Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Metal ion effects on Polyphenol Oxidase Covalently immobilized on a Bio-Composite

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Received March 19 2020; Accepted June 5, 2020; Published August 31, 2021

Doi: http://dx.doi.org/10.14715/cmb/2021.67.2.8

Abstract: Biosensors can be developed using different immobilization methods. Interest in immobilization methods have increased because biosensors have been important for science. Polyphenol oxidase (PPO) was used generally in biosensor applications. For this purpose, Polyphenol oxidase from banana was purified and covalently immobilized on chitosan-gelatin bio-composite. The properties of immobilized enzyme were investigated and compared to free enzyme. Various parameters were studied such as pH, temperature and storage stability on immobilized and free enzyme. Kinetic parameters were also evaluated by different substrates on immobilized and free enzyme. Catechol was determined the best substrate for immobilized enzyme with optimum condition. *In vitro* effects of metal ions were studied on immobilized enzyme. Concentration range of metal ions is 1.0-10.0 $x10^{-6}$ mol/L. The activity of immobilized PPO was increased by Fe^{+2} and Ag^{+1} ion. Co^{+3} and Cu^{+1} had very strong inhibitory effects with IC_{50} values of 19.69$x10^{-3}$ mol/L and 23.49 $x10^{-3}$ mol/L, respectively. Inhibition constants (Ki) and inhibition types of metal ions were determined with immobilized enzyme. Zn^{+2} and Cr^{+3} ions were showed competitive inhibition and Pb^{+2} ions were determined non-competitive inhibition with immobilized enzyme. Mixed type inhibition was obtained with Co^{+3} ion using catechol as substrate with 3.33$x10^{-5}$ mol/L Ki value on immobilized PPO. Immobilized PPO can be evaluated for biosensor for the purpose of measurements of metal ions.

Key words: Polyphenol oxidase; Immobilization; Bio-composite; Metal ions; Optimum pH; Optimum temperature; IC_{50} values; Kinetic constants; Inhibition type.

Introduction

Immobilized enzymes have an advantage highly economically developed with their catalytic properties in industrial applications (1). Enzyme immobilization was based on industrial applications, biosensors, bioaffinity chromatography and many biotechnological products in medicines (2). Immobilization techniques have increased significantly at the last 30 or 40 years. To improve the applicability of immobilized enzymes to other practical processes, it is necessary to develop new methods and to better understand and develop existing techniques.

The quality of the support material is crucial for the design of immobilized enzyme systems. An ideal support material should have features such as physical resistance to pressure, bioavailability, resistance to microbial attacks, being hydrophilic, increasing enzyme selectivity, reducing product inhibition (3, 4). A great number of support material and methods can be applied for the enzyme immobilization. Therefore, it is important that the choice of suitable matrix and immobilization method over the free enzyme should be well justified (5).

Chitosan is a natural carbohydrate biopolymer derived from deactylation of chitin with reactive amino and hydroxyl groups which link with enzymes easily. This support material is inexpensive, abundant and high mechanical strength for enzyme immobilization (6). Gelatin is a nontoxic natural biopolymer with an adhesion quality. It has a wide range of uses in food and pharmaceuticals (7). Many fruits and vegetables contain polyphenol oxidase (EC 1.14.18.1; PPO) which a bifunctional, copper containing enzyme widely distributed in the phylogenetic. It catalyses both the o-hydroxylation of monophenols to give o-diphenols (cresolase activity). The further oxidation of o-diphenols to o-quinones (catecholase activity) using molecular oxygen (8-12). There has been much interest in PPO among biochemists and food technologists (9, 12-16). PPO obtained from different source demonstrates different substrate specificities and property of inhibition (16). Therefore, characterization of the enzyme could help to develop more effective methods for controlling browning of plants and products (17, 18).

Immobilization has the potential to increase enzyme stability. Soluble and immobilized forms of PPO are used for dephenolization. Many researchers have been studied phenol degradation of the immobilized PPO (6). Chitosan-gelatine bio-composite had never been investigated to be used as a support material for PPO immobilization. However, many workers have tried immobilization of PPO isolated from different sources with different support materials (19-21).

Metal ions are the leading cause of environmental pollution and increasingly dangerous factors. Soil contamination of metal ions (such as Cu, Pb, Zn, Ag, Cd, Co, Fe, Ni, Cr) and damage to the environment have become very important current issues (22). The toxic effect of metal ions is known for living things including

microorganisms, plants, animals and humans (23, 24). Metal ions known to an important contaminant group; toxic and carcinogenic effects, as well as accumulation in living organisms (23). In general, the environmental problems of metal ions are important to human, animal and plant health and the effects on water ecosystems (24).

In the present study, PPO from banana (*Musa carevendishi*) was covalently immobilized onto chitosan-gelatin bio-composite. Kinetic constants were determined using different substrate on immobilized and free enzyme. Optimal pH, optimal temperature and storage stability was studied on the immobilized enzyme. The immobilized PPO was evaluated *in vitro* effects of metal ion such as Ag^{+}, Fe^{+2}, Ba^{+2}, Hg^{+2}, Co^{+3},Cu^{+1}, Pb^{+2}, Zn^{+2}, Ni^{+2},Cr^{+3}, Cd^{+2}, and Mn^{+2}. Inhibition constants (K_i) and inhibition types were calculated for every metal ion for immobilized PPO. Concentration range of metal ions is 1.0-10.0 x10^{-6} mol /L. The biocomposite was evaluated for suitability for the PPO. The PPO immobilization system can be developed in biosensors for metal ions.

Materials and Methods

Enzyme assays were carried out with the aid of a Biotek Power Wave XS UV-visible spectrophotometer. Spectrophotometric measurements were measured with PerkinElmer Lambda Spectrophotometer. Sepharose 4B, L-tyrosine, protein assay reagents, enzyme purification chemicals for buffers, enzyme assay chemicals for buffers were obtained from Sigma-Aldrich (St Louis, MO, USA) and Merck Chem Co. (Darmstadt, Germany). All other chemicals and reagents were of the highest quality available.

Extraction and purification procedure

Banana (50 g) sample was homogenized using a blender for 2 minute in 100 mL of 0.1 mol/L phosphate extract buffer (pH: 6.8) containing 10x10^{-3} mol/L ascorbic acid and 5% poly(ethylene glycol). The homogenate was filtered and the filtrate was centrifuged at 15000*g* for 30 min at 4 °C. The supernatant was brought to 80% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. The precipitated PPO was separated by centrifugation at 15000*g* for 60 min. The precipitate was dissolved in a small amount of homogenization buffer and dialyzed at 4 °C in the same buffer for 24 h with three changes of buffer during dialysis. After dialysis, the fraction was purified with affinity chromatography by Sepharose 4B-L-tyrosine-p-aminobenzoic acid gel (25). We determined enzyme activity and 280 nm protein determination for all tube (Figure 1).

Determination of protein content

The protein content was determined according to the Bradford (26) method using bovine serum albumin as a standard.

Polyphenol oxidase enzyme activity assay

Kinetic assays were carried out by measuring the increase in absorbance at 420 nm for catechol with a Biotek Power Wave XS UV-VISIBLE spectrophotometer at 25 °C. The reaction was carried out in a quartz cuvette. The sample cuvette contained 0.950 mL of substrates in various concentrations prepared in 0.1 M phosphate buffer (pH: 6.8) and 0.050 mL of the enzyme. For each measurement, the volume of solution in the quartz cuvette was kept constant at 1 mL. The reference cuvette contained all of the components except the substrate, with a final volume of 1 (25).

In vitro inhibition kinetic studies

PPO enzyme activity was assayed by following the oxidation of catechol. Activity % values of PPO for six different concentrations of each metal ions were determined by regression analysis using the Microsoft Office 2000 Excel. PPO activity without metal ion was accepted as 100% activity. The inhibitor concentration causing up to 50% inhibition (IC_{50} values) on enzyme were determined from the graphs. Inhibition constants (Ki) were calculated from the Lineweaver–Burk plots for each inhibitor.

Bio-composite preparation

Chitosan (1 g) and gelatin (1 g) were prepared separately by dissolving in acetic acid and then were mixed together in 1:1 proportion. This mixture was stirred for 3 hour at room temperature for drying. The bio-composite was stored at 4°C with 0.1M phosphate buffer (pH: 7.0) before use (27).

Immobilization of polyphenol oxidase on bio-composite

Purified polyphenol oxidase (2 mL) was suspended in chitosan-gelatin bio-composite in a 0.1 mol/L cold phosphate buffer (pH: 7.0). The solution was mixed by mild shaking for at 4 °C for overnight incubation. The unbound enzyme was washed with distilled water, after it was washed with 1M sodium phosphate buffer (pH: 7.0) until free polyphenol oxidase disappeared. Immobilized preparation was kept in the phosphate buffer (pH: 7.0) at 4 °C till further use (7).

Optimum pH and temperature

The enzyme assay was carried out at different pH values (5.0-10.0) at 25 °C. Optimum temperature was determined by a enzyme activity assay in the temperature range from 20-70 °C.

Results

Polyphenol oxidase was purified from banana (*Musa carevendishi*) by ammonium sulfate precipitation, dialysis and affinity chromatography, respectively (Figure

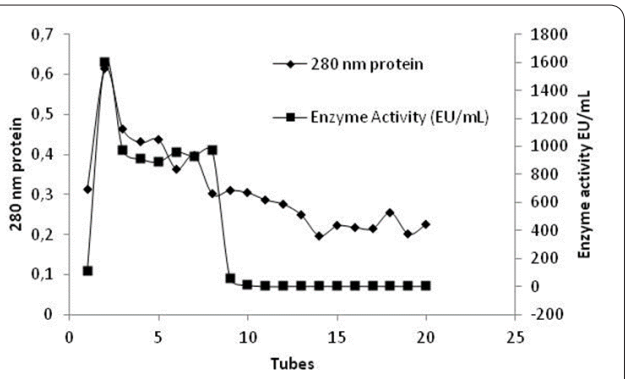

Figure 1. Purification of PPO enzyme isolated from banana (*Musa carevendishi*)

1) (25). Immobilization method was carried out with the purified enzyme. PPO was covalently immobilized on chitosan-gelatin bio-composite (Figure 2).

Firstly, chitosan-gelatin bio-composite was formatted by Chen et al. (28) but this bio-composite had never been studied to be used as a support material for polyphenol oxidase immobilization. However, many researchers have studied to immobilize polyphenol oxidase on to calcium alginate beads (29), antimony doped tin oxide matrix (30), polypyrrole nanotubes (31), mesoporous silica materials (32) and Fe_3O_4-chitosan nanoparticles (33). In this study, polyphenol oxidase from banana (*Musa carevendishi*) was immobilized on to chitosan-gelatin bio-composite.

Free and immobilized enzymes were stored in 0.1 M phosphate buffer (pH: 6.8) at 4 °C and the enzyme activities were measured for a period of 12 weeks. Free PPO enzyme activity was reduced more rapidly than the immobilized enzyme activity. Free enzyme was lost its activity within four weeks, but the immobilized enzyme activity was maintained about 51 % its activity during 12 weeks storage period (Figure 3). This decrease in enzyme activity is normal for both the free enzyme and the immobilized enzyme. These results are compatibility with the literature (34).

Kinetic constants (K_M and Vmax) of immobilized and free enzyme were obtained from Lineweaver-Burk graph using catechol, pyrocatechol, pyrogallol and 4-methlycatechol as a substrate (Table 1, Table 2). Kinetic constants (K_M and V_{max} values) of free enzyme were found to 0.023 mol/L 3333.33 EU/mL.min, respectively (Table 2). K_M and V_{max} values were determined to be 0.086 mol/L and 14285 EU/mL.min for immobilized enzyme and immobilized enzyme's catalytic activity was higher than free enzymes for catechol (Figure 4). Of these four substrates, catechol was the best substrate because of the highest V_{max}/K_M value, followed by 4-methylcatechol, pyrogallol and pyrocatechol (Table 1, Table 2).

Three dimensional structures in the enzyme can occur and cause the change in the kinetic parameters of the immobilized enzyme during the covalent immobilization (5, 35-37). Immobilized PPO showed higher kinetic constants compared to its free enzyme. Immobilization of PPO with the bio-composite increased the enzyme activity. Because, bio-composite bind appropriately to the three-dimensional structure of PPO, enzyme's active site became suitable for binding of the substrate. In addition, the activity of immobilized enzyme was determined by the amount of enzyme interacting with the bio-composite.

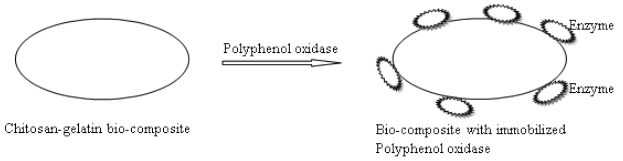

Figure 2. Schematic representation of immobilization of PPO onto chitosan-gelatin bio-composite

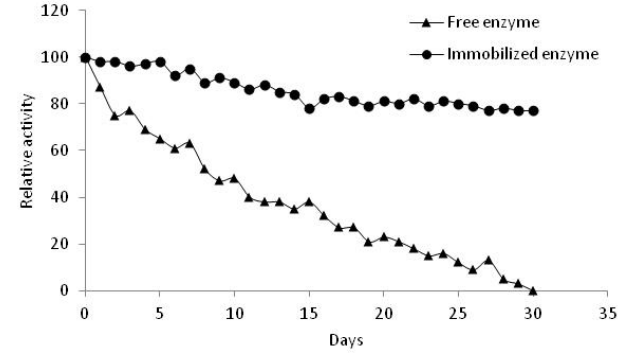

Figure 3. Storage stabilities of free and immobilized PPO at 4°C for 12 weeks.

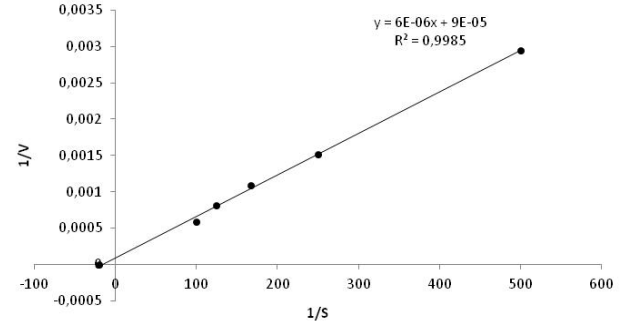

Figure 4. Kinetic constants of immobilized PPO were obtained using catechol (Other graphics are not shown here).

The effect of pH on the activity free and immobilized PPO was studied within the pH range of 5.0-10.0 at 25 °C. The enzyme activities are shown in Figure 5. The maximum activity was found at 6.5 for free and immobilized enzyme. The activities of free and immobilized PPO were assayed at varied temperatures (20-70 °C).

Table 1. Immobilized PPO's kinetic constants.

Substrates	V_{max}(EU $mL^{-1}min.^{-1}$)	K_M (mM)	V_{max}/K_M(EU mL^{-1} $min.^{-1}$ mM^{-1})
4-methylcatechol	1250	10	125
Catechol	14285.71	85.69	166.71
Pyrogallol	1428.57	14.28	100.04
Pyrocatechol	11111.11	400	27.77

Table 2. Free PPO's kinetic constants.

Substrates	V_{max}(EU $mL^{-1}min.^{-1}$)	K_M (mM)	V_{max}/K_M(EU mL^{-1} $min.^{-1}$ mM^{-1})
4-methylcatechol	1111.11	0.01	111.11
Catechol	3333.33	0.023	144.93
Pyrogallol	1250	80	15.62
Pyrocatechol	250	40	6.25

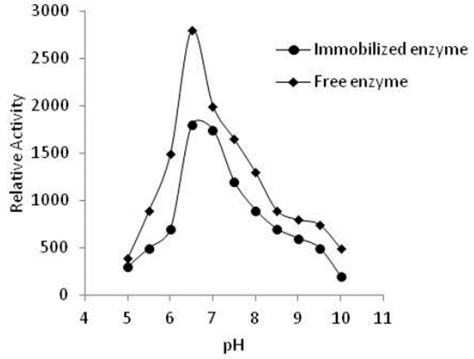

Figure 5. The effect of pH on the activity of free and immobilized enzyme.

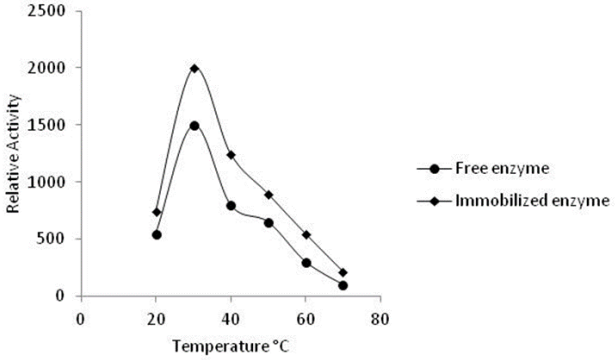

Figure 6. The effect of temperature on the activity of free and immobilized enzyme.

The effect of temperature on the activity of free and immobilized PPO is shown in Figure 6. It was determined that the maximum catalytic activity was found at 30 °C for free and immobilized enzymes.

IC_{50} values in Table 2, Co^{+3} ions showed the greatest inhibition with the 19.69 x10^{-3}mol/L IC_{50} value on immobilized PPO (Figure 7). In Table 3, Ba^{+2} and Hg^{+2} was found IC_{50} values approximate to each other with 32.81 and 32.56 x10^{-3}mol/L, respectively. Ni^{+2} ions were showed less inhibition effect in studied heavy metal ions with 53.37 x10^{-3}mol/L IC_{50} value. The activity of immobilized PPO was increased by Fe^{+2} and Ag^{+1} ions. IC_{50} values of other metal ions are in Table 2.

Ki values and inhibition type (in Table 3) were determined for metal ions on immobilized and free PPO. Co^{+3} ions were showed mixed type inhibition with 3.33x10^{-5} mol/L Ki value on immobilized PPO. Ni^{+2}, Ba^{+2}, Hg^{+2}, Cd^{+2}, Mn^{+2} and Cu^{+1} ions was found the mixed type inhibition with 10 x10^{-5}, 2.0 x10^{-5}, 15 x10^{-5}, 2.27 x10^{-5}, 10 x10^{-5} and 6.25 x10^{-5} mol/L Ki values, respectively. Zn^{+2} and Cr^{+3} ions were showed competitive inhibition with 26.3 and 3.33 x 10^{-5} mol/L Ki values, respectively. Pb^{+2} ions were determined non-competitive inhibition with 1.09x10^{-5} mol/L Ki value.

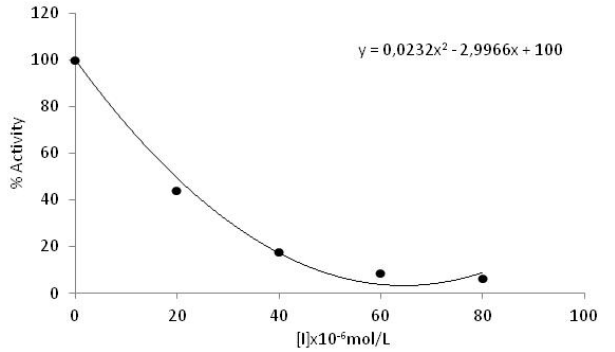

Figure 7. Determination of IC_{50} value on immobilized PPO for Co^{+3} ions.

Discussion

Purification of enzyme contains expensive and time consuming methods. So, reuse enzymes are important for industrial applications. The problem of reuse of free enzymes has been increasingly decreased with immobilized enzyme. It is important that the enzyme activity in the immobilization of the enzyme is maintained for a long time and that there is no significant decrease in the enzyme activity. The reusability of bio-composite immobilization system may provide many advantages for biotechnological applications.

Selecting support material for using enzyme immobilization is important to stability and cost for scientific studies. Therefore, chitosan and gelatin are commercially available and inexpensive materials for bio-composite. PPO has been attracted a lot attention among scientists.

In this study, the PPO enzyme was purified from banana covalently immobilized on bio-composite. Kinetic constants were determined using different substrate. Optimal pH, optimal temperature and storage stability

Table 3. IC_{50} and Ki values and inhibition type of immobilized PPO for some metal ions.

Metal ions	IC_{50} values (μM)	Ki x10^{-5} M	Inhibition type
Cu^{+1}	23.49	6.25	Mixed
Ba^{+2}	32.81	2.0	Mixed
Hg^{+2}	32.56	15	Mixed
Zn^{+2}	51.73	26.3	Competitive
Cd^{+2}	35.90	2.27	Mixed
Co^{+3}	19.69	3.33	Mixed
Cr^{+3}	42.94	3.33	Competitive
Pb^{+2}	50.85	1.09	Non-competitive
Mn^{+2}	30.61	10	Mixed
Ni^{+2}	53.37	10	Mixed
Fe^{+2}	Activator		
Ag^{+1}	Activator		

was studied. Immobilized PPO was evaluated *in vitro* effects of metal ions. Inhibition constants and inhibition types were calculated for every metal ion. Finally, bio-composite can be promising alternative as support materials for PPO immobilization. The immobilization system can be used in biosensors about measuring concentration of metal ions.

Acknowledgments
This work was supported by Balikesir University Research Grant No.: 2018/51.

Conflicts of Interest
All authors declared that they have no conflicts of interest.

Authors' contribution
Prof. Dr. Oktay Arslan, Assoc. Dr. Serap Beyaztaş Uzunoğlu and Assoc. Dr. Tayfun Uzunoğlu conceived and designed the study. Assoc. Dr. Serap Beyaztaş Uzunoğlu, Assoc. Dr. Tayfun Uzunoğlu, Assoc. Dr. Murat Evyapan and Samet Koçsuz performed the experiments. Assoc. Dr. Tayfun Uzunoğlu, Assoc. Dr. Serap Beyaztaş Uzunoğlu and Assoc. Dr. Murat Evyapan wrote the paper.

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