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Ascorbic Acid Biosensor Using Ascorbate Oxidase Immobilized on Alkylamine Glass Beads

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ABSTRACT

A biosensor for ascorbic acid based on enzyme kinetics of ascorbate oxidase (E.C.1.10.3.3.) was developed. The enzyme was extracted from *Cucurbita maxima*, or jerimun and immobilized by covalent bounding, using glutaradehyde as a bifunctional agent, on alkylamine glass beads, with and without enzyme active site protection. A low-cost, home-made oxygen electrode was applied as a transducer. The system has sensitivity from 62.5 up to 500 μM of ascorbic acid with satisfactory operation for more than 2 mo.

Index Entries: Ascorbic acid; biosensor; oxygen electrode; *Cucurbita maxima.*

INTRODUCTION

Several methods for the assay of ascorbic acid based on color have been applied, however, they suffered from a lack of specificity. In addition, they required expensive manipulations and separation techniques (*1*). More expensive techniques such as high-pressure liquid chromatography (HPLC) also have been used to detect ascorbic acid in biological fluids and fruit juices, e.g., lemon and orange juices. This report describes a biosensor based on ascorbate oxidase (E.C.1.10.3.3.) from *C. maxima* and

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immobilized on alkylamine glass beads that measures oxygen consumption. The enzyme was fixed onto a bowel chamber where the level of oxygen was constantly measured by a low-cost oxygen electrode (*2*). The biosensor was based on oxygen consumption following the reaction.

$$\text{Ascorbic acid} + O_2 \rightarrow \text{Dehydroascorbic acid} + H_2O$$

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Merck (Germany) and were reagent grade or better. The alkylamine glass beads were obtained from H. H. Weetall (Ciba-Corning Diagnostics, Medfield, MA).

Enzyme

The ascorbate oxidase (ascorbic oxidoreductase E.C.1.10.3.3.) was extracted from *C. maxima* and partially purified by ammonium sulphate precipitation at 40–60% (w/v) following the procedure of Carvalho et al. (*3*). Protein concentrations were determined by the Lowry method using bovine serum albumin as the standard (*4*).

Immobilization

The enzyme was immobilized using glutaraldehyde as the bifunctional agent through Shiff base formation with alkylamine glass beads (500 A pore size 80–120 mesh) according to the Weetall method (*5*) following two different procedures:

1. The enzyme-active site was protected with ascorbic acid solution 500 m*M* in citrate phosphate buffer (pH 6.0 0.1*M* with Etilenodiaminotetracetico acid (EDTA 2 m*M*) during immobilization.
2. The enzyme-active site was not protected during immobilization.

The support plus enzyme was sorted in a semipermeable compartment made by nylon net in citrate phosphate buffer (pH 6.0 0.1*M* with EDTA 2 m*M*) at 4°C. This was fixed into a 3mL cuvette used as a reaction chamber (Fig. 1).

Experimental Procedure

The electrode was calibrated from 0–100% of oxygen saturation following the Marques and Lima Filho procedure at 28°C (*2*). A 3mL cuvette was used as the reaction chamber. A vol of approx 25 mg of glass beads with 0.5 mg of immobilized enzyme was sealed into a nylon net bag and

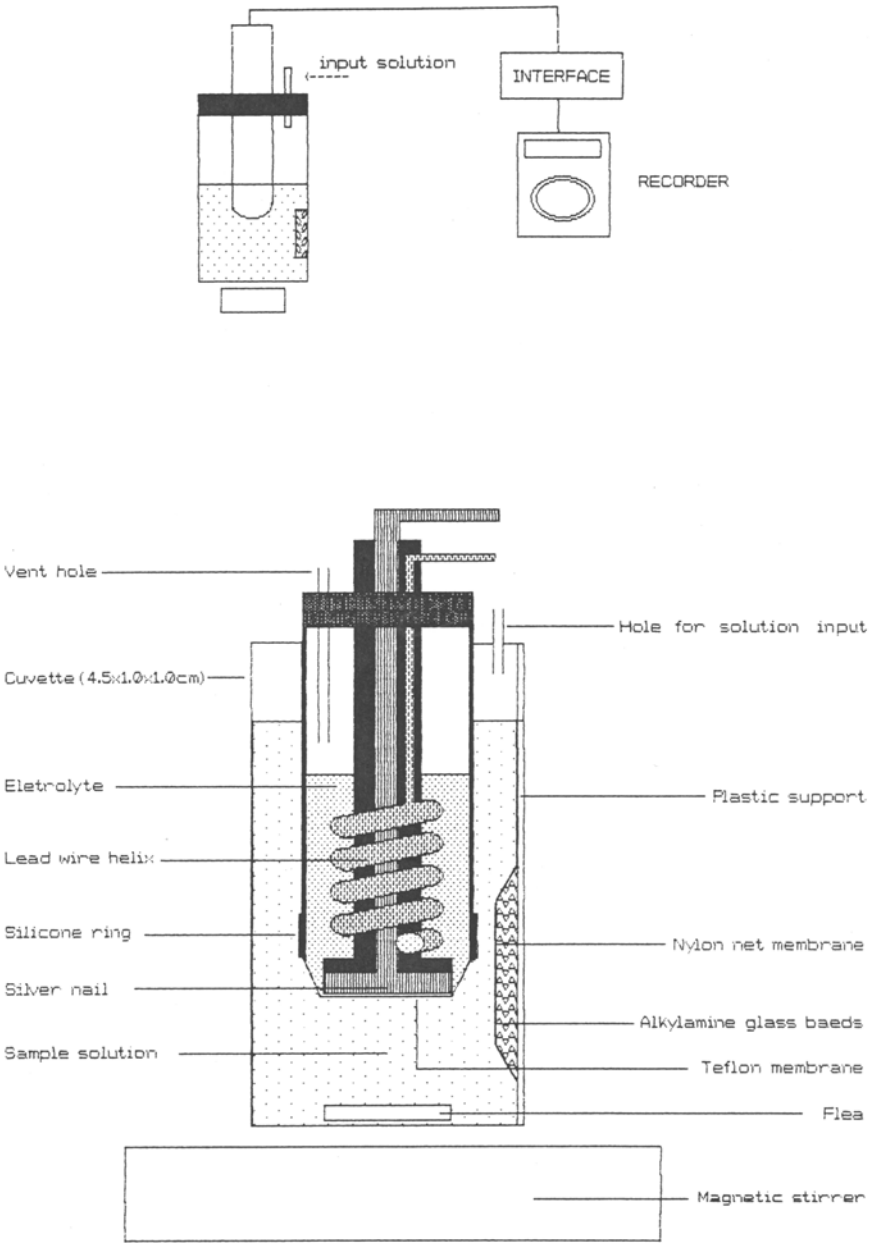

Fig. 1. The ascorbic acid biosensor design.

then fixed in the internal wall of the chamber (Fig. 1). A vol of 1.3 mL of citrate-phosphate buffer was added in the chamber containing the oxygen electrode, and the solution was stirred until stabilized, indicated by a constant signal from the electrode. The reaction was carried out by addition of 0.1mL of substrate giving final concentrations of 62.5, 125, 250, 500, 750 and 1000 μM.

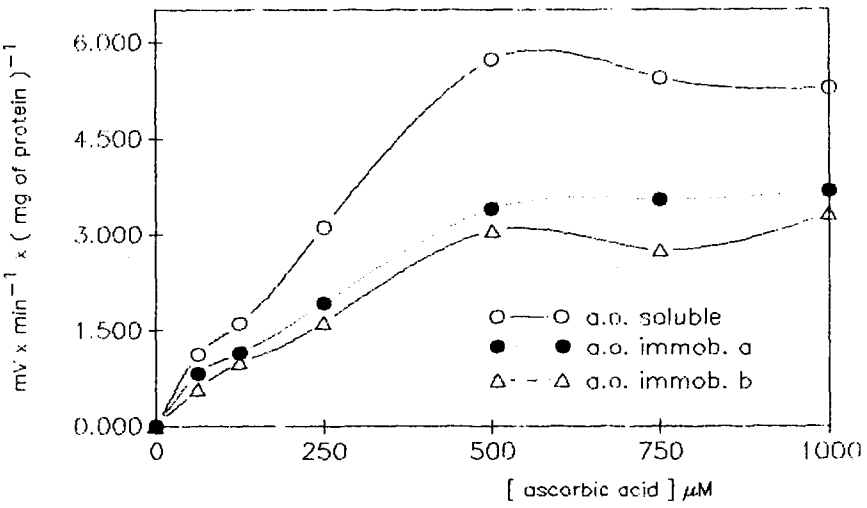

Fig. 2. K_m determinations of ascorbic oxidase (ao) carried out at 28°C and pH 6.0 under different conditions: O—O soluble; Δ—Δ immobilized onto glass beads without active site protection; and ●—● immobilized onto glass beads with active site protection.

Enzyme Activity and pH Curve

The enzyme activity and pH curve were determined by following the enzyme reaction in 0.1*M* citrate-phosphate pH 4.0, pH 5.0, pH 6.0, and pH 7.0 and 0.1*M* boric acid-borax buffer pH 8.0 and pH 9.0.

RESULTS AND DISCUSSION

The concentration of the protein extracted from *C. maxima* was 2.7 mg/mL. The amount of protein immobilized was estimated by measuring free protein found in the solution after incubation with the active support. The levels immobilized protein on the glass bead supports were 21.7 μg/mg of support and 22.1 μg/mg of support for immobilization with active site protection and without protection, respectively. The enzyme affinity constants (Michaelis-Menten, or K_m) were 349 μ*M* for soluble ascorbate oxidase and 384 μ*M* for the immobilized derivative with active site protection. The K_m for the derivative prepared without active site protection was 408 μ*M*. These differences are not statistically significant (Fig. 2).

The presence of substrate during immobilization did not change the amount of protein bound to the support and did not increase the enzyme

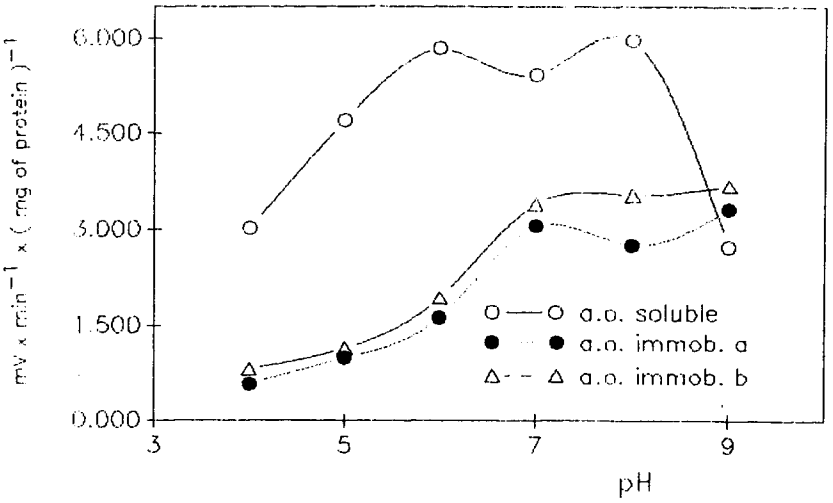

Fig. 3. pH profiles of ascorbate oxidase (ao) carried out at 28°C and pH 6.0 under different conditions: O—O soluble; Δ—Δ immobilized onto glass beads without active site protection; and ●—● immobilized onto glass beads with active site protection.

activity significantly as shown in Fig. 2. This can be accounted for if the link between the enzyme, and the support does not occur in or near the active site such that the tertiary structure of the enzyme is modified.

The soluble ascorbate oxidase showed highest activity at pHs between 6.0 and 8.0, decreasing drastically at pH 9.0. However, the immobilized enzyme had higher activity up to pH 7.0 and remained stable until pH 9.0. At pH values below 7.0, the immobilized ascorbate oxidase had very low activity. The differences in pH profiles between the soluble and immobilized enzyme are probably the result of microenvironment differences caused by H^+ diffusion and partition coefficient bias as well as changes in quaternary structure (*6*) (Fig. 3).

With a simple, inexpensive system, we have attempted to characterize the range for ascorbate, between 62.5 and 500 μM, which is greater than the Halvalzis and Potamia potentiometric method (*7*). They found a range of 1.7–280 μM of ascorbic acid using a bromide-selective electrode. However, Shaffer and colleagues, applying a FIA system connected to an oxygen optrode, has measured concentrations of as much as 6 mM in ascorbic acid (*8*). The reading time of 4 min was similar to that of Mason and co-workers, who used an HPLC system with an electrochemical detector, which is much more expensive than this biosensor system. Another advantage of this system is that it can be connected to an on-line digital system. The biosensor described here operated satisfactorily for more than 2 mo.

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