

CALCIUM ALGINATE AS A SUPPORT MATERIAL FOR IMMOBILIZATION OF L-AMINO ACID OXIDASE ISOLATED FROM ASPERGILLUS FUMIGATUS

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ABSTRACT

Crude L-amino acid oxidase (L-ao) of *Aspergillus fumigatus* was immobilized in various solid supports namely, entrapment in calcium alginate gel and gelatin gel, and adsorption in nylon membrane by cross linking with glutaraldehyde. Immobilization of *A. fumigatus* L-ao in calcium alginate gel was the most favourable since the percent entrapped activity was maximal in calcium alginate beads (31.77%) as compared to the adsorption in nylon membrane (26.88%). Maximum enzyme activity was found with 3 % sodium alginate. Reaction time for immobilized beads increases from 2h reaching a maximum at 6h. The activity of the calcium alginate entrapped *A. fumigatus* L-ao beads was assayed for four cycles with DL-alanine as the substrate. The enzyme showed 87.52 % activity during the second reuse and 72.37 % activity on its third use. Immobilization in gelatin gel was ineffective since the immobilized gel cubes were unstable and disintegrated during the enzyme reaction. As for immobilization of the L-ao by adsorption in nylon membrane, only 26.88% activity was retained.

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KEY WORDS

L-amino acid oxidase; *Aspergillus fumigatus*; immobilization; calcium alginate.

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[I] INTRODUCTION

Immobilization of enzymes is a technique, adopted to reduce the cost and increase the utilization of enzymes. Immobilized enzymes are widely used in different industries, especially in food and pharmaceutical and offer several advantages over bulk or free enzymes. Advantages include high productivity, automation, continuous processing, precise control of the extent of reaction, easy product recovery and non-contamination of the final product by the enzyme [1].

The determination of amino acids is important in several applications, and it is often the determination of single amino acids which is desired. In the clinical laboratory, determination of certain amino acids in physiological fluids or tissue may be useful indicator of certain diseases or disorders. Besides numerous chromatographic and electrophoretic methods developed for the determination of amino acids, including also chiral determinations, a method for the efficient determination of amino acids of particular chiral configuration can be developed by employing the stereoselective L-ao as biosensors or enzyme electrodes [2].

Two basic factors, crucial to the success of construction of biosensors are the method of immobilization of the enzyme and the selection of the most suitable signal transducer. Enzymatic biosensors for amino acids have already been described in analytical and biochemical literature, differing as to the kind of enzyme used, the method of enzyme immobilization employed and the type of transducers utilized. Immobilized L-ao degrades L-amino acids to 2-oxo acid, ammonia and hydrogen peroxide

[3]. Amperometric biosensors for the determination of D- and L-enantiomers of L-leucine were developed by physically immobilizing L-amino acid oxidase in diamond paste [4].

Aspergillus fumigatus L-ao is an important enzyme that causes the racemic resolution of DL-alanine to produce D-alanine [5]. In the present study, different methods of immobilization were investigated with *A. fumigatus* L-ao. These include entrapment in calcium alginate gel, entrapment in gelatin gel, and adsorption in nylon membrane by cross linking with glutaraldehyde. The efficiency of an immobilization process can be measured by the following criteria. Most important of all, a high percentage of the enzymes must be initially retained in the gel matrices. Secondly, the enzyme activity must be preserved. And thirdly, the enzymes must be physically restrained from diffusing back into the substrate solution at a later time. Studies were done looking into the above aspect.

[II] MATERIALS AND METHODS

2.1. Isolation of crude L-ao of *A. fumigatus*

A. fumigatus cells (96 h old) were harvested by centrifugation at 9400 g and 10°C, for 10 minutes. The cells were then homogenized in a French pressure cell press (Thermo spectronic, USA) at 1500 psi for 5 minutes in ice cold condition. The cell suspension obtained was centrifuged at 13600 g for 15 minutes at 4°C. The cell debris was discarded and the clear supernatant was taken as the crude enzyme.

2.2. Entrapment in Calcium alginate beads

Crude L-aaO (5mL or 7.2 mg) was mixed with 3% sodium alginate solution in 1: 2 ratio. The immobilized enzyme beads were formed by dripping the enzyme-alginate mixture from a height of approximately 20 cm, drop wise into the calcium chloride (0.2 M) solution with continuous shaking. The beads were washed 3-4 times with distilled water and finally with 50 mM sodium phosphate buffer, pH 7.2. The beads were dried and weighed for further studies.

2.2.1. L-amino acid oxidase activity of the calcium alginate beads

The beads (2 g) were added to 10 mL of 50 mM sodium phosphate buffer, pH 7.2 containing 50 mM of the substrate i.e. DL-alanine. The reaction was performed for 2-7 h at 30°C, 200 rpm. The enzyme assay was performed with 0.5 mL of the reaction filtrate as described previously [5]. Briefly, 0.5 mL of the reaction mixture was diluted 5 times with distilled water and reacted with 0.4 mL of 2, 4-dinitrophenylhydrazine (0.2% saturated in 2N HCl) for 10 minutes. To this 1.5 mL of 3M NaOH was added and absorbance at 550 nm was recorded after 15 minutes. One unit of L-aaO activity was defined as the amount of enzyme producing 1 μmol pyruvate/min under the standard assay conditions.

2.2.2. Effect of sodium alginate concentration

Various concentration of sodium alginate (1% - 4%) were used to acquire beads with greater stability. Sodium alginate solution (1- 4 %) were prepared. The rest of the procedure for enzyme immobilization was same as stated previously.

2.2.3. Reusability of the calcium alginate immobilized beads

The L-aaO activity of the calcium alginate immobilized beads was assayed for four cycles with 50 mM DL-alanine as the substrate, in order to find out the reusability of the entrapped enzyme. The enzyme reaction was performed for 6h under the conditions described earlier.

2.3. Entrapment in gelatin gel

Crude L-aaO (5mL or 7.2 mg) was mixed with the 10 % gelatin solution in 1: 2 ratio. 2mL of the hardening solution (20 % vol. formaldehyde, 50 % vol. ethanol and 30 % vol. water) was added to the enzyme-gelatin mixture. The solution was poured into a mold or a small beaker and frozen at 0°C for 4 h to facilitate the gel formation. When the gel was set, it was cut into small cubes of approximately 3mm per side. The gel cubes were washed once with distilled water and finally with 50 mM sodium phosphate buffer, pH 7.2. The enzyme activity was checked for the gelatin beads.

2.4. Cross linkage in nylon membrane

To a mixture of the L-aaO (100 μL) and BSA (166 μL of 40 mg/mL), 166 μL of 2.5 % glutaraldehyde was added to initiate cross linking. The mixture was layered onto a prewetted nylon membrane of pore size 0.22 μm and the solution was allowed to cross link at room temperature (20-25°C) for 2-3 h until a yellowish hard gel layer is formed. The enzymic membrane was washed with 50 mM sodium phosphate buffer, pH 7.2 to remove excess glutaraldehyde and the L-aaO activity was determined.

2.4.1. L-aaO activity of the enzymic membrane

The enzymic membrane is immersed in 4 mL of 50 mM sodium phosphate buffer, pH 7.2 containing 50 mM of substrate i.e. DL-alanine. The reaction was performed for 3-5 h at 30°C, 200 rpm. The enzyme assay was performed with 0.5 mL of the reaction filtrate as described previously and the enzyme activity was recorded.

2.4.2. Reusability of enzyme immobilized nylon membrane

The L-aaO activity of the enzymic membrane was assayed for two cycles with 50 mM DL-alanine as the substrate, in order to find out the reusability of the entrapped enzyme. The enzyme reaction was performed as before.

2.5. Statistical analysis

The experiments were done in triplicate and the data are expressed as mean ± standard deviation.

[III] RESULTS

3.1. Effect of sodium alginate concentration on the stability of the calcium alginate beads

Various concentration of sodium alginate (1% - 4%) was used to acquire beads with greater stability. The maximum enzyme activity was found with 3 % sodium alginate. At lesser concentrations of sodium alginate i.e. 1% and 2%, the beads were not formed properly and were very unstable. Also, maximum leakage of the enzyme occurred owing to the large pore size of the less tightly cross linked fragile calcium alginate beads. When the sodium alginate concentration was increased to 4%, the enzyme activity was comparatively very low, which might be due to the high viscosity of the enzyme entrapped beads, which decreased the pore size and thus hindered the penetration of the substrate into the beads.

3.2. Calculations of the L-aaO activity after entrapment in calcium alginate beads

The efficiency of an immobilization process can be measured by the criteria that high percentage of the enzyme must be initially retained in the gel matrices. Keeping this in mind, the percent entrapped activity of the immobilized enzyme was calculated. The enzyme activity was recorded as the Units/g bead.

Initial activity of the free enzyme = 0.03024 μmol/min/ml or Units/mL

Volume of enzyme solution taken for immobilization = 5 mL

Weight of beads formed after immobilization of enzyme solution = 14 g

Enzyme solution entrapped in 2 g beads = 0.714 mL

Therefore enzyme entrapped = 0.02156 Units/ 2g beads (on the basis of free enzyme)

Activity of immobilized enzyme obtained in 2g beads = 0.00685 Units/ 2 g beads

Therefore enzyme activity after entrapment = 31.77 %

3.3. Effect of reaction time on the activity of the calcium alginate beads

The effect of the reaction time on the activity of the immobilized enzyme was investigated by performing the enzyme reaction for different time periods. The enzyme activity, recorded as the Units/g bead increases from 2h reaching the maximum at 6h and then decreases as the reaction time is increased to 7h [Figure-1].

3.4. Reusability of the calcium alginate beads

The activity of the entrapped enzyme was assayed for four cycles with DL-alanine as the substrate. The enzyme showed 87.52 % activity during the second reuse and 72.37 % activity on its third

use [Figure-2]. Loss of the enzyme activity of the entrapped enzyme was observed during the fourth cycle. This decrease in the enzyme activity was due to the leakage of the enzyme from the beads. Thus the loss in activity on the second reuse was only 27 %.

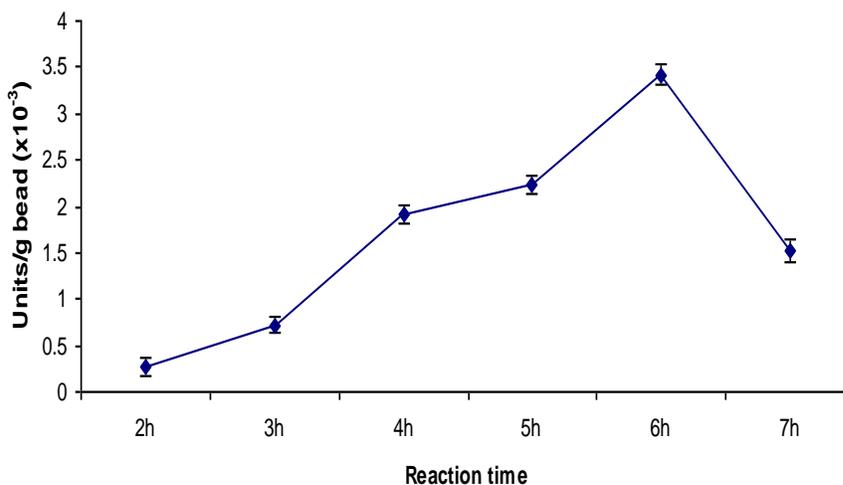


Fig. 1. Enzyme activity of the calcium alginate beads at different time periods. Reaction was performed from 2-7h under the standard conditions. Data are expressed as mean of three individual experiments \pm standard deviation.

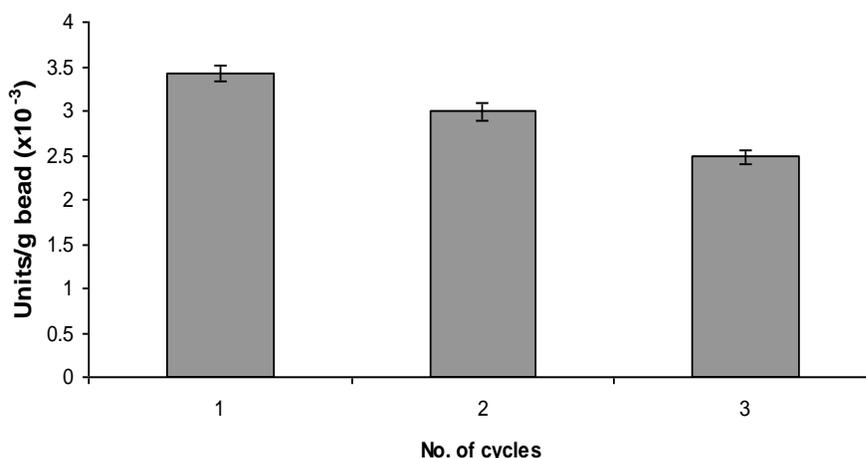


Fig. 2. Repeated use of immobilized L-ao beads showing the number of times the immobilized enzyme can be used. Data are expressed as mean of three individual experiments \pm std. deviation.

3.5. Immobilization of L-ao of *A.fumigatus* in gelatin gel

The immobilization of L-ao in gelatin gel was ineffective since the immobilized gel cubes were unstable and disintegrated during the enzyme reaction. Thus, this method of immobilization is not feasible for the enzyme.

3.6. Immobilization of L-ao of *A.fumigatus* by cross linking in nylon membrane

3.6.1. L-ao activity of enzymic membrane

L-ao was immobilized in 0.22 μm nylon membrane by cross linking with 2.5 % glutaraldehyde. L-ao activity was assayed for the enzymic membrane and was recorded as the μmol of pyruvate formed /min / membrane or Units/ membrane.

Calculation of enzyme activity:

Initial activity of free enzyme = 0.03024 Units/mL

Therefore, enzyme activity in 100 μ L enzyme = 0.003024 Units/mL

Enzyme activity of the enzymic membrane = 8.13×10^{-4} Units / membrane (Reaction performed for 5 h)

Therefore, enzyme activity after cross linking = 26.88 %

Thus after cross linking in nylon membrane, only 26.88 % of the enzyme activity is obtained.

3.6.2. Reusability of the enzymic membrane

The L-ao activity of the membrane was assayed for two cycles in order to check the reusability of the cross linked enzyme. Complete loss of the enzyme activity was observed at the second use of the membrane. This may be due to leakage of the enzyme from the membrane during the enzyme reaction. This suggests that the cross linking of *A. fumigatus* L-ao by glutaraldehyde on nylon membrane is not suitable for reuse.

[IV] DISCUSSION

The determination of amino acids is important in several applications, and it is often the determination of single amino acids which is desired. Besides numerous chromatographic and electrophoretic methods developed for the determination of amino acids, including also chiral determinations, a method for the efficient determination of amino acids of particular chiral configuration can be developed by employing the stereoselective L-ao as biosensors or enzyme electrodes [2].

Two basic factors, crucial to the success of construction of biosensors are the method of immobilization of the enzyme and the selection of the most suitable signal transducer. Enzymatic biosensors for amino acids have already been described in analytical and biochemical literature, differing as to the kind of enzyme used, the method of enzyme immobilization employed and the type of transducers utilized. A specific multi enzyme biosensor for L-alanine has been developed with the use of alanine dehydrogenase combined with salicylate hydrolase and pyruvate oxidase on a Teflon membrane covering a Clark amperometric oxygen sensor [6].

The immobilization of *A. fumigatus* L-ao in calcium alginate gel was the most favorable since the percent entrapped activity was maximal in calcium alginate beads (31.77%) as compared to the adsorption in nylon membrane (26.88%). The entrapment of enzyme in calcium alginate is one of the important methods of immobilization. Alginates are commercially available as water soluble sodium alginates and they have been used for more than 65 years in the food and pharmaceutical industries as thickening, emulsifying and film forming agent. Entrapping within insoluble calcium alginate gel is recognized as a rapid, non-toxic, inexpensive and versatile method for immobilization of enzymes as well as cells [7].

The maximum enzyme activity was found with 3 % sodium alginate concentration. At lesser concentrations of sodium alginate i.e. 1% and 2%, the beads were not formed properly and were very unstable. Also, maximum leakage of the enzyme occurred owing to the large pore size of the less tightly cross linked fragile calcium alginate beads. When the sodium alginate concentration was increased to 4%, the enzyme activity was comparatively very low, which might be due to the high viscosity of the enzyme entrapped beads, which decreased the pore size and thus hindered the penetration of the substrate into the beads. Thus, 3 % sodium alginate concentration is most suitable for the immobilization of the *A. fumigatus* L-ao. This is in agreement with Farag and Hassan, 2004 [8] who report that the sodium alginate concentration ranging from 2-3 % was suitable for the immobilization of keratinase, lipase and protease.

The enzyme activity increases from 2h reaching the maximum at 6h; whereas, the unimmobilized or the free enzyme shows the maximum enzymatic activity at 1 h of reaction time. Thus the immobilized enzyme achieves the maximum enzyme activity at a reaction time 6 times higher as compared to the free enzyme. This increase in time is due to the time required by the substrate molecules to penetrate into the beads and reach the active sites of the enzyme. This observation is supported by Qader et al., 2007 [9], who reported that calcium alginate entrapped dextranucrase took 60 minutes to achieve the maximum enzyme activity, which was 4 times higher than the free enzyme.

Reusability of immobilized enzyme is a very important criterion for assaying its applicability in industry and is also a characteristic of immobilization. Immobilized L-ao in calcium alginate beads showed 87.52 % activity during the second reuse and 72.37 % activity on its third use. Loss of the enzyme activity of the entrapped enzyme was observed during the fourth cycle. This decrease in the enzyme activity was due to the leakage of the enzyme from the beads. The loss in activity on the second reuse was only 27 %. This observation coincides with the fact that alpha-amylase entrapped in calcium alginate beads was reused for 6 cycles with approximately 30 % loss in activity [10]. However, the immobilized *A. fumigatus* L-ao calcium alginate beads can be effectively used for 3 cycles.

[V] CONCLUSION

Thus, 3 % calcium alginate was the suitable support for immobilization of *A. fumigatus* L-ao since the percent entrapped activity was maximal in calcium alginate beads (31.77%) as compared to the adsorption in nylon membrane (26.88%).

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CONFLICT OF INTEREST

Authors declare no conflict of interest

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