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
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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 Fe²⁺ and Ag⁺ ion. Co³⁺ and Cu²⁺ had very strong inhibitory effects with IC₅₀ values of 19.69 x10⁻⁶ mol/L and 23.49 x10⁻⁶ mol/L, respectively. Inhibition constants (Ki) and inhibition types of metal ions were determined with immobilized enzyme. Zn²⁺ and Cr³⁺ ions were showed competitive inhibition and Pb²⁺ ions were determined non-competitive inhibition with immobilized enzyme. Mixed type inhibition was obtained with Co³⁺ ion using catechol as substrate with 3.33 x10⁻⁶ mol/L Ki value on immobilized PPO. Immobilized PPO can be evaluated for biosensor for the purpose of measurements of metal ions.

Keywords: Polyphenol oxidase; Immobilization; Bio-composite; Metal ions; Optimum pH; Optimum temperature; IC₅₀ 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, biodegradability 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 deacetylation 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 carevendishii) 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⁺, Fe²⁺, Ba²⁺, Hg²⁺, Co³⁺, Cu²⁺, Pb²⁺, Zn²⁺, Ni²⁺, Cr³⁺, Cd²⁺, and Mn²⁺. Inhibition constants (Kᵢ) 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 10x10⁻² 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% (NH₄)₂SO₄ saturation with solid (NH₄)₂SO₄. 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 (IC₅₀ 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 carevendishii) by ammonium sulfate precipitation, dialysis and affinity chromatography, respectively (Figure 1).
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$_3$O$_4$-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 $V_{max}$) of immobilized and free enzyme were obtained from Lineweaver-Burk graph using catechol, pyrocatechol, pyrogallol and 4-methylcatechol 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.

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 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                            |
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$^{-3}$, 15 x10$^{-5}$, 2.27 x10$^{-5}$, 10 x10$^{-5}$ and 6.25 x10$^{-5}$mol/L K$_i$ 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.

**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 being increasingly decreased with immobi

| 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 Koçsu performed the experiments. Assoc. Dr. Tayfun Uzunoğlu, Assoc. Dr. Serap Beyaztaş Uzunoğlu and Assoc. Dr. Murat Evyapan wrote the paper.

References
1. Liu XP, Zhang ZJ, Zhang Y, Guan YJ, Liu Z, Ren JS, Qu XG. Artificial metalloenzyme-based enzyme replacement therapy for the treatment of hyperuricemia. Adv Funct Mater 2016;43:7921-8.
2. Schulze B, Wubbolts MG. Biocatalysis for industrial production of fine chemicals. Curr Opin Biotechnol 1999; 10:609-15.
3. Fessner WD, Anthonsen T. Modern Biocatalysis: stereoselective and environmentally friendly reactions. Berlin: Wiley-VCH, 2008.
4. Rozzell JD. Biocatalysis at commercial scale: myths and realities. Chimica Oggi, Vol 6/7, 1999: 42.
5. Colak U, Gençer N. Immobilization of paraxanthin onto chitosan and its characterization. Artif Cells Nanomed Biotechnol 2012; 40:290-5.
6. Dinçer A, Becerik S, Aydemir T. Immobilization of tyrosinase on chitosan-clay composite beads. Int J Biol. Macromol 2012; 50:815-20.
7. Agarwal P, Dubey S, Singh M, Singh RP. Aspergillus niger PA2 tyrosinase covalently immobilized on a novel eco-friendly bio-composite of chitosan-gelatin and its evaluation for l-dopa production. Front Microbiol 2016 doi: 10.3389/fmicb.2016.02088.
8. García-Carmona F, Valero E, Cabanes J. Effect of L-proline on mushroom tyrosinase. Phytochemistry 1988; 27:1961-4.
9. Arslan O, Doğan S. Inhibition of polyphenol oxidase obtained from various sources by 2,3-diaminopropionic acid. J Sci Food Agric 2005; 85:1499-504.
10. Perez-Gilabert M, Garcia-Carmona F. Characterization of catecholase and cresolase activities of egg plant polyphenol oxidase. J Agr Food Chem 2000; 48:695-700.
11. Arslan O, Temur A, Tozlu I. Polyphenol oxidase form Allium sp. J Agric Food Chem 1997;45:2861-3.
12. Rocha AMC, Morais MMB. Influence of controlled atmosphere storage on polyphenoloxidase activity in relation to colour changes of minimally processed 'Jonagored' apple. Int J Food Sci Tec 2001;36:425-32.
13. Zhou XR, Xiao YJ, Meng XH, Liu BJ. Full inhibition of Whangkeumbae pear polyphenol oxidase enzymatic browning reaction by L-cysteine. Food Chem 2018; 266:1-8.
14. Hithamani G, Medappa H, Chakkaravarti A, Ramalakshmi K. Raghavaran KSMS. Effect of adsorbent and acidulants on enzymatic browning of sugarcane juice. J Food Sci Tech Mys 2018; 10:4356-62.
15. Marques L, Fleuret A, Macheix J. Characterization of multiple forms of polyphenoloxidase from apple fruit. Plant physiol Bioch 1995; 33:193-200. 16. Paul B, Gowda L.R. Purification and characterization of polyphenol oxidase from the seeds of field bean (Dolichos lablab). J Agr Food Chem 2000; 48:3839-46.
17. Yoruk R, Marshall MR. Physicochemical properties and function of plant polyphenol oxidase: a review. J. Food Biochem 2003; 27:361-422.
18. Yue-Ming J, Zauberman G, Fuchs Y. Partial purification and some properties of polyphenol oxidase extracted from litchi fruit pericarp. Postharvest Biol Tec 1997; 10:221-8.
19. Munjal N, Sawhney SK. Stability and properties of mushroom tyrosinase entrapped in alginate, polyacrylamide and gelatin gels. Enz Microb Technol 2002; 30:613-9.
20. Saini AS, Kumar J, Melo JS. Microplate based optical biosensor for L-DOPA using tyrosinase from Amorphophallus campanulatus. Anal Chim Acta 2014; 849:50-6.
21. Micheloni OB, Farroni AE, Garcia P, Furlan RLE. Rapid autographic method for detection of enzymatic browning inhibitors based on enzyme immobilization. Food Chem 2018; 269:638-43.
22. Rai UN, Tripathi RD, Vajpayee P. Bioaccumulation of toxic metals (Cr, Cd, Pb and Cu) by seeds of euryale ferox salisb. (makhana). Chemosphere 2002; 46:267-72.
23. Gu YG, Ning JJ, Ke CL, Huang HH. Bioaccessibility and human health implications of heavy metals in different trophic level marine organisms: A case study of the South China Sea. Ecotoxicol Environ Saf 2018; 163: 551-7.
24. Pandey N, Sharma CP. Effect of Heavy metals Co2+, Ni2+ and Cd2+ on growth and metabolism of cabbage. Plant Sci 2002; 163:753-8.
25. Arslan O, Erzengin M, Sinan S, Ozensoy O. Purification of mulberry (Morus alba L.) polyphenol oxidase by affinity chromatography and investigation of its kinetic and electrophoretic properties. Food Chem 2004; 88:479-84.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-51.
27. Tembe WS, Karve M, Inamdar S, Haram S, Melo J, D’Souza SF. Development of electrochemical biosensor based on tyrosinase immobilized in composite biopolymeric. Anal Biochem 2006; 349:72–7.
28. Chen T, Embree HD, Brown EM, Taylor MM, Payne GF. Enzyme-catalyzed gel formation of gelatin and chitosan: potential for in situ applications. Biomaterials 2003; 24:2831-41.
29. Arabaci G, Uslugulo A, Cesko C. Immobilization of polyphenol oxidase from princess tree leaf and its applications in dye decolorization. Res J Biotechnol 2018; 4:5-10.
30. Turkan A, Faiz O, Kaya ED, Kocyigit A. Immobilization of polyphenol oxidase enzyme on new matrix antimony doped tin oxide (SnO2:Sb) thin film. Fresen Environ Bull 2018; 7:4844-56.
31. Li HQ, Hu X, Zhu HM, Zang Y, Xue HG. Amperometric phenol biosensor based on a new immobilization matrix: polypyrrole nanotubes derived from methyl orange as dopant. Int J Electrochem Sci 2017; 7:6714-28.
32. Escuin PC, Garcia-Bennett A, Ros-Lys JV, Foix AA, Andres A. Application of mesoporous silica materials for the immobilization of polyphenol oxidase. Food Chem 2017; 217:360-3.
33. Lei SC, Xie MH, Hu B, Zhou L, Sun Y, Saeeduddin M, Zhang HC, Zeng XX, . Effective synthesis of theaflavin-3,3’-digallate with epigallocatechin-3-O-gallate and epicatechin gallate as substrates by using immobilized pear polyphenol oxidase. Int J Biol...
34. Bayramoglu G, Akbulut A, Arica MY. Immobilization of tyrosinase on modified diatom biosilica: Enzymatic removal of phenolic compounds from aqueous solution. J Hazard Mater 2013; 244-245:528-36.

35. Arica MY. Immobilization of polyphenol oxidase on carboxymethylcellulose hydrogel beads: preparation and characterization. Polym Int 2000; 49:775-81.

36. Madoery RR, Gattone CG, Fidelio G. Bioconversion of phospholipids by immobilized phospholipase A(2). J Biotechnol 1995; 401:145-53.

37. Martinek K, Kilbanov AM, Goldmacher VS, Berezin IV. The principles of enzyme stabilization. Biochimica et Biophysica Acta 1977; 4851-912.