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L-Dopa Synthesis on Conducting Polymers

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With regards to the synthesis of L-Dopa (l-3,4-dihydroxy phenylalanine) two types of biosensors were designed by immobilizing tyrosinase on conducting polymers; polypyrrole (PPy) and poly(3,4-ethylenedioxythiophene) (PEDOT). PPy and PEDOT were synthesized electrochemically and tyrosinase immobilized by entrapment during electropolymerization. The kinetic parameters of the designed biosensors, maximum reaction rate of the enzyme (V_{max}) and Michaelis Menten constant (K_m) were determined. V_{max} were found as 0.013 for PPy matrix and 0.041 μmol/min.electrode for PEDOT matrix. K_m values were determined as 3.7 and 5.2mM for PPy and PEDOT matrices respectively. Calibration curves for enzyme activity vs. substrate concentration were drawn for the range of 0.8 to 2.5 mM L-Tyrosine. Optimum temperature and pH, operational and shelf life stabilities of immobilized enzyme were also examined.

Keywords: Conducting polymers, electrochemical biosensors, enzyme immobilization, L-dopa synthesis, tyrosinase

1 Introduction

Recently, conducting polymers have attracted much interest in many areas. They are widely utilized in electrochromic devices (ECDs) or 'smart windows' (1). Conducting polymers are used in organic light emitting diodes (OLEDs) (2). These polymers exhibit numerous features, which enables them to act as proper immobilization matrices of biomolecules and fast electron transfer for the construction of biosensors (3), gas sensors (4), solar cells (5), EMI shielding (6), and polymer batteries (7).

Among these, polypyrrole (PPy) and poly(3,4-ethylenedioxythiophene) (PEDOT) have been studied extensively due to their unique properties such as easy preparation, high conductivity, and high environmental stability (8). Several studies have shown that PPy could be easily electropolymerized in water (9). PPy is of great interest owing to its potential uses in microelectronics, microsystems, optical sensors, and photoelectronic chemistry (10–12). It is also a promising material for drug delivery and sensing applications (13). In the last few years, PEDOT attracted researchers for its interesting properties like environmental stability (14). Besides, it has high conductivity and fascinating spectrochemical properties (15). Polymer has a low band gap due to its conjugated, planar EDOT rings and alkanedioxy substituents (16). PEDOT, PPy and their derivatives were used as immobilization matrices for enzymes such as glucose oxidase (17), polyphenol oxidase (18), invertase (19), and alcohol oxidase (20). In order to use polymer films as biosensors or actuators in drug release applications, it is necessary to eliminate surfactants or organic solvents. This can be achieved by synthesizing the matrix polymers in aqueous media (8).

L-Dopa (l-3,4-dihydroxy phenylalanine) has attracted much attention as a drug in Parkinson's disease treatment since 1967 (21). Parkinson's disease is a disorder characterized by bradykinesia, rigidity, tremor at rest, postural instability, micrographia and shuffling gait (22). This disease occurs when dopamine level in the substantia nigra of the brain is not enough. Dopamine, cannot cross the blood–brain barrier, hence, does not have the ability to reach the dopaminergic cells of the brain, whereas its precursor, L-Dopa can (23).

L-Dopa can be produced from L-Tyrosine by the help of the enzyme, tyrosinase. Polyphenol oxidase or tyrosinase (E.C. 1.14.18.1) is a copper containing enzyme which is one of the most versatile enzymes in nature (24). It is commonly found in mushrooms, yeast, bananas, grapes, apples, potatoes, frogs, and mammals (25). Moreover, tyrosinase has widespread applications in industry; electrochemical biosensor for dopamine (26), construction of sensors to determine the phenolic amount in waste water (27), detection of catechols in urine (28), obtaining the concentration of total phenolics in red wine (29).

Tyrosinase catalyzes three oxygen-dependent reactions. First one is called cresolase activity (Eq. 1); the

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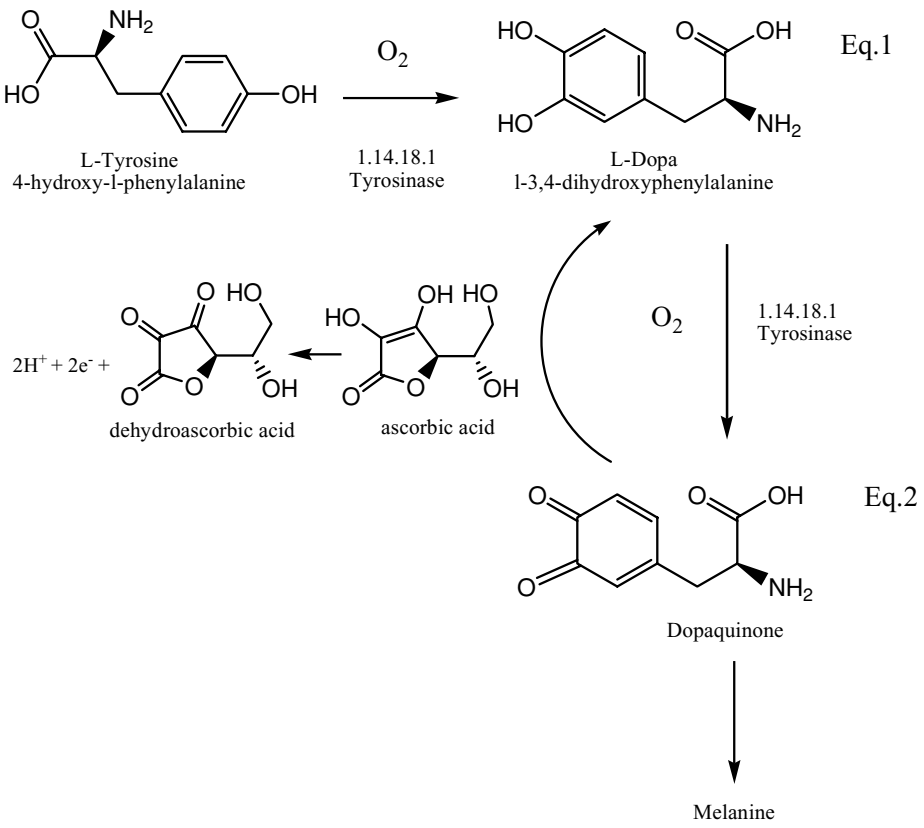

Sch. 1. Route for reactions of tyrosinase and production of L-Dopa.

orthohydroxylation of monophenols, second one is catecholase activity (Eq. 2) which is the oxidoreduction of orthodiphenols to orthoquinones (Scheme 1) (30) and the last one is the conversion 5,6-dihydroxyindole to melanochrome (31). Polymerization of dopaquinone into melanin depends on dopaquinone's high reactivity. To eliminate this conversion and to obtain the desired product L-Dopa, ascorbic acid is used as the reducing agent (30).

So far, L-Dopa has been synthesized by various methods: immobilizing tyrosinase on zeolite (32), chitin (33), enzacryl AA supports (34), chitosan matrices (35), metal nano particles (36), nylon 6, 6 (22), Cu-alginate gel (27), polyacrylamide and gelatin gels (37), modified polystyrene matrices (38) and silk fibroin matrix (39). To the best of our knowledge, L-Dopa has not been synthesized on a conducting polymer platform. In this paper, conducting polymers of pyrrole and 3,4-ethylenedioxythiophene matrices were used to obtain the L-Dopa as the product.

2 Experimental

2.1 Materials

Tyrosinase (E.C. 1.14.18.1), L-Tyrosine, L-ascorbic acid, hydrochloric acid, sodium hydroxide, sodium molybdate, and sodium nitrite were purchased from Sigma and used as received without further purification. Sodium dodecylsulfate (SDS) was supplied from Merck. Pyrrole (Py) and 3,4-ethylenedioxythiophene (EDOT) were purchased from Aldrich and used without further purification. $NaH_2PO_4.H_2O$ (Sodium Phosphate Monobasic) and $NaHPO_4.7H_2O$ (sodium phosphate dibasic) were purchased from Fisher Scientific Company.

2.2 Instrumentation

Potentioscan Wenking POS-73 potentiostat, Shimadzu UV-1601 spectrophotometer, and Memmert D-91126 model water bath were used.

2.3 Preparation of Biosensors

Biosensors based on PPy and PEDOT were constructed via electrochemical polymerization of Py and EDOT. Electrochemical polymerizations were achieved by constant potential electrolysis carried out in a three electrode cell compartment, containing platinum plates (1 cm^2) as working, counter electrodes, and an Ag wire as a pseudoreference electrode.

For the PPy based biosensor, polymerization of pyrrole (0.01M) was achieved by applying 1.0V for 20 min. SDS (2% w/v) was used as the supporting electrolyte. For the

PEDOT based biosensor, electropolymerization of EDOT (0.005M) was achieved by applying 0.8 V for 15 min in the presence of 1% w/v SDS. Electropolymerizations were done in the presence of phosphate buffer (pH 7) and tyrosinase (1% w/v). Entrapment of tyrosinase was achieved during polymerization.

2.4 Measurements

All experiments were done in constant temperature water bath while shaking. The activities of biosensors were determined for both free and immobilized enzyme. For free enzyme activity, 0.01M tyrosinase solution was added to L-tyrosine solutions (0.5 to 2.5 mM) containing L-ascorbic acid. After 30 min reaction time, enzymatic assay was performed by adding 1ml HCl (2M), 1ml NaOH (2M) and 1 ml %15 $NaMo_4$ and $NaNO_2$ solutions in specific reaction times (5, 10 and 15 min). For determining the immobilized tyrosinase activity, different concentrations of L-tyrosine and L-ascorbic acid solutions were prepared. Electrodes were put into test tubes containing substrate solutions. To get the desirable concentration of L-Dopa, 1 h was required for PPy matrix, whereas it was found as 50 min for PEDOT matrix. Then, the enzymatic assay mentioned above was performed. Since formation of L-Dopa complex is time dependent, L-Dopa concentrations were determined by spectrochemical analysis at 460nm exactly after 1 hour. Enzyme electrodes were kept in phosphate buffer at 4°C when not in use and daily prepared electrodes were used in all experimental steps.

3 Results and Discussion

In this paper, reasons behind using PPy and PEDOT as the immobilization matrices to construct biosensors are suitable film thicknesses, high environmental stability, and good conductivities (16, 14, 40). Moreover, the polymerization of Py and EDOT reveals polymers as immobilization supports on the electrode surface. Therefore, they are proper materials to entrap the enzyme and also maintain the stability of enzyme against changes in the environment. Optimization experiments were done to determine the conditions affecting tyrosinase activity and hence the production of L-Dopa.

3.1 Kinetic Parameters forFree and Immobilized Enzyme

An enzymatic reaction reaches a maximum velocity (V_{max}) when the substrate concentration is increased to a level where there is a constant rate of product formation. From Lineweaver-Burk plot, V_{max} can be determined. The substrate concentration corresponding to the half of the maximum velocity is called Michaelis–Menten constant (K_m). This refers to the affinity of enzyme to its substrate. Increase in the K_m value indicates that the affinity between the substrate and enzyme is low.

Table 1. Kinetic parameters of free enzyme and developed PPy and PEDOT enzymatic biosensors

	Vmax (μmol/min. electrode)	*Km (mM)*	*Efficiency Factor (η)*	*Catalytic efficiency (k_b)*
Free enzyme	0.014	4	—	0.0035
PPy biosensor	0.013	3.7	0.93	0.0035
PEDOT biosensor	0.040	5.2	2.86	0.0077

In order to determine V_{max} and K_m values for immobilized enzyme, activity assay was applied for different concentrations of L-Tyrosine solutions. V_{max} and K_m values were determined from Lineweaver-Burk plot at 25°C and pH 7. (Table 1) K_m values are generally related to the microenvironment of the enzyme and porosity of the matrices. According to Table 1, PPy matrix exhibits a smaller K_m value than the one for free enzyme. This result indicates that PPy matrix provides a suitable microenvironment compared to the bulk. In the case of PEDOT matrix, it reveals a bit higher K_m value than the one for free PPO. The microenvironment provided by this matrix does not prevent enzyme and substrate to come together. Besides, there is also a significant increase in the V_{max} values. It is obvious that whenever the enzyme substrate comes together, the complex generates the product. The efficiency factor (η) is the ratio of the maximum reaction rates of the immobilized enzyme over that of the free enzyme. Values of η were calculated from this ratio and were found as 0.93 for PPy and 2.86 for PEDOT matrices. V_{max}/K_m is the catalytic efficiency (k_b) of an enzyme–substrate pair. Catalytic efficiency of the free tyrosinase was calculated as 0.0035. It was found as 0.0035 and 0.0077 for PPy and PEDOT matrices, respectively.

3.2 Optimum Ph

Optimum pH determination was carried out by changing pH between pH 6 and 7.5. The same procedure mentioned in section 2.4 was applied for both free and immobilized enzyme matrices. Tyrosinase has an isoelectric point of 6.1 (31). For free enzyme, PPy and PEDOT matrices, maximum activity was observed around pH 7.0 (Fig. 1). At pH 7.0 phosphate buffer, entrapped enzyme was negatively charged for both matrices. This protects the enzyme against denaturation. Both matrices have suitable environment for enzyme and both biosensors have the advantages in medical applications since the blood pH is 7.4.

3.3 Optimum Temperature

Enzymes are sensitive to the changes in temperature. The effect of temperature on relative enzyme activity was examined between 5 and 50°C and illustrated in Figure 2.

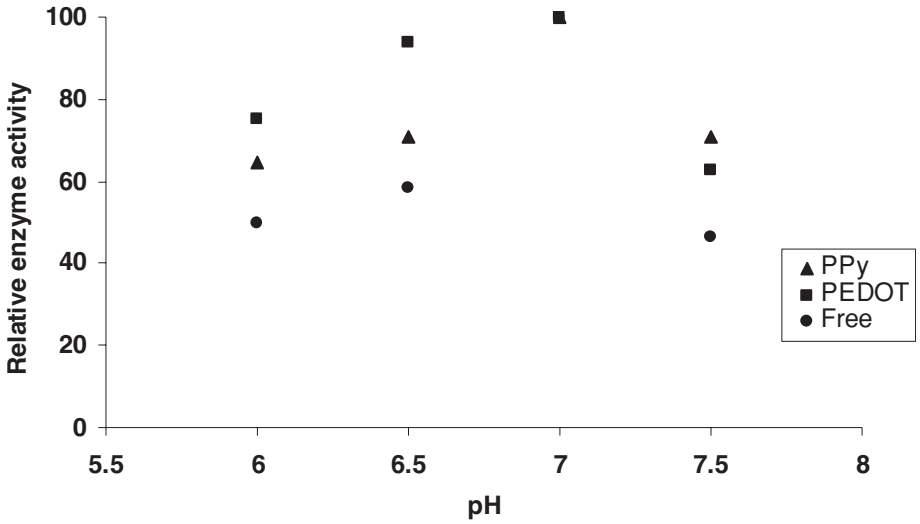

Fig. 1. Effect of pH on PPy and PEDOT based enzymatic biosensors (6–7.5 Potassium phosphate buffer with 2.5 mM L-Tyrosine).

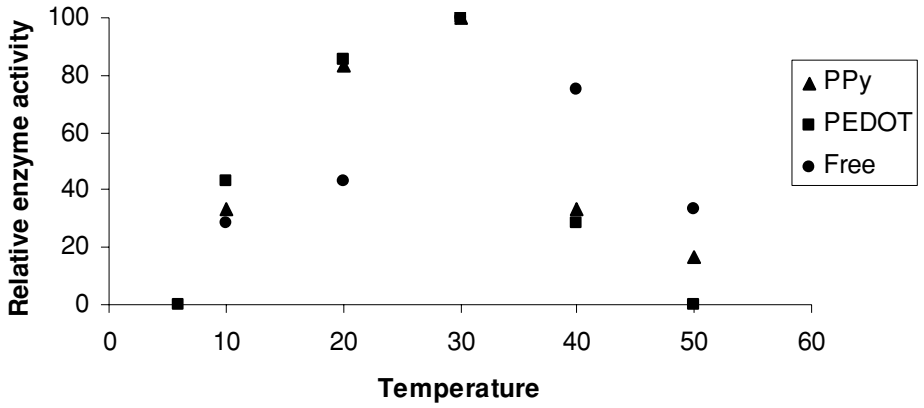

Fig. 2. Effect of temperature on PPY and PEDOT based enzymatic biosensors (5–50°C Potassium phosphate buffer (pH 7) with 2.5 mM L-Tyrosine).

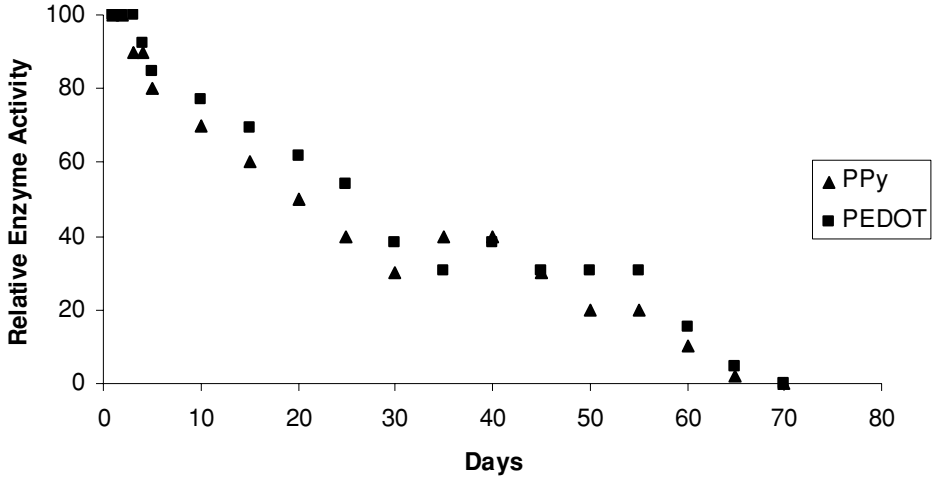

Fig. 3. Shelf life of PPy and PEDOT based enzymatic biosensors (25°C Potassium phosphate buffer (pH 7) with 2.5 mM L-Tyrosine).

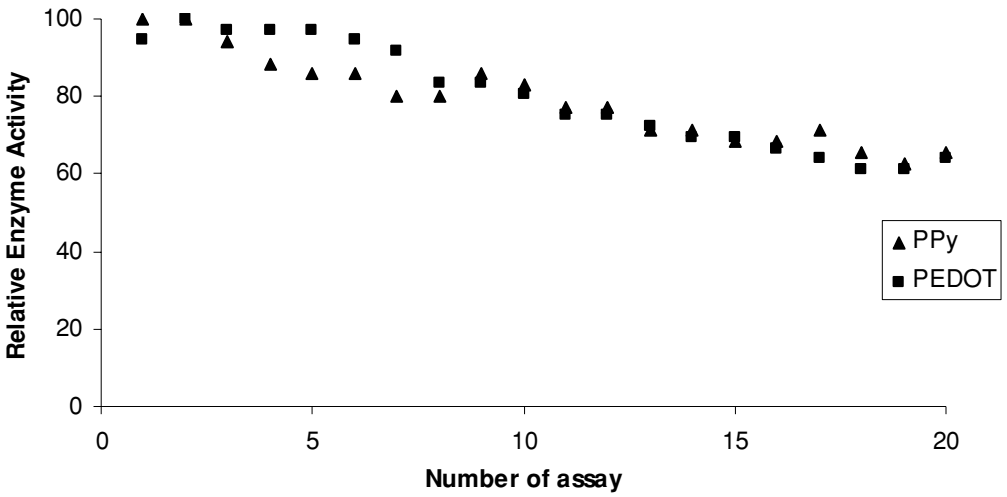

Fig. 4. Operational stability of PPy and PEDOT based enzymatic biosensors (25°C Potassium phosphate buffer (pH 7) with 2.5 mM L-Tyrosine).

For free, PPy and PEDOT matrices, maximum activity was found at 30°C. PPy matrix shows a reasonable activity in a wider temperature range compared to PEDOT matrix. PPy and PEDOT based biosensors can be used at temperatures lower than 30°C, whereas free enzyme does not reveal an appreciable activity at 20°C.

3.4 Analytical Characteristics

The analytical characteristics of the enzymatic biosensors were examined using L-tyrosine as the substrate in potassium phosphate buffer (pH 7) at 25°C. Calibration curves were plotted for enzyme activities versus substrate concentrations. For the PPy based biosensor, a linear relationship was observed between 0.8 and 2.5 mM L-Tyrosine as given by the equation; $y = 1.55x + 0.0012$. For PEDOT based biosensor, a linear relationship was observed between 0.8 and 2.5 mM L-Tyrosine as shown by the equation $y = 4.98x + 0.0023$ (x is the concentration of L-Tyrosine, y is the enzyme activity). At higher concentrations for both systems, enzyme activity remained constant reaching to saturation.

For the shelf life measurements of the enzyme electrodes, the activity of PPy and PEDOT electrodes were checked every 5 days for 65 days (Fig. 3). Both matrices retain 40% of original activity even after the 50th day.

The operational stabilities of the enzyme electrodes were given in Figure 4. To achieve these results, 20 repetitive experiments were performed in one day. When we compare their operational stabilities, PPy matrix shows 70% activity even at the 20th measurement. PEDOT matrix reveals almost the same results.

4 Conclusions

Production of L-Dopa was achieved using tyrosinase immobilized on conducting polymers PPy and PEDOT. Kinetic parameters, calibration curves, operational and shelf life stabilities, optimum temperature and pH were investigated for the matrices. K_m values for immobilized tyrosinase were almost the same for matrices and free enzyme. Maximum velocity was found to be higher compare to free enzyme in PEDOT matrix. This shows that enzyme in PEDOT matrix have higher ability to form product. Tyrosinase has a maximum activity at 30°C and pH 7 for both PPy and PEDOT. PPy supplies better operational stability and higher relative activity for the production of L-Dopa. This study proves that conducting polymers; PPy and PEDOT can be used as immobilization matrices for tyrosinase in the production of L-Dopa.

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