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Characterization of TPO Enzyme and its Immunogenecity in New Zealand White Rabbits (*Oryctolagus cuniculus)*

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Abstract: Hypothyroidism is characterized by inadequate production of thyroid hormones, chiefly thyroxine (T4) and triiodothyronine (T3). Thyroid peroxidase (TPO), is an enzyme and crucial for producing thyroid hormones, and also serves as a primary autoantigen in autoimmune thyroid conditions, such as Hashimoto's thyroiditis and Graves' disease. The existence of anti-thyroid peroxidase antibodies (anti-TPO) indicates an autoimmune response in which the immune system TPO which is normally recognized as part of the body (self), begins to be identified by the immune system as a something foreign (non-self). This study aimed to characterize, measure TPO activity, and evaluate the immunogenicity of TPO protein derived from patient serum and its ability to induce TPO antibody production in New Zealand White Rabbits. TPO protein isolation was achieved through SDS-PAGE and confirmed by western blot technique have molecular weight of 52 kDa. In samples from normal patients, the average TPO enzyme activity was 31.34 U/mL, while in hypothyroid patients, the average activity increased to 49.46 U/mL. TPO polyclonal antibody production in rabbits indicated its immunogenicity, peaking in week eight post-immunization, with an ELISA absorbance of 0.555. Dot blot analysis further confirmed anti-TPO sensitivity at various dilutions, anti-TPO has a high sensitivity at 1:80 dilution still detects TPO in sera patient.

Keywords: Autoimmune Thyroiditis; Dot Blot; ELISA; Hypothyroidism; TPO

Introduction

Hypothyroidism is a medical disorder marked by a deficient thyroid gland, resulting in inadequate synthesis of thyroid hormones, chiefly thyroxine (T4) and triiodothyronine (T3). This deficit may lead to several metabolic abnormalities and clinical symptoms, such as fatigue, weight gain, cold sensitivity, dry skin, and cognitive impairments (Jain et al., 2022; Mathur, 2018; Trifanescu & Poiana, 2019). The condition can be categorized into various kinds, including primary hypothyroidism, commonly caused by autoimmune disorders like Hashimoto's thyroiditis, and secondary hypothyroidism, which arises from pituitary dysfunction (Tang et al., 2021).

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The epidemiology of hypothyroidism differs worldwide, shaped by factors like iodine deficiency, genetic predispositions, and environmental influences. The global prevalence of hypothyroidism is estimated to be between 0.2% and 5.3% in the general population, with elevated rates noted in women, especially during their reproductive years (Hu et al., 2020; Mirahmad et al., 2023). A systematic analysis revealed that the frequency of hypothyroidism varies considerably across different countries, with certain studies documenting rates as high as 36.3% in select patient categories, including individuals with non-alcoholic fatty liver disease (Taylor et al., 2018; Abboodi, 2016).

The incidence of hypothyroidism in Indonesia significantly exceeds the global average. A study indicated that the prevalence of hypothyroidism among the reproductive-age population is approximately 2%, with notable gender differences, as thyroid problems are more prevalent in women (Marimuthu & Loganathan, 2017; Pushpagiri et al., 2015). In addition, hormonal factors also contribute, where the hormone estrogen during the menstrual cycle and pregnancy can affect thyroid function (Tarigan & Siahaan, 2021). Additionally, congenital hypothyroidism screening in Indonesia has indicated concerning prevalence rates, estimated between 1:2916 and 1:1167, markedly exceeding the global average of 1:3000 to 1:4000 (Setyaningsih & Wulandari, 2022).

The diagnosis of hypothyroidism generally requires the assessment of serum thyroid-stimulating hormone (TSH) and free thyroxine (FT4) concentrations. Increased TSH levels along with diminished FT4 levels substantiate the diagnosis of primary hypothyroidism (Simonsick et al., 2009; Trifanescu & Poiana, 2019). The identification of hypothyroidism, especially in relation to autoimmune thyroiditis, has progressively depended on the assessment of thyroid peroxidase antibodies (anti-TPO antibodies). These antibodies function as a vital biomarker for the diagnosis of autoimmune thyroid disorders, such as Hashimoto's thyroiditis, a predominant cause of primary hypothyroidism. Research demonstrates that anti-TPO antibodies are detected in over 90% of individuals with Hashimoto's thyroiditis, rendering them a very sensitive marker of thyroid autoimmunity (Mehanathan et al., 2019; Patagar & Rudrappa, 2022).

Thyroid peroxidase (TPO) is an essential enzyme involved in the production of thyroid hormones, it catalyzes the iodination of tyrosine residues in thyroglobulin, enabling the incorporation of iodine into the hormone precursor, which is crucial for the synthesis of active thyroid hormones. The enzyme resides on the apical surface of thyroid follicular cells and is essential for the organification of iodide, a step crucial for appropriate thyroid function. A decrease in TPO activity might result in dyshormonogenesis, marked by an inadequate synthesis of thyroid hormones, leading to increased thyroid-stimulating hormone (TSH) levels and possible goiter development (GHasan et al., 2011). Besides its enzymatic function, TPO is acknowledged as a major autoantigen in autoimmune thyroid disorders, such as Hashimoto's thyroiditis and Graves' disease. The existence of anti-TPO indicates an autoimmune response in which the immune system TPO which is normally recognized as part of the body (self), begins to be identified by the immune system as a something foreign (non-self) (Bresson et al., 2005; Siriwardhane et al., 2018; Wright-Pascoe, 2010).

The screening for hypothyroidism in Indonesia faces several challenges, including insufficient training for healthcare providers, inadequate funding, and a focus on high-risk patients. Inefficient sample collection and management can lead to overlooked diagnoses and postponed treatment, which are crucial for mitigating hypothyroidism's negative effects, especially in newborns. Financial constraints also limit the scope and efficacy of screening initiatives, causing discrepancies in access to care across Indonesia. The screening mechanism often prioritizes high-risk patients, neglecting a significant number of cases, especially in rural or disadvantaged regions. Promptness of screening results is also a critical concern, as delays can impede timely diagnosis and treatment, especially for congenital hypothyroidism (Karunarathna & Hettiarachchi, 2021; Pulungan et al., 2021).

This study aims to characterized the 52 kDa thyroid peroxidase (TPO) protein from sera patient, measure TPO activity, and its interaction with antibodies in New Zealand White Rabbits. These findings contribute to a better understanding of TPO's immunogenic properties and support its application in detecting hypothyroidism conditions.

Method

The collection of sera patient samples received approval from the Ethics Committee of the Faculty of Medicine at Universitas Brawijaya (KEPK FKUB) under No. 187/EC/KEPK/04/2022, with tested by Anti-TPO AccuBind ELISA Microwells (Product Code: 1125-300) and Elisa RSR™ TRAb 3rd Generation (Cardiff CF14 5DU United Kingdom). Additionally, the animal studies were approved by the Research Ethics Committee of Brawijaya University under No. 215-KEP-UB-2023.

Protein Isolation

Sera patients were isolated by preparing serum and adding 0.4 M PBST-PMSF (5x volume). Sonication was performed at 400 Hz for 10 minutes, followed by centrifugation at 6000 rpm for 15 minutes at 4°C to form

a pellet and supernatant. The supernatant was transferred to a microtube, and cold ethanol (1:1 ratio) was added. After incubation at -20°C for 1 hour, centrifugation was done at 10000 rpm for 15 minutes at 4°C. The pellet was air-dried, and Tris-Cl pH 6.8 was added.

Molecular Weight Analysis

The SDS-PAGE method involves two main stages: a 12% separating gel and a 5% stacking gel are prepared. The separating gel is created by mixing water, acrylamide, Tris HCl, SDS, APS, and TEMED, while the stacking gel is made with a similar mixture. Samples are prepared with RSB and Tris HCl at pH 6.8, heated, and loaded into the wells after the gels set. Electrophoresis is performed at 150V until the samples reach the bottom of the separating gel, after which the gel is removed for staining and analysis.

Confirmation of TPO Molecular Weight by Western Blot Technique

After SDS-PAGE without staining, the gel was transferred to an NC membrane and filter paper soaked in blotting buffer and stored at 4°C. The sandwich setup, consisting of the gel, NC membrane, and filter paper, was arranged in a Mini Trans Blot Cell and transferred at a constant voltage of 30 V with a current below 85-90 mA for 7 minutes. Proteins on the membrane were examined, then blocked with 3% BSA-PBS and incubated for 1 hour at room temperature. After washing with PBST, the primary antibody (Mouse Monoclonal TPO) was added at a 1:250 concentration and incubated overnight at 4°C. Further washing with PBST was performed, and the secondary antibody (Goat anti mouse IgG AP) was added at a 1:1000 concentration, followed by another wash. BCIP-NBT substrate was added, and the resulting bands were observed. The reaction was stopped with distilled water, and the membrane was dried and documented.

Isolation of TPO with electroelution method (Aulanni'Am et al., 2018; Aulanni'am, 2023)

The gel containing the 52 kDa protein band from SDS-PAGE was subjected to electroelution. The results were cut and placed in a cellophane bag with 0.2 M phosphate buffer, which was then put into a chamber with 0.1 M phosphate buffer. Electroelution was performed at 30 amperes for 17 hours at 4°C. The results were collected from the cellophane and placed in a microtube, where cold ethanol was added in a 1:1 ratio and mixed, then frozen overnight. The mixture was centrifuged at 10,000 rpm for 15 minutes, the supernatant was discarded, and the pellet was air-dried and resuspended in Tris-Cl pH 6.8.

TPO Activity Measurement

To measure peroxidase enzyme activity using the Thyroid Peroxidase Assay Kit from Sigma-Aldrich (Cat. MAK092) using ELISA reader at a wavelength of 507 nm, with a temperature of 37°C and an incubation time of 30 minutes.

TPO Polyclonal Antibody Production (Aulanni'am, 2023)

Blood was collected from the auricular vein of rabbits, with 3 mL taken per rabbit. Intramuscular injections were administered in the right and left semimembranosus muscles at a dose of 250 µL each. The collected blood was placed in a vacutainer tube, tilted at 45˚ for 1 to 2 hours to allow separation of blood cells and serum. After centrifugation at 3000 rpm for 10 to 15 minutes at room temperature (RT), the serum was isolated.

Serum purification for IgG collection involved adding 50% SAS to the serum in a 1:1 ratio, followed by vortexing and centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected, and the process was repeated with additional SAS. The resulting pellets were resuspended in 0.2 M phosphate buffer and dialyzed for 15 hours at 4°C using 0.1 M phosphate buffer. Absolute ethanol was then added in a 1:1 ratio, and the solution was refrigerated for 24 hours. After discarding the supernatant, the pellet was dried until the ethanol odor disappeared, and Tris HCl pH 6.8 was added to each microtube.

Analysis of TPO Antibody Titer

The indirect ELISA (iELISA) was conducted by coating a microplate with 1 µg/mL antigen in carbonate-bicarbonate buffer and incubating it at 4°C overnight. After washing with PBS Tween-20, blocking buffer was added, followed by a second wash. Primary antibodies (1:200 in 1% BSA) were added and incubated for 1 hour. After another wash, secondary anti-rabbit IgG AP was added (1:2500 dilution) and incubated for 1 hour. The plate was washed, and p-NPP substrate was added, incubated in the dark for 30 minutes, followed by 3M NaOH to stop the reaction. Absorbance was measured at 405 nm, and antibody titers were analyzed in Excel.

Laboratory Sensitivity Test using Dot Blot Method

In the Dot Blot test, sera patient were diluted with coating buffer (1:10, 1:20, 1:40, and 1:80) and applied (5 µL/well) onto a nitrocellulose membrane pre-moistened with PBS. After blocking with 1% PBS BSA for 30 minutes and washing with 0.5% PBS Tween-20, primary antibodies (diluted 1:10, 1:20, 1:40, and 1:80) were added (5 µL/well) and incubated for 1 hour. Following another wash, Anti-Rabbit IgG Alkaline Phosphatase (1:2500) was applied for 1 hour, and the membrane was washed again. Western blue substrate was added for 30 minutes

in the dark, then rinsed with distilled water and dried for spot examination.

Result and Discussion

TPO is an important enzyme in the synthesis of thyroid hormones, and its characterization in this method through techniques such as SDS-PAGE and Western blotting SDS-PAGE is a technique for separating proteins based on their molecular weight (Major et al., 2015).

The determination of TPO's molecular weight was carried out using the SDS-PAGE method with a gel concentration of 12%. The protein profile revealed a 50-52 kDa protein in patient serum is thought to be the molecular weight of TPO (Fiqriyana et al., 2013). Proteins were also detected around 260 kDa, 169.10 kDa, 103.29 kDa, 80 kDa, 62.64 kDa, 70 kDa, and 25 kDa as shown in Figure 1. A molecular weight confirmation test for the TPO protein was then conducted using the western blot method.

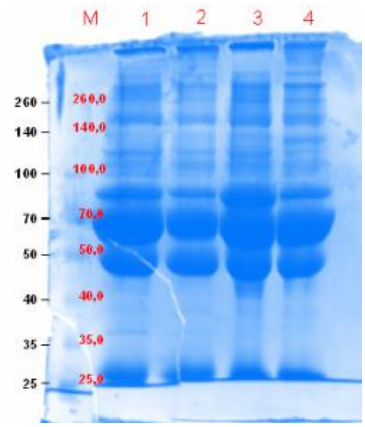

Figure 1 Protein Profile from sample using SDS-PAGE 12%

The presence of complex proteins can also affect the molecular weight. TPO, which is involved in complex enzymatic reactions appears at 52 kDa, because it can be part of a larger protein complex or have several subunits (Meisrimler et al., 2014).

After SDS-PAGE, the next method is western blotting which is used to confirm the molecular weight of TPO from SDS-PAGE by providing specificity through the use of antibodies. This method involves transferring proteins from the SDS-PAGE gel to a membrane, followed by incubation with primary antibodies and detection using secondary antibodies conjugated with enzymes (Sule et al., 2023).

In the western blotting method, patient serum as the antigen reacted with TPO monoclonal antibodies, with a positive antigen-antibody reaction indicated by the appearance of a purple-blue stain (Figure 2). This result confirmed a positive reaction of the antigen against TPO monoclonal antibodies, visualized as a purplish-blue band in the 50-52 kDa region (Fiqriyana et al., 2013). This band is assumed to be the TPO protein. TPO appearing at around 50-52 kDa in Western blot analysis can be influenced by the specific antibodies used in the assay. The use of TPO monoclonal antibodies has been shown to produce specific bands corresponding to TPO, assists in the differentiation of TPO from other peroxidases present in thyroid tissue (Jankovic et al., 2021).

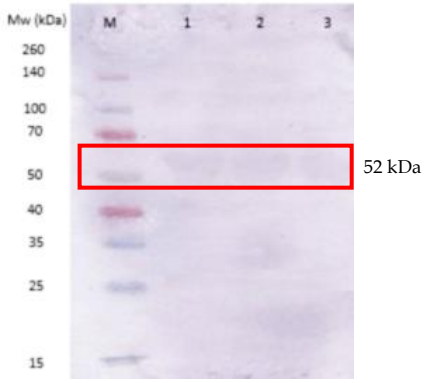

Figure 2 The Result of confirmation of TPO by Western Blotting

The activity of the TPO enzyme from sera patients was measured at a wavelength of 507 nm, with a temperature of 37 °C and an incubation time of 30 minutes, using the colorimetric method. The results obtained can be seen in Table 1.

Table 1 Average of TPO Activity From Sera Patient

Sample	Average of TPO Activity (U/mL)	Standard Deviation
Normal	31.34	0.155
(+) Hypothyroidism	49.46	23.56

In the normal sample, the average of TPO activity is 31.34 U/mL with a standard deviation of 0.155. This indicates that in the normal group, TPO activity is relatively low, and there is minimal variation between samples. This low TPO activity may indicate stable thyroid gland function without any autoimmune conditions affecting the gland. While in the positive hypothyroid sample, average of TPO activity increases to 49.46 U/mL with a standard deviation of 23.56. The high standard deviation suggests greater variation between samples in this group. The positive sample's increased TPO activity could suggest a heightened immune reaction to the TPO enzyme, a common occurrence in autoimmune thyroid conditions (Capuzzo, 2021; Jafaar & Meena, 2016).

High TPO activity in the body with autoimmune conditions, such as Hashimoto's thyroiditis, there is an increase in anti-TPO antibody levels that can damage

thyroid cells and interfere with the process of thyroid hormone synthesis (Shimizu et al., 2020). In the case of Hashimoto's thyroiditis, increased anti-TPO antibodies actually contribute to decreased thyroid hormone production, indicating that high TPO enzyme activity does not always correlate with increased thyroid hormone production (Plesker & Hintereder, 2021). Factors that influence TPO activity include environmental conditions, such as temperature and pH, which can influence the stability and activity of the enzyme (Major et al., 2015).

To prove that TPO is immunogenic by titer measurement method with indirect ELISA method at 405 nm wavelength using ELISA reader. The principle of the ELISA method works by coating the microplate with TPO, followed by the addition of antibodies. The TPO will bind to the coated antigen. Secondary antibodies, conjugated to enzymes, are then added and will bind to the primary antibody. The enzymatic reaction produces a measurable signal, usually a color change (Eick et al., 2020).

In this method, we have collected 10 antibodies against TPO induction in rabbits. The highest titer measurement aimed to determine at which blood sampling the highest TPO antibody production occurred. The results can be seen in the Table 2 and Figure 4.

Table 2 Absorbance Value of TPO Antibody

Week-…	Absorbance Value (405nm)
Pre-imun	0.152
Bleeding 1	0.33
Bleeding 2	0.401
Bleeding 3	0.477
Bleeding 4	0.421
Bleeding 5	0.393
Bleeding 6	0.475
Bleeding 7	0.494
Bleeding 8	0.555
Bleeding 9	0.473
Bleeding 10	0.412

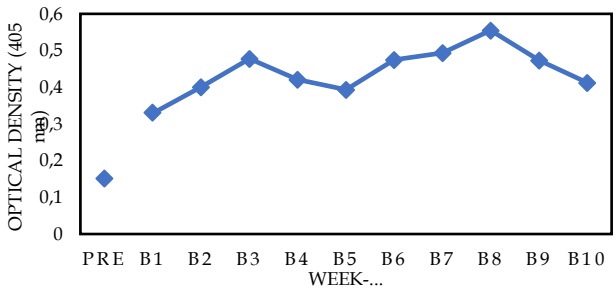

Figure 3 TPO Antibody Titer

From the first to the tenth week, the antibody levels in the rabbits fluctuated, as shown in Figure 3. The highest antibody titer after the first booster was in the 3rd week and in the 8th week after second booster. This because the antigen levels in the rabbit's body reached their highest point so that the antibodies produced also reached their highest levels after the booster. After comparing the highest titers in the first booster and the second booster, it can be seen that the highest antibody titer occurred in the 8th week after the second booster with an absorbance value of 0.555. This is caused by the number of memory B cells and plasma B cells being greater than after the first booster so that more B cell proliferation occurred and the immunoglobulin G produced was also high (Darwin, 2021).

The immune response involving IgG is characterized by the production of TPO antibodies occurring several days after contact with the antigen. IgG responses are faster and more long-lasting, with TPO antibodies having higher titers, better specificity, and greater binding affinity. In secondary responses, following a booster injection, TPO antibody production is faster, with higher titers and greater affinity. This booster injection, also known as the anamnestic response, is driven by memory cell stimulation, and the addition of adjuvants can enhance antibody titers and prolong the duration of the immune response (Delahaut, 2017).

In the laboratory sensitivity test using dot blot for TPO and anti-TPO. The dot blot method is a valuable technique for detecting TPO antibodies due to its high sensitivity. This approach enables the visualization of antibodies bound to the TPO antigen on the membrane. The accuracy of this test is further enhanced by the use of an enzyme-linked immunosorbent assay (ELISA) to measure TPO antibodies, which can complement the results obtained from the dot blot method (Abdrabo et al., 2022; Dalia et al., 2022). Figure 5, a purple-colored spot is visible, and its density was measured using ImageJ software, as shown in Table 3.

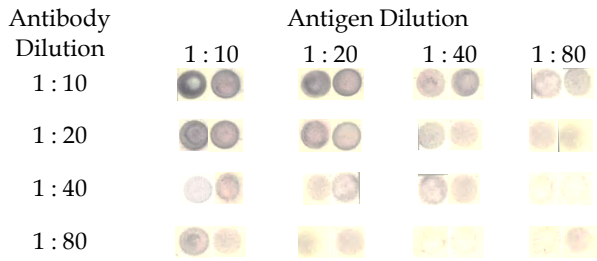

Figure 4 Laboratory Sensitivity Test of TPO and Anti-TPO using Dot Blot Method

Table 3 Intensity of TPO and Anti-TPO by ImageJ Software

Antibody Dilution	Antigen Dilution			
	1:10	1:20	1:40	1:80
1:10	40772.26	33313.44	22014.77	13444.44
1:20	40707.34	27634.98	11256.43	9550.83
1:40	18853.61	14676.74	10968.11	527.44
1:80	15980.99	5234.80	527.43	272.894

In this method, antigen dilutions of 1:10, 1:20, 1:40, and 1:80 were carried out, as well as antibody dilutions of 1:10, 1:20, 1:40, and 1:80. A mixture of antigen dilution 1:10 and antibody 1:10 produced the highest color intensity and the most concentrated purple color with an intensity value of 40772.26. Conversely, antigen and antibody dilution 1:80 produced the lowest color density with the brightest color with an intensity value of 272,894.

The dot blot test operates on the principle of applying proteins to a membrane, followed by analysis with specific antibodies. This method allows for the measurement of TPO antibody levels, which is crucial for diagnosing autoimmune thyroid conditions. The sensitivity of the dot blot test can be enhanced by optimizing reagents and detection conditions, ensuring that even low levels of TPO antibodies can be accurately detected (Qi et al., 2018; Taesuji et al., 2022).

The color reaction in antigen and antibody dilutions showed that TPO and anti-TPO in small amounts could still bind and react. Therefore, it can be concluded that TPO and anti-TPO can still be detected at a dilution of 1:80 (Nurmahdi et al., 2013). Factors that influence antigen-antibody interactions include antigen-antibody ratio, pH, electrolyte concentration, temperature, and time (Aulanni'am, 2023).

Conclusion

The conclusion of this study is that TPO isolated from patient serum has a molecular weight of 52 kDa with TPO enzyme activity in normal patient samples having an average TPO activity value of 31.34 U/mL, while in positive hypothyroid patients the average TPO activity value was 49.46 U/mL. TPO is immunogenic, as evidenced by the highest antibody production in the 8th week after second booster with an absorbance value of 0.555. Then the anti-TPO has high sensitivity, where at a dilution of 1:80 it still detects the TPO from sera patient.

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Author Contributions
All authors contributed the article and approved the submitted version.

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Conflicts of Interest
The authors declare no conflict of interest.

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