

การตรึงเอนไซม์ไทโรซิเนสบนแผ่นเมมเบรนโพลีอีเธอร์ซัลโฟนและการประยุกต์ใช้

The immobilization of tyrosinase enzyme on polyethersulfone membrane and its applications

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บทคัดย่อ

การวิเคราะห์ประสิทธิภาพของเอนไซม์ไทโรซิเนสที่ตรึงบนแผ่นเมมเบรนและวิธีวิเคราะห์หาปริมาณของสารยับยั้งเอนไซม์ไทโรซิเนสที่เหมาะสมด้วยเทคนิคทางโครมาโทกราฟีได้ถูกพัฒนาขึ้น ประสิทธิภาพของเอนไซม์ไทโรซิเนสที่ถูกตรึงบนแผ่นเมมเบรนวัดได้จากการย้อมติดสีดำของสารผลิตภัณฑ์เมลานินบนแผ่นเมมเบรนซึ่งเกิดจากการเร่งปฏิกิริยาสารละลายแอล-โดปาโดยเอนไซม์ไทโรซิเนส จากการศึกษาพบว่า การตรึงเอนไซม์ทำได้โดยง่ายด้วยการปิเปตต์เอนไซม์ลงบนแผ่นเมมเบรนโพลีอีเธอร์ซัลโฟน สารประกอบคอปเปอร์ซัลเฟตมีผลเพิ่มประสิทธิภาพของเอนไซม์ที่ถูกตรึง สารไตรเมทิลไฮโดรควิโนนยับยั้งประสิทธิภาพของเอนไซม์ไทโรซิเนสที่ถูกตรึงสังเกตเห็นเป็นวงสีขาวบนพื้นดำ ความสามารถในการยับยั้งนี้ขึ้นกับความเข้มข้นของไตรเมทิลไฮโดรควิโนน การวิเคราะห์ความสามารถของไตรเมทิลไฮโดรควิโนนในการยับยั้งเอนไซม์ด้วยเทคนิคทางโครมาโทกราฟีทำได้โดยวัดขนาดของวงด่างด้วยเครื่อง Gel doc และเทคนิคดังกล่าวสามารถนำมาประยุกต์ใช้ในการวิเคราะห์หาปริมาณของไตรเมทิลไฮโดรควิโนน โดยวัดความสามารถในการยับยั้งประสิทธิภาพของเอนไซม์ไทโรซิเนสที่ถูกตรึงบนแผ่นเมมเบรน ซึ่งจากเทคนิคที่พัฒนาขึ้นนี้สามารถตรวจวัดปริมาณของไตรเมทิลไฮโดรควิโนนได้ในช่วงความเข้มข้น 40 ถึง 80 ไมโครกรัม

Abstract

On-membrane analysis of tyrosinase activity and chromatographic detection of tyrosinase inhibitor was developed and optimized. The activity of immobilized tyrosinase was determined from the conversion of L-dopa to melanin staining on polyethersulfone (PES) membrane. Tyrosinase could be immobilized on PES membrane by simply using the pipetting technique. Copper sulfate was found to enhance the immobilized tyrosinase activity. Trimethylhydroquinone (TMHQ) inhibited immobilized tyrosinase activity and was observed as white spots in contrast to the dark background of stained melanin on the PES membrane. The sizes of inhibitory zones were quantified by using Gel Doc. The tyrosinase inhibitory effect of TMHQ was concentration dependent. TMHQ at the concentration range of 40–80 g could be detected by using an optimized on-membrane chromatographic detection of tyrosinase activity on PES membrane.

คำสำคัญ: ไทโรซิเนส, การตรึงเอนไซม์, เมมเบรนโพลีอีเธอร์ซัลโฟน

Keywords: Tyrosinase, Immobilization, Polyethersulfone membrane.

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Introduction

Tyrosinase enzyme (EC 1.14.18.1) belongs to a large group of proteins named type-3 copper proteins (Dur n et al., 2002). The inhibition of tyrosinase activity will lead to the inhibition of melanin formation as it is a key enzyme in the melanin biosynthetic pathway (Briganti et al., 2003). The inhibition of melanin formation results in a whitening effect, therefore many research studies have taken place for a new safe whitening compound. Generally tyrosinase enzyme activity is tested *in vitro* from the reaction of a mixture of enzymes and its substrates, L-Dopa or L-tyrosine. To test the tyrosinase inhibitory effect, the experiment can be performed by incubating the tyrosinase inhibitor with the reaction mixture of tyrosinase and its substrates (Jeon et al., 2005) or by treating the melanoma cell lines directly with the tyrosinase inhibitors (Usuki et al., 2003). However, these methods either require several operations or are expensive.

Various methods exist for enzyme immobilization on solid supports. The immobilization of enzymes on a membrane can be divided into two main categories: (i) physical methods based on molecular interactions between enzyme and support, and (ii) chemical methods based on the formation of covalent bonds. The supports and the methods for enzyme immobilization were studied to ensure the highest retention of enzyme activity, its stability and durability (Arica et al., 2004). As an example, tyrosinase was immobilized on polyethersulfone (PES) membrane for the development of a new amperometric biosensor to detect the phenolic compounds (Climent et al., 2001). PES membrane has been widely used for ultrafiltration systems (Duarte et al., 2003) and enzyme immobilization (Gomes et al., 2004). PES was also used in an on-membrane quantitative analysis system for

glycyrrhizin in licorice roots and traditional Chinese medicines (Morinaga et al., 2005a). Similarly, it was used in the detection and quantification of ginsenoside Re in ginseng samples via an immunostaining method using monoclonal antibody against ginsenoside Re (Morinaga et al., 2006). PES membrane was shown to be successfully used for the eastern blotting of steroidal saponins in an extract of *Panax quinquefolium* (Morinaga et al., 2005b). The existence of positive charge and active aldehyde groups of the PES membrane let it react with free amino groups, which allows covalent bonding of proteins, peptides, or other amino-containing compounds to the membrane.

In our study, the PES membrane was selected and used as a solid support for the reaction between the tyrosinase enzyme and L-dopa. This method was developed for the chromatographic detection of tyrosinase inhibitor based on on-membrane analysis and aimed at facilitating the detection of tyrosinase inhibitor where instruments, time, cost and human resources are limited.

Experimental

1. Materials

Mushroom tyrosinase was purchased from Fluka (Switzerland). The 3,4-dihydroxy-L-phenylalanine (L-dopa) was bought from Sigma (USA). Analytical grade trimethylhydroquinone (TMHQ) was purchased from Fluka (Switzerland) and dissolved in methanol as a stock solution. PES membrane was distributed by Pall Corporation (USA). Gel doc from SYNGENE Bio IMAGING (England) and UV-Vis spectrophotometer from Shimadzu 1240 mini (Japan) were used for the tyrosinase activity studies. Other substances and solvents were analytical grade and were used without further purification.

2. Immobilization of tyrosinase on PES membrane

The immobilization of enzyme on PES membrane by the pipetting technique has been previously reported (Climent et al., 2001). Therefore, we adopted this technique and further determined factors that might enhance the activity of immobilized tyrosinase. Many factors such as copper sulfate, skim milk, tween-phosphate buffer solution (T-PBS), and temperature were independently tested on the immobilization process at different steps and compared with the untreated membranes.

The on-membrane analysis of tyrosinase activity was performed via two steps. In step 1, twenty microliters of tyrosinase enzyme (from the stock concentration of 678 units/ml) was spotted on PES membrane and left for 2 hr at room temperature (30°C). In step 2, the immobilized tyrosinase was soaked in 2 ml of L-dopa solution (stock concen-

tration of 10 mM) for 1 hr with continuous shaking at room temperature. The immobilized tyrosinase catalyzed L-dopa led to dopachrome which was further converted to melanin product. The formation of melanin in solution was measured by UV-Vis spectrophotometer at a wavelength of 400 nm (Choi et al., 2004), whereas the intensity of melanin stained on the membrane was measured by Gel doc. To compare the activity of immobilized enzyme with free enzyme, the same concentration of tyrosinase enzyme on the membrane and same concentration of L-dopa were mixed in the test tube and the melanin formation was measured after 1 hr by using a UV-Vis spectrophotometer. The effect of each factor on the enzyme immobilization process was performed with the similar method mentioned above and summarized in Figure 1.

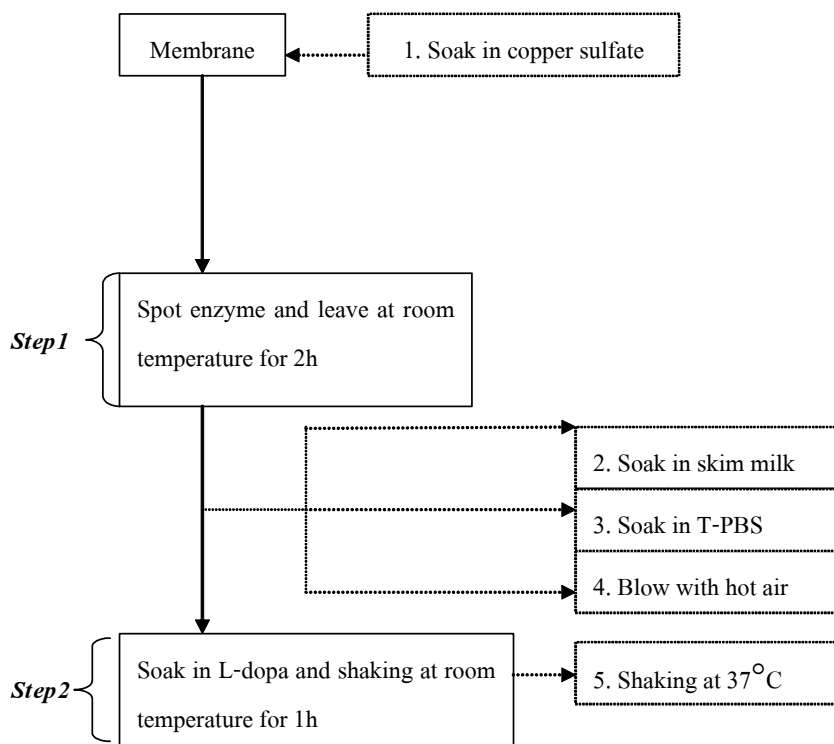


Figure 1. Schematic diagram represented the techniques used in immobilization of tyrosinase enzyme on PES membrane.

2.1. Effect of copper sulphate on tyrosinase immobilization on PES

Immobilization of tyrosinase onto the polyethyleneimine-grafted and Cu (II) chelated poly (HEMA-co-GMA) membrane was enhanced by the Cu (II) ion (Arica et al., 2004). Therefore, copper sulfate was studied to determine whether it enhanced the activity of immobilized tyrosinase on PES membrane. The experiment was conducted by treating PES membrane with copper sulfate solution (100 ppm) for 1 hr with continuous shaking at room temperature. After the PES was dried under an atmosphere for 30 min, the enzyme immobilization process steps 1 and 2 were performed and compared to the untreated membrane. Then the melanin formation in solution was measured by the UV-Vis spectrophotometer, while the melanin intensity stained on PES membrane was measured by the Gel doc.

2.2. Effect of skim milk on tyrosinase immobilization on PES

Skim milk was used to reduce the non-specific binding that might occur on the PES membrane (Morinaga et al., 2006). After the tyrosinase was spotted on PES membrane (step 1), skim milk solution (5%w/v) in 0.1 M phosphate buffer (PBS) pH 6.8 was used to soak the immobilized enzyme for 1 hr with continuous shaking at room temperature. After the membrane was dried under an atmosphere, it was further soaked in L-dopa solution (step 2). The membrane treated with skim milk was compared with the untreated membrane. Finally, the melanin formation in solution and that stained on membrane were measured respectively for absorbance and intensity.

2.3. Effect of T-PBS on tyrosinase immobilization on PES

Generally, T-PBS has been used along with bovine serum albumin or skim milk to wash the excess amount of the protein from the membrane (Morinaga et al., 2005a). Thus, T-PBS was also tested for its influence on tyrosinase immobilization in this study. After spotting tyrosinase enzyme on PES membrane (step 1), the membrane with immobilized enzyme was soaked twice with 0.1 M PBS pH 7.4 containing 0.05 % v/v of tween for a duration of 30 min each time with continuous shaking at room temperature. After the membrane was dried under an atmosphere, the membrane was soaked in L-dopa solution for another 1 hr (step 2). The membrane untreated with T-PBS was also tested for comparison. The absorbance and intensity of melanin formation were measured.

2.4. Effect of temperature on the drying of membrane with immobilized enzyme in step 1

Based on the method optimization, the enzyme on PES membrane was left to dryness for 2 hr at room temperature which was not an appropriate microenvironment for the enzyme due to the lack of water content to restore the structure and function of the enzymes. Therefore, the effect of temperature was also tested to determine whether it influenced the immobilized tyrosinase activity. Tyrosinase was spotted on PES membrane (step 1), the tyrosinase allowed to be adsorbed on PES for 2 hr, and then blown to dryness within a few seconds. Then the membrane was soaked in L-dopa solution (step 2). The un-blown membrane was also evaluated for comparison.

2.5. Effect of temperature on substrate catalysis by immobilized tyrosinase in step 2

To determine whether the temperature affects the activity of immobilized enzyme in catalyzing its substrate, the immobilized membrane from step 1 was continuously soaked in L-dopa solution for 1 hr in a water bath (37°C) compared to the immobilized membrane which was soaked in L-dopa solution at room temperature (30°C). Then the melanin absorbance and melanin intensity of stained melanin on PES membrane were recorded and compared.

3. Chromatographic detection of tyrosinase inhibitor on PES membrane

The optimization of on-membrane analysis of tyrosinase activity was used and applied for the chromatographic detection of tyrosinase inhibitor. TMHQ is a hydroquinone derivative which possesses a tyrosinase inhibitory effect. Different concentrations of TMHQ have been chromatographically detected on PES membrane. The experiment was performed by loading the TMHQ solution on PES membrane as spots 1 to 5 corresponding to the concentrations of 40, 50, 60, 70 and 80 g, respectively. Then the membrane was developed with acetonitrile: water: formic acid (50:50:2 by volume). The developed membrane was continuously treated with copper sulfate, enzyme and L-dopa solutions according to step 1 and step 2 as mentioned above. All experiments were performed at room temperature. The membrane stained with melanin was completely dried by hot air to stop the reaction between immobilized enzyme and its substrate. Finally the membrane was photographed and analyzed by Gel doc. All experiments were done in triplicate consecutively within one day.

Results and Discussion

Our study demonstrated the immobilization of tyrosinase enzyme on PES membrane as observed from the stained melanin on membrane where the tyrosinase enzyme was spotted (Figure 2). The melanin absorbance of free enzyme was considered as 100% enzyme activity and the absorbance of melanin formation from the immobilized enzyme was calculated as enzyme activity compared with free enzyme.

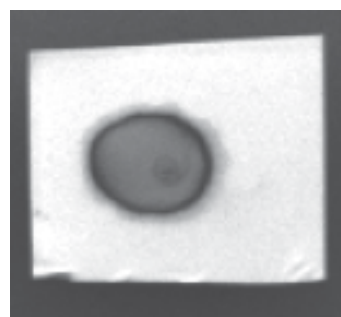


Figure 2. The melanin spot on the PES membrane after spotting tyrosinase, soaked in L-dopa solution for 1 h at room temperature and dried at room temperature. The melanin spot was photographed with Gel doc.

To confirm that the immobilized enzyme had not leaked out from the membrane during the experiment, the immobilized tyrosinase enzyme was soaked and continuously shaken in PBS for 1 hr. Then, L-Dopa solution was added into the PBS, and the melanin formation in the solution mixture was measured by using UV-Vis spectrophotometer. The control study was also performed by using free enzyme to catalyze the L-dopa solution. Results showed that the absorbance of melanin in the solution mixture of PBS-L-Dopa solution mixture was 0.25% compared to the melanin absorbance catalyzed by the free enzyme. This result indicates that the enzyme was still intact and had not leaked from the membrane.

Another study was also performed by soaking the immobilized tyrosinase enzyme in the L-Dopa solution. We found that melanin was formed and existed as solution as well as stained on the PES membrane. Thus, we had to measure these two forms of melanin formation via two ways. The absorbance of melanin in the solution was measured by UV spectrophotometer and the stained melanin on PES was measured by using Gel doc.

1. Effect of copper

The results in Figure 3 show that the membrane treated with copper sulfate significantly increased the activity of immobilized tyrosinase and melanin intensity on PES. The activity of immobilized tyrosinase increased from 1.15% to 21.85% in the absence and presence of copper sulfate respectively. The melanin intensity on PES increased from 2,420,245 to 3,123,857 in the absence and presence of copper sulfate, respectively. Our result demonstrates that the Copper (II) ion enhances the binding of tyrosinase onto PES, which might be due to Copper (II) ion maintaining the structural feature of the tyrosinase active binding site (Arica et al., 2004).

Our results are similar to those of the study of Arica et al. (2004) who proved that the incorporation of Cu^{++} ions onto poly (HEMA-co-GMA)-PEI membrane significantly increased tyrosinase adsorption capacity of the membrane from 19.3 to 26.6 mg/m^2 due to the epoxide group of the membrane being able to form covalent linkages with different side chains of proteins. This study was the first to report using copper sulfate on a PES membrane to enhance the activity of immobilized tyrosinase enzyme.

2. Effect of skim milk

Skim milk did not enhance the activity of immobilized enzyme since the immobilized enzyme activity and melanin intensity were 5.57 and 1,149,868 respectively in the absence of skim milk compared to 3.49 and 936,743 respectively in the presence of skim milk (Figure 4). The skim milk might wash out or block the tyrosinase enzyme on PES membrane from catalyzing the L-dopa solution. Therefore, the immobilized enzyme activity and intensity resulting from immobilized enzyme treated with skim milk were less than from the untreated immobilized enzyme.

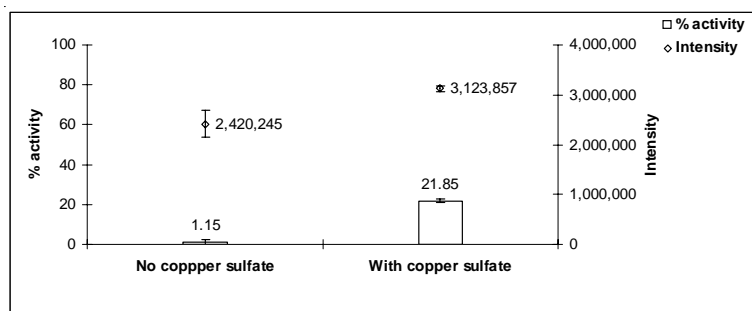


Figure 3. Effect of copper sulfate to tyrosinase immobilization on PES membrane. Percent activity of the immobilized tyrosinase was calculated in comparison to the activity of the mobilized enzyme. The melanin absorbance from UV reading in the absence and presence of copper sulphate are represented by bar graph using the left Y-axis. While the melanin intensity stained on membrane detected by Gel doc is also plotted on the same graph using the right Y-axis.

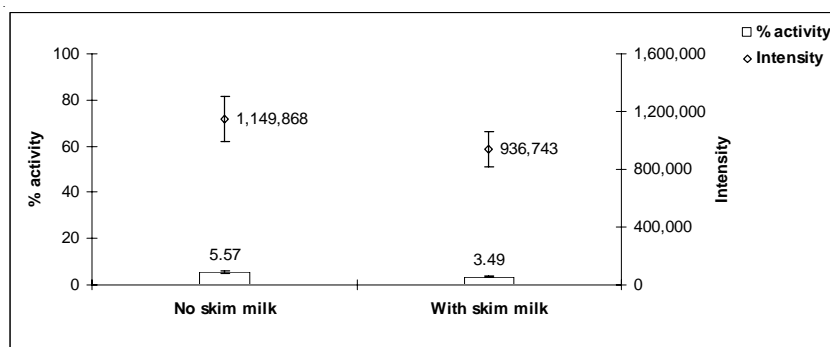


Figure 4. Effect of skim milk to tyrosinase immobilization on PES membrane. Percent activity of the immobilized tyrosinase was calculated in comparison to the activity of the mobilized enzyme. The melanin absorbance from UV reading in the absence and presence of skim milk are represented by bar graph using the left Y-axis. While the melanin intensity stained on membrane from Gel doc is also plotted on the same graph using the right Y-axis.

3. Effect of T-PBS

The immobilized enzyme activity and melanin intensity on the membrane were 2.09 and 1,656,462 respectively in the absence of T-PBS compared to 1.47 and 1,565,392 respectively in the presence of T-PBS (Figure 5). T-PBS might wash out or block the tyrosinase enzyme on PES membrane when the immobilized enzyme was soaked in the T-PBS solution. Therefore, skim milk and T-PBS did not enhance the immobilized enzyme activity on PES membrane. Hence, skim milk and T-PBS were not further used for tyrosinase immobilization on PES membrane.

4. Effect of temperature on the drying of immobilized enzyme in step 1

After the tyrosinase was allowed to be adsorbed on PES for 2 hr, the hot air from the blower was blown onto the membrane aiming to dry the membrane prior to soaking into the L-dopa solution. Figure. 6 illustrates the decrease of immobilized enzyme activity and melanin intensity on the membrane. The immobilized enzyme activities were decreased

from 2.62 to 1.91 in the absence and in the presence of hot air, respectively. The melanin intensity decreased from 1,512,784 to 1,348,634 in the absence and in the presence of hot air, respectively. Therefore, this hot air might denature immobilized enzyme. Thus, for drying the immobilized membrane, the membrane was just left to dryness at the room temperature.

5. Effect of temperature on substrate catalysis by immobilized tyrosinase

Figure 7 shows that the immobilized enzyme activity and melanin intensity on the membrane also decreased from 2.63% and 1,555,340 respectively when the immobilized enzyme was soaked in L-dopa at room temperature (30°C), compared to 1.92% and 1,430,433 respectively when the immobilized enzyme was soaked in L-dopa solution in a water bath (37°C). Thus, the temperature also had an effect on the immobilized enzyme in catalyzing its substrate. Therefore, the enzyme immobilization process must be conducted at room temperature or at a temperature not exceeding 30°C.

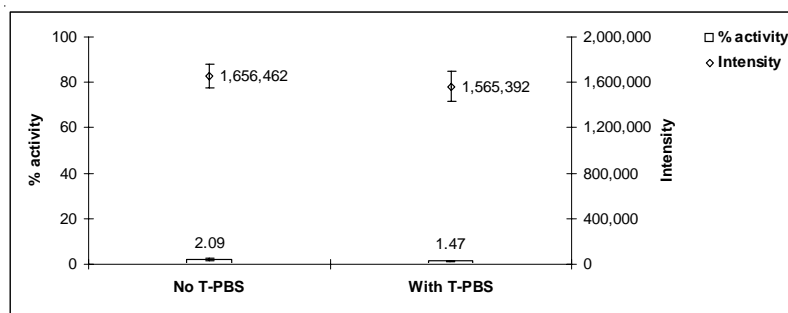


Figure 5. Effect of T-PBS to tyrosinase immobilization on PES membrane. Percent activity of the immobilized tyrosinase was calculated in comparison to the activity of the mobilized enzyme. The melanin absorbance from UV reading in the absence and presence of T-PBS are represented by bar graph using the left Y-axis. While the melanin intensity stained on membrane from Gel doc is also plotted on the same graph using the right Y-axis.

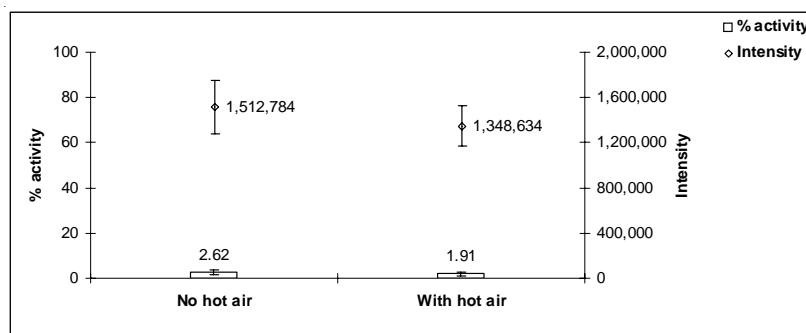


Figure 6. Effect of temperature on drying step of tyrosinase immobilization on PES membrane. Percent activity of the immobilized tyrosinase was calculated in comparison to the activity of the mobilized enzyme. The melanin absorbance from UV reading in the absence and presence of hot air are represented by bar graph using the left Y-axis. While the melanin intensity stained on membrane from Gel doc is also plotted on the same graph using the right Y-axis.

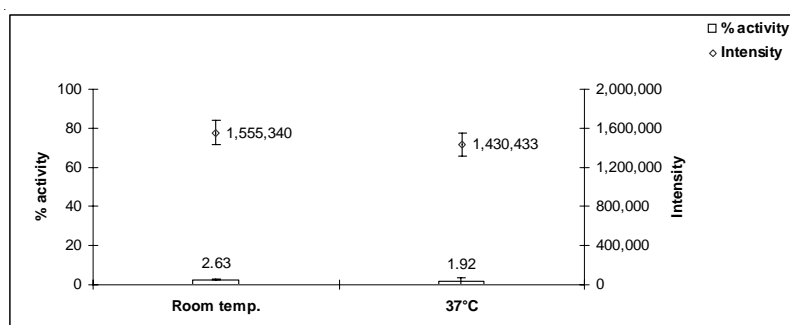


Figure 7. Effect of temperature on substrate catalysis by immobilizes tyrosinase. Percent activity of the immobilized tyrosinase was calculated in comparison to the activity of the mobilized enzyme. The melanin absorbance from UV reading in the different conditions is represented by bar graph using the left Y-axis. While the melanin intensity stained on membrane from Gel doc is also plotted on the same graph using the right Y-axis.



Figure 8. Chromatographic detection of TMHQ on PES membrane. Spot 1–5 indicate the inhibitory zone of TMHQ on tyrosinase activity with the loaded amount of 40, 50, 60, 70 and 80 g, respectively.

6. Chromatographic detection of tyrosinase inhibitor

A photograph of the chromatographic detection of TMHQ on PES membrane (Figure.8) was analyzed by Gel doc. TMHQ is a hydroquinone derivative and was tested previously in our laboratory for its tyrosinase inhibitory effect. TMHQ inhibited tyrosinase activity with IC_{50} of 31.67 ± 0.87 g/ml.

Thus, TMHQ was used as a representative of hydroquinone for chromatographic detection of tyrosinase inhibitor. The white spots caused from the inhibitory effect of TMHQ on immobilized tyrosinase were observed and measured. The standard curve between the concentrations against the inhibition zones was plotted. The chromatographic detection of TMHQ on PES membrane showed inhibition zones that increased gradually according to the TMHQ concentration. The average of TMHQ inhibition zones from three separate experiments on different membranes was calculated and is shown in Table 1. The standard curve of TMHQ was plotted between the concentrations against the average for the inhibition zone. Under these conditions, the full linear range of the assay was extended from 40 to 80 g as shown in Figure. 9.

High variations between replicates from membrane to membrane might occur from many factors such as the techniques of applying spot, edge effects from the chromatographic technique, or uneven temperature on each day. To minimize this limitation, a new standard curve should be prepared at each time of determination.

Table 1. The average of TMHQ inhibitory zone from three different membranes analyzed by Gel doc corresponds to each concentration.

Concentration per spot (g)	Average of inhibition zone	% RSD
40	401 132.08	32.97
50	671 80.91	12.06
60	786 65.45	8.32
70	909 94.68	10.41
80	1024 129.81	12.67

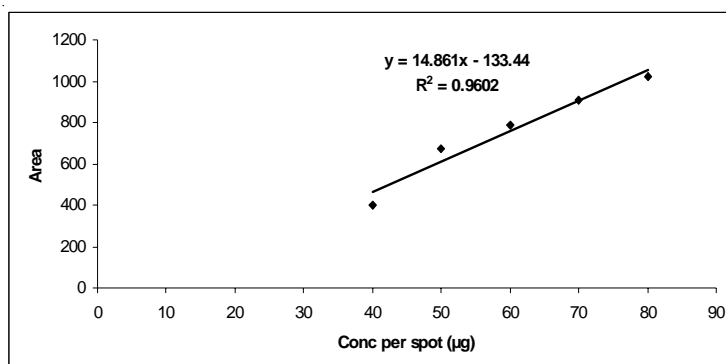


Figure 9. Standard curve of TMHQ inhibitory effect on immobilized tyrosinase plotted between the concentrations against the averages of TMHQ inhibitory zones of three different membranes performed consecutively within one day.

Conclusion

Tyrosinase enzyme was successfully immobilized on PES membrane simply by the pipetting technique. The pre-treatment of PES membrane by copper sulphate enhanced the immobilized tyrosinase enzyme activity. Each step of experiment can be performed at room temperature and should not exceed 30°C. This information is useful for the development of a screening technique for tyrosinase activity or the on-membrane analysis of tyrosinase inhibitor. Apparently our study is the first report of the chromatographic detection of tyrosinase inhibitor on PES membrane using the immobilized tyrosinase enzyme. The TMHQ standard is a tyrosinase inhibitor model for further development of on-membrane analysis of other tyrosinase inhibitor via chromatographic detection.

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