Chem. Anal. (Warsaw), **48**, 283 (2003)

Polarographic Determination of Tyrosinase Activity in Enzymatic Reaction with Tyrosine as a Substrate

by Józef Hurek*, Kornel Nowak and Beata Gąsowska

Institute of Chemistry, University of Opole,
ul. Oleska 48, 45–052 Opole

Key words: tyrosinase, polyphenol oxidase, electrochemical detection

A voltammetric investigation of tyrosinase activity is proposed as an alternative to the spectrophotometric method. Voltammetric approach provides low detection limit, high sensitivity and resistivity to the solution inhomogenity, as well as enables one to simultaneously monitor the dioxygen consumption in the reaction, which is an additional indicator of the tyrosinase enzymatic activity.

W pracy przedstawiono metodę woltamperometryczną jako alternatywną do spektrofotometrycznej metody badania aktywności tyrozynazy. Charakteryzuje ją niska granica oznaczalności, wysoka czułość oraz niewrażliwość na niejednorodność roztworów. Wskazano ponadto na możliwość równoczesnego pomiaru zużycia tlenu wskutek reakcji jako dodatkowego wskaźnika aktywności enzymatycznej tyrozynazy.

* Corresponding author.

Tyrosinase (EC 1. 14. 18. 1; phenolase, poliphenol oxidase) is a versatile, copper-containing enzyme present in the majority of living organisms — from microorganisms to mammals [1,2]. Tyrosine appears mostly in melanocytes, the cells that produce pigments. Moreover, this enzyme is responsible for the browning of fruits and plants, which unfavourably lowers their quality [3–5]. Its activity is often altered in the cases of skin cancer in higher organisms.

Tyrosinase inhibitors are of therapeutic importance since they can regulate some disturbances in pigmentation. Also in cosmetics they are used as additives in the preparations for skin whitening [6,7]. Hence it appears clear that the investigations of tyrosinase, its inhibitors, and also research and improvement of activity determination methods are of great importance.

In the presence of dioxygen tyrosinase catalyses two processes: hydroxylation of monophenols to orthodiphenols and oxygenation of orthodiphenols to the respective orthoquinones (Fig. 1). These two processes are usually examined in order to estimate the tyrosinase activity and therefore tyrosine and L-DOPA are mostly used as the substrates [8–10]. The studies on tyrosinase activity usually involve spectrophotometry [8,10,11]. However, the reaction mixture tends to become turbid. Moreover, it is almost impossible to determine selectively a single reaction product. Therefore the polarographic (or voltammetric) method [12] seems promising for the determination of tyrosinase activity.

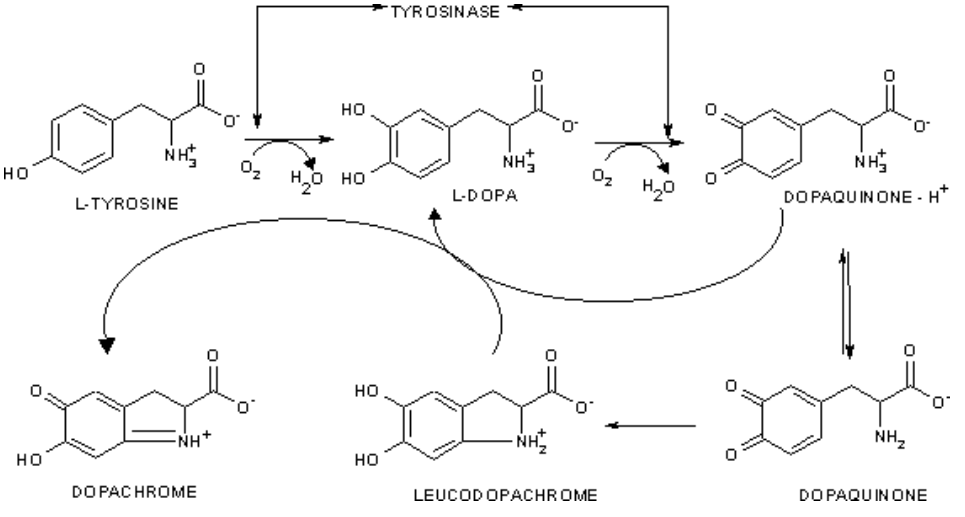

Figure 1. The scheme of tyrosinase-catalysed reaction sequence from tyrosine to dopachrome

Quinones can be reversibly reduced at the mercury electrode, at the potential of *ca* –0.2 V, and their current responses are easy to measure, which has been already employed for the construction of the tyrosinase amperometric biosensors [13–16].

In this paper the predominance of the polarographic (voltammetric) methods of tyrosinase activity determination over the spectrophotometric ones has been proved.

EXPERIMENTAL

Reagents

Tyrosinase (Mushroom tyrosinase; EC 1.14.18.1), Sigma Chemical What. (USA). Tyrosine, POCh – Gliwice (PL). Sodium phosphate ($Na_2HPO_4 \cdot 2H_2O$), anal. grade, POCh Gliwice.

Sodium phosphate ($NaH_2PO_4 \cdot 2H_2O$), anal. grade, POCh Gliwice. Mercury (Hg), anal. grade, POCh Gliwice.

Apparatus

Beckman Spectrophotometer, Model DU 640B coupled with JULABO thermostat. Spectrophotometer Specord M40 (Germany). Pulse polarograph PP–04, Unitra Telpod Cracow, equipped with the digital data acquisition mode. Digital pH-meter CP 215, Elmetron Zabrze. Automatic micropipettes LABMATE; 10–100 μL and 20–200 μL.

Methods

Enzymatic reaction. Enzyme and substrate (tyrosine) were dissolved in the phosphate buffer (0.1 mol L^{-1} N_2HPO_4/NaH_2PO_4), pH = 6.8. Enzyme activity was determined in 2.6 mL of the reaction mixture, initially containing tyrosine (concentrations ranging from 0.05 mmol L^{-1} to 0.15 mmol L^{-1}); phosphate buffer 0.1mol L^{-1}, pH = 6.8 and 20 μL of the enzyme (tyrosinase) added as the last component. Reaction was carried out at the fixed temperature 25°C.

Spectrophotometric measurement. The gradual dopachrome formation in time was monitored with spectrophotometer at the wavelength of 475 nm. The absorbance was measured for 10 min to let its values be better stabilised.

Polarographic measurements. A two-electrode arrangement with calomel electrode as a reference and HMDE or MD–DME as an indicatory was employed to electrochemical measurements obeying the following techniques: DPP, DPV, NPP and NPV. Electrodes were shielded from external fields. The mercury drop duration time was precisely controlled. Every sample was diluted twice with the phosphate buffer.

Experimental parameters:

- pulse amplitude in DPP and DPV — $\Delta E = 20$ mV
- pulse duration — $t_p = 40$ ms
- pulse base potential in NPP and NPV — $U_R = 0.00$ V
- starting potential — $U_o = 0.00$ V
- temperature — T = 293 K

MD–DME properties:

– drop duration	$t_1 = 3$ s
– mercury pressure	h = 24 cm
– mercury flow rate	m = 1.45 mg Hg s^{-1}
– scan rate	v = 0.0026 Vs^{-1}

HMDE properties:

– scan rate	v = 0.025 Vs^{-1}
– mercury drop area	S = 0.0326 cm^2

Potential values are referred to the SCE.

RESULTS AND DISCUSSION

Change in the spectrum due to reaction progress. Due to the enzymatic reaction progress the maximum absorption appears in the range from 450 to 500 nm. Tyrosinase converts tyrosine to orthoquinone, which finally transforms to dopachrome whose concentration gradually increases and in consequence its absorbance rises within the aforementioned range. In fact the wavelength of 475 nm was assigned to the maximun absorbance.

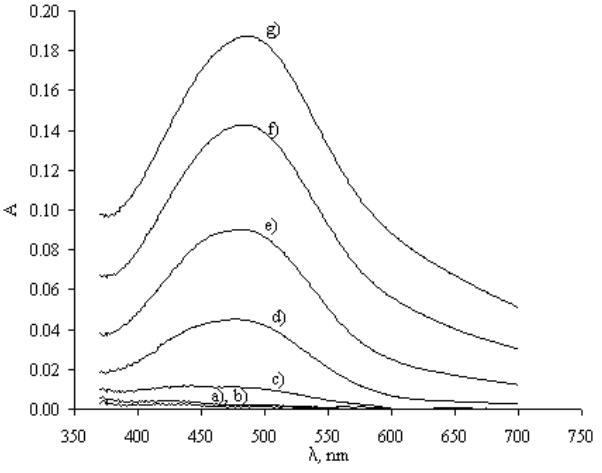

Figure 2. Spectra of the reaction mixture at various stages of the reaction progress: a) no enzyme added; b) after 1 min; c) after 3 min; d) after 5 min; e) after 6 min; f) after 8 min; g) after 10 min

It was observed (Fig. 2) that the higher the orthoquinone concentration in the solution, the more absorbance peak shifts to the larger wavelengths. However, the

absorbance peak is rather wide and its shift can not arouse any errors although the absorbance was measured at the selected wavelength. More alarming obstacle is the tendency of reaction mixture to become turbid due to the precipitates formed. Therefore, spectrophotometrical measurements have to be performed immediately after the sample is prepared. Thus it is recommended to prepare the samples just before investigation.

In contrast to spectrophotometry the polarographic approach allows one to eliminate the unwanted influence of the sample turbidity.

In enzymology the polarographic methods are relatively less common than spectroscopic ones. Therefore a detailed description of their advantages in the investigation of tyrosinase activity is presented.

Polarographic curves registered before and after enzymatic reaction. Before the enzymatic reaction was initialised the background NP polarograms were recorded (Fig. 3). In order to eliminate the two-step oxygen reduction – curve (a) the solution was deoxygenated – curve (b) and electroinactivity of the enzymatic reaction substrate (tyrosine) and possible trace pollutions was confirmed – curve (c).

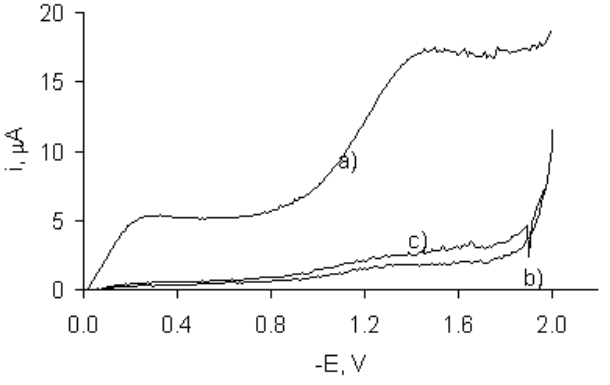

Figure 3. NP polarograms recorded in the studied solution before enzymatic reaction: a) 0.1 mol L^{-1} phosphate buffer (pH = 6.8); before removal of oxygen: b) 0.1 mol L^{-1} phosphate buffer (pH = 6.8); after removal of oxygen: c) 0.1 mol L^{-1} phosphate buffer (pH = 6.8); after removal of oxygen; tyrosine concentration: CTYR = 0.075 mmol L^{-1}

After the enzymatic reaction is completed the NP polarographic responses change their shapes are altered (Fig. 4). Without deoxygenation of the solution the two-step oxygen reduction is still well pronounced – curve (a), yet its second wave is of smaller height than the respective curve in Figure 3. Moreover, even if the solution was deoxygenated, the first oxygen wave was still apparent. In fact this wave refers to the reduction of quinone, which is formed in the enzymatic reaction.

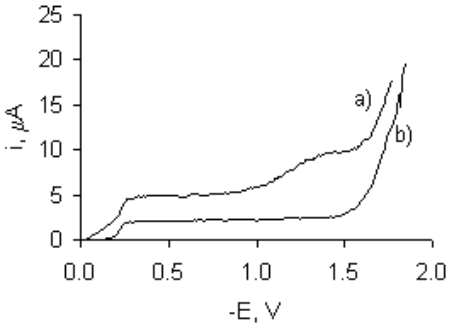

Figure 4. NP polarograms recorded in the studied solution 10 min after the enzymatic reaction was initialised a) before removal of oxygen; b) after removal of oxygen. Tyrosine concentration: C_{TYR} = 0.075 mmol L^{-1}

According to Figure 5 it is possible to measure the oxygen consumption degree in the studied reaction, which is not possible with the spectrophotometrical method. In order to determine the tyrosinase activity the quinone reduction NP polarographic wave height should be considered. However, DPP technique provides one with more precise results, since the NP waves are replaced with easily measurable peaks of a maximum at the potential about –0.2V. DP current responses are of smaller magnitude in comparison to those recorded with NP technique, which may be of some importance at low enzyme activity. Since the quinone reduction is reversible, the decrease is of one order of magnitude can be compensated with the appropriately improved sensitivity.

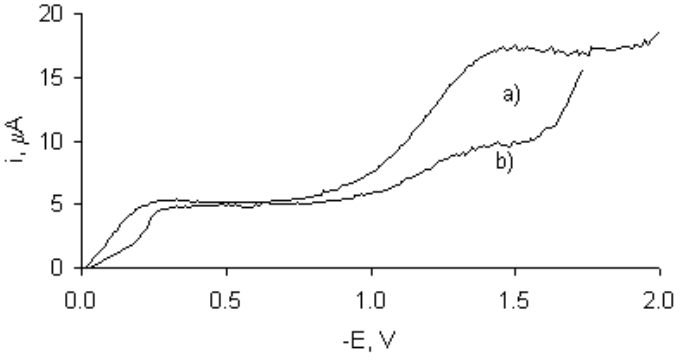

Figure 5. NP polarograms recorded before (a) and after (b) the enzymatic reaction, without the preliminary oxygen removal. Tyrosine concentration: C_{TYR} = 0.075 mmol L^{-1}

Figure 6 exhibits exemplary DP cathodic voltammograms for different initial substrate concentrations and the same enzyme activity. To provide satisfactory sensitivity a sufficiently high concentration of the substrate is required.

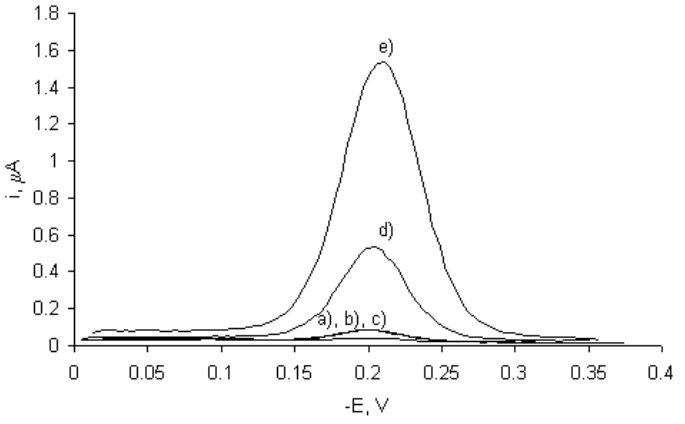

Figure 6. DP voltammogramms for the increasing initial substrate concentration: a) 0.05 mmol L^{-1}, b) 0.055 mmol L^{-1}, c) 0.06 mmol L^{-1}, d) 0.075 mmol L^{-1}, e) 0.09 mmol L^{-1}

From Figure 7 it is evident that this concentration in the reaction mixture should not be lower than 0.06 mmol L^{-1}. It seems to correspond to the detection limit of the formed quinone for the method used.

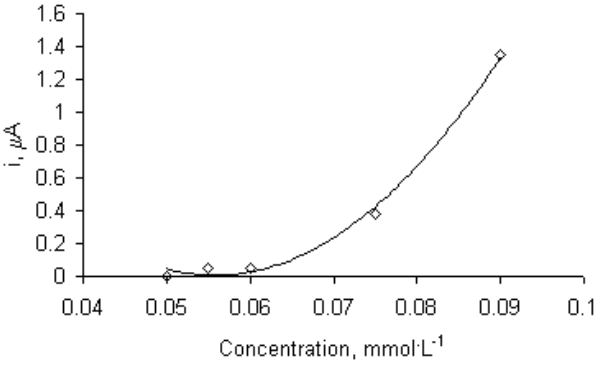

Figure 7. The dependence of quinone reduction NP currents on the initial substrate concentration (conditions as in Figure 5)

Statistical Analysis

In order to estimate the precision and accuracy of the proposed method for determination of the tyrosinase activity an exemplary enzyme solution was examined. The DPV and DPP measurements were performed for five identical samples, and the measurements were repeated after 24 h. The negative influence of the long storage time of the samples on the analysis results was observed (a gradual decrease of the peak height) as a consequence of sequent chemical reactions in the mixture. The quality of determinations was also worsened especially for DPV (at HMDE). For DPP (at MD–DME) the accuracy and precision did not change significantly in time and equaled to 8% and 3%, respectively (for the peak height) and 10% and 4% (for the peak area). The statistical data treatment for HMDE is presented in Table 1 (HMDE introduces the largest variability into the results). The accepted confidence level was $\alpha = 0.95$.

Table 1. Statistical analysis of the results from voltammetric and spectroscopic measurements

Parameter	Method				
	voltammetry (HMDE)				spectroscopy
	peak high, μA		peak area		absorbance
	a	b	a	b	a
n	4*	5	4*	5	4
x	0.1630	0.0954	0.0308	0.022	0.5709
s	0.0088	0.0080	0.0008	0.0053	0.0637
t*s	0.0279	0.0223	0.0026	0.0148	0.2026
s_r, %	5.36	8.41	2.67	23.84	11.15
$(t*s)_r$, %	17.08	23.36	8.50	66.18	35.49
C_{TYR}, $mmolL^{-1}$	0.07				0.5

a – determined immediately, b – determined after 24 h, n – number of samples, x – mean value of measured signal, s – standard deviation, t*s – confidence interval, s_r – relative standard deviation, $(t*s)_r$ – relative confidence interval, 4* – one strongly differing point was removed, C_{TYR} – tyrosine (substrate) concentration.

The determination requires the voltammetric and spectrophotometric ana-lysis to be performed just after the enzymatic reaction is initialized (after 24 h the ma-ximum absorbance changes even by 100%). Hence, the usage of MD-DME together with DPP seems to be an optimum choice providing one with the satisfactory precision and accuracy despite a slight decrease in time of the measured current response.

CONCLUSIONS

The tyrosinase activity in the reaction with tyrosine as a substrate can be estimated polarographically (voltammetrically). Accuracy, sensivity and detection limit improved in comparison to the usually used spectrophotometric method. In addition, the problems with the turbidity of the solution can be eliminated. There is a necessity to deoxygenate the reaction mixture before the measurement, which lengthens the time of analysis but makes possible to measure the oxygen consumption during enzymatic reaction at the same time. The oxygen consumption can be an additional indicator of the tyrosinase activity.

REFERENCES

1. Kong K.-H., Lee J.-L., Park H.-J. and Cho S.-H., *Biochem. Mol. Biol. Int.*, **45**, 717 (1998).
2. Espin J.C. and Wichers H.J., *J. Agric. Food Chem.*, **47**, 3518 (1999).
3. Espin J.C. and Wichers H.J, *J. Agric. Food Chem.*, **47**, 2638 (1999).
4. Kahn V., Shalom N.B. and Zakin V., *Pigm. Cell Res.*, **8**, 234 (1995).
5. van Leeuwen J., Peppelenbos H., van Uden W., Pras N. and Wichers H.J., *Recent Res. Devel. Phytochem*., **2**, 455 (1999).
6. Virador V.M., Kobayashi N., Matsunaga J. and Hearing V.J., *Anal. Biochem.*, **270**, 207 (1999).
7. Kubo I. and Yokokawa Y., *Phytochem.*, **31**, 1075 (1992).
8. Sakuma K., Ogawa M., Sugibayashi K., Yamada K.-I., Yamamoto K., *Arch. Pharm. Res*., **22**, 335 (1999).
9. Rescigno A., Sanjust E., Soddu G., Rinaldi A.C., Sollai F., Curreli N. and Rinaldi A., *Biochim. and Biophys. Acta*, **1384**, 268 (1998).
10. Lejczak B., Kafarski P., Makowiecka E., *Biochem. J.*, **242**, 81 (1987).
11. Schved F. and Kahn V., *Pigm. Cell Res.*, **5**, 58 (1992).
12. Jimenez M., Garcia-Canovas F., Garcia-Carmona F., Tudela J. and Iborra J.L., *Int. J. Biochem*., **18**, 39 (1986).
13. Coche-Guerente L., Desprez V., Diard J.-P. and Labbe P., *J. Electroanal. Chem*., **470**, 53 (1999).
14. Campanella L., Favero G., Sammartino M.P. and Tomassetti M., *J. Mol. Catal. B-Enzym.*, **7**, 101 (1999).
15. Campanella L., Favero G., Sammartino M.P. and Tomassetti M., *Anal. Med. Acta*, **393**, 109 (1999).
16. Viviana Campo Dall'Orto, Danilowicz C., Rezzano I., Michele Del Carlo, Mascini M., *Anal. Lett.*, **32**, 1981 (1999).

Received October 2001
Accepted October 2002