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ISOLATION OF THE THYROID PEROXIDASE COMPLEX

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SUMMARY

A procedure for the isolation and purification of a thyroid peroxidase complex is presented. The preparation can readily be completed in 1–2 days and gives yields of 30–50% of the peroxidase present in the thyroid. This preparation has a high molecular weight of approx. 200 000 as estimated by gel filtration. Disc gel electrophoresis in sodium dodecyl sulfate, a procedure which gives monomeric subunit size shows that the monomer is approx. 70 000. The enzyme preparation is free of contaminating hemoproteins and appears to have a protoheme prosthetic group. The preparation has a turnover number for guaiacol oxidation of $8 \cdot 10^3$ moles/min per heme prosthetic group, and for the iodination of tyrosine of 200 moles/min per heme. When the preparation is treated with trypsin there is a marked loss in the ability of the enzyme to catalyze iodination while the ability of the enzyme to catalyze guaiacol oxidation is unaffected.

INTRODUCTION

Recent work has directly implicated thyroid peroxidase in thyroxine biosynthesis. This enzyme catalyzes the iodination of tyrosine residues in thyroglobulin[1–4,10] and may be involved in a second catalytic function, the oxidative coupling of these iodinated tyrosines to produce thyronine residues[10].

Since the enzyme activity was found in the microsomal fraction of the cell[9], a number of isolation procedures have employed thyroid microsomes as starting material[3,4]. Alternatively extracts of whole thyroid tissue homogenates have been used by others as the starting material[1,4–8]. A major short coming of these earlier attempts at isolation was low yields of the final enzyme preparation. This paper describes a rapid isolation procedure for pig thyroid peroxidase which provides a high percent yield of active enzyme. Some properties of the preparation are discussed.

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MATERIALS AND METHODS

All chemicals were of reagent grade and solutions were made using triple distilled water. Enzyme activity was assessed either as ability to oxidize guaiacol or iodinate amino acids, peptides and proteins. Guaiacol activity was followed spectrophotometrically at 470 nm as previously described[3]. Iodination was assayed potentiometrically using an iodide specific electrode[10–12].

Absorption spectra were recorded with a Cary 14 spectrophotometer. Heme concentration was determined by the pyridine hemochromogen method[13] using a millimolar extinction of 34.4 at 557 nm for the reduced chromophore. Non-heme iron was estimated by the method of KURUP AND BRODIE[14]. Flavin content was determined by the method of RAO *et al.*[15] and acid labile sulfide by the method of KING AND MORRIS[16]. The copper content was estimated by the method of BRUMBY AND MASSEY[17].

Rabbit antisera was produced by injection of purified peroxidase in Freund's adjuvant according to the method described by ALLEN AND MORRISON[18,19]. Protein concentration was estimated by the biuret method[20].

Enzyme isolation

Pig thyroids were obtained fresh from the slaughter house and trimmed of extraneous fat and connective tissue. This material could then be stored at −20° up to 1 month without loss of peroxidase activity. All steps in the isolation procedure were performed at 4°. One kg of the trimmed tissue was washed several times with 0.9% NaCl solution and then suspended in 250 ml of 1.0 M Tris–HCl (pH 7.4) and homogenized in a blender at 20 000 rev./min for 30 sec. The homogenate was filtered through a double layer of No. 60 cheesecloth to remove large particles. The red filtrate was centrifuged at 30 000 × *g* for 70 min which gave a brown pellet. This pellet was suspended in 130 ml, 0.1 M phosphate buffer (pH 7.4) and made 1% with respect to cholate. After 2 h of stirring the suspension was centrifuged at 78 000 × *g* for 45 min. The turbid supernatant was subjected to trypsin digestion (0.13 mg/ml) for 45 min in an ice bath. At the end of this time 0.26 mg/ml soybean trypsin inhibitor was added and extraneous protein removed by centrifugation at 140 000 × *g* for 45 min. $ZnCl_2$ was dissolved in 0.1 M Tris–HCl (pH 7.4) to give a 0.1 M solution. Enough $ZnCl_2$ solution was added to the red filtrate to produce a 0.017 M solution with respect to the zinc. After stirring for 15 min the resultant precipitate was removed by centrifugation at 78 000 × *g* for 30 min. To the supernatant the same volume of $ZnCl_2$ as above was added. The stirring and centrifugation were repeated. The resulting golden second supernatant was passed through a DE-52 column (5 cm × 4 cm) equilibrated with 0.1 M phosphate (pH 7.4). The active fractions were collected and lyophilized. The anhydrous powder was dissolved in 5 ml cold distilled water to give a turbid solution. This solution was dialyzed *versus* distilled water for 2 h at 4° and then centrifuged at 140 000 × *g* for 30 min to yield a clear golden solution of active peroxidase. This represented a 30% recovery of activity originally present in the homogenate. The purified preparation was stored at −20°.

Heme extraction

The purified enzyme was treated with 5 times its volume of acidified acetone

(2 vol. of 12 M HCl, 98 vol. of acetone) and washed with an equal volume of ether. This treatment releases the heme into the ether phase. After evaporating the ether extract to dryness, the pyridine hemochromogen was formed by addition of 1 vol. of pyridine and 2 vol. of 0.05 M NaOH.

Molecular size

The molecular size was estimated by gel filtration on a Sepharose 6B column (2.5 cm × 120 cm) equilibrated with 0.1 M phosphate buffer (pH 7.4). Samples of known molecular weight were applied to the column separately in a mixture and the elution pattern plotted *versus* the log of the molecular weight to obtain the standard curve. Molecular weights of thyroid peroxidase samples were determined from the standard curve when the peroxidase was run with known standard.

Molecular weights by disc gel electrophoresis were done according to the method of WEBER AND OSBORN[21].

RESULTS

The preparation of soluble thyroid peroxidase is outlined in Table I. It was necessary to combine in sequence the cholate treatment and a short trypsin digestion in order to more efficiently solubilize the enzyme. $ZnCl_2$ step was used to selectively precipitate the major amount of contaminating hemoglobin. This step was very effective in removing contaminating pseudoperoxidase. Passage through the DE-52

TABLE I

ISOLATION OF THYROID PEROXIDASE

State of preparation	*Total protein (g)*	*Total guaiacol activity (mmoles oxidized/sec)*	*% Activity recovered*
Homogenate	60.2	21.9	100
Filtrate	34.6	23.3	107
30 000 × *g* precipitate	5.4	14.4	65
Cholate extract	4.9	16.1	73
Trypsin digest	4.4	17.9	82
1st $ZnCl_2$ precipitation	3.6	14.7	67
2nd $ZnCl_2$ precipitation	3.1	11.1	50
Final product	0.99	5.8	26

column removed a brown material which was enzymatically inactive. This brown material was not easily removed from the ion exchanger and was not studied further. The final soluble enzyme was found to be stable when stored at −20° for at least 12 months.

Two distinct advantages are associated with this preparation; first, the relatively short time of preparation when compared with previously reported isolation methods[1,4–8] and second the consistant high yields which represent a significant increase in total enzyme recovered.

Analysis of various tissues by Ouchterlony double diffusion methods clearly

TABLE II

IMMUNODIFFUSION ANALYSIS OF PORCINE TISSUE

Pig tissue extract	*Protein (mg/ml)*	*Thyriod peroxidase*
Thyroid	0.5	+
Thymus	24.5	—
Liver	61.5	—
Lung	41.5	—
Esophagus	18.5	—
Pancreas	78.8	—
Urinary bladder	46.0	—
Lacrimal	37.5	—
Salivary	28.0	—

indicated that this protein was present only in the thyroid gland. Table II shows the results of these studies. Tissue protein concentrations were high in order to detect small quantities of the enzyme, although different concentrations have also been tried in order to eliminate inhibitory reactions which sometimes occur in studies of this type. In no case was the enzyme protein detectable in tissue other than thyroid. However, rat thyroid tissue did cross react with the antisera to pig thyroid peroxidase.

In order to more fully characterize the hemoprotein nature of the peroxidase a study of the spectral properties was undertaken. As summarized in Table III, this preparation had similar spectral properties to those previously described[1,3,4,6].

TABLE III

SORET MAXIMA OF THYROID PEROXIDASE

Derivative	*Thyroid peroxidase (nm)*
As prepared	410
As prepared + CN^-	416
CN^- difference spectra	
peak	428
trough	403
Reduced	425
Reduced + CO	420

Azide, fluoride, and iodide did not form spectrophotometrically detectable ligands when used at concentrations as high as 0.1 M, in the pH range from 5 to 8.

The reduced pyridine hemochromogen of thyroid peroxidase had absorption maxima at 557, 524, and 418 nm. The maxima are typical for photoheme IX. The prosthetic group could be extracted by the acid–acetone method[13]. When the pyridine hemochromogen of the extracted heme was reduced, absorption maxima identical with those for the holoenzyme were observed.

Examination of the enzyme for its non-heme iron, flavin, acid-labile sulfide and copper content revealed that in the final preparation none of these were present.

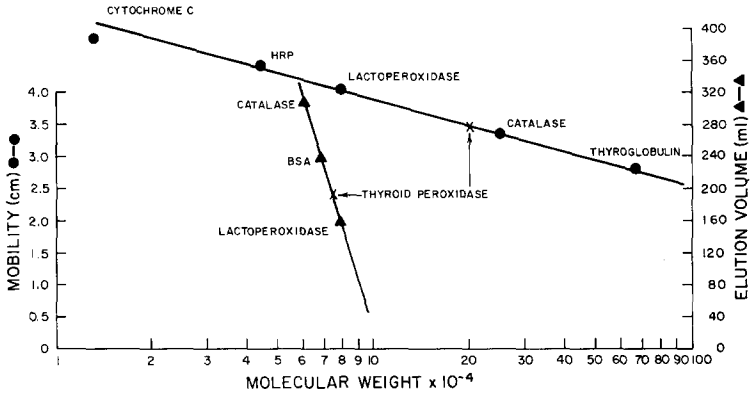
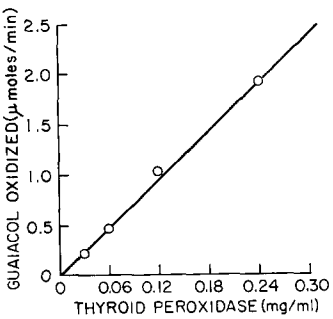


Fig. 1. Determination of size of the thyroid peroxidase. The size was estimated on a standardized column of Sepharose 6B, and by disc gel electrophoresis in sodium dodecyl sulfate as outlined under MATERIALS AND METHODS. HRP, horse radish peroxidase; BSA, bovine serum albumin.

It was found that in less pure preparations and during the initial steps in the procedure, some non-heme iron and flavin as FAD could be detected.

Molecular weight determinations on this preparation were performed by two methods. Column chromatography, designed to provide data on the molecular weight of the active component and disc gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol for elucidation of subunit size. Fig. 1 illustrates the results of these two determinations. The data from chromatography at pH 7.4 on Sepharose 6B revealed an active component with a molecular weight around 200 000, while gel electrophoresis showed a subunit of 70 000 mol. wt.

Enzymatically thyroid peroxidase will catalyze the oxidation of phenolic compounds such as guaiacol. This has been used as a means for monitoring the purification during isolation. A second catalytic function is the iodination of tyrosine



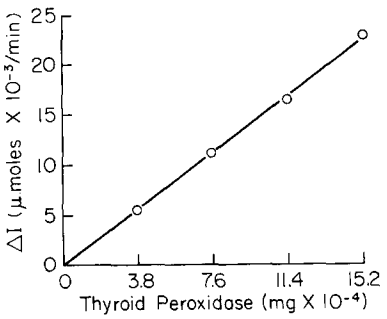


Fig. 2. The rate of catalysis of guaiacol oxidation *versus* concentration of thyroid peroxidase. The rate of oxidation was monitored at 470 nm. The assay was carried out in a cuvette with 10-mm light paths containing 33 mM guaiacol, 33 mM phosphate buffer (pH 7.4), 0.4 mM H_2O_2 and varying amounts of the enzyme in 3 ml.

Fig. 3. The rate of catalysis of iodination of tyrosine *versus* enzyme concentration. The assay was monitored using iodide specific electrode as described in the text. The reaction mixture contained 0.05 M phosphate buffer (pH 8.0); $1 \cdot 10^{-4}$ M, KI, $8 \cdot 10^{-4}$ M L-tyrosine, $3 \cdot 10^{-4}$ M H_2O_2 and varying concentrations of enzyme in a final volume of 5 ml.

residues. Since most proteins are readily iodinated, but at different rates, it is difficult to accurately assess iodination activity during the early steps of isolation when large amounts of extraneous protein are present. In the impure preparations, iodide uptake can be obtained even in the absence of added substrate which can be iodinated. In the final preparation no iodination takes place without the addition of a substrate which can be iodinated.

The optimum iodide concentration observed with tyrosine as substrate is $5 \cdot 10^{-4}$ M similar to the optimum reported by TAUROG *et al.*[6]. The pH optimum around 8.5 is somewhat higher than previously reported[1,4] although the iodide and peroxide optimum agrees well with published values[1,4].

As seen in Figs. 2 and 3 both enzyme activities are pseudo first order with respect to enzyme concentration. When iodination, with tyrosine as substrate, is compared to guaiacol activity a constant ratio between the two activities is observed. The two activities, however, have different pH optimum (Fig. 4) and a difference of one order of magnitude for the optimum peroxide concentration.

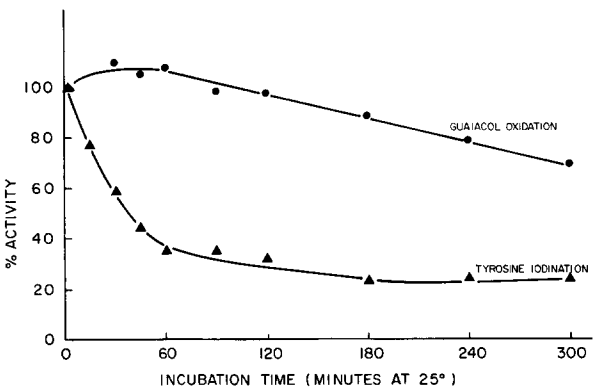


Fig. 4. Effect of incubation with trypsin on peroxidase activity. The thyroid peroxidase (100 mg) was incubated with 8.3 mg trypsin at 25°. At varying time intervals soybean trypsin inhibition was added to stop the digestion. Assays were performed as described in MATERIALS AND METHODS. The results are presented as percentage of activity at zero time. ●—●, catalysis of guaiacol oxidation; ▲—▲, catalysis of tyrosine iodination.

The turnover numbers for these activities based on the heme content are 200 moles/min per mole heme for iodination and $8 \cdot 10^3$ moles/min per mole heme for guaiacol.

In order to establish whether the two activities attributable to this enzyme have identical active sites, the enzyme was treated with trypsin. Fig. 4 shows the effect of the exposure of the preparation to trypsin for various times at room temperature. The preparation loses its ability to catalyze the iodinate of tyrosine within 60 min while the ability to catalyze guaiacol oxidation remains relatively unchanged over this same time period.

DISCUSSION

Although several reports on the isolation of thyroid peroxidase are present in

the literature[1-8] they all suffer from the fact that they are long procedures. The preparation described here can be completed easily in a relatively short time. Perhaps more important is the recovery of activity in the present preparation. Routinely 30% of that activity found in the original homogenate is recovered and yields as high as 50% have been obtained. This represents better than a 10-fold increase over yields reported by the more recent workers[1,3,4,6]. These higher yields help to ensure the fact that the major peroxidase of the thyroid is being isolated and not a contaminating peroxidase such as might be present in the white cell.

Several factors in this preparation appear to influence the yield of active enzyme. Storage at any of the early stages of the preparation was found to lead to inactivation of the enzyme. After the trypsin digestion step, however, activity is maintained when the preparation is frozen. $(NH_4)_2SO_4$ was likewise found to cause appreciable inactivation of the peroxidase along with chromatography on molecular sieves. To remove the largest contaminant, hemoglobin, $ZnCl_2$ was used to selectively precipitate this pseudoperoxidase. The passage through DE-52 at pH 7.4 increased the iodination activity but had little effect on guaiacol activity. A dark band was bound to the resin which could represent a naturally occurring inhibitor present in thyroid tissue. As yet the bound material has not been characterized but preliminary data indicates that when added back to purified thyroid peroxidase inhibition of iodination results which suggests that this fraction may be involved *in vivo* in the control of iodination.

Since guaiacol oxidation leads to the coupling of this phenolic compound the guaiacol assay may measure the ability of the peroxidase to oxidize phenols and hence the ability to catalyze thyronine formation. This activity shows an optimum at pH 8.0 similar to previously reported values[4,22] and also has the same peroxide optimum[22] previously reported.

The two catalytic functions attributed to the peroxidase may involve two different active centers. This is evidenced by the fact that iodination can be selectively destroyed without apparent alteration in guaiacol activity (see Fig. 4). Both activities do, however, appear to involve the heme prosthetic group. Removal of this group causes inactivation of both functions. The differences probably, therefore, involve the binding sites of the enzyme. The data indicate that the iodination binding site is not identical to the phenol oxidation site, and that the catalysis of phenol oxidation is less specific than the iodination. This is consistant with the findings with lactoperoxidase[10-12] which showed that in the catalysis of iodination the phenolic compound also formed an enzyme substrate complex. In the case of thyroid peroxidase the binding site for the iodide may be destroyed by the trypsin treatment. Phenol oxidation (*i.e.*, guaiacol oxidation) however may not involve the same binding site or the same kind of orientation as iodination hence guaiacol activity is uneffected.

Although the present preparation is not purer on a protein basis than the other preparations reported in the literature the high turnover number on the basis of the heme contant indicate that it is not grossly contaminated with other heme proteins. The results with the pyridine hemochromogen of the prosthetic group of the present preparation are different than the observations of TAUROG *et al.*[6]. The heme in their preparation has a pyridine hemochromogen at 562 mm. The differences in these observations are not readily explained. It may be that there are two peroxidases in the thyroid. The peroxidase isolated in the present study is certainly the predominant

one in the thyroid, since we have obtained yields approaching 50% of the activity present in the thyroid while other preparations have one tenth that yield of the total activity.

The difference in the molecular weight reported by the various investigators[1,3,6] may be explained on the basis of two peroxidases or dissociation of a high molecular weight enzyme into its subunits[23]. Thus, the 200 000 molecular weight found in the present study does dissociate into 70 000 subunits in sodium dodecyl sulfate. The high molecular weight complex may have important properties involved in regulation of enzyme activity and its specificity.

The biosynthesis of thyroxine is clearly regulated through the hypothalamus and pituitary while other hormones such as growth hormone also appear to play a role. Nervous stimuli and blood flow are also involved in the complex control of biosynthesis. The thyroid itself appears to have autonomous regulation of biosynthesis. Thus it would appear likely that multiple sites of control for hormone biosynthesis are involved. The peroxidase could well be one point of control. The high molecular weight complex isolated in these studies represents a state of the enzyme which lends itself well to allosteric control mechanisms.

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