Purification of the human thyroid peroxidase and its identification as the microsomal antigen involved in autoimmune thyroid diseases

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Human thyroid peroxidase (TPO) has been purified from thyroid microsomes by immunoaffinity chromatography using a monoclonal antibody (mAb) to TPO. The eluted material had a specific activity of 381 U/mg and exhibited a peak in the Soret region. The ratio of A_{411} to A_{280} ranged from 0.20 to 0.25. Upon SDS-polyacrylamide gel electrophoresis, the purified enzyme gave two contiguous bands in the 100 kDa region. Further, it has been demonstrated that sera with anti-microsomal autoantibodies from patients presenting Graves' or Hashimoto's thyroiditis diseases were able to bind to purified TPO and to inhibit in a dose-dependent manner the mAb binding to purified TPO. This suggests that TPO is the thyroid antigen termed to date the microsomal antigen.

Microsomal antigen *Thyroid peroxidase* *Autoantibody* *Monoclonal antibody* *Autoimmunity*

1. INTRODUCTION

Thyroid peroxidase (TPO) plays a major role in biosynthesis of thyroid hormones [1,2]. Current methods for TPO purification allow one to recover only a few percent of the enzyme activity [3–8]. Taken together with the scarcity of human tissue, this explains why no specific information is available on human TPO. Recently, TPO has been involved in autoimmune thyroid diseases [9,10] which are associated with the presence of autoantibodies (aAb) directed to various components of the gland [11]. The nature of several of these autoantigens remains unknown [11].

In a previous paper, we have shown that the microsomal antigen is antigenically related to human TPO [10]. Taking advantage of an anti-human TPO monoclonal antibody (mAb) produced in our laboratory [10,12], we present here the first data on preparation and partial characterization of purified human TPO. Furthermore, we give evidence that TPO is an autoantigen recognized by anti-thyroid aAb and shares most of the properties attributed to the microsomal antigen [13–15].

2. MATERIALS AND METHODS

2.1. *Preparation of solubilized human thyroid microsomes*

Human thyroid tissue was obtained from patients undergoing surgery for various diseases of the gland. The microsomal fraction was prepared according to [6] with minor modification. Microsomes were incubated overnight at 4°C in 50 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM KI and 0.3% sodium deoxycholate (DOC). After 100000 × *g* centrifugation for 1 h at 4°C, the solubilized material was treated by a neutralized solution of $(NH_4)_2SO_4$ at 60% saturation. The precipitated material was dissolved in and extensively dialyzed against 10 mM Tris-HCl

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buffer, pH 7.8, 0.1 mM KI. The preparation was stored at −80°C until use.

2.2. *Affinity-purification of human TPO*

10 mg anti-TPO mAb was coupled to 5 ml CNBr activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. 100 mg solubilized microsomes corresponding to 38 g thyroid tissue were passed through the affinity column previously equilibrated with 10 mM Tris-HCl buffer, pH 7.8, 0.1 mM KI, 0.5 M KCl and 0.1% DOC (buffer B.1). Washing was achieved with the previous buffer followed by 0.1 M borate buffer, pH 9.0, 0.1 mM KI, 1 M KCl and 0.5% DOC (buffer B.2). Human TPO eluted with 0.1 M NH_4OH, 0.1 mM KI, 1 M KCl and 0.5% DOC (buffer B.3). The eluate fractions were extensively dialyzed against 10 mM Tris-HCl buffer, pH 7.5, 0.1 m KI and stored in liquid nitrogen until use.

2.3. *TPO activity assay*

The enzymatic activity of TPO was measured using the guaiacol assay [4]. Briefly samples containing TPO were dissolved in 0.067 M phosphate buffer (pH 7.4) containing 40 mM guaiacol in a final volume of 2.0 ml. The reaction was initiated by addition of H_2O_2 at a concentration giving 0.3 mM in the final volume. The assay was performed at room temperature. The oxidation of guaiacol was followed spectrophotometrically at 470 nm. One unit of the enzyme activity was defined to oxidize 1 μmol of guaiacol/min [6].

2.4. *Immunoprecipitation assay*

Sera from patients with Graves' disease or Hashimoto's thyroiditis were mixed with various amounts of affinity purified hTPO in 10 mM Tris-HCl buffer (pH 7.5) in a total volume of 0.3 ml. After overnight incubation at 4°C, samples were centrifuged at 10000 × *g* for 5 min. Supernatants were incubated with 0.5 ml protein A-Sepharose 4B gel (Pharmacia) for 1 h at 4°C. After centrifugation 15 min at 2000 × *g*, TPO activity was measured in supernatants and precipitates.

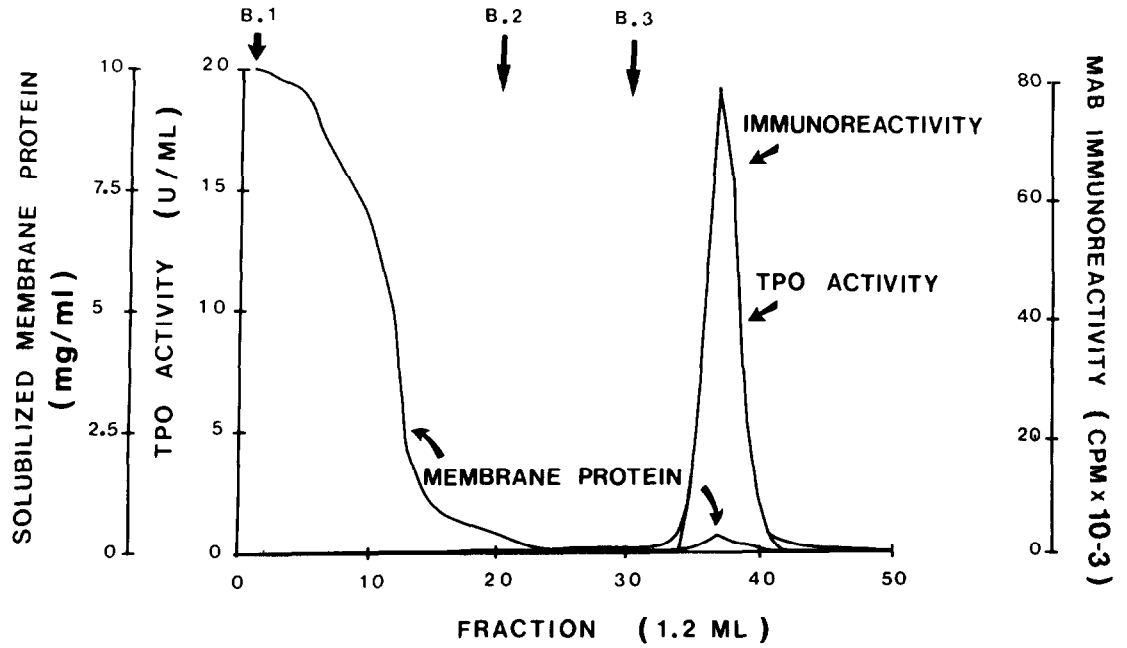

Fig.1. Immunoaffinity chromatography profile of a crude preparation of the human thyroid peroxidase. The ammonium sulfate precipitated fraction (100 mg) was applied to 5 ml of the mAb-CNBr Sepharose column. The arrows indicate the buffers used: B.1 = 10 mM Tris-HCl buffer, pH 7.8, 0.1 mM KI, 0.5 M KCl and 0.1% DOC; B.2 = 0.1 M borate buffer, pH 9.0, 0.1 mM KI, 1 M KCl and 0.5% DOC and B.3 = 0.1 M NH_4OH, 0.1 mM KI, 1 M KCl and 0.5% DOC.

2.5. *Radioimmunoassay*

Affinity-purified hTPO was coated overnight at 4°C on flexible microtiter plates (Falcon). Each well received 50 μl of hTPO solution at 0.002 mg/ml in phosphate buffered saline, pH 7.3. After washing and saturation with bovine serum albumin, anti-hTPO mAb was incubated alone or mixed with serial dilutions of patient's sera for 90 min at 37°C. After numerous washings, mAb binding to hTPO was evidenced by incubating radiolabeled affinity purified F(ab)$_2$ sheep anti-mouse antibodies for 90 min at 37°C. After numerous washings, wells were dried, cut and counted.

2.6. *Other methods*

Electrophoresis in SDS-polyacrylamide slab gels (gradient 8–18%) was achieved according to [16]. Immunoblotting was carried out according to [17] and isoelectrofocusing according to [18]. Protein concentration was determined by the method described in [19].

3. RESULTS

The affinity-column procedure eliminated more than 99% of the proteins which appeared essentially devoid of TPO activity (fig.1). Elution with NH_4OH allowed recovery of the TPO activity. Noteworthy, the enzyme activity profile was almost superimposable on the immunoreactivity assayed with the mAb (fig.1). Assays for protein concentration and TPO activity were carried out at successive steps of the purification procedure (table 1). Data for the initial homogenate and the particulate fraction are not presented since there was too much interference with the guaiacol assay in these crude preparations. On the basis of previous papers from other groups [3–6], a purification of about 10-fold over the starting material to microsomes may be assumed. Beginning with microsomes, the purification factor was 331-fold. Therefore the overall purification would be about 3000- to 3500-fold.

Table 1

Activity of thyroid peroxidase at various stages of purification

Stage of purification	Total protein (mg)	Total units	Specific activity (U/mg)	Purification factor
Microsomes	791	915	1.1	1
Solubilisate	383	861	2.2	2
Affinity chromatography	1.6	610	381	331

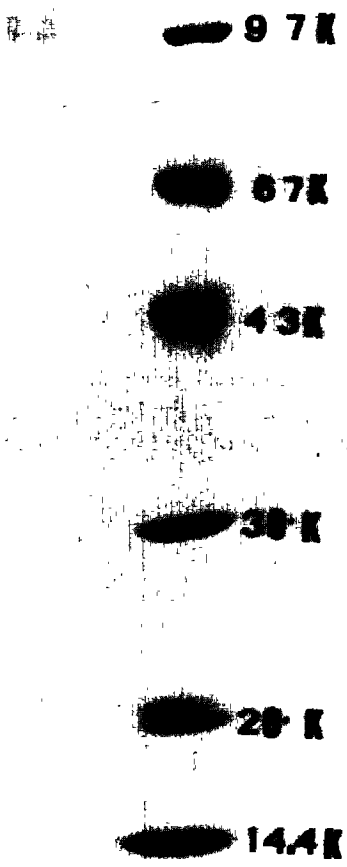

Fig.2. SDS-polyacrylamide gel electrophoresis of purified human thyroid peroxidase. Samples were treated with 5% 2-mercaptoethanol. After electrophoresis gel was stained with Coomassie brilliant blue R-250. The numbers in the margin indicate the molecular masses ($\times 10^{-3}$) of the standards.

Electrophoresis in SDS-polyacrylamide slab gels of TPO showed two contiguous bands of apparent molecular mass of about 95 and 105 kDa (fig.2). Immunoblotting of such gels using the mAb did not distinguish the two bands which appeared to retain the antigenicity toward the mAb. Further separation by isoelectrofocusing yielded two-dimensional electrophoretic patterns indicating that these two bands differed slightly by their pH_i: pH 7.0–7.4 for the 105 kDa band and pH 6.5–7.2 for the 95 kDa band. Absorption spectra of the purified TPO exhibited a peak in the Soret region which shifted from 411 to 422 nm by reducing agents. The $A_{411}:A_{280}$ ratio was 0.20 to 0.25 while that for a commercial preparation of bovine lactoperoxidase was 0.72. Preliminary experiments of adsorption-elution of the affinity-purified TPO on concanavalin A-Sepharose gel and assay for the enzyme activity demonstrated that human TPO is a glycoprotein.

Sera from healthy subjects and patients with autoimmune thyroid diseases were assayed for their ability to immunoprecipitate the purified TPO. After incubation with the affinity-purified material, IgG were precipitated by protein A coupled-Sepharose and the TPO activity assayed in the supernatant. With normal sera, devoid of anti-microsomal and anti-thyroglobulin aAb, the immunoprecipitation procedure elicited a decrease of about 20% in the TPO activity in the supernatant (fig.3). This result could be accounted for by some nonspecific precipitation or inhibition of TPO. In contrast, sera from patients with Graves' disease or Hashimoto's thyroiditis immunoprecipitated

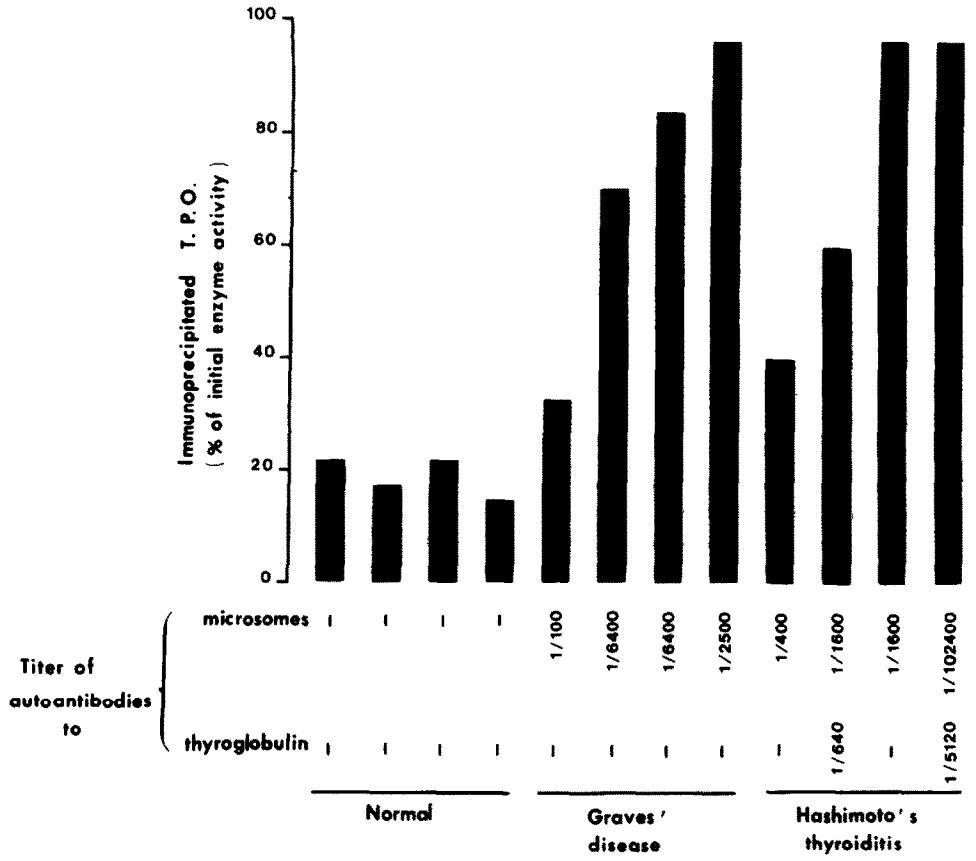

Fig.3. Human thyroid peroxidase activity after precipitation by various sera from patients with Hashimoto's and Graves' diseases and normal subjects. Each bar represents individual serum. Anti-microsomal and anti-Tg titers were measured by hemagglutination tests (Thymune M and Thymune T, Wellcome, Dartford).

TPO to an extent ranging from 30 to 100% of the initial enzyme amount. Noteworthy, the TPO immunoprecipitating activity of sera matched the titer of anti-microsomal aAb and was not related to the titer of anti-thyroglobulin aAb (fig.3).

The interaction of anti-microsomal autoantibodies with human TPO was further studied by experiments of inhibition of the mAb binding to purified TPO by pools of sera and purified IgG. As shown in fig.4, normal sera and IgG did not interfere with mAb binding to TPO. In contrast, pools of sera and IgG from patients with anti-microsomal aAb inhibited in a dose-dependent manner the binding of the anti-TPO mAb to the affinity-purified material (fig.4).

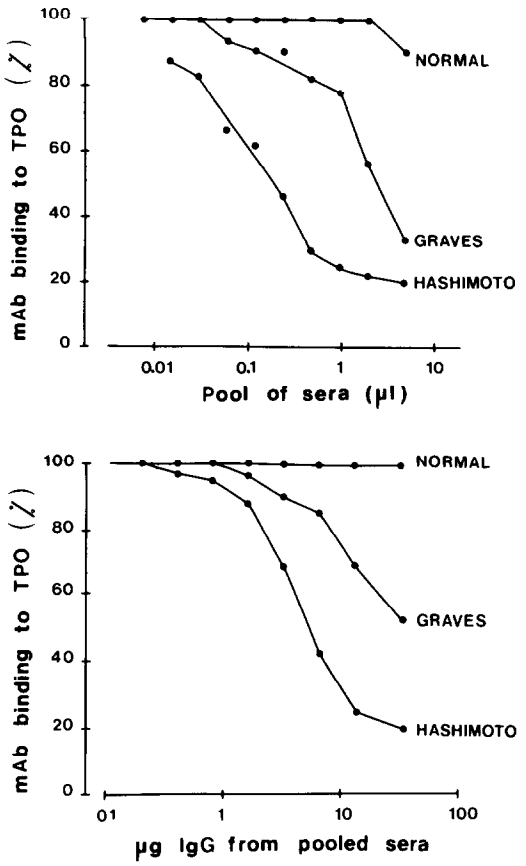

Fig.4. Inhibition of mAb binding to TPO by pools of sera (upper panel) and IgG (lower panel) from normal subjects and patients with Hashimoto's and Graves' diseases. The initial mAb binding was 3725 cpm.

4. DISCUSSION

The cumbersome procedures for purification of TPO involve numerous cumbersome steps [3–7]. The availability of mAb to TPO allows one to purify the enzyme much more rapidly and with a higher yield than the procedures currently in use [8]. Another problem encountered in the purification of TPO is the choice of the solubilization method. The most successful method for solubilizing TPO has been trypsin digestion in combination with detergents [3–6]. This procedure, however, may lead to variable cleavages of the enzyme molecule and to purification of trypsin-modified TPO [7,8]. The procedure used here allows high purification (~3500-fold) of the native human TPO. This is obtained with a reasonable yield (~10 mg TPO/kg thyroid tissue).

Upon SDS-polyacrylamide electrophoresis, TPO gives two contiguous bands of M_r 95000 and 105000 which differ by their pH_i but retain their antigenicity toward the anti-TPO mAb. In keeping with previous reports on purified native porcine TPO [7,8], this could suggest that TPO has suffered limited degradation by thyroid protease and/or glycosidases. Absorption spectra of purified human TPO compare well with those of bovine and porcine TPO [3–8]. The ratio of absorbance at 413 nm to that at 280 nm is about half that of purified trypsinized TPO [3–6] but similar to that of the native enzyme from porcine thyroid [8].

Recognition of TPO by anti-thyroid aAb is unquestionably demonstrated by aAb immunoprecipitation of TPO activity, and inhibition of mAb binding to TPO. Immunoprecipitation of TPO activity by aAb in autoimmune thyroid diseases has been recently reported [9]. However, the use of solubilized thyroid particulate fraction by these investigators is highly questionable. Effectively, aAb in thyroid diseases are directed against numerous antigens and coprecipitation of TPO with other autoantigens cannot be excluded. In our case, the TPO preparation used presents almost all the characteristics required in the literature for highly purified TPO [3–8]. Accordingly, our experiments of immunoprecipitation clearly demonstrate, for the first time, the direct interaction of aAb with the TPO molecule. This finding is further confirmed by inhibition of mAb binding to

TPO by sera and IgG from patients with autoimmune thyroid diseases but not from normal subjects.

Only recently have some characteristics of the microsomal antigen been disclosed [13–15]. The microsomal antigen was reported as a glycoprotein of 105 kDa [14,15] present in the cytoplasm and on the apical membrane of human thyroid cells [11–13]. Noteworthy, TPO has been localized on the apical border of thyroid follicular cells [20] and has been also found widely distributed in the cytoplasm [21]. It thus appears that TPO shares all the properties attributed to the microsomal antigen. Taking into account all the information available on the microsomal antigen and TPO, the data presented here strongly suggest that human TPO would be the microsomal antigen involved in autoimmune thyroid diseases.

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