes of bromine for use in conjunction with computer-assisted positron tomography.

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Radioisotopes of iodine (especially I-125 and I-131) have been used extensively as protein labels because of their availability and the relative ease of attaching iodine to proteins to produce labeled agents of high specific activity. For use in nuclear medicine, however, radioiodinated proteins are less than ideal due to the high radiation dose to a patient receiving proteins labeled with I-125 or I-131 and the rapid rate of deiodination of iodine-labeled proteins (*1,2*) due to the weak bond between carbon and iodine. The chemical problems associated with iodine can be overcome by using a radioisotope of bromine as the label for proteins (*3*), since bromine forms a stronger bond with carbon than does iodine (*4*).

Bromine-77 decays >99% by electron capture and <1% by positron emission, with major gamma emissions at 239 keV (23%) and 521 keV (22%) (*5*). The 57-hr half-life of Br-77 makes it better suited to most nuclear medicine studies than the longer-lived I-125 and I-131 that are commonly used. Assuming long biological half-lives of proteins, the whole-body dose delivered by Br-77 in the form of a plasma tracer would be about one-tenth the dose delivered by either I-125 or I-131 (*3*). The use of I-123 as a protein label would lead to a reduced radiation dose (*6,7*), but its 13-hr half-life is too short for some studies, and hydrolysis still occurs with this radionuclide. Also, because iodide concentrates in the thyroid and bromide does not (*8*), the avoidance of an acute radiation dose to the thyroid by the use of Br-77 is even more significant.

Carrier-free radioiodine may be attached to proteins by several methods, all of which involve oxidation of iodide to a higher oxidation state. Bromination of proteins, on the other hand, is more difficult to accomplish because of the lower oxidation potential of bromide (*4*). Hence, it is difficult to find an oxidizing agent that will oxidize bromide without causing extensive oxidative

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damage to a protein. Tyrosyl, histidyl, and cysteinyl residues in proteins are susceptible to halogenation (*9*), the tyrosyl sites being the most stable (*10*). The effect of pH on the sites of halogenation of proteins has been investigated (*11,12*), and it has been shown that a pH of 7 is necessary for labeling to occur primarily at tyrosyl residues.

Studies have been carried out to evaluate various iodination methods with respect to the physicochemical and biological properties of the labeled proteins (*1,2,10–15*). The use of halogenating enzymes is the mildest method for effecting iodination of proteins, but lactoperoxidase, the enzyme most commonly used for catalyzing radioiodination (*16,17*), does not catalyze bromination. Chloroperoxidase has been used to attach radiobromine to a variety of substrates (*3*), but this enzyme has a pH optimum of 2.8 and does not function at all above pH 4.5. Proteins can be halogenated indirectly by attaching a radiobrominated acylating agent, SHPP [N-succinimidyl-3-(4-hydroxyphenyl)-propionate] (*18*). The SHPP can be brominated at low pH using the chloroperoxidase method, and then attached to the protein under mild conditions (*19*). This indirect method is more difficult than direct bromination, the labeling yields are rather low, and the protein may be adversely affected by the substitution of this large group.

A new brominating enzyme, bromoperoxidase (BPO), has recently been isolated from the red algae *Bonnemaisonia hamifera* (*20*) and *Penicillus capitatus* (*21*). Extracts of *B. hamifera* have been shown to contain large quantities of brominated derivatives of 2-heptanone (*22*); thus the algae apparently use the enzyme to produce brominated ketones. Bromoperoxidase appears to catalyze bromination and iodination and has a pH optimum near neutrality (*20,21*).

MATERIALS AND METHODS

Materials. Canine fibrinogen was isolated from fresh plasma either by the glycine precipitation and ethanol fractionation method of Mosesson and Sherry (*23*) and Blombäck and Blombäck (*24*) or by triple ammonium sulfate precipitation (*25*). The protein was stored below −70°C. Crude preparations of the enzyme were isolated from *B. hamifera* or *P. capitatus* (*20*). The standard assay for bromoperoxidase (BPO) activity (*26*) is based on the rate of formation of 2-bromo-2-chloro-5,5-dimethyl 1,3-cyclohexanedione (bromochlorodimedone) from 2-chloro-5,5-dimethyl-1,3-cyclohexanedione (monochlorodimedone).

Bromine-77 was produced in the Washington University 52-in. cyclotron as previously described (*3*), and was also obtained as a spallation product from Los Alamos Scientific Laboratories (*27*).

Labeling methods. Human serum albumin and canine fibrinogen were radiobrominated enzymatically at neutral pH using bromoperoxidase (*28,29*). The procedure for enzymatic bromination involves mixing the protein of interest, radiobromide, and the bromoperoxidase in 0.1 *M* phosphate buffer (pH 7.0); the reaction is initiated by the addition of a small amount of H_2O_2, and the reaction mixture is incubated at 37°C. All of the reaction parameters (including the concentration of protein, the amount of bromoperoxidase, the concentration of hydrogen peroxide, the pH, and the time of incubation) were varied to determine those conditions giving the highest labeling yields. Labeling efficiencies were determined by trichloroacetic acid (TCA) precipitation (10% TCA w/v) of the labeled protein. Since radiobrominated enzyme molecules will also precipitate with TCA, radiobromination reaction mixtures were also analyzed to check for the amount of radiobrominated enzyme present. HSA was analyzed by gel permeation chromatography on Ultrogel A-44* (1.5 × 200 cm) eluted with 0.9% NaCl, and canine fibrinogen by ammonium sulfate precipitation. In all cases, the percentage of labeled BPO was negligible, thus confirming the validity of TCA precipitation as a method for determining the yield of labeled protein. The dependence of the enzymatic halogenation reactions on the pH of the labeling solution was investigated using the following buffer systems: pH 4.5 (0.05 *M* citrate-0.05 *M* phosphate), pH 5.0–6.0 (0.1 *M* acetate), pH 6.5–7.5 (0.1 *M* phosphate), and pH 8.0–9.0 (0.1 *M* borate). Dependence on radiobromide concentrations was not studied because carrier-free radiobromine was used (1 μCi Br-77 is $\sim 10^{-10}$ moles of bromine). Iodinated fibrinogen was prepared using a modification (*1*) of the iodine monochloride method originally described by McFarlane (*25*). Halogenated proteins were purified of any remaining free radiohalide before use in any experiments. Labeled albumin was separated from free radiohalide by gel permeation chromatography using a 1 × 6 cm Sephadex G-10-120 column eluted with 0.1 *M* phosphate buffer (pH 7.0). Fibrinogen was purified by reversible precipitation with ammonium sulfate at 25% of saturation.

In vitro studies. The biologic integrity of radiohalogenated fibrinogen was measured by determining the percentage of radioactivity found in the clot formed in vitro upon addition of bovine thrombin† (*1,30*). Clottability was also monitored spectroscopically by measuring the uv absorbance of the clot and supernatant at 280 nm. The molecular-weight profile of radiolabeled fibrinogen was determined by gel permeation chromatography. A Sepharose-4B column (0.9 × 50 cm) was eluted with saline-citrate buffer (0.15 *M* NaCl, 0.05 *M* sodium citrate, 0.05 *M* ε-amino caproic acid, pH 7.0).

The stability of radiohalogen labels on proteins was studied by measuring the protein-bound radioactivity over a period of time. The in vitro hydrolysis experiments were performed by placing the labeled protein in either

0.1 *M* phosphate buffer (pH 7.0) or human serum, and storing at 37°C for 7–10 days. Aliquots were withdrawn daily and assayed for bound radioactivity by TCA precipitation.

In vivo studies. In vivo clearance studies of labeled fibrinogen were carried out in normal dogs. Before injection, the radiobrominated fibrinogen was purified by gel permeation chromatography on Sepharose-4B, and screened for in vitro clottability >80%. The labeled fibrinogen (25–100 μCi) was administered intravenously; I-125 fibrinogen prepared by the iodine monochloride method was injected into each dog as a standard of comparison for the Br-77 fibrinogen prepared enzymatically using BPO. Blood samples were withdrawn from the dogs several times on the day of injection, and at least once a day for the following 4–7 days, and tested for clottability. The results were expressed as counts per gram of clottable radioactivity.

Thrombus uptake of labeled fibrinogen was also studied in dogs. A thrombus was induced in the femoral vein with an electrical current, described by Coleman and coworkers (*15*). Labeled fibrinogen (1–2 mCi) was injected 3 hr after the thrombus was induced. Fibrinogen labeled with I-125 by the ICl method was used for comparison with Br-77 fibrinogen prepared using BPO. Scintillation images were obtained 1 and 2 hr after injection of the labeled fibrinogen using a medium-energy, parallel-hole collimator. Also, the thrombus and a blood sample were removed and counted at 24 hr after injection.

RESULTS

Reaction parameter variation studies. Each of the essential reaction parameters was varied, keeping all other parameters constant. The reaction yields are expressed as the percent labeling, i.e., the percentage of radioactivity that is protein-bound. Parameter variation experiments were carried out with various combinations of reactant concentrations to investigate the possibility of alterations in the optimum concentration of one reactant resulting from changes in the concentrations of the others. Such co-variance is not observed for the radiobromination reactions catalyzed by BPO_p (*P. capitatus*); reaction parameter dependence curves are plotted for several combinations of reactant concentrations to illustrate this absence of co-variance. In the case of the radiobromination reaction catalyzed by BPO_b (*B. hamifera*), co-variance is observed between the hydrogen peroxide and bromoperoxidase concentrations.

Bromoperoxidase isolated from B. hamifera. Figure 1 shows the effect of hydrogen peroxide concentration on the bromination of albumin catalyzed by BPO_b (*B. hamifera*). The radiobromination reactions were incubated at 37°C in pH 7 phosphate buffer for 30 min; the reaction mixtures contained 0.1 mg/ml human serum albumin and 0.1, 0.2, or 0.4 units BPO_b activity. The

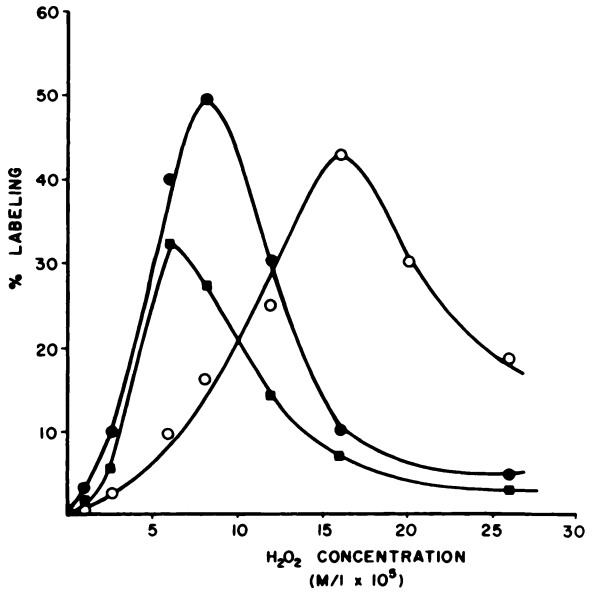

FIG. 1. Effect of hydrogen peroxide concentration on BPO_b catalyzed radiobromination of human serum albumin at pH 7. Reaction mixtures contained ■ 0.1 units BPO, ● 0.2 units BPO, O 0.4 units BPO.

hydrogen peroxide concentration has a pronounced effect on the radiobromination labeling efficiency, with the optimum H_2O_2 concentration being 80 μM, when 0.2 units BPO_b are present in the reaction mixture.

The effect of protein concentration (whether human serum albumin or canine fibrinogen) on the labeling yield is similar to that shown for the reaction catalyzed by BPO_p (*vide infra*). Maximum labeling efficiencies are obtained when the protein concentration is 0.1 mg/ml.

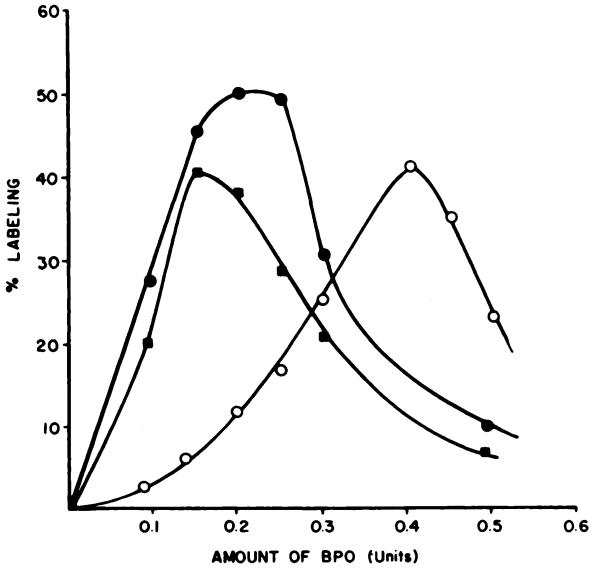

FIG. 2. Effect of amount of bromoperoxidase on BPO_b catalyzed bromination of albumin at pH 7. Reaction mixtures contained ■ 60 μM H_2O_2, ● 80 μM H_2O_2, O 160 μM H_2O_2.

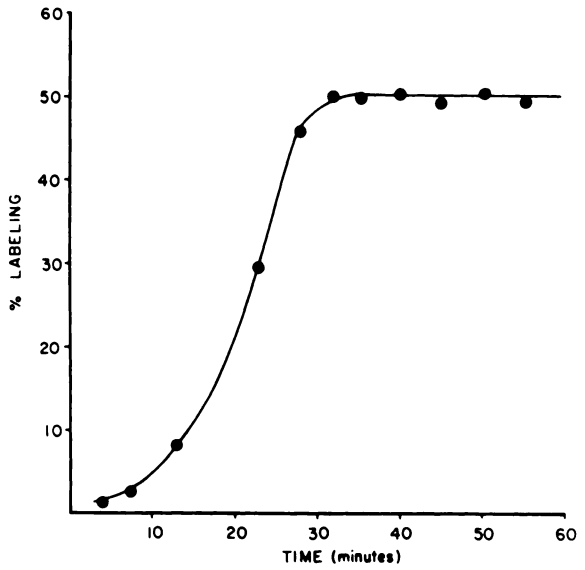

FIG 3. Time dependence of BPO_b catalyzed radiobromination of albumin at pH 7.

The amount of bromoperoxidase used to catalyze radiobromination of proteins also affects the labeling yield, as shown in Fig. 2. The radiobromination reactions were incubated at 37°C for 30 min in 0.1 *M* phosphate buffer containing 0.1 mg/ml HSA and 60, 80, or 160 μM H_2O_2. Optimum labeling efficiencies occur when 0.2–0.25 units of BPO activity are used in the presence of 80 μM H_2O_2.

The pH-dependence curve for the BPO_b-catalyzed radiobromination of proteins is bell-shaped, with an optimum near 6.5 and no labeling below pH 4 or above pH 8. The labeling yields at pH 7 are still relatively high, and proteins labeled at this pH are thought to be the most stable toward loss of the radiolabel by hydrolysis. Accordingly, 0.1 *M* phosphate buffer (pH 7) was used for all of the labeling reactions.

Thus the optimum conditions for radiobromination of proteins that the BPO_b catalyzed at neutral pH require a reaction mixture that contains the following: 0.1 mg/ml protein, 0.2 units BPO_b 80 μM H_2O_2, and carrier-free $^{77}Br^-$ in 0.1 *M* phosphate buffer (pH 7.0). The labeling mixture, with a final volume of 0.25 ml, is incubated at 37°C for 30–35 min, at which time the reaction is complete (Fig. 3).

Bromoperoxidase isolated from P. capitatus. Variation of reaction parameters was also carried out for this bromoperoxidase preparation. The effect of hydrogen peroxide concentration on the labeling yield is shown in Fig. 4. The reaction mixtures were incubated at 37°C for 30 min in 0.1 *M* phosphate buffer (pH 7.0) containing 0.05, 0.15, or 0.25 mg/ml HSA and 0.075, 0.2, or 0.3 units BPO_p activity. The labeling yields reach a maximum and level off when the H_2O_2 concentration reaches 80 μM in the presence of 0.2 units BPO_p and 0.15 mg/ml HSA.

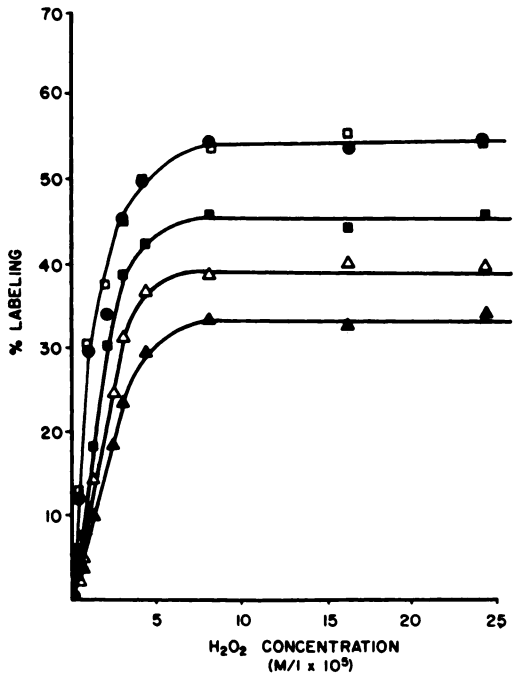

FIG. 4. Effect of hydrogen peroxide concentration on BPO_p catalyzed radiobromination of albumin at pH 7. Reaction mixtures contained △ 0.05 mg/ml HSA and 0.2 units BPO, ■ 0.15 mg/ml HSA and 0.075 units BPO, ● 0.15 mg/ml HSA and 0.2 units BPO, □ 0.15 mg/ml HSA and 0.3 units BPO, and ▲ 0.25 mg/ml HSA and 0.2 units BPO.

The effect of protein concentration on BPO_p bromination at pH 7 is plotted in Fig. 5. The reaction mixtures contained 30, 80, or 160 μM H_2O_2 and 0.025, 0.2, or 0.25 units BPO_p in 0.1 *M* phosphate buffer (pH 7.0). They were incubated at 37°C for 30 min. The highest

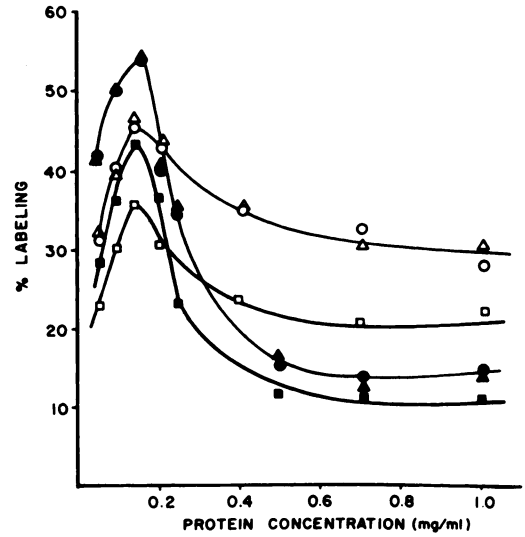

FIG. 5. Effect of protein concentration on BPO_p catalyzed radiobromination at pH 7. Reaction mixtures contained human serum albumin: ■ 0.2 units BPO_p and 30 μM H_2O_2, ● 0.2 units BPO_p and 80 μM H_2O_2, ▲ 0.25 units BPO_p and 80 μM H_2O_2; and canine fibrinogen: □ 0.025 units BPO_p and 80 μM H_2O_2, ○ 0.2 units BPO_p and 80 μM H_2O_2, △ 0.2 units BPO_p and 160 μM H_2O_2.

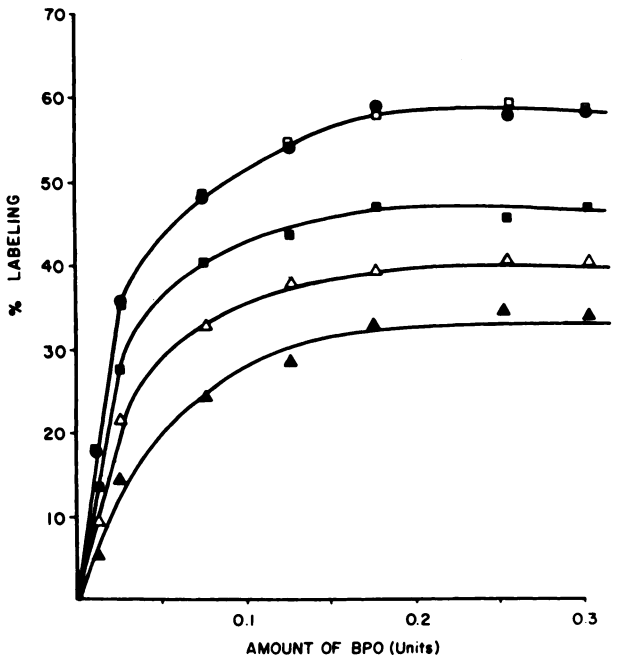

FIG. 6. Effect of amount of bromoperoxidase on BPO_p catalyzed radiobromination of albumin at pH 7. Reaction mixtures contained △ 0.05 mg/ml HSA and 80 μM H_2O_2, ■ 0.15 mg/ml HSA and 30 μM H_2O_2, ● 0.15 mg/ml HSA and 80 μM H_2O_2, □ 0.15 mg/ml HSA and 160 μM H_2O_2, ▲ 0.25 mg/ml HSA and 80 μM H_2O_2.

labeling efficiencies are observed at protein concentrations of 0.15 mg/ml in the presence of 0.2 units BPO_p and 80 μM H_2O_2.

Altering the amount of BPO_p used to catalyze the radiobromination of albumin at pH 7 also affects the labeling yields, as shown in Fig. 6. The reaction mixtures, containing 0.05, 0.15, or 0.25 mg/ml HSA and 30, 80, or 160 μM H_2O_2 in 0.1 M phosphate buffer (pH 7.0), were incubated at 37°C for 30 min. The labeling yields reach a plateau at 0.15–0.2 units of BPO_p activity in the presence of 0.15 mg/ml HSA and 80 μM H_2O_2.

The pH-dependence of radiobromination catalyzed by BPO_p is very similar to that observed with BPO_b, except that it exhibits a broader maximum with the optimum pH at 6.0 and it gives negligible yields below pH 3 and above pH 9. Again, the labeling yields at pH 7 are quite high.

To summarize, the optimum conditions for bromination of proteins at pH 7 using BPO_p require reaction mixtures containing 0.15 mg/ml protein, 0.2 units BPO_p 80 μM H_2O_2, and carrier-free radiobromine in 0.1 M phosphate buffer (pH 7.0). The reaction is essentially complete after incubation at 37°C for 15 min. The time dependence curve is similar to that shown in Fig. 3 for BPO_b except there is no initial delay in the rise of the labeling yields.

The behavior of BPO_b toward reaction-parameter dependence studies was different from that of BPO_p, and from other peroxidases that have been studied (*3,10,31*). The only apparent difference between the BPO isolated from *B. hamifera* and that found in *P. capitatus* was the purity of the enzyme extract. BPO_b was a relatively crude extract that was found to contain significant quantities of carrier bromine. Quantitative analysis of the two enzyme extracts showed that BPO_b contained 0.04 moles/l bromide; this is at least fifteen times that found to be present in BPO_p.

Addition of carrier bromine to the radiobromination reaction mixture catalyzed by the BPO_p was carried out in an attempt to simulate the conditions present in the BPO_b radiobromination reaction. The amount of carrier bromine added was proportional to the amount of BPO_p used to catalyze the reaction; the relative concentrations of enzyme and bromide were equivalent to the ratio in the BPO_b enzyme extract. Figure 7 shows the dependence of the radiobromination reaction yield on enzyme concentration with carrier bromine added. This behavior is analogous to that observed for the enzyme concentration dependence in the case of the radiobromination reaction catalyzed by BPO_b (Fig. 2).

In vitro studies. In vitro hydrolysis experiments were carried out to determine the percentage of the radiolabel that remains bound to protein over a period of time. Samples of labeled protein were kept in human serum at 37°C to simulate in vivo conditions, or in 0.1 M phosphate buffer (pH 7). These two hydrolysis media yielded identical results. Table 1 is a compilation of the data obtained for in vitro hydrolysis of human serum albumin radiobrominated using the bromoperoxidase methods, and results for in vitro hydrolysis of I-131 HSA prepared using lactoperoxidase (*19*).

Table 2 summarizes the in vitro clottabilities of canine and human fibrinogen radiobrominated using the BPO

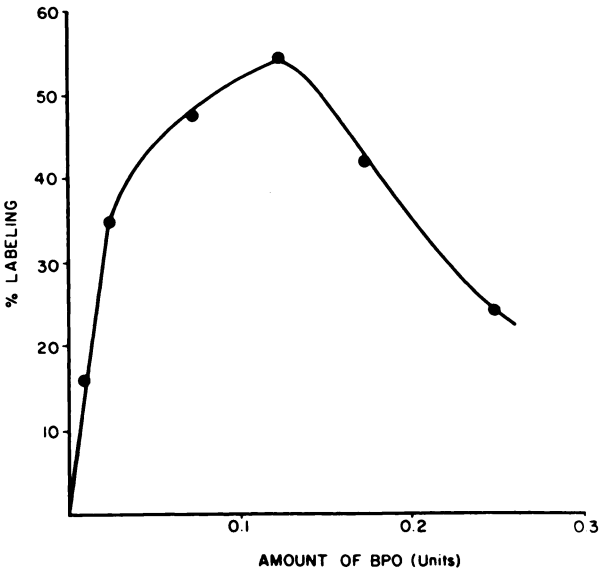

FIG. 7. Effect of carrier bromine on the BPO_p catalyzed radiobromination of HSA at pH 7.

TABLE 1. IN VITRO HYDROLYSIS OF RADIOHALOGENATED ALBUMIN

Label	Br-77		I-131
Method used	BPO	BPO	
Time elapsed (days)	*B. hamifera* % bound	*P. capitatus* % bound	LP (*19*) % bound
0	100	100	100
1	100	100	94
2	100	100	91
3	100	100	89
4	100	100	87
7	100	100	83
10	100	100	—
Average % hydrolyzed per day	0.0 ± 0.0	0.0 ± 0.0	3.4 ± 1.3

method; all of the values reported are averages of three determinations.

In vivo studies. The integrity of radiobrominated canine fibrinogen prepared using BPO_p was tested by in vivo clearance experiments in dogs. The labeled fibrinogen samples were first analyzed by gel chromatography on Sepharose-4B to detect molecular weight variations in the protein. Only samples of unaltered molecular weight were tested in vivo. A typical in vivo clearance curve for Br-77 fibrinogen prepared using BPO_p is shown in Fig. 8.

Thrombus uptake studies were carried out on Br-77 canine fibrinogen labeled by the BPO_p method. The thrombus-to-blood ratios (defined as cpm/g of thrombus, divided by cpm/g of blood) are compared with thrombus-to-blood ratios obtained for I-125 fibrinogen in the same animals. Thrombus-to-blood ratios ranged from 15 to 20 for Br-77 canine fibrinogen labeled using BPO_p. The mean ratio of thrombus-to-blood ratio for Br-77 fibrinogen prepared with the BPO_p technique, compared with fibrinogen iodinated conventionally, is 1.04 ± 0.02. Figure 9 shows scintigrams of the site of thrombosis in the hind limb of a dog. The images were obtained 1 and 2 hr after injection of Br-77 canine fibrinogen labeled using BPO_p.

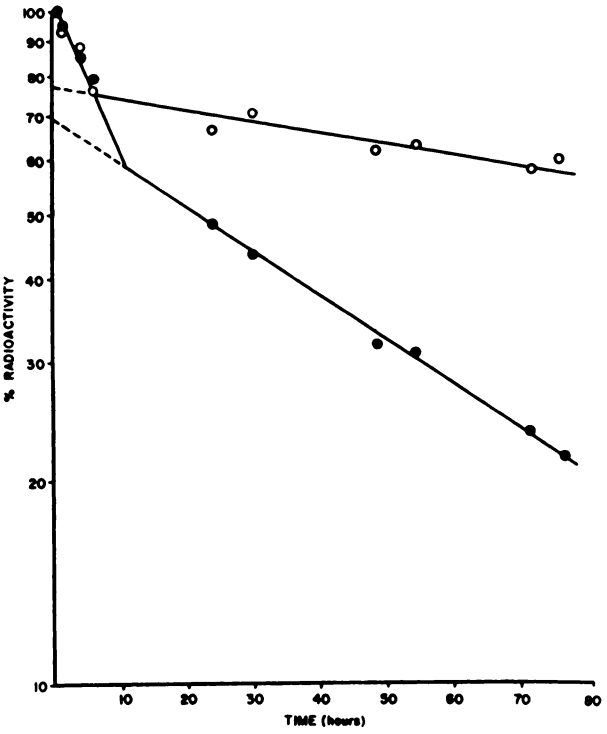

FIG. 8. In vivo clearance of Br-77 canine fibrinogen BPO_p (O) compared with I-125 fibrinogen (ICI) (●). Percent of initial clottable radioactivity present in plasma (log scale) against time after injection of labeled fibrinogen.

DISCUSSION

The reaction parameters for these enzymatic radiohalogenations were varied in order to develop a practical procedure for the radiobromination of protein molecules. The behavior exhibited by BPO_b with respect to reaction parameter dependence is unlike that observed for all other peroxidase-catalyzed radiohalogenations that have been studied (*3,10,31*). The anomalous behavior of BPO_b appears to be the result of the presence of carrier bromine in the BPO_b extract. As shown in Fig. 7, the presence of carrier bromine in the BPO_p-catalyzed bromination reaction decreases the yield of labeled protein; thus it seems likely that the decrease in yields of radiobrominated protein at high BPO_b concentrations (Fig. 2) is the result of carrier bromine that is present in

TABLE 2. IN VITRO CLOTTABILITY OF Br-77 FIBRINOGEN

Labeling method	Canine Fibrinogen*		Human Fibrinogen†	
	Isotopic clottability	Spectroscopic clottability	Isotopic clottability	Spectroscopic clottability
BPO (*B. hamifera*)	85 ± 3	94 ± 2	95 ± 2	96 ± 1
BPO (*P. capitatus*)	85 ± 2	95 ± 1	94 ± 2	95 ± 1

* Mean ± s.d. for nine samples from five animals.

† Mean ± s.d. for six samples from three humans.

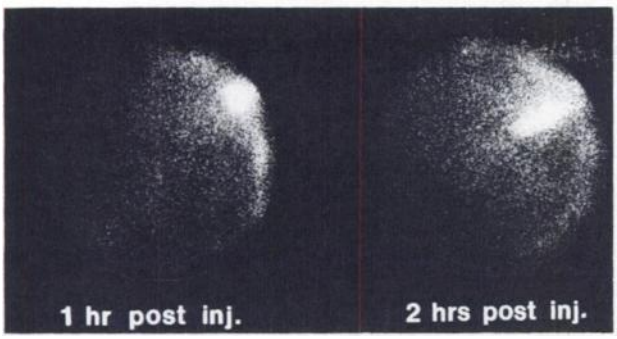

FIG. 9. Scintigrams of site of thrombosis in hind limb of dog. Images were taken at 1 and 2 hr after injection of 2.0 mCi of Br-77 canine fibrinogen prepared using BPO_p.

the enzyme extract. The sensitivity of BPO_b-catalyzed halogenation reactions to hydrogen peroxide at high H_2O_2-to-enzyme ratios (Fig. 1) may result from oxidative damage to the enzyme and/or inactivation of the BPO_b molecule by extensive self-bromination. The extent of such a self-bromination "suicide" reaction is likely to be significant only for BPO_b, since there is a high concentration of carrier bromine in the reaction mixture (~4 m*M*) as compared with halogen acceptor (~2 μM tyrosine). When only carrier-free radiohalide is present (i.e., in the reaction catalyzed by BPO_p), inactivation of the peroxidase through such a "suicide" reaction is not as likely to occur.

The integrity of proteins labeled at neutral pH with bromine-77 using bromoperoxidases isolated from *B. hamifera* and *P. capitatus* was studied by a variety of methods, including in vitro hydrolysis, in vitro clottability, in vivo clearance, and in vivo thrombus uptake. The results of in vitro hydrolysis studies on HSA, radiobrominated using these halogenating enzymes (Table 1) showed the labeled HSA to be completely stable toward in vitro hydrolysis of the radiolabel. Thus, the proteins radiobrominated by these two enzymatically catalyzed halogenation reactions at neutral pH are the most stable halogenated proteins that have been prepared.

In vitro clottability measurements carried out on both human and canine fibrinogen labeled by either BPO method (Table 2) gave results consistent with those reported by other workers for radioiodinated fibrinogen (*1,10*). The difference in clottabilities of canine fibrinogen determined isotopically and spectroscopically—which is most likely a result of the cleavage of the tyrosyl-containing fibrinopeptide B during the fibrinogen/fibrin conversion (*32*)—has also been observed for I-125-labeled canine fibrinogen iodinated under mild conditions (*1,10*). The isotopically and spectroscopically determined clottabilities for human fibrinogen radiobrominated with these two enzymes are identical. Human fibrinopeptides A and B contain no tyrosyl residues, and thus no radiobromine is lost as a result of the cleavage of these fragments during the clotting process.

The in vivo clearance curve for Br-77-labeled canine fibrinogen prepared using BPO_p (Fig. 8) shows similar *y* intercepts for the Br-77 fibrinogen and the I-125 fibrinogen standard. Thus the amount of Br-77-labeled fibrinogen that is rapidly cleared—due to slight changes in the protein molecule and equilibration of intra- and extravascular protein—is similar to that observed for I-125 fibrinogen prepared by the conventional iodine monochloride method. The second components of the clearance curves, however, show half-times quite different for the Br-77- and I-125-labeled fibrinogen molecules. The Br-77 fibrinogen clears at a much slower rate that the standard, with a half-time ~3.5 times that of the standard I-125 fibrinogen. This disappearance rate is similar to that of canine fibrinogen containing Se-75 methionine incorporated in vivo during protein biosynthesis, the half-time for Se-75 canine fibrinogen clearance being ~3.1 times that of I-125 fibrinogen (ICl) in the same dog (*33*). Thus Br-77-labeled canine fibrinogen prepared by the BPO_p method exhibits in vivo clearance behavior that appears to be superior to that of standard I-125 fibrinogen.

The results of thrombus uptake studies carried out on Br-77-labeled canine fibrinogen prepared with BPO_p indicate that the radiobrominated proteins prepared by this enzymatic technique behave as well as or better than the standard I-125 fibrinogen that is routinely used. In other words, the biologic function of the labeled fibrinogen molecules is retained.

In summary, both in vivo and in vitro studies carried out to test the integrity of canine fibrinogen, when radiobrominated using the halogenating enzymes discussed here, indicate that the labeled proteins (a) are stable toward loss of the radiolabel by hydrolysis; (b) retain their biologic function in the clotting process as determined by in vitro and in vivo methods; and (c) are not denatured to an appreciable extent during the labeling process, as evidenced by their in vivo clearance rates. Due to the absence of carrier bromine and a more abundant supply of the enzyme (Lowell P. Hager, personal communication), the BPO isolated from *P. capitatus* is superior to that from *B. hamifera* for preparing radiobrominated proteins. The extension of the enzymatic radiobromination method described here to attach Br-77 to other proteins to measure turnover rates, and to other types of compounds to produce clinically useful radiopharmaceuticals, is promising. Also, short-lived isotopes of bromine (such as Br-75 and Br-76), which decay by positron emission, can be attached to the same types of compounds and used in conjunction with computer-assisted positron tomography.

FOOTNOTES

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