

## Stabilization of Tyrosinase-Bovine Serum Albumin Crystals by Glutaraldehyde

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**Abstract:** Tyrosinase and bovine serum albumin were co-crystallized by saturated ammonium sulfate solution (65%) and 20% polyethylene glycol (PEG) 6000 and n-propanol as co-solvents. The obtained crystals were cross linked by glutaraldehyde solution (1% v/v). Polyethylene glycol 6000 was found to be better co-solvent than n-propanol. The developed biocatalyst could be recycled 6 times without further loss of tyrosinase activity. No loss of activity of cross linked tyrosinase-bovine serum albumin crystals was observed upon storage of the developed CLECs at refrigerator for six months.

**Key words:** Tyrosinase, bovine serum albumin, PEG 6000, n-propanol, crystallization, glutaraldehyde, stability

### INTRODUCTION

Tyrosinase is a biocatalyst (EC 1.14.18.1) containing two atoms of copper per active site with mono-oxygenase activity that is responsible for the biosynthesis of melanins and other polyphenolic compounds<sup>[1]</sup>. Mushroom tyrosinase as a biocatalyst, in the presence of ascorbic acid<sup>[2]</sup>, has been employed to produce valuable compounds like hydroxytyrosol (an anti oxidant), l-dopa (a drug used in the treatment of Parkinson's disease)<sup>[3-5]</sup>. Other compounds like coumestrol and caffeic acid are reported to be produced by such biocatalyst<sup>[6,7]</sup>. Lenhart *et. al.*<sup>[8]</sup> and Aberg *et. al.*<sup>[9]</sup> used tyrosinase for biocatalytic grafting of phenolic moieties or protein onto chitosan and eventually the modified chitosan could be used as hydrogels for skin substitutes<sup>[10]</sup>, adhesives<sup>[11]</sup>, matrices for drug delivery and for tissue engineering<sup>[12,13]</sup>. The enzymatic conversion of substrate to product under mild conditions has made the enzymes of prime importance to be used in biotechnological processes. Most of the enzymatic or biocatalytic processes occur in environmentally mild conditions with specificity and efficiency. There are obstacles in employing biocatalysts in enzyme catalysed processes, the major problems in such processes are biocatalyst instability and reuse of it in processes leading to conversion/synthesis of substrates/products. To circumvent such obstacles and in view of bioengineering advances, biocatalysts could be stabilized/immobilized due to following two main reasons.

- \* Easy separation of the enzyme from the product that simplifies enzyme application and permits reliable and efficient technology.
- \* Enzyme reuse provides number of cost advantages that are often an essential prerequisite for establishing an economically viable enzyme catalyzed processes<sup>[14]</sup>.

Tyrosinase has been immobilized to convert l-tyrosine to l-dopa in the presence of ascorbic acid that the later substance inhibits the conversion of l-dopa to quinone<sup>[15-19]</sup> and removal of phenols from the industrial wastewater<sup>[20,21]</sup>. Furthermore, tyrosinase is also immobilized in order to construct biosensors for the quantification of phenol and related compound in the environment<sup>[22]</sup>. Cross linked enzyme crystals (CLECs) have several characteristics that confer significant advantages over conventional enzyme immobilization methods like enhanced temperature stability, absence of an inert support, catalysis under harsh conditions such as temperature, pH and organic solvents. The insoluble nature of CLECs facilitates easy separation of the enzyme from the reaction mixture which increases the reuse efficiency of the enzyme<sup>[23]</sup>. Immobilization/stabilization of tyrosinase by cross-linking crystallized tyrosinase and bovine serum albumin complex is a method employed to develop a biocatalyst, which transforms l-tyrosine in the presence of ascorbic acid to l-dopa.

### MATERIALS AND METHODS

Tyrosinase, bovine serum albumin (fraction V) and glutaraldehyde were purchased from Sigma. L-

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Tyrosine, l-dopa and ascorbic acid were procured from Merck. Other reagents used were of analytical grade.

**Assay of tyrosinase activity:** Tyrosinase activity was determined colorimetrically from the amount of l-dopa produced by tyrosinase action on l-tyrosine in the presence of ascorbic acid and molecular oxygen as reported by Munjal and Sawhney<sup>[24]</sup>. The reaction mixture contained 1ml of 2.5 mM tyrosine and ascorbic acid in 0.05M phosphate buffer pH7 to which 0.1 ml (or 0.1 g of stabilized tyrosinase-bovine serum albumin) of appropriately diluted tyrosinase solution was added. The reaction mixture was incubated at 25 °C for 25 minutes. To the above solution, 1 ml of the following reagents were added: 2 M HCl, 2 M NaOH, 15% (w/v) sodium molybdate and finally 15% (w/v) sodium nitrite. Absorbance was recorded at  $\lambda$  450nm after 1 h against blank which contained inactivated free tyrosinase solution. Units of enzyme activity can be defined as the amount of enzyme that produces 1 $\mu$  mole of l-dopa in one minute under the above assay conditions, employing l-tyrosine as the substrate. The protein content of free tyrosinase solution was measured by Lowry's method, using bovine serum albumin as standard.

**Co-crystallization of tyrosinase and bovine serum albumin:** Batch co-crystallization of tyrosinase and bovine serum albumin was carried out at refrigerator. To tyrosinase solution containing 0.356 mg/ml protein was added 2 mg/ml of bovine serum albumin (fraction V) to give a protein solution containing 2.356 mg/ml. Ammonium sulfate at concentration of 65% saturation along with 20% polyethylene glycol 6000 or 20% n-propanol as co solvents were added to tyrosinase solution containing bovine serum albumin (fraction V) at 4 °C separately. The mixtures were kept on magnetite stirrers overnight and they were then left in refrigerator. The obtained crystals from each mixture were assayed for tyrosinase activity as mentioned above. The obtained crystals from each reaction mixture were cross linked with 1% (v/v) glutaraldehyde solution prepared in phosphate buffer pH 7, for 3 hours at 4 °C separately. The cross linked crystals were washed several times with distilled water and assayed for tyrosinase activity.

## RESULTS AND DISCUSSION

Tyrosinase and bovine serum albumin were co-crystallized due to low protein content of tyrosinase. The final concentration of bovine serum albumin in the enzyme solution was adjusted to 2 mg/ml. N-Propanol

and polyethylene glycol 6000 were separately used as co-solvents to crystallize the protein solution containing tyrosinase and bovine serum albumin. It was observed that PEG was a better co-solvent in crystallizing concomitantly tyrosinase and BSA complex, and the loss of activity of tyrosinase-BSA complex crystals cross linked with 1% glutaraldehyde using n-propanol was almost three times more than PEG as a co-solvent. There are reports indicting the use of single enzyme like chloroperoxidase, glucoamylase, subtilisin and penicillin G acylase to be crystallized or aggregated and then cross linked with glutaraldehyde solution respectively<sup>[25,26,27,28]</sup>.

Table 1: Loss of tyrosinase activity during crystallization and post-crystallization processes

Activity of free tyrosinase	2.17 U/ml/min $\pm$ 0.057
Activity of free tyrosinase after addition of BSA	1.36 U/ml/min $\pm$ 0.046
Activity of tyrosinase-BSA complex crystals dissolved in buffer and dialyzed.	0.27 U/g/min $\pm$ 0.011
Activity of tyrosinase-BSA complex crystals after cross linking with glutaraldehyde	0.093 U/g/min $\pm$ 0.007

Table 1 indicates the loss of tyrosinase activity during crystallization process of tyrosinase and BSA complex. Fig. 1 shows the scanning electron micrograph of the developed biocatalyst. CLECs are highly stable biocatalyst and this could be due to compactness of enzyme molecules almost to the theoretical limit, stabilization is a consequence of intense polar and hydrophobic interactions<sup>[29]</sup>.

The operational stability and storage stability of newly developed tyrosinase biocatalysts were studied. The operational stability was performed in batches containing 1g of stabilized tyrosinase in a solution containing 5 mM l- tyrosine and ascorbic acid at 25°C and 150 rpm. Duration of each cycle was 2 hours, after each cycle the developed biocatalyst was separated from the reaction mixture by centrifugation at 4000 rpm, 4°C for 10 min and the crystals were washed with 0.05 M phosphate buffer pH 7. The washed out crystals were checked for tyrosinase activity and protein content, negligible leakages of enzyme and protein in first 3 cycles were observed. During further cycles no loss in activity of developed biocatalyst could be seen (Fig. 2).

The enzyme was stored in 0.05 M phosphate buffer pH 7 for 6 months at refrigerator and every month, the crystals were taken out to check the enzyme activity, during the storage no loss in enzyme activity could be detected. Tyrosinase and BSA complex were kept in the assay buffer containing 2.5 mM ascorbic acid in order to determine tyrosinase action on tyrosine content of BSA in the formed crystalline complex.

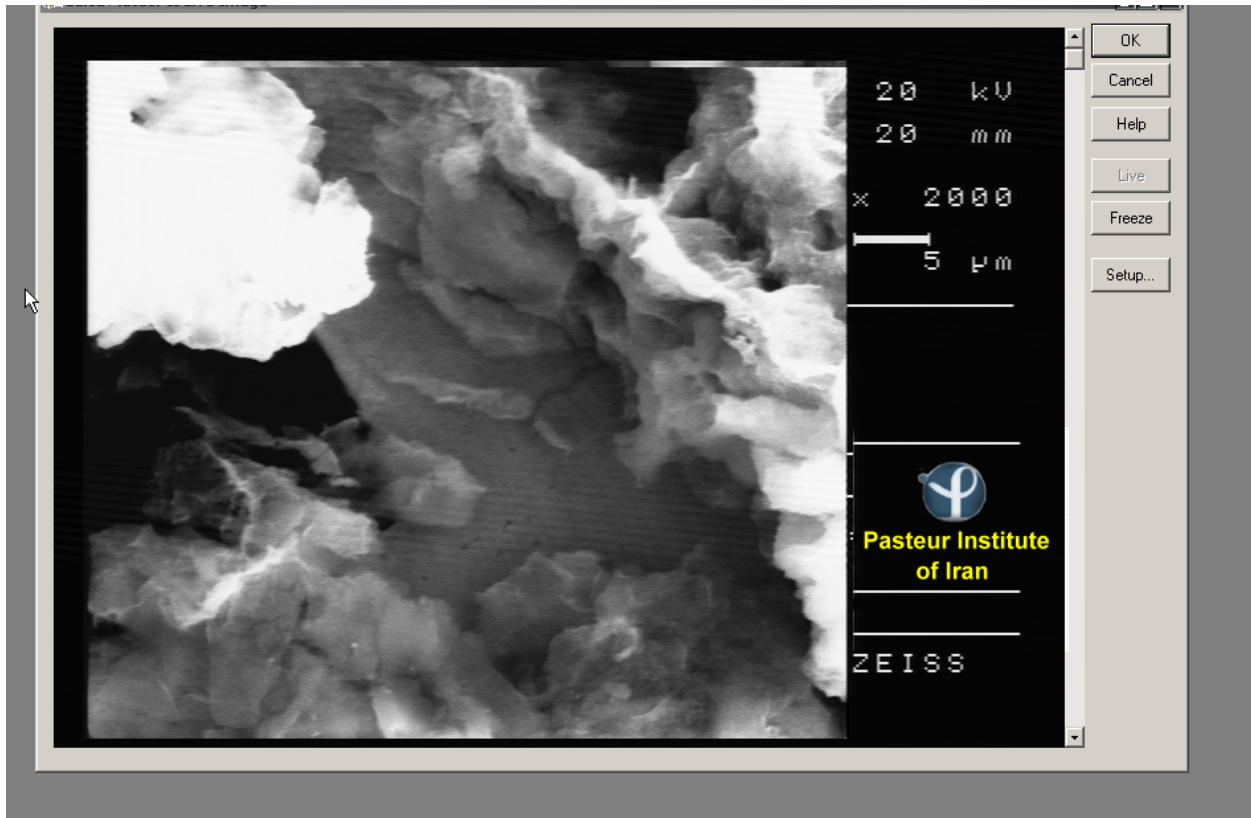


Fig. 1: Scanning electron micrograph of cross-linked tyrosinase-bovine serum albumin crystals

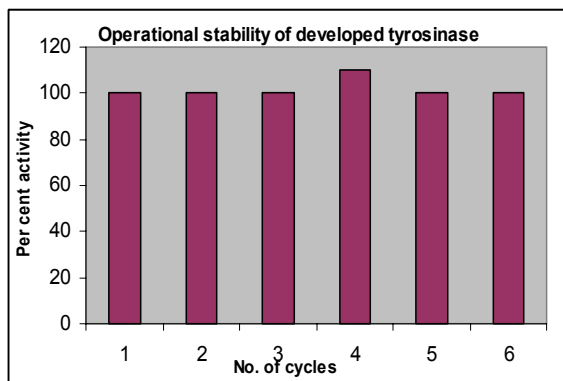


Fig. 2: Operational stability of cross-linked tyrosinase-bovine serum albumin crystals in batch operation

There was no tyrosinase activity in tyrosinase - BSA complexes. Therefore, due to low protein content of tyrosinase, bovine serum albumin was used to enrich tyrosinase to develop highly stable cross-linked tyrosinase crystals containing bovine serum albumin.

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