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Mushroom Tissue-based Biosensor for Inhibitor Monitoring

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Summary

A mushroom-carbon paste tissue electrode was used for amperometric measurements of inhibitors of tyrosinase. Measurements are carried out in the presence of the catechol substrate. The influence of the tissue loading and location in the plant are explored, and possible response mechanisms are discussed. The resulting inhibitor biosensor is inexpensive, characterized by high sensitivity and speed, offers micromolar detection limits, and requires no incubation period. Flow injection monitoring of diethyldithiocarbamate, benzoic acid and thiourea is illustrated. Such inhibition tissue electrode holds great promise for field monitoring of pollutants.

Keywords: tissue electrode, inhibition, tyrosinase, mushroom, biosensor

Introduction

There has been considerable interest in replacing isolated enzymes with tissue materials as the biocatalytic entity of electrochemical sensors *(1–3)*. The major advantages accrued from the use of tissue materials are high stability and activity (associated from the presence of the enzyme in large quantities in its natural environment) and low cost. Useful schemes for sensing important substrates (such as phenols, hydrogen peroxide, urea, ascorbic acid or glutamine) have thus been developed in connection with different animal or plant tissues. In addition to substrate detection, such whole-cell materials have offered effective elimination, *via* enzymatic digestion, of potential interferences *(4)*.

The goal of the present study is to expand the concept of tissue biosensors towards the monitoring of important enzyme inhibitors. The modulation of enzymatic activities by inhibitors, such as pesticides or heavy metals, has been widely used for monitoring these toxins *(5,6)*. Such inhibition assays have previously relied on the depressed activity of isolated enzymes. The coimmobilization of a potato tissue and isolated glucose oxidase onto a Clark oxygen electrode was employed earlier for detecting the inhibitory action of phosphate and fluoride anions *(7)*. The rich activity of tyrosinase in the mushroom tissue *(8)*, embedded in the carbon paste host, is employed in the following sections for fast and sensitive monitoring of low levels of important inhibitors, such as diethyldithiocarbamate, thiourea or benzoic acid. Such operation of whole-cell bioelectrodes should lead to effective toxicity testings based on the interaction of natural biocomponents with toxin pollutants.

Experimental

Apparatus

Amperometric experiments were carried out with a BAS CV27 voltammetric analyser (Bioanalytical Systems-(BAS), W. Lafayette), in connection with a BAS X-Y-t recorder. The carbon paste working electrode, reference electrode (Ag/AgCl, Model RE-1, BAS), and platinum wire auxiliary electrode joined the 10 mL electrochemical cell (Model VC-2, BAS) through holes in its PTFE cover. The flow injection system consisted of a carrier reservoir,

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an Alitea C-4V pump, a Rainin Model 501 sample injection valve (20 μL loop), interconnecting PTFE tubings, and a 'homemade' large volume wall-jet amperometric detector.

Electrode Preparation

The mushroom-based carbon paste electrode was prepared as follows. First a section of mushroom, removed using a scalpel, was ground with a mortar and pestle. The desired amount of ground mushroom was then mixed thoroughly with 0.90 g of graphite powder (Fisher), with a mortar and pestle. Subsequently, 0.60 g of mineral oil (Aldrich) was added and hand mixed with a spatula, in a weighing boat. A portion of the resulting paste was packed into the electrode cavity (3 mm diameter, and 1 mm depth), of a PTFE sleeve.

Electrical contact was established *via* a copper wire. The paste surface was smoothed on weighing paper.

Reagents and Procedure

The supporting electrolyte used was 0.05 M phosphate buffer (pH = 7.4). The mushrooms used in this study were purchased from a local grocery store and were grown in J. M. Farms Inc (Miami, OK). Catechol (Sigma), sodium diethyldithiocarbamate (Aldrich), thiourea (Fisher), benzoic acid (Baker), were used without further purification. All measurements were performed at room temperature, by applying the desired operating potential and allowing the transient current to decay prior to the amperometric monitoring. A stirring rate of 300 rpm was employed in the batch experiments, while a carrier flow rate of 2.0 mL/min was used in the flow injection analysis.

Results and Discussion

Response Characteristics and Proposed Mechanisms

The mixed tissue-carbon paste electrode configuration, shown previously to be extremely useful for substrate detection *(9)*, has been employed in the present study for the measurements of inhibitors. The perturbed biocatalytic activity of the mushroom containing tyrosinase, in the presence of different inhibitors (and a fixed level of its catechol substrate), is used for amperometric detection of these toxins. Fig. 1 shows current-time recordings at the mushroom-modified electrode for $1 \cdot 10^{-4}$ M addition of catechol(s), followed by successive $2 \cdot 10^{-5}$ M additions of sodium diethyldithiocarbamate (A,i), thiourea (B,i) and benzoic acid (C,i). The relative decrease of current due to inhibition versus the initial substrate response, for compounds A, B and C, in Fig. 1, range from 34.7% to 98%, 11.7% to 47.8% and 12% to 37.4%, respectively for inhibitor concentrations ranging from 20 to 160 μM.

The tissue electrode responds very rapidly to these micromolar increments of the inhibitor level, as indicated from the decreased substrate signal. Steady-state currents are achieved within approximately 12 s (benzoic acid), 15 s (thiourea) and 30 s (diethyldithiocarbamate); no incubation period is required. Such a fast response characterizes also substrate measurements at tissue-carbon paste electrodes *(9)*, and is attributed to the absence of an external layer hindering the transport of the inhibitor and substrate. Detection limits of around $5 \cdot 10^{-6}$ M diethyldithiocarbamate, $8 \cdot 10^{-6}$ M thiourea and $1.2 \cdot 10^{-5}$ M benzoic acid can be estimated from the signal-to-noise characteristics of these data (S/N = 3). Such micromolar detection limits are comparable with those common at inhibitor biosensors based on isolated tyrosinase *(10)*.

Also shown in Fig. 1 (top) are the resulting calibration plots. As expected for inhbition processes, such plots are not linear with a significant curvature at high inhibitor concentrations. A proper computational approach will need to be developed to provide some method of linearisation of the inhibitor calibration curve in order to facilitate practical use of this biosensor.

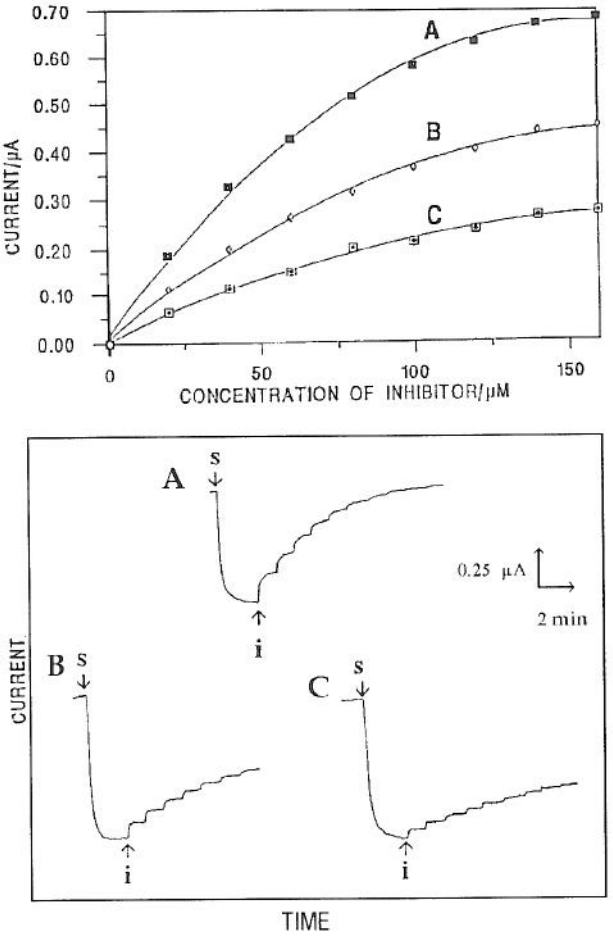

Fig. 1. Current-time recordings at mushroom modified carbon paste electrodes, upon addition of tyrosinase substrate(s), ($1 \cdot 10^{-4}$ M catechol). Followed by successive increments of inhibitor (i), in $2 \cdot 10^{-5}$ M steps, (A) sodium diethyldithiocarbamate, (B) thiourea and (C) benzoic acid. Also shown are the corresponding calibration plots. Operating potential, –0.20 V; electrolyte solution, 0.05 M phosphate buffer, pH = 7.4, stirred at 300 rpm; mushroom loading 25% (mass fraction).

The trend in sensitivity, diethyldithiocarbamate > thiourea > benzoic acid, reflects the extent of inhibitor-enzyme interaction, i. e., the degree of inhbition. These profiles can be used to estimate the coefficient of inhibition, $I_{0.5}$: $4.0 \cdot 10^{-5}$ M (diethylthiocarbamate), $5.8 \cdot 10^{-5}$ M (benzoic acid) and $6.2 \cdot 10^{-5}$ M (thiourea).

The preparation of the tissue carbon paste electrode has a profound effect upon its inhibitor detection. Fig. 2A displays the influence of the tissue loading in the biocomposite upon the calibration plots for sodium diethyldithiocarbamate. While no response is observed using the 5% (mass fraction) mushroom content (e), the sensitivity increases dramatically between 7.5% (d) and 25% (b) and then more slowly. The relative current decrease versus the initial substrate response, for sensors a-d are as follows: a: 13.7% to 69.4%; b: 22.8% to 94.4%; c: 19.4% to 93.1%; d: 18.2% to 81.9% for inhibitor concentrations ranging from 20 to 160 μM. Notice also that the dynamic range is extended upon lowering the tissue loading. Such behaviour indicates increased mass-transport limitations at lower mushroom contents. A 25% (mass fraction) loading yielded the most favorable signal-to-noise characteristics and was used in most subsequent work.

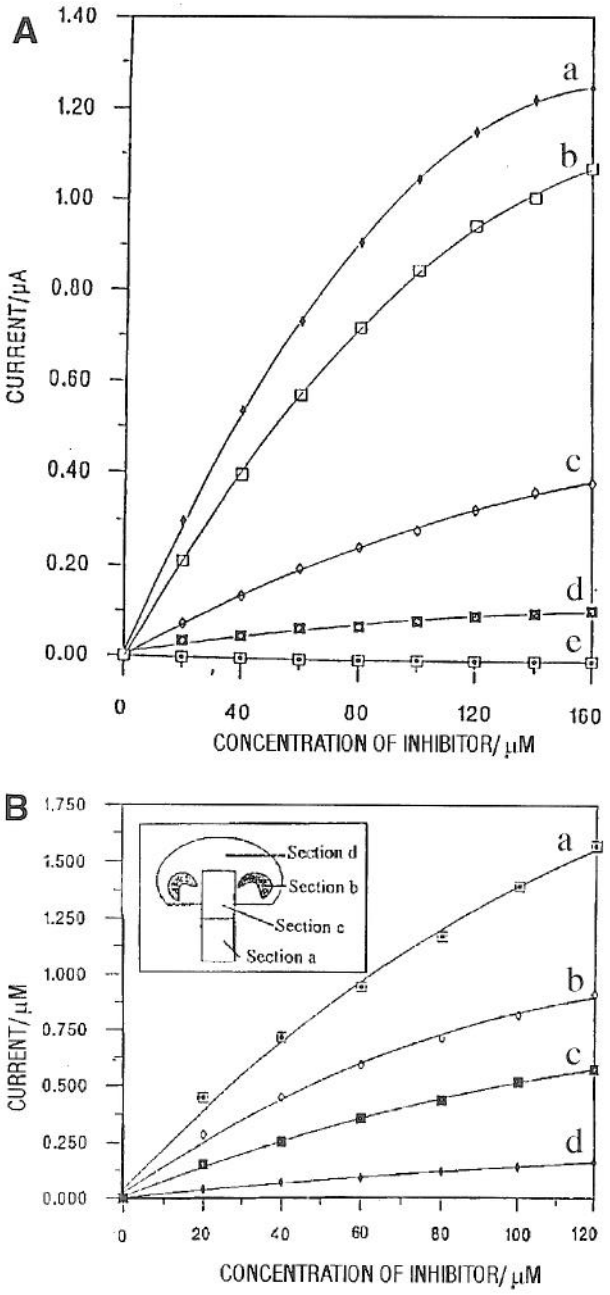

Fig. 2. (A) Effect of mushroom tissue loading on inhibition responses of sodium diethyldithiocarbamate: (a) 37.5%; (b) 25%; (c) 12.5%; (d) 7.5%; (e) 5%. (B) Investigation of inhibition responses of sodium diethyldithiocarbamate using different tissue sections (a, b, c, d). Also shown (inset) a schematic illustration of the mushroom sections. Conditions as given in Fig. 1.

The sensitivity and dynamic range are influenced also by the mushroom section used for constructing the electrode. It is well known *(8)* that the distribution of the tyrosinase activity in the whole mushroom is not uniform. Fig. 2B shows calibration plots for the diethyldithiocarbamate inhibitor using different tissue sections, in accordance to the mushroom schematic shown in the inset. While all tissue sections exhibit a useful reponse, the stalk tissue (section a) appears to contain more active enzyme than other morphological parts. The sensitivity trend: section a > section b > section c > section d, thus reflects the enzyme distribution patterns, in a manner described by Rodriquez and Flurkey *(8)*. Notice also the changes in the linear dynamic range associated with the use of different sections. Such changes reflect the different kinetic characteristics of different isoenzyme forms of tyrosinase in different mushroom sections *(8)*.

The inhibition of tyrosinase appears to proceed through interaction with its active copper site *(11)*. Yet, no information is available regarding the complex mechanism of the operation of inhibitor tissue electrodes. The exact mechanism should depend on whether the immobilized cells remain intact in the carbon paste matrix or broken down during the electrode preparation. In the first case, the mechanism must involve transport of the inhibitor into, within and from the immobilized cells. The inhibitor diffusion into and out of the cell may be facilitated by channels formed in the outer cell membrane during the electrode construction. Alternately, if the cells are structurally broken down, the enzyme of interest is released, and the inhibitor entrance into the cells is not required. The fast response of the present electrode may support the latter mechanism. Such models are analogous to those suggested for the detection of substrates at tissue electrodes *(2)*.

Tissue – Electrode as Flow Injection Detector of Inhibitors

The attractive dynamic properties of the inhibitor tissue electrode can be exploited for on-line applications, as desired for monitoring toxins in flowing streams. Fig. 3 displays flow injection measurements of various inhibitors of the mushroom tyrosinase (diethydithiocarbamate (B), thiourea (C) and benzoic acid (D)) in solutions containing the catechol substrate. Such detection scheme relies on the decrease of the cathechol peak, compared to that observed without the inhibitor (A). Such defined suppressions of the substrate peak allow convenient detection of submilimolar concentrations of the inhibitor. As the shape and width of the catechol peak are not affected by the presence of these inhibitors, high injection rates of 60 samples/h can be realized.

Similar to its batch counterpart, the tissue-based flow detector responds in a non-linear fashion to changes in the inhibitor concentration. For example, Fig. 4A

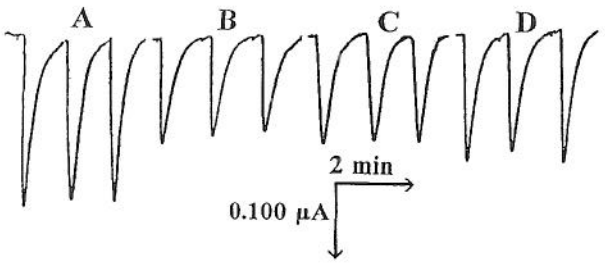

Fig. 3. Flow injection peaks, at a mushroom modified carbon paste electrode, for $5 \cdot 10^{-4}$ M catechol solutions containing $2 \cdot 10^{-4}$ M sodium diethyldithiocarbamate (B), thiourea (C), and benzoic acid (D), as well as without inhibitor (A). Carrier solution, 0.05 M phosphate buffer, (pH = 7.4). Applied potential, –0.20 V, flow rate, 2.0 mL/min. Mushroom loading 25% (mass fraction).

shows flow injection current peaks for catechol solutions containing increasing levels of diethyldithiocarbamate $2 \cdot 10^{-4}$ M – $1 \cdot 10^{-3}$ M (b – f), along with the catechol response without the inhibitor (a). The peak decreases rapidly upon increasing the inhibitor concentration at first, and then more slowly. The precision of the this flow injection operation is indicated from Fig. 4B, that displays peaks for 15 repetitive injections of a $2 \cdot 10^{-4}$ M diethyldithiocarbamate solution. This series yielded a mean peak current of 197 nA and a relative standard deviation of 2.4%. Such behavior suggests rapid dissociation of the enzyme-inhibitor complex, i.e., renewal of the enzymatic activity.

In summary, it has been illustrated that a tissue electrode can be employed for rapid monitoring of micromolar levels of inhibitors. Such tissue electrodes are extremely inexpensive compared with their enzymatic counterparts. Other pollutants inhibiting the tyrosinase activity, including heavy metals, pesticides or cyanide, may be monitored in a similar fashion.

Such bioelectrode thus holds great promise for rapid low-cost toxicity testing using nature as a guide. The operation of mushroom electrodes in nonaqueous solvents *(12)* can expand the scope of such inhibitor measurements to new environments. While the concept is illustrated in connection with the mushroom tissue, it could be expanded to other plant and animal tissues, and to whole cells, in general. Fundamental studies concerning the complex structure of the tissue-carbon paste biocomposite, and the inhibitor transport and interactions are needed.

Acknowledgment

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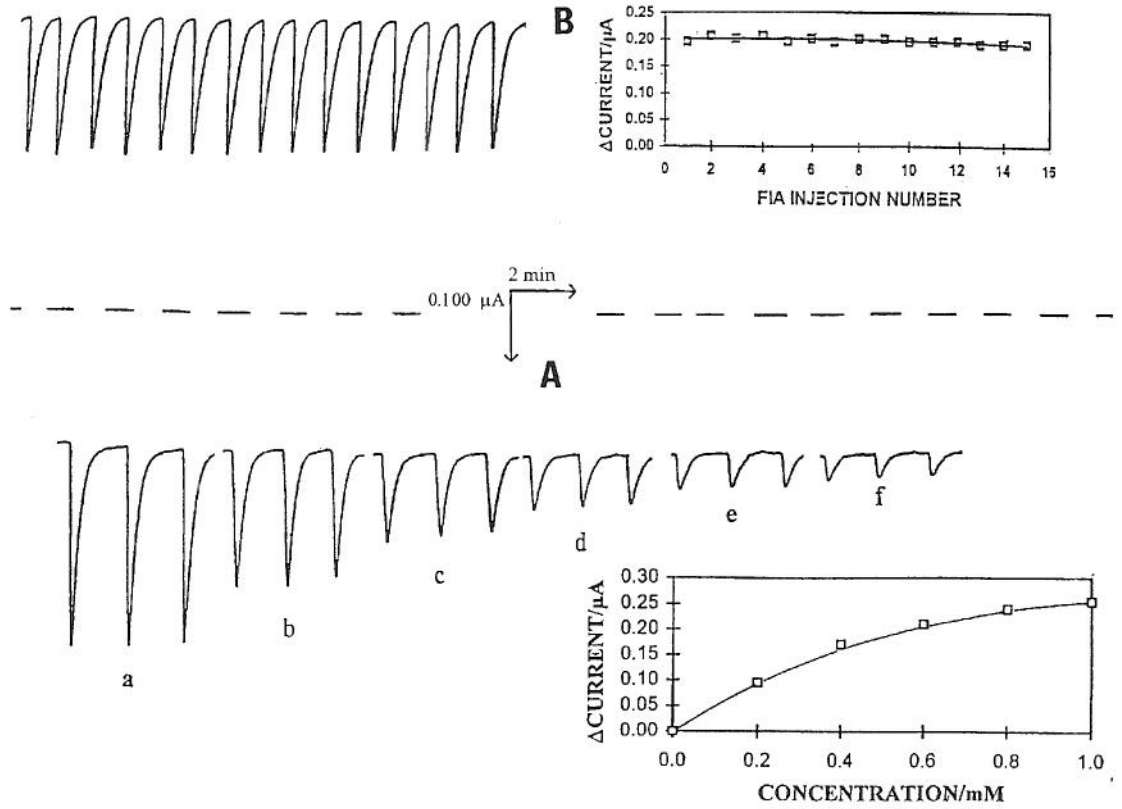

Fig. 4. Flow injection calibration (A) and precision (B) data at mushroom modified carbon paste electrodes. In (A), $5 \cdot 10^{-4}$ M catechol solution containing ($0 - 1 \cdot 10^{-3}$ M) sodium diethyldithiocarbamate (a–f), were used. In (B), $5 \cdot 10^{-4}$ M catechol solution containing $2 \cdot 10^{-4}$ M sodium diethyldithiocarbamate was used. Conditions as given in Fig. 3.

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Biosenzor izgrađen na osnovi tkiva pečurke za određivanje inhibitora

Sažetak

Primijenjena je elektroda od grafitne paste modificirana dodatkom tkiva pečurke za amperometrijska mjerenja inhibitora tirozinaze. Mjerenja su provedena uz katehol kao supstrat. Ispitan je utjecaj količine tkiva i dijela gljive, odakle je ono uzeto, a razmatrani su i mogući mehanizmi odgovora. Proizvedeni inhibitorski biosenzor je jeftin, odlikuje se velikom osjetljivosti i brzinom odziva, omogućava otkrivanje inhibitora u mikromolarnim količinama, a nije mu potrebna inkubacija. Prikazano je određivanje dietilditiokarbamata, benzojeve kiseline i tioureje postupkom injektiranja u protok. Takve inhibicijske tkivne elektrode imaju sve izglede da se primijene za terensko određivanje polutanata.