# NON-COVALENT IMMOBILIZATION OF QUINCE (*Cydonia oblonga*) POLYPHENOL OXIDASE ON ALUMINA

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## Abstract

Polyphenol oxidase enzyme purified partially from quince (*Cydonia oblonga*) was immobilized on alumina ($Al_2O_3$) by simple adsorption at pH 6.8. The properties of immobilized enzyme were compared to those of the free enzyme. Optimum pH (8.5) and temperature (45 °C) were determined, showing the alteration of pH and temperature profiles by immobilization. Catechol, L-DOPA, p-cresole and pyrogallol were tested as substrate and it was established that affinity was highest for catechol. $K_m$ constant was 5 mM for catechol. Thermal and storage stability were carried out. It was observed that the immobilized enzyme had storage stability for a period of one year.

## Introduction

Alumina is a suitable matrix for protein immobilization by simple adsorption and has been very often utilised for this purpose. Although the covalent attachment has been more extensively utilised,[1–3] recently there has been renewed interest in immobilization by non-covalent methods. Non-covalent method is a very economic and simple method and the enzymatic activity retains during immobilization. The main disadvantage of this method is the weak binding so that enzymes immobilized by this method may not be used industrially. However the stability of the enzyme generally increases following this application. In this method, the stability of the adsorbed enzyme derivative will depend on the strength of the noncovalent bonds formed between the support and the amino acid residues on the surface of the protein. Two principal types of bonds can be formed between the enzyme and the inorganic support and they are electrostatic bonds and hydrogen bonds.

Polyphenol oxidases which are isolated from various sources have been immobilized on different supports such as zeolite, sepiolite, bentonite,[4] chitin,[5] glass beads[6] by non-covalent method. Some of this immobilized polyphenol oxidases have been used to control the pollution in water,[7] to remove and transform toxic compounds

of industrial process[8,9] and to determine catechol and other biologically active catecholamines in the biologic liquids as enzymatic biosensor.[10,11]

In this present paper, we aimed to immobilize the quince (*Cydonia oblonga*) polyphenol oxidase (EC.1.14.18.1) on alumina by adsorption method and to characterise it in terms of kinetic constant, optimum pH and temperature, substrate specificity, thermal and storage stability.

## Experimental

*Materials*

Quinces were obtained from local market in Edirne, Turkey. Aluminum 60 ($Al_2O_3$), ascorbic acid, ammonium sulphate, catechol, pyrogallol, p-cresole were purchased from Merck (Darmstad, FRG). Polyvinylpyrrollidone (PVP), L-DOPA, Triton X-100, Folin Ciocalteau reagent were supplied by Sigma Chem. Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

*Methods*

*Extraction of Polyphenol Oxidase*

The extraction method previously described[17,18] was followed in a modified form. 100 g of quince tissue was cut quickly in to thin slices and homogenised with 100 ml of 0.1 M phosphate buffer, pH 6.8 containing 10 mM ascorbic acid, 0.1% PVP and 0.5% Triton X-100 in a Waring Blender for 3 min. The homogenate was filtered through glass wool and the filtrate was centrifuged at 160 000xg for 30 min at 4 °C by a Sanyo MS 60 Ultrasantrifuge. The supernatant was brought to 90% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. The precipitated PPO was separated by centrifugation at 160 000xg for 30 min. The precipitate was dissolved in a small amount of 0.1 M phospate buffer, pH 6.8 and dialysed at 4 °C in the same buffer for 6 h with three changes of buffer during dialysis. The dialysate was used as the active enzyme.

*Immobilization of Quince Polyphenol Oxidase on Alumina by Adsorption*

Polyphenol oxidase was immobilized by adsorption on alumina using an organic solvent (acetone) as an immobilization medium. Prior to immobilization, alumina was dried at 105 °C for 2 h.

For the immobilization 4 ml dialysate, containing 0.650 mg protein/ml, was mixed with 5.2 g alumina under low stirring for 30 min at 4 °C. After this period, 8 ml of cold acetone was added to this mixture. For the completion of adsorption, the mixture was kept at 4 °C for 1 h by shaking continously and slightly, and the final mixture was separated by vacuum filtration. Then the solid was washed with 15 ml of cold acetone and distilled water until the filtrate was free from any unbound enzyme and finally dried *in vacuo*.[19,20] Further experiments were carried out to select appropriate enzyme loading by using an immobilized amount of polyphenol oxidase for different amounts of support.

*Determination of the Amount of Enzyme Bound on the Supports*

Protein in the filtrates and dialysate were estimated by employing Lowry method using bovine serum albumine as standart.[21] Finally, the amount the protein bound on the support was found by subtracting unbound protein from total protein.

*Assay of Immobilized Polyphenol Oxidase Activity*

The activity of the immobilized enzyme was assayed by using the modified Burton method.[22] 20 mg biocatalyst (enzyme+support) was added to a solution of catechol (0.02 M; 5 ml) prepared in phosphate buffer (0.05 M, pH 6.8) at 25 °C in a tube. The mixture was briefly but rapidly shaken to dissolve the enzyme off support, filtered, and monitored to observe the increase in absorbance due to catechol at 420 nm for 1 min using a Shimadzu UV 160 A spectrophotometer. The blank sample contained only 3 ml catechol solution. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO activity was defined as the amount of enzyme+support that caused an increase in absorbance of 0.001 per min. Relative spesific activity was calculated using the following formula:

Relative Specific Activity = Unit / mg immobilized protein

*Effect of pH and Temperature*

Immobilized/free polyphenol oxidase activities were assayed at various pH values with catechol as substrate. The phosphate buffers were used between pH 5-10 in this purpose.

In optimum temperature assay, immobilized/free polyphenol oxidase were incubated at temperatures ranging from 5 °C to 70 °C prior to measurement of its residual activity.

*Determination of Kinetic Constants*

The Michaelis constant ($K_m$) of the immobilized/free polyphenol oxidase for catechol were calculated from Lineweaver-Burk plots.

*Substrate Specificity of Immobilized Polyphenol Oxidase*

Immobilized/free polyphenol oxidase activities were determined using four different substrates (catechol, pyrogallol, L-DOPA, p-cresole). All substrate solutions were prepared in 0.05 M phosphate buffer, pH 6.8.

*Thermal and Storage Stability*

Immobilized polyphenol oxidase was kept for 1 h at temperatures from 10 °C to 90 °C before enzyme assay. Then those activity assays were carried out using catechol as a substrate.

To determine the activity change of the immobilized enzyme with time, the activity assay of immobilized preparate, which was stored in deep freeze at –40 °C were done for a period of one year.

## Results and Discussion

Polyphenol oxidase (EC.1.14.18.1) catalyses the regioselective aerobic oxidation of monophenols to o-diphenols and their eventual dehydrogenation to o-quinons. Undesirable browning in fresh fruits and vegetables, resulting from the oxidations of

polyphenolic compounds occurs during post harvest storage and processing.[12] To clear up this phenomenon, considerable number of study on purification, structure and mechanism of action of polyphenol oxidase enzyme has been realized.[13,14] Recently immobilized polyphenol oxidases have been used in the several industrial processes and in the various analytical and monitoring devices. Mazzei *et al* and Nisto *et al* have designed enzymatic biosensors by using immobilized polyphenol oxidase.[10,15] Wada *et al* and Crecchio *et al* have investigated controlling the pollution in waste water with immobilized polyphenol oxidases.[8,16] While most of these studies have been carried out with mushroom enzymes, we have utilized the enzyme which is isolated from quince. Quince polyphenol oxidase is about as active as that of isolated from mushrooms. Alumina has been considered to be a good candidate matrix in the immobilization by simple adsorption for quince polyphenol oxidase since it is cheap and economical support.

In the present study partially purified polyphenol oxidase from quince was immobilized on alumina by adsorption. As an increasing amount of enzyme was bound, the activity was decreased. This has been explained by "crowding" of the enzyme on the polymer surface.[5] At higher enzyme concentrations, the loss of activity on immobilization was about 40%. The preparation with an activity yield of 99% was used in the present work (Table 1).

**Table 1**. Immobilization of Quince Polyphenol Oxidase on Alumina at pH 6.8

| *Protein of quince PPO (mg) / 5 g bentonite* ***(x)*** | *Protein of PPO in washing (mg)* ***(y)*** | *Bound protein of PPO (mg)* ***(x-y)= A*** | *Imm. yield* ***(%)*** **A/x . 100 = %** | ***Relative spesific activity*** *(U/ mg imm. protein)* |
|---|---|---|---|---|
| 2.60 | 0.03 | 2.57 | 99 | 5447 |
| 5.20 | 0.36 | 4.84 | 93 | 2692 |
| 10.4 | 3.96 | 6.44 | 62 | 1346 |

The immobilized enzyme gave a $K_m$ value of 5 mM as compared with 7.5 mM for free enzyme by using catechol as substrate. For immobilized and free potato polyphenol oxidase, these values were reported to be 37 mM and 34 mM respectively.[5] We found that the highest activity of immobilized and free polyphenol oxidase were at

pH 8.5 and pH 9.0, respectively. The activity of immobilized enzyme was observed higher than that of free enzyme at alkaline pH and its pH profile was found to be different from the free enzyme (Figure 1). For immobilized mushroom polyphenol oxidase, optimum pH was given as 7.0 and its activity was high at alkaline pH as similar to our findings.[6]

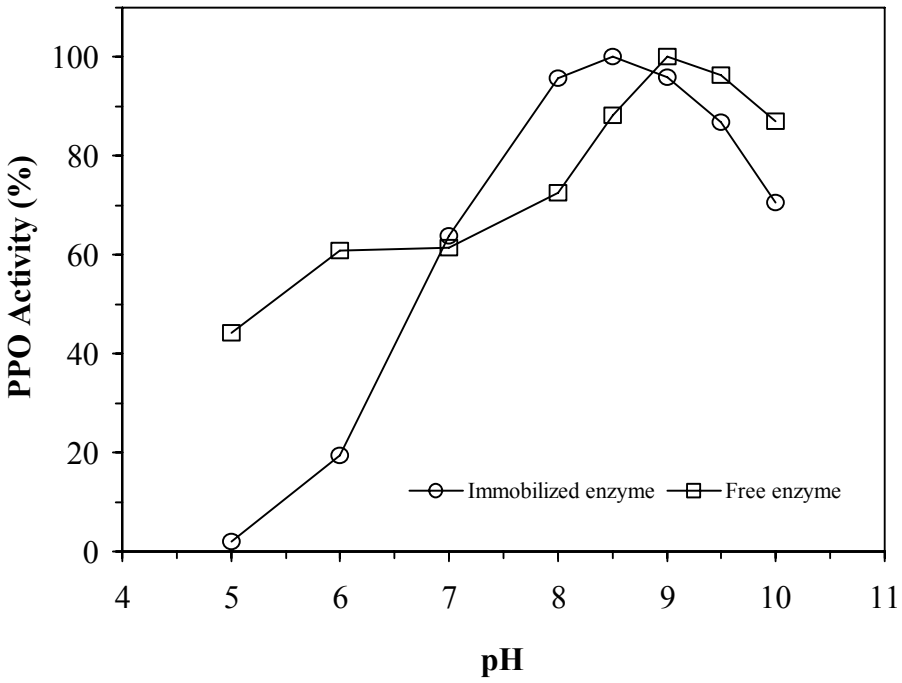


**Figure 1.** pH profiles of immobilized and free enzyme

The temperature profile of immobilized enzyme was shown in Figure 2 where it is seen that the optimum temperature is 45 °C. For immobilized mushroom polyphenol oxidase, this value was found as 30 °C.[6] Optimum temperature of free enzyme from quince is about 40 °C, and has broader temperature profile than that of immobilized enzyme (Figure 2).

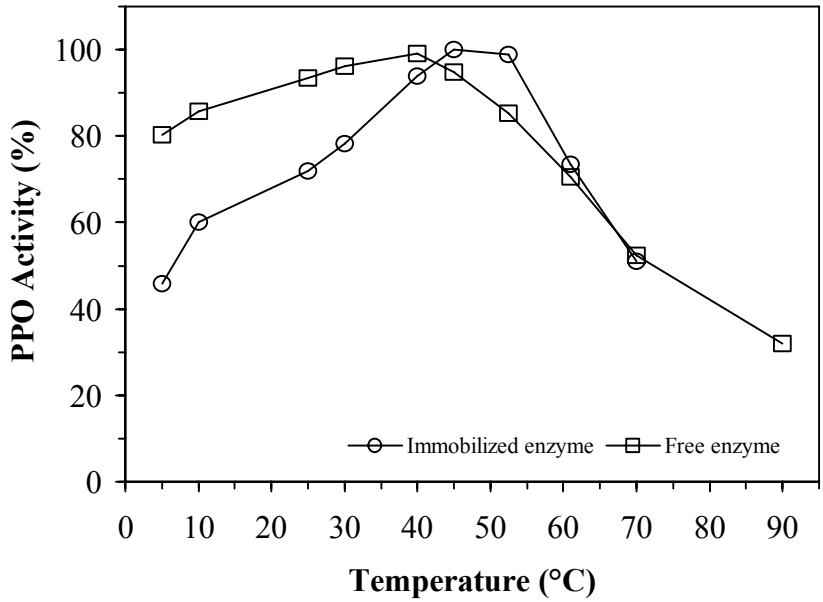


**Figure 2.** Temperature profile of immobilized and free enzyme

Catechol, L-DOPA, p-cresole and pyrogallol were tested as substrates and the results of activity assays were summarized Table 2. It was observed that while catalytic efficiency increased in the order of catechol, p-cresole, L-DOPA, no activity observed for pyrogallol. However, for free polyphenol oxidase, while pyrogallol is a good substrate, p-cresole is not a substrate at all, showing that immobilization may cause a change in substrate specificity.

**Table 2.** Substrate Specificity of Immobilized and Free Polyphenol Oxidase

| Substrate | *Immobilized enzyme (U/ mg immobilized protein)* | *Free enzyme (U/ mg protein)* |
|---|---|---|
| Catechol | 5236 | 2179 |
| L-DOPA | 1879 | 786 |
| p-cresole | 4263 | *NA* |
| Pyrogallol | **NA** | 1230 |

*NA: No activity*

Immobilized polyphenol oxidase activity was determined at 11 different temperatures ranging from 10 to 90 °C following 1 h incubation. Thermal stability profile was shown in Figure 3. Immobilized polyphenol oxidase showed almost the same activity for each temperature up to 60 °C. These results indicate that immobilization preserved the enzyme structure from thermal inactivity. The resistance of immobilized polyphenol oxidase to temperature is an important potential advantage for practical applications of this enzyme.

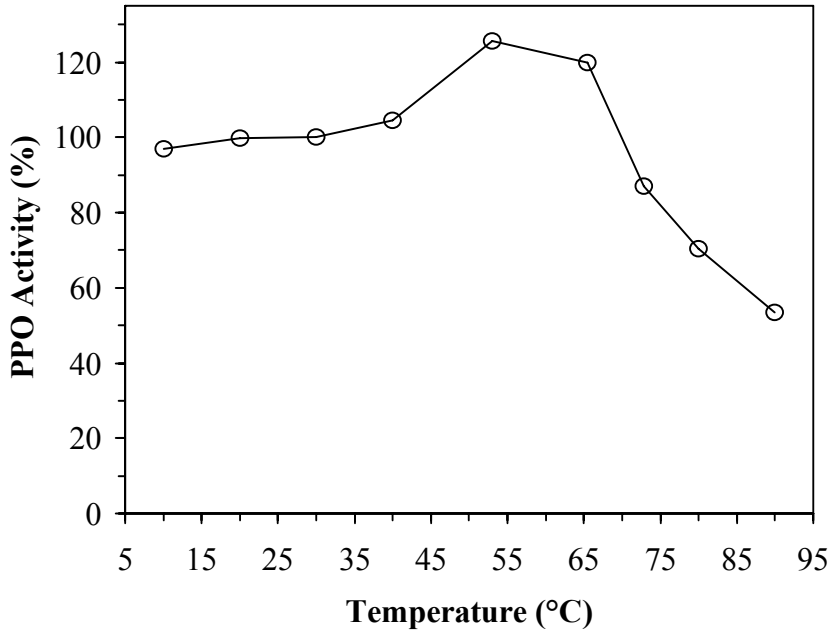


**Figure 3.** Thermal stability of immobilized enzyme. 20 mg of immobilized enzyme dissolved in 1.0 ml 0.05 M phosphate buffer, pH 6.8 and was incubated at different temperatures for 30 min. It was cooled to 25 °C immediately, and the activity was measured as described in Material and Methods

The activity assays of immobilized enzyme were carried out to determine the effect of time on the polyphenol oxidase activity for a period of one year. We observed that stability did not change considerably during this period (Figure 4). This is an advantage for the industrial usage, ecological treatments and biosensor applications.

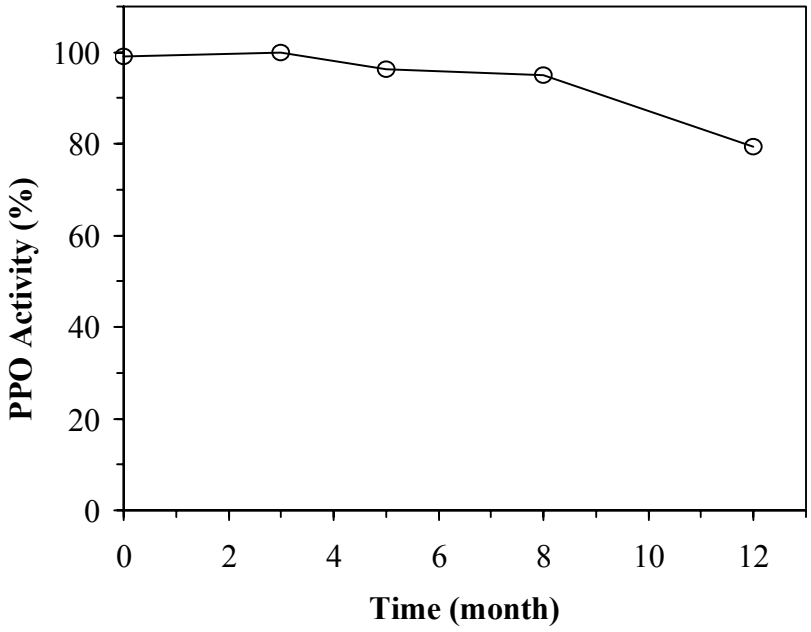


**Figure 4.** The storage stability profile of immobilized enzyme

## Conclusions

Polyphenol oxidase enzyme was partially purified from quince *(Cydonia oblonga)* by 90% $(NH_4)_2SO_4$ precipitation and dialysis. By using the dialysate as the enzyme source, immobilization was realized on alumina by non-covalent imobilization method. The yield of immobilization was 99%. Optimum pH and temperature were determined, 8.5 and 45 °C, respectively. While L-DOPA, catechol and p-cresole were a good substrate no activity was observed with pyrogallol. It was determined that catechol was the better substrate to another, its $K_m$ constant was 5 mM. In the storage and thermal stability assays it was seen that immobilized enzyme had storage and thermal stability Having thermal and storage stability the practical applications of enzyme is a potential advantage. The detailed study of immobilization on the same support with the different methods, such as the covalent attachment, and the usage of this immobilized polyphenol oxidase, which has high storage stability in the batch, and continous processes will be further investigated.

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## Povzetek


Encim polifenol oksidazo smo izolirali iz *Cydonia oblonga* in imobilizirali na aluminijevem oksidu z adsorpcijo pri pH 6.8. Lastnosti imobiliziranega encima smo primerjali s prostim encimom in ugotovili, da se z imobilizacijo spremenita pH in temperaturna profila. Študirali smo tudi termično stabilnost in trajnost imobiliziranega encima. Imobiliziran encim lahko hranimo do enega leta.