*Endocrine Journal* 1993, **40** (1), 141–148

# Individual Difference in Toxic Diffuse Goiters Assessed by Heme-based Specific Activity and Km Value#

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**Abstract.** Thyroid peroxidase (TPO) was purified from each thyroid of 10 patients (experiment 1) and 4 patients (experiment 2) with toxic diffuse goiter by a simplified method with monoclonal anti-TPO antibody-assisted immunoaffinity column chromatography. The final preparations were used to measure the heme concentration based on the cyanide difference spectrum, and to determine the Km and $k_{cat}$ values from double reciprocal plots in the assay employing guaiacol and iodide as the second substrates. The heme-based specific activities of TPO purified from thyroids in experiment 1 were higher than those in experiment 2, which were probably impaired by freezing-thawing, and those of porcine TPO previously reported. There were some differences in the kinetic properties between experiment 1 and experiment 2, but the individual differences within each group were relatively small, the values for CV (=SD/mean) being 0.16–0.48.

*Key words*: Thyroid peroxidase, Toxic diffuse goiter, Heme content, Michaelis constant.
(*Endocrine Journal* **40**: 141–148, 1993)

**THE THYROID** peroxidase (TPO) activity of patients with toxic diffuse goiters has been examined by several research groups [1–7]. The majority of the groups reported that the activity was enhanced in the toxic diffuse goiters, in accordance with histochemical observations in stimulated localization of the enzyme in endoplasmic reticulum and microvilli areas [6, 8, 9]. A quantitative increase in TPO is therefore believed to be responsible for the high production of thyroid hormones in toxic diffuse goiters.

On the other hand, the protein-based specific TPO activity of the particulate fractions obtained from toxic diffuse goiters varied from thyroid to thyroid [7] and some hypothyroids had markedly low TPO activity per molecule compared with the normal value [10, 11]. Thus, the possibility cannot be denied that some qualitative change as well as quantitative variation may occur in TPO in toxic diffuse goiters. To determine these changes, the enzyme must be purified to have its heme concentration determined. However, since the TPO content is very low in each thyroid and solublilization with detergents and proteases from the microsomes is a prerequisite for purification, it was difficult to purify the enzyme from a thyroid gland. Thus, the purification of TPO has been

Received: July 17, 1992
Accepted: December 28, 1992
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#A preliminary report of portions of this work was presented at the 63rd Annual Meeting of the Japanese Biochemical Society on September 15th, 1990 (Abstract: *Seikagaku* 62: 724).
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performed by starting from pooled thyroids of animals [12–15] and patients with toxic diffuse goiter [16]. However, recent improvement in conventional column chromatography [13–15] and monoclonal anti-TPO antibody-assisted affinity chromatography [17–20] has stimulated the development of more efficient procedures for purification of human TPO.

In the present study, we have attempted to improve the efficiency of purification of TPO by one-step conventional column chromatography followed by monoclonal antibody-assisted affinity column chromatography. Since the method requires only 15 g of thyroid tissue to obtain a final TPO preparation, the heme content of which can be determined, we could isolate TPO from each patient with toxic diffuse goiter and compare the catalytic properties on the basis of heme content.

## Materials and Methods

*Materials*

Trypsin (1:300) was purchased from ICN NBC, Cleveland, OH, U.S.A. Soybean trypsin inhibitor (Type II) and 4-chloro-1-naphthol were from Sigma, St Louis, MO, U.S.A. CNBr-activated Sepharose was from LKB-Pharmacia Biotechnology, Uppsala, Sweden, DE52 (microgranular DEAE cellulose) was from Whatman International Ltd, Maidstone, England, the avidin-biotin-peroxidase complex was from Vector Laboratories Inc. Burlingame, CA, U.S.A. Sodium cholate and Tween 20 were from Nakarai Tesque Co., Kyoto, Freund's adjuvant was from DIFCO Laboratories, Detroit, Michigan, U.S.A. The cell culture medium used was RPMI 1640 medium obtained from Nissui Seiyaku Co., Tokyo. Bovine fetal serum was purchased from Wheaton Co., penicillin G and streptomycin sulfate were from Meiji Seika Co., Tokyo and 7% sodium hydrogen carbonate was from Hikari Seiyaku Co., Osaka. Other reagents were of analytical grade obtained from Wako Pure Chemicals, Osaka.

*Thyroid tissues*

Patients with toxic diffuse goiter underwent partial thyroidectomy at Ito Hospital, Tokyo (10 patients, experiment 1) and Kuma Hospital, Kobe (4 patients, experiment 2). Table 1 shows the clinical data for these patients. The thyroid tissues were frozen with dry ice and kept in the freezer at −70°C until use.

*Homogenization, fractionation of toxic diffuse goiter and assay for peroxidase activity in the mitochondria-microsomes fraction*

A part (usually 1 g) of each toxic diffuse goiter was cut off in the frozen state and used for homogenization, fractionation and for peroxidase assay [22]. The remaining tissues were stored at −70°C and used for purification of TPO by affinity column chromatography as described below. The peroxidase activity of the mitochondria-microsomes obtained from the 1 g of thyroid tissue was measured, after treatment with cholate, by "ordinary assay" method using guaiacol and iodide as the second substrate as described previously [22].

*Assay for guaiacol oxidation and iodide oxidation activities of TPO*

For the guaiacol oxidation assay, the reaction mixture (3.02 m*l*) contained 33 mM guaiacol, 0.27 mM $H_2O_2$ and 33 mM sodium phosphate (pH 7.4), and for the iodide oxidation assay, it (3.02 m*l*) contained 0.135 mM $H_2O_2$, 4.95 mM potassium iodide, and 33 mM sodium phosphate (pH 7.0). The reaction was started by adding 20 $\mu l$ of $H_2O_2$. When the mitochondria-microsomes were used as the enzyme source, they were treated with 0.7% cholate before the reaction as described [22]. The amount of enzyme which gave a change of 1.0 absorbance unit per second was taken as 1 unit and expressed as guaiacol unit (GU) or iodide unit (IU). To calculate the enzyme turnover number, an extinction coefficient of $5.57\times10^3$ $M^{-1}cm^{-1}$ was employed for oxidized guaiacol [23, 24] and $26\times10^3$ $M^{-1}cm^{-1}$ for the oxidized iodide, triiodide [25]. To analyze double reciprocal plots, the concentrations of the enzyme and the substrates were varied as described in each experiment.

*Purification of TPO from each thyroid of patients with toxic diffuse goiter*

A part of the frozen thyroid (about 15 g) was

**Table 1.** Clinical data for patients with toxic diffuse goiters

| Patients No. | Sex | Age | Size of goiter[a] (g) | $FT_4$ (pg/ml) | $FT_3$ (ng/dl) | TSH ($\mu$U/ml) | TRAb (%) | TGAb[b] | MCAb[b] |
|---|---|---|---|---|---|---|---|---|---|
| *Experiment 1* | | | | | | | | | |
| 1 | F | 13 | 141 | 4.3 | 0.85 | 15.7 | 77.6 | 400 | 1600 |
| | | | | 5.6 | 1.54 | 0.05 | 72.6 | 400 | 400 |
| 2 | F | 26 | 68 | 25.0 | 10.0 | ND[c] | 52.7 | (−) | (−) |
| | | | | 7.1 | 1.69 | 0.05 | 48.0 | (−) | (−) |
| 3 | F | 15 | 75 | 13.3 | 4.94 | 0.05 | 20.3 | (−) | 100000 |
| | | | | 7.7 | 1.25 | 0.12 | 62.5 | (−) | 25000 |
| 4 | F | 21 | 65 | 21.7 | 10.0 | 0.45 | 83.1 | ND | ND |
| | | | | 3.6 | 0.60 | 9.6 | 50.7 | 400 | 100000 |
| 5 | M | 33 | 73 | 3.3 | 1.19 | 9.4 | 16.2 | (−) | (−) |
| | | | | 3.9 | 1.43 | 0.54 | 11.5 | (−) | (−) |
| 6 | F | 20 | 40 | 13.9 | 5.51 | <0.05 | 67.4 | 400 | 6400 |
| | | | | 4.0 | 1.26 | 9.1 | 59.5 | 100 | 400 |
| 7 | F | 34 | 65 | ND | 0.73 | 4.5 | ND | (−) | 400000 |
| | | | | 4.1 | 1.16 | 0.28 | 23.7 | (−) | (−) |
| 8 | F | 36 | 143 | 11.3 | 4.33 | <0.05 | 61.4 | (−) | 25000 |
| | | | | 9.4 | 2.13 | <0.05 | 23.9 | (−) | 6400 |
| 9 | F | 30 | 38 | 15.9 | 5.53 | <0.05 | 23.9 | (−) | 1600 |
| | | | | 5.8 | 2.29 | <0.05 | 13.8 | (−) | 400 |
| 10 | M | 29 | 75 | 17.5 | 6.09 | <0.05 | 73.2 | (−) | 1600 |
| | | | | 25.0 | 10.0 | <0.05 | 74.1 | (−) | 6400 |
| *Experiment 2* | | | | | | | | | |
| 11 | F | 22 | 181 | 7.21 | 0.44 | <0.05 | 73.0 | (−) | 3200 |
| | | | | 6.12 | 1.28 | <0.05 | ND | (−) | 800 |
| 12 | M | 43 | 82 | 22.0 | 9.9 | <0.05 | ND | (−) | 1600 |
| | | | | 3.62 | 0.87 | <0.05 | ND | (−) | ND |
| 13 | F | 31 | 110 | 4.44 | 0.68 | 13.71 | ND | (−) | (−) |
| | | | | 3.89 | 0.81 | 4.14 | 77.8 | (−) | (−) |
| 14 | F | 27 | 158 | 18.0 | 4.95 | <0.05 | 17.6 | 1600 | 25600 |
| | | | | 4.60 | 1.25 | <0.05 | ND | 1600 | ND |
| Normal | | | | 2.8–5.5 | 0.8–1.9 | 0.3–3.5 | <10 | (−) | (−) |

The data in the upper and lower lines of each patient indicate those obtained at the time of the first medical examination and the operation, respectively.
a: The size of goiters was estimated by the method described by Takamatsu *et al.* [21]. b: The level of the antibody is shown in titers. The sign (−) indicates that two-fold diluted serum does not react with thyroglobulin and microsomal proteins.

thawed, sliced and washed with 0.9% NaCl containing 10 mM phosphate buffer (phosphate buffered saline, PBS). After blotting with filter paper, the tissues were homogenized in about 45 m*l* of 0.25 M sucrose/PBS with Ultra-Turrax and the homogenate was centrifuged at 2000× g for 10 min. The cytosol obtained was centrifuged at 105,000× g for 60 min and the pellet (mitochondria-microsomes) was suspended in buffer A (PBS containing 10 mM KI) (0.2–0.5 m*l* per g tissue). After the protein concentration of the suspension was measured, an appropriate volume of buffer A was added to the suspension to make it contain 20 mg protein/m*l*, and the mixture was preincubated for 5 min at 37°C. Then to the mixture, a trypsin (1:300) (10 mg/m*l*) solution and a cholate (10%) solution were added to make a concentration of 3% (3 mg trypsin (1:300) to every 100 mg protein) and 0.5%, respectively, followed by incubation for 60 min at 37°C [26]. At the end of incubation, a trypsin inhibitor (10 mg/m*l*) was added to obtain a trypsin:inhibitor ratio of 1:1.5 and the mixture was chilled for 15 min. The supernatant was applied to a DEAE-cellulose column (12×80 mm) which was previously equilibrated with buffer A, and the column was washed with buffer A until the

absorbance at 280 nm of the eluate was below 0.1. Gradient elution (0–0.5 M NaCl in buffer A containing 0.1% cholate) was then performed and the active fractions were combined. The solution was centrifuged at 500× g for 10 min several times in a Centriflow (CF 25 membrane, Amicon Div., W.R. Grace Co.) to concentrate the enzyme and to replace the medium with 10 mM Tris-HCl (pH 7.8) containing 0.1 mM KI, 0.5 M KCl and 0.1% cholate. The solution (3–5 m*l*) was then applied to an anti-TPO antibody-associated Sepharose column which was prepared as described below, washed with 5 m*l* of 0.1 M borate buffer (pH 9.0) containing 0.1 mM KI, 1 M KCl and 0.5% cholate, and TPO was eluted with 0.1 M $NH_4OH$ containing 0.1 mM KI, 1 M KCl and 0.5% cholate. The active fractions eluted were pooled for ultrafiltration in a Centriflow at 500× g for 10 min several times to replace the medium with 10 mM buffer A. The final preparation was usually yellow in color and about 3 m*l* in volume. An aliquot of the solution was immediately subjected to cyanide difference spectrum analysis to determine the concentration of heme.

*Culture of hybridoma and preparation of affinity column*

*Preparation of Hybridoma*: Hybridoma cells producing the anti-TPO antibody were prepared as described previously [9, 20]. Thyroid microsomes solubilized with n-octyl-$\beta$-D-glucoside from toxic diffuse goiters were used as the antigen. Five BALB/C mice were immunized with the antigen and the spleen cells were fused with NS1 myloma cells at a ratio of 5 : 1 in the presence of 50% polyethylene glycol 4000. Fused cells were distributed to 96 wells in microculture plates and selected for binding activity by HAT medium, and culture supernatants were screened for binding activity by dot immunobinding assay under conditions where they bound to human thyroid microsomes but not to human thyroglobulin or kidney microsomes. Consequently, 7 wells were selected and cloned at least twice by limiting dilution. Seven clones compatible with the above conditions which secreted immunoglobulin G (IgG) class antibodies were finally expanded and injected intraperitoneally into BALB/C mice to collect ascites fluid by Sephacryl S-300 gel filtration and DEAE-cellulose chromatography and tested for their binding ability by micro-ELISA. The clone which produced an antibody with the highest inhibitory action on TPO was TM-Mo 3.

*Preparation of Sepharose-bound mAb and affinity column*: The clone TM-Mo 3 was injected into BALB/C mice to collect the ascites fluid containing the mAb. The mAb was purified on a Protein-A Sepharose column, and coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions. One milliliter beads coupled with 4 mg mAb are packed into a column (6×35 mm), and the column was washed with 20 m*l* PBS.

*Determination of heme concentration*

The concentration of TPO was determined from the cyanide-difference spectrum [12], except that a newly established extinction coefficient of the peak minus trough, $85\times10^3$ $M^{-1}cm^{-1}$ [27], was employed. Small cuvettes (0.45 m*l*, 5 mm wide and 10 mm light path) were used.

*Preparation of polyclonal anti-TPO antibodies and Western blotting*

Anti-human TPO polyclonal antibodies were prepared as described previously [28]. SDS-PAGE and western blotting experiments were performed as reported [29].

*Determination of protein content*

Protein was determined by the method of Lowry *et al.* [30] with the bovine serum albumin as the standard.

## Results

Starting with about 1 g tissue of each, the mitochondria-microsomes fractions were prepared and assayed for TPO activity. The protein-based specific activity of each thyroid in experiment 1 varied from 3.76 to 55.0 mGU/mg protein and from 3.68 to 55.0 mIU/mg protein, the mean±SD (n=10) being 24.5±17.3 mGU/mg protein and 21.8±16.3 mIU/mg protein. These values were similar to those obtained in the guaiacol assay and higher than those obtained in the iodide assay previously for normal thyroid (the mean value was 30.0 mGU/mg protein and 6.6 mIU/mg protein, n=10) [20].

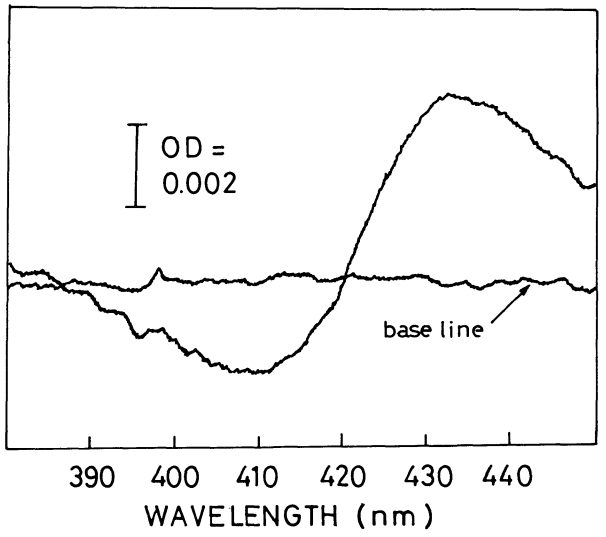


**Fig. 1.** Difference spectrum of TPO-cyanide complex. TPO preparation used in this experiment was from patient 2 in experiment 1. Both cuvettes contained 80 μg protein per ml (pH 7.4). Potassium cyanide was added to the sample cuvette to make a concentration of 100 μM.

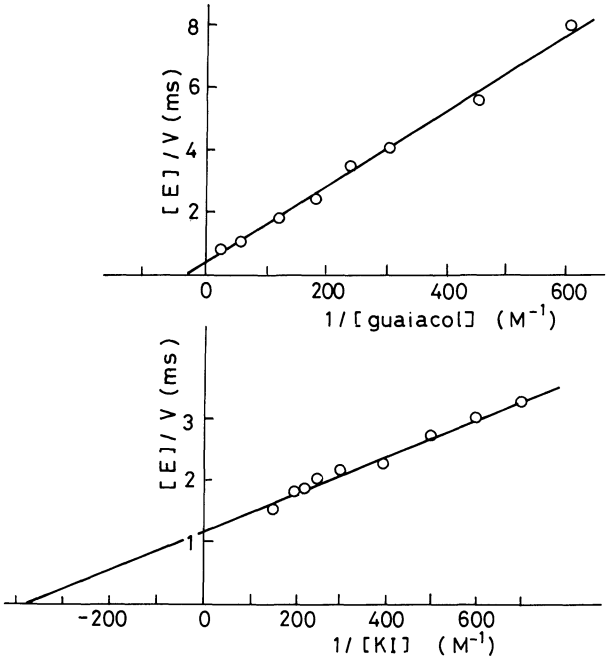


**Fig. 2.** Double-reciprocal plots of guaiacol oxidation (upper panel) and iodide oxidation (lower panel) catalyzed by TPO purified the thyroid of patient 2. The TPO concentrations used were 0.24–0.61 nM (upper panel) and 0.12–0.30 nM (lower panel).

TPO in the mitochondria-microsomes obtained from about 15 g of each thyroid from 14 patients was solubilized with cholate plus trypsin. Then it was purified by DEAE-cellulose chromatography and affinity chromatography. The final preparation each was subjected to determination of the heme concentration and the Km values for the second substrates, guaiacol and iodide. The former is based on the observation of the difference spectrum for TPO-CN versus TPO [12] and the latter, on the analysis of double reciprocal plots. Figs. 1 and 2 show some examples of these experiments.

In the latter figure, the intercepts of the line with the abscissa and the ordinate are $-1/Km$ and $[E]/V_{max}$, respectively. Thus, the values for Km and $k_{cat}$ ($=V_{max}/[E]$) were calculated and are shown in columns b and c in Table 2. The values shown in column a are the values for peroxidase activity obtained under standard assay conditions divided by heme content. In the table, data for two groups, experiment 1 (10 patients) and experiment 2 (4 patients), are summarized. The values for Pa 8 were discarded by Smirnoff's analysis. The data in the same group resemble each other as will be seen from the comparatively low coefficient of variation (the ratio of SD to the mean), but those in the two groups were somewhat different, especially the values obtained in the guaiacol assay. The cause of the difference will be discussed below.

## Discussion

The procedure for TPO extraction and purification from a large amount of thyroid tissues has already been established [12–15], but a more efficient and less time-consuming method was required to fulfil the purpose of the present study to compare the heme-based activity of TPO preparation derived from each thyroid. To accomplish this, we re-examined each step in the purification procedures and finally employed the procedures described in Materials and Methods which include an effective solubilization with trypsin and cholate and two-step column treatment: ion exchange chromatography and immunoaffinity chromatography. By these procedures, we were able to isolate the enzyme with an RZ ($A_{412}/A_{280}$) of 0.1–0.3 within 12 h, starting with less than 15 g of

**Table 2.** The values for (a) heme-based specific activity, (b) Km and (c) $k_{cat}$ of TPO purified from toxic diffuse goiters

| Patients No. | Guaiacol assay | | | Iodide assay | | |
|---|---|---|---|---|---|---|
| | (a)[a] | (b) | (c) | (a) | (b) | (c) |
| *Experiment 1* | | | | | | |
| 1 | 0.44 | 1.23 | 214 | 0.83 | 1.42 | 145 |
| 2 | 0.67 | 0.57 | 743 | 1.59 | 0.85 | 212 |
| 3 | 0.34 | 0.37 | 424 | 1.71 | 2.30 | 359 |
| 4 | 0.73 | 1.15 | 466 | 1.41 | 1.68 | 251 |
| 5 | 0.43 | 0.33 | 517 | 2.54 | 1.76 | 381 |
| 6 | 0.42 | 1.52 | 373 | 1.65 | 1.72 | 293 |
| 7 | 0.35 | 1.14 | 308 | 1.48 | 2.41 | 306 |
| 8[b] | (2.58) | (15.7) | (2174) | (2.97) | (2.83) | (609) |
| 9 | 0.52 | 0.62 | 226 | 0.79 | 1.93 | 143 |
| 10 | 0.62 | 0.90 | 305 | 1.25 | 1.51 | 184 |
| Mean | 0.50 | 0.88 | 397 | 1.47 | 1.73 | 253 |
| ±SD (n=9) | 0.14 | 0.42 | 166 | 0.52 | 0.47 | 88 |
| CV[c] | 0.28 | 0.48 | 0.42 | 0.35 | 0.27 | 0.35 |
| *Experiment 2* | | | | | | |
| 11 | 3.03 | 41.7 | 3143 | 5.27 | 2.24 | 996 |
| 12 | 2.51 | 45.0 | 3522 | 5.51 | 2.56 | 840 |
| 13 | 3.23 | 100.8 | 5501 | 3.38 | 2.40 | 553 |
| 14 | 4.17 | 85.3 | 6007 | 3.87 | 1.76 | 636 |
| Mean | 3.25 | 68.9 | 4543 | 4.51 | 2.24 | 756 |
| ±SD (n=4) | 0.69 | 29.4 | 1421 | 1.04 | 0.35 | 200 |
| CV | 0.21 | 0.43 | 0.31 | 0.23 | 0.16 | 0.26 |

a: (a), (b) and (c) stand for heme-based specific activity (GU or IU/nmole heme), Km (mM) and $k_{cat}$ ($s^{-1}$), respectively. b: The data for Pa 8 in parentheses were excluded in the calculation of the mean ± SD by Smirnoff's analysis. c: CV (coefficients of variation) means the ratio of SD to the mean.

thyroid tissue. It has been reported that the almost pure TPO preparation had an RZ of 0.55–0.6 [13, 19]. Thus, the purity of the TPO preparations obtained here was estimated to be 15–50%.

The heme concentration in the final preparation was determined by the method which employs a cyanide difference spectrum [12, 26], since the concentration was so low (below 1 $\mu$M) that determination by the usual method (measuring the absorbance at the Soret band, i.e. $A_{412}/\varepsilon_{413}$) tended to cause some systematic errors due to slight turbidity in the solution.

After observation of the cyanide difference spectrum, the remaining samples of purified preparation were used for kinetic studies. As Table 2 shows, higher activities were always found in experiment 2 than in experiment 1, especially in the guaiacol assay. In the case of experiment 1, the homogenization of the tissues was started late in the morning, and the measurement of kinetics was postponed to the next day, with the sample being frozen until the measurement. The tissues in experiment 2 were subjected to homogenization early in the morning and the activity could be measured immediately after the final step of purification. It is therefore likely that the freezing-thawing of the enzyme with extremely low concentrations in the case of experiment 1 might have decreased its activity, and this may be the main cause of the difference between the two groups in kinetic data. The loss of activity in the iodide assay was less than that in the guaiacol assay. TPO is known to have two binding sites for the second substrates, one for iodide and the other for aromatic substrates [11, 16], and it is possible that these sites differ in stability.

As described above, the TPO activities in experiment 1 seem to be more or less impaired, and the levels of heme-based specific activities of TPO in toxic diffuse goiters may be represented by the activities of experiment 2. It is noteworthy that these are several times higher than those of porcine TPO previously reported [31]. It is not yet determined, however, whether the heme-based specific activities of toxic diffuse goiter's TPO are equal to those of normal human thyroid TPO, although it is highly probable in view of the results of cloning experiments [32, 33]. In addition, all the samples of experiment 1 and experiment 2 showed a band at approximately 100 kDa on western blotting as in the case of normal human thyroid tissues (not shown).

In the present paper, the degree of divergence of data in one group was expressed by the use of the coefficients of variation, CV. The CV values for the heme-based specific activities of purified TPO, 0.28 and 0.21 for experiment 1 (n=9) and experiment 2 (n=4), respectively, which should theoretically converge to zero, are considered to reflect some variation in the determination of activity and protein content. On the other hand, the CV value for protein-based specific activity of TPO in the mitochondria-microsomes was 0.66, which is higher than that in heme-based specific activity. The individual difference of the protein-based specific activity in the mitochondria-microsomes may be therefore ascribed to, in addition to experimental errors, some variation in such a factor as TPO content per unit tissue. However, these individual differences were generally small, as in the case of normal thyroid tissues [22]. On the other hand, both toxic diffuse goiters and normal tissues were similar as to the protein-based TPO specific activities of mitochondria-microsomes. It is therefore considered that the remarkable increase in TPO activity in toxic diffuse goiters is mostly due to the hyperplasia in thyroid tissues with almost the same TPO content per unit tissue.

## Acknowledgments

The authors are grateful to Dr. K. Ito (Ito Hospital, Tokyo) and Dr. K. Kuma (Kuma Hospital, Kobe) for supplying thyroids of patients with toxic diffuse goiters, and Koichi Sato (Daiichi Pharmaceutical Co.) for helpful discussion.

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