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# PURIFICATION OF THYROID PEROXIDASE BY MONOCLONAL ANTIBODY-ASSISTED IMMUNOAFFINITY CHROMATOGRAPHY

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SUMMARY: A rapid method was developed for purification of hog thyroid peroxidase by immunoaffinity chromatography on a column of Sepharose 4B coupled to a monoclonal antibody to the peroxidase. The purified enzyme had a specific activity of 194 units/mg and showed the same absorption spectrum in the Soret and visible regions as that of the enzyme purified after trypsin treatment. The ratio of $A_{413\ nm}$ to $A_{280\ nm}$ was 0.24, being much less than that for the trypsinized enzymes. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, it gave a broad protein band in the 100,000-dalton region. It is concluded that the preparation purified in this study represents a native form of thyroid peroxidase. 

Thyroid peroxidase, which is a membrane-bound hemoprotein, has been purified by procedures involving trypsin digestion (1-3). The enzyme thus purified has been used for studies of its reaction mechanism(s) (3-8). Further improvements in the purification method are, however, awaited for two reasons: a) the procedures currently in use are tedious and time-consuming, and b) the thus purified peroxidase seems to be a trypsin-modified form. In this communication, we report a monoclonal antibody (mAb)-assisted immunoaffinity chromatography method which permits rapid purification of hog thyroid peroxidase at a high yield.

## MATERIALS AND METHODS

Production and purification of a mAb against hog thyroid peroxidase will be described in detail elsewhere. Briefly, the peroxidase purified by the

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Abbreviations used were: mAb, monoclonal antibody; Tg, thyroglobulin; PHR, phosphorylase b; BSA, bovine serum albumin; OVA, ovalbumin.

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previous method (3) was injected to BALB/c mice and splenic lymphocytes isolated from the immunized animals were fused with mouse myeloma cells, P3-X63-Ag8-U1, with the aid of polyethylene glycol 1,500 (9). A hybridoma cell exhibiting a high antibody activity was transplanted to mouse ascites. An anti-peroxidase mAb was purified from the resulting ascitic fluids by ammonium sulfate fractionation, Sephacryl S-300 gel filtration and DEAE-cellulose chromatography. The purified mAb (15 mg) was coupled to 1 g of CNBr-activated Sepharose 4B which had been washed according to the manufacturer's instruction. Sodium dodecyl sulfate polyacrylamide gel (7.5 % cross-linked) electrophoresis (SDS-PAGE) was conducted as described by Laemmli (10). After electrophoresis, the gel was stained with Coomassie brilliant blue or subjected to electrophoretic transfer (Western blotting) to a nitrocellulose membrane (Bio-Rad Lab.) by the method of Burnette (11). Following the transfer and blocking, the nitrocellulose membrane was immersed in a solution containing the mAb, and then reacted with goat anti-mouse IgG antibodies conjugated with horseradish peroxidase (Tago Inc., Burlingame). The membrane thus treated was then stained for peroxidase activity with 4-chloro-1-naphthol and $H_2O_2$. Thyroid peroxidase activity was measured with guaiacol as substrate; one unit of the enzyme oxidized 1 μmol of guaiacol per min (3). Protein was determined by the method of Lowry et al. (12) using bovine serum albumin as a standard.

## RESULTS

Hog thyroid peroxidase was partially purified by the previously published method (3) up to the DEAE-cellulose chromatography step. When this partially purified peroxidase, which possessed a specific activity of 50 units/mg and had suffered trypsin digestion, was applied to an anti-peroxidase mAb-coupled Sepharose 4B column, the enzyme activity was tightly adsorbed on the column and could not be eluted with a buffer containing 1 M KCl. However, the activity could be readily eluted with 0.1 M $NH_4OH$ plus 1 M KCl (Fig. 1). The recovery of the activity in this step was

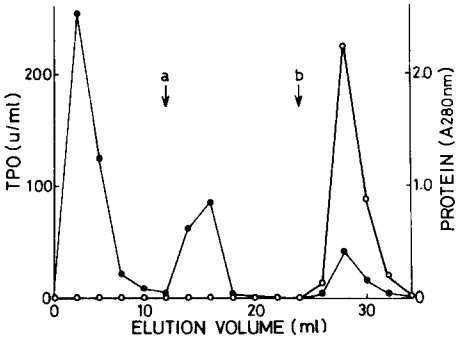


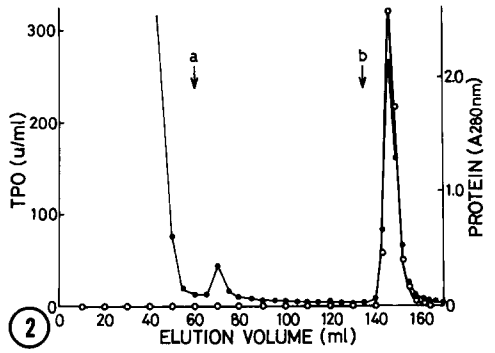

Fig. 1. Immunoaffinity chromatography of partially purified thyroid peroxidase. The enzyme, partially purified by the previous method (3) up to the DEAE-cellulose chromatography step (50 u/mg, 660 units), was applied to 2.4 ml of the immunoaffinity column equilibrated with 10 mM phosphate buffer (pH 7.5) containing 0.1 mM KI, and the column was washed with the same buffer. At Arrow a, the washing buffer was changed to 10 mM phosphate buffer (pH 7.5) containing 1 M KCl and 0.1 mM KI, and at Arrow b, elution of the enzyme was started with 0.1 M $NH_4OH$ containing 1 M KCl and 1 mM KI.
—●— , protein; —○— , thyroid peroxidase activity.

almost quantitative ( > 95 %). The peroxidase thus eluted (termed "trypsinized enzyme") had a specific activity of about 500 units/mg, a value of which is comparable to that of the best preparation of trypsinized peroxidases previously obtained (3).

Having thus confirmed the effectiveness of the immunoaffinity method, we attempted to purify a native form of the peroxidase by this method. For this purpose, the peroxidase was solubilized from thyroid microsomes with deoxycholate and fractionated with ammonium sulfate, as described previously (3). The ammonium sulfate fraction (specific activity, 1.5 units/mg) was charged to the immunoaffinity column, equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.2 % sodium cholate, 0.1 M KCl and 0.1 mM KI, and the column was washed with the equilibration buffer and also with 50 mM borate buffer (pH 9.0) containing 0.5 % sodium cholate, 1 M KCl and 1 mM KI. The enzyme was then eluted with 0.1 M $NH_4OH$ containing 0.5 % sodium cholate, 1 M KCl and 1 mM KI (Fig. 2). The recovery of activity in this step was again higher than 95 %, and the purified enzyme had a specific activity of 194 units/mg. Both this preparation (termed "native enzyme") and the trypsinized enzyme obtained above were dialyzed against 25 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM KI and stored at -70°C.

Fig. 3 shows the absorption spectra of the native enzyme and the trypsinized enzymes purified in this study and by the previous method (3). Their absorption spectra in the Soret and visible regions were practically the same, exhibiting a Soret peak at 413 nm and three minor peaks at 543, 585 and 638 nm. However, the 280 nm peak, relative to the Soret peak, was much more intense in the native enzyme than in the two trypsinized preparations. The ratios of absorbance at 413 nm to that at 280 nm (RZ value) were 0.24, 0.53 and 0.50, respectively for the native enzyme, the trypsinized enzyme obtained in this study, and the trypsinized enzyme purified by the previous method (3).

Upon gel filtration through a Sepharose 6B column, the native enzyme was eluted as a single, broad peak at a position corresponding to a molecular



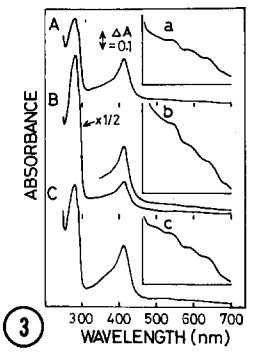




Fig. 2. Immunoaffinity chromatography of a crude preparation of thyroid peroxidase. Peroxidase was solubilized from thyroid microsomes with deoxycholate and fractionated with ammonium sulfate as described previously (3). The ammonium sulfate fraction (1.5 u/mg, 1950 units) was applied to 10 ml of the immunoaffinity column equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.2 % sodium cholate, 0.1 M KCl and 0.1 mM KI. After washing the column with the equilibration buffer, the washing buffer was changed to 50 mM borate buffer (pH 9.0) containing 0.5 % sodium cholate, 1 M KCl and 1 mM KI at Arrow a. Elution was then started at Arrow b with 0.1 M $NH_4OH$ containing 0.5 % sodium cholate, 1 M KCl and 1 mM KI. —●—, protein; —○—, thyroid peroxidase activity.

Fig. 3. Absorption spectra of purified thyroid peroxidase preparations. A, Trypsinized enzyme purified by immunoaffinity chromatography (from Fig. 1). B, Untrypsinized enzyme purified by immunoaffinity chromatography (from Fig. 2). C, Trypsinized enzyme purified by the previous method (3). Spectra were recorded at a full scale of 1.0, except that the spectrum marked with x1/2 was recorded at a full scale of 2.0 and the spectra shown in insets at a full scale of 0.1.

weight of about 400,000 (data not shown). When the native enzyme was subjected to SDS-PAGE, it gave a broad protein band in the 100,000-dalton region together with 4 faint bands, 2 of which in the 200,000-dalton region (Group a) and the other 2 in the 85,000-dalton region (Group b) (Fig. 4, lane B). A Western blotting experiment indicated that not only the main band but also the 4 minor bands retained antigenicity toward the mAb (Fig. 4, lane D). As expected, thyroid microsomes yielded a large number of protein bands (Fig. 4, lane A), but only one band having a molecular weight of 100,000 could react with the mAb (Fig. 4, lane C). On SDS-PAGE and subsequent Western blotting for the trypsinized enzyme, at least 5 immunoreactive bands (c through g) were observed (Fig. 4, lane E), although the intensity of individual band varied from experiment to experiment. Polypeptide c, a very faint band, had a molecular weight of 77,000. The main polypeptide, marked f, had a molecular weight of 52,000 and resembled

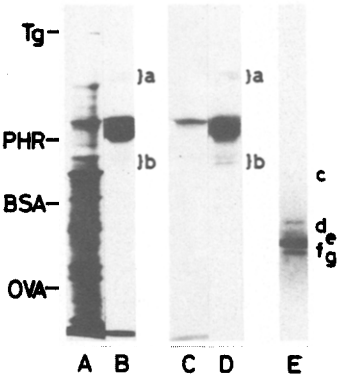



Fig. 4. SDS-polyacrylamide gel electrophoresis of purified thyroid peroxidase preparations. Samples used were: thyroid microsomes (500 μg) for lanes A and C; untrypsinized enzyme purified by immunoaffinity chromatography (25 μg) for lanes B and D; and trypsinized enzyme purified by immunoaffinity chromatography (22.5 μg) for lane E. Samples were treated with 5 % 2-mercaptoethanol. Electrophoresis was performed as described under Materials and Methods. After electrophoresis, gels A and B were stained with Coomassie brilliant blue, and gels C, D and E were subjected to Western blotting and stained for activity to react with anti-peroxidase mAb as described under Materials and Methods.

the 60,000-dalton subunit of the peroxidase reported by Rawitch et al. (2). And the molecular weights of the other bands (d, e and g) ranged from 58,000 to 49,000. When gel E was stained with Coomassie brilliant blue, a polypeptide chain of 30,000 daltons was also seen as reported by Rawitch et al. (2). Unlike the case of Rawitch et al. (2), our enzyme preparation did not show a clear profile on SDS-PAGE without addition of 2-mercaptoethanol. Trypsin treatment of the native enzyme resulted in the appearance of the same molecular weight species on gel filtration as that reported for the trypsinized enzyme (13). We concluded that a molecular weight of about 71,000 is given for these trypsinized enzymes irrespective of the difference in the way of trypsin treatment.

## DISCUSSION

The immunoaffinity chromatography method developed in this study for purification of thyroid peroxidase is superior to the procedures currently in use (1-3) in rapidity, high yield and intactness of the enzyme. The new procedure involves only three steps, solubilization, ammonium sulfate fractionation and immunoaffinity chromatography, and therefore can be completed in a much shorter time than the previous ones which consist of 7 to 8

cumbersome steps. A second advantage of this method is the high yield of peroxidase (> 75 %); in the previous method only a few % of the enzyme activity can be recovered in the final preparation (1-3). It is now possible to obtain about 25 mg of purified native peroxidase from 1 kg of thyroid glands. The most important feature of the present purification method is that it involves no trypsin digestion step and thus the risk of proteolytic modifications of the peroxidase is minimized. This is in sharp contrast to the previous methods (1-3) in which trypsin digestion at or after solubilization of microsomes is required for further purification of the peroxidase.

The results shown in Fig. 4 indicate that thyroid peroxidase preparations so far purified by procedures involving trypsin digestion do not represent the native form of the enzyme. Upon SDS-PAGE the native peroxidase gives a major polypeptide band having a molecular weight of about 100,000, whereas the trypsinized preparation yields several polypeptide bands all having molecular weights much lower than 100,000. This fact is explicable by assuming a tryptic attack causing partial cleavages of polypeptide chain(s) of the enzyme. Since the molecular weight of the trypsinized enzyme was 71,000, the RZ value and the specific activity per weight measured according to Lowry *et al.* (12) are irregularly low for the native enzyme. This may be explained by assuming that the leaving polypeptide during tryptic digestion is rich in tyrosine and tryptophan.

The above arguments, however, do not necessarily justify that the enzyme purified in this study is wholly a native one, as can be seen in its SDS-PAGE profile. In this profile, the major band in the 100,000-dalton region is very broad (Fig. 4, lanes B and D) in contrast to the anti-peroxidase mAb-reactive band detected in microsomal preparation (Fig. 4, lane C). Furthermore, two faint bands (Group *b*) are seen in the 85,000-dalton region. These observations suggest the possibility that the peroxidase purified in this study has also suffered limited proteolysis by a thyroid protease(s) during the purification procedure. The nature of the two faint bands in

the 200,000-dalton region (Group a) is unclear, but at least one of them is likely to be a dimer of the 100,000-dalton polypeptide. It is to be stressed that all these polypeptide bands retain antigenicity toward anti-peroxidase mAb, indicating that the preparation is freed from non-peroxidase polypeptides.

The information accumulated in this study is still insufficient to estimate the molecular weight of the native peroxidase. Since the enzyme contains only one polypeptide having a molecular weight of about 100,000 and is eluted at a position corresponding to a molecular weight of about 400,000 on gel filtration, the native enzyme may exist as tetramer.

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