A Biosensor for the Determination of Cyanide in Cassava

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ABSTRACT

A simple biosensor for the determination of total cyanide in cassava is demonstrated. The biosensor was developed based on the use of a cyanide ion selective electrode made from AgI and Ag_2S and the enzyme linamarase which was isolated from cassava root cortex. Results are reported on a sensing system which relies on the catalytic hydrolysis of cassava linamarin by the enzyme linamarase immobilised on calcium alginate beads deposited on the ion selective electrode membrane. The biosensor is able to measure cyanide in solution over the 10^{-6} to 10^{-2} M concentration range as well as cyanide liberated as a result of the action of linamarase on linamarin over the 10^{-4} to 10^{-2} M range.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important tropical crop finding significant use as a food source. It ranks as the most important root crop in the world and ranks second among African staple crops [1]. Unfortunately, cassava contains two cyanogenic glycosides, linamarin and lotaustralin, the former being the principal cyanogenic glycoside. In addition, cassava contains the enzyme linamarase. Catalysed by linamarase, linamarin is rapidly hydrolysed to glucose and acetonecyanohydrin. Under neutral conditions, acetonecyanohydrin decomposes to acetone and hydrogen cyanide [2] as shown in Figure 1.

This cyanide producing potential of cassava poses a threat to man and animals with respect to food safety. Several health problems have been reported which have been attributed to high dietary cyanide exposure. Some of these include acute poisoning [3], goiter and cretinism [4], konzo, a paralytic disease of the legs [5] and tropical ataxic neuropathy (TAN) [6]. This potential toxicity of cassava calls for the need to ensure food safety in the consumption of cassava.

In an attempt to manage the health risk to humans posed by cassava consumption and to promote food safety, it is important that simple, rapid, and inexpensive analytical methods for

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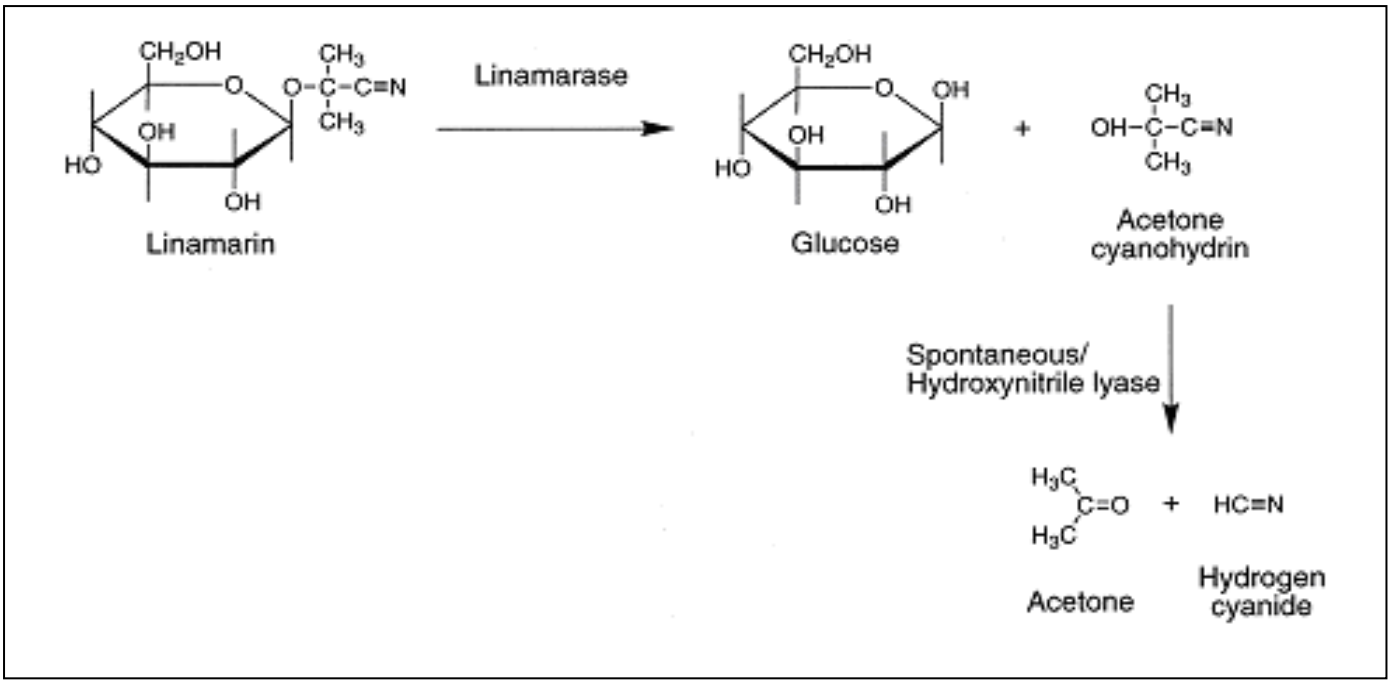

Figure 1: Hydrolysis of linamarin

determining cyanide content in cassava be made available. Many analytical methods have been developed, most of which have been based on the principle of the hydrolysis of linamarin by linamarase and the determination of the cyanide released. Cyanide in cassava has been reported to have been determined spectrophotometically [7], amperometrically [8], and potentiometrically [9]. The methods however, have been suitable only for laboratory use and are devoid of the portability and simplicity that would allow sampling and analysis to be undertaken reliably on-site. In an attempt to overcome this, a dip-stick method [10] was developed for use in the fields: however, it is limited by the fact that on the spot results cannot be obtained.

In this report, a simple and inexpensive design of a hybrid enzyme electrode for the quantitative determination of the amount of cyanide in cassava is reported. This biosensor is based on consolidating linamarase and a co-precipitate of AgI and Ag_2S to produce an all-solid state composite electrode. The principle behind this design is that when the electrode comes in contact with the analyte solution, the enzyme linamarase will catalyze the hydrolysis of linamarin thus producing glucose, acetone and hydrogen cyanide. The cyanide released in solution reversibly reacts with the surface of the membrane of the electrode exchanging with the iodide ions. A potential is generated at the surface of the electrode which is measured against a Ag/AgCl electrode. The potentiometric measurements can be used to determine the concentration of the cyanide ions in solution. This electrode is not only simple but also highly selective for cyanide ions.

EXPERIMENTAL

Materials

All chemicals used in this study were purchased from Alfa Aesar (Ward Hill, MA 01835 USA) and were of analytical grade unless otherwise stated. They were used without further purification. Ag_2S and AgI were used in the preparation of the mixed electroactive salt used to make the membrane pellet. Phosphate buffer was prepared with Na_2HPO_4 and Na_2HPO_4, and stock cyanide and iodide solutions were prepared using KCN and NaI respectively. The electrode body was constructed using conductive silver epoxy

(Epoxies, etc.), 18-gauge copper wire, and standard epoxy resin housed in a 6.35 mm PVC rod (McMaster-Carr, Cleveland, OH). The membrane pellets were pressed in a 3 mm pellet press, and 3M wet/dry silicon carbide sandpaper was used to polish the membrane surface. The reference electrode was a silver/silver chloride wire with a porous Vycor glass frit encased in a glass tube to which was added a saturated NaCl solution. The electrode was purchased from Bioanalytical Systems (West Lafayette, IN). Potentiometric measurements were conducted using a unique eight channel high input impedance 12 bit A/D converter designed by the Naval Research Laboratory (NRL) co-author. The electrodes were attached to pin receptacles on the device, which was then interfaced with a computer through a serial port. Visual Basic and C++ software programs designed at the NRL were used to collect sensor data from the device.

Enzyme preparation

The enzyme, linamarase, was extracted and purified from freshly harvested cassava cortex as described by Nok and Ikediobi [11]. The cortex was sliced and homogenized in acetate buffer (pH 5.5). The extract was precipitated with cold acetone and the supernatant dialyzed. The supernatant was purified by gel filtration using Sephadex G-200 and ion-exchange chromatography. Fractions collected were assayed for linamarase activity and protein using alkaline picrate solution and Bradford's method[12], respectively. The partially purified enzyme was immobilized in calcium alginate beads.

Fabrication of the cyanide ion selective electrode transducer and evaluation of response

The cyanide ion selective membrane was prepared by mixing equimolar amounts of Ag_2S and AgI. The salt mixture was pulverized using an agate mortar. The resulting powder was pressed into a 3 mm pellet in a pellet press.

The membrane pellet was polished with silicon carbide sandpaper and glued into a 3 mm hole on the bottom side of the PVC rod using standard epoxy. A copper wire was connected to the pellet with silver epoxy and passed through a drilled hole in the top of the electrode so that it could be inserted into the NRL 12 bit A/D converter. The hole was sized to allow an 18-gauge wire to fit tightly into the PVC rod and to be fixed in place with standard epoxy. The electrode as depicted in Figure 2 was conditioned before use in 10^{-3}M solution of analyte for 15 min. The response of this transducer was tested against a Ag/AgCl reference electrode. The fabricated sensor and a silver-silver chloride reference electrode were connected to pin receptacles on the NRL board having a 12 bit A/D converter and interfaced with a computer. The electrode was immersed in the test solution and evaluated with cyanide and iodide solutions by standard addition. The electrode serves as a cyanide electrode as well as an iodide selective electrode. Volumes of either stock NaI or KCN (10^{-6} M to 10^{-1} M) were added to the initial buffer solution to bring the analyte concentration from $10^{-7.5}$ M to 10^{-2} M by 0.5 log [concentration] changes.

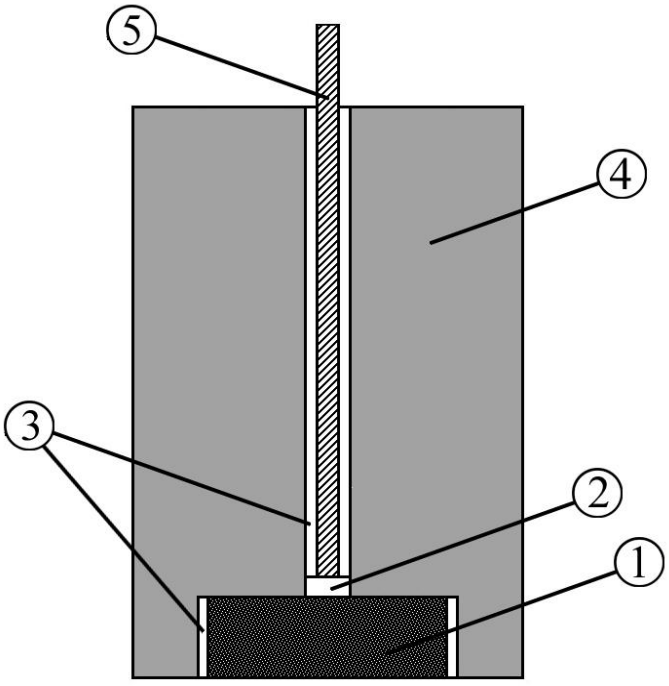

Figure 2: Biosensor design. (1) Composite membrane pellet (2) Conductive silver epoxy. (3) Standard epoxy. (4) PVC body. (5) Copper wire.

Additions were made every 200 s, and electrode responses observed were plotted against the logarithmic concentrations of the test solutions according to the Nernst equation. The results were then analyzed to determine the linearity, slope, and limit of detection (LOD) for the fabricated ion selective electrode (ISE).

LODs were calculated by the intersection of a line parallel to the x-axis through the mean of the potential value measured at the lowest concentration and the upper 95% confidence value of the best fit slope in the linear region of the calibration curve. Thus, LODs reported are those which correspond to the maximum limit of detection per the IUPAC recommended method[13].

Biosensor performance and evaluation of response

The enzyme linamarase was selected because of its specificity for the substrate linamarin which is the principal cyanogens found in cassava. The electroactive silver salt mixture and the alginate immobilized linamarase were mixed thoroughly in a 10:1 ratio using an agate mortar. A 5-mm diameter biosensor membrane was produced with 0.5 mm thickness using a 5-mm pellet die, manufactured in a Washington State University Machine Shop, in a hand press. This enzyme electrode was constructed as previously described in 2.3 above.

Iodide, cyanide and linamarin solutions with concentrations of 10^{-4} to 1 M were prepared. The response of the biosensor to iodide was tested in order to compare the transducer response in the presence of the immobilized enzyme response. A pH 8 tris-glycine buffer solution was used for measuring the response to cyanide and linamarin, as pH 8 has been shown to have the smallest variation in electrode response due to dissociation of HCN [14]. Also, at higher pH levels dissociation causes the release of I^- ions resulting in unpredictable potential changes. Furthermore, the enzyme has been reported to retain 85% of the maximum activity between pH 5and 8[15].

RESULTS AND DISCUSSION

Responses of transducer to iodide and cyanide

The electrode response characteristics to iodide and cyanide are summarized in Table 1. Figure 3 displays the average (n = 3) potentiometric response and the dynamic linear range of the transducer. It was necessary to zero the mV readings of the calibration curves at the lowest concentration ($10^{-7.5}$ M) to account for differences in initial readings caused by the measuring device.

Table 1: I^- and CN^- Response Characteristics of Sensor.

	CN^-	I^-
Average slope	59.6 mV/log	68.8 mV/log
LOD(M)	$10^{-4.5}$ M	10^{-5} M
Linear range (M)	$10^{-4} – 10^{-2}$ M	10^{-5} - 10^{-2} M

Theoretically, every halide membrane electrode can be converted into a cyanide electrode, but in practice, the silver iodide based cyanide electrode is the most suitable for the determination of cyanide because of its selectivity[16]. It has been suggested that the response of this electrode is due to the complexation reaction which liberates iodide at the membrane surface. Liberated iodide establishes equilibrium with the silver ions and a potential is generated[14,17].The response of the sensor to cyanide ions is due to the release of iodide ions into the solution as a result of the following complexation reaction occurring at the interface of the membrane and solution:

$$AgI\ (m) + 2CN^- \rightleftharpoons [Ag\ (CN)_2]^- + I^-$$

Chemisorption of the primary ion (CN^-) from the solution phase onto the surface of the electrode leads to the release of iodide ion which establishes an equilibrium with the silver ion in the solid state composite electrode. The establishment of

electrode potential results from an I^- driving force that leads to transport of I^- across the electrode body and charge separation across the electrode that creates a measurable Nernst potential.

The values obtained for both analytes are indeed Nernstian, with the iodide response slope ranging from 59.6 to 65.7 mV/decade and cyanide response ranging from 51.7 to 58.4 mV/decade. Such responses are suitable for analytical responses. The lower LOD of the sensor is $10^{-5.7}$ M when sensing iodide and $10^{-4.9}$ when sensing cyanide. The linear ranges are 10^{-5} - 10^{-2} M for I^- and 10^{-4} –10^{-2} M for CN^-. Thus this cyanide ISE is suitable for the construction of a cyanide biosensor.

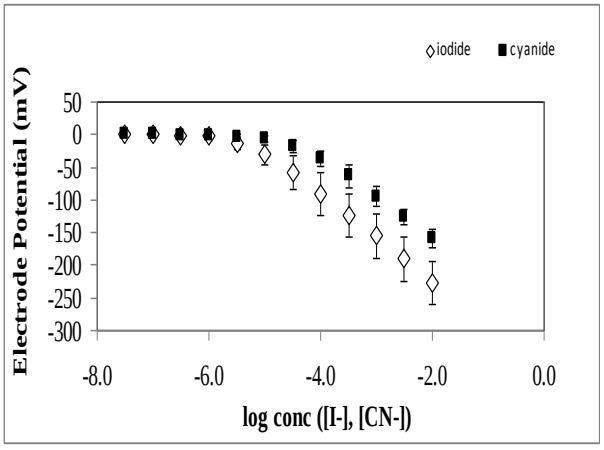

Figure 3: Average Potentiometric Response of Cyanide ISE Constructed From AgI and Ag_2S to Standard Iodide and Cyanide Solutions.

The biosensor response.

The response of the biosensor in the presence of the iodide ions was investigated to see if it retained the same response in the presence of the immobilized enzyme. The biosensor sensitivity was not as the AgI/Ag^2S electrode alone, having a maximum slope of 10 mV/decade over the 10-2.5 - 10-2 range and a working non-linear range of 10^{-4}-10^{-2} M with an average slope of 6.5mV/decade as shown in Figure 4.

It was expected that the sensor containing linamarase would have a similar response to that of the AgI/Ag_2S electrode for the I^- ion. When used to sense linamarin, the electrode demonstrated a linear range of 10^{-4} to 10^{-2} M as shown in Figure 5, with a response slope of 2.5 mV/decade. The low response slope is likely due to low concentrations of enzyme at the electrode surface and mass transfer resistance within the immobilization matrix. This will limit the turnover rate of linamarin making the CN^- concentration near the surface and within the matrix very low translating to a low I^- ion concentration produced as a result of the I^- and CN^- exchange. It may also be that the resulting CN^- and/or I^- could have diffused away into the bulk solution. The electrode response may be limited either because of limited exchange of CN^- with I^- or limited I^- concentration near the membrane electrode surface. Furthermore, the ionic nature of the polymeric immobilization matrix, calcium alginate may be a factor contributing to diminished sensitivity; as calcium ions may complex with the free cyanide ions in solution produced from the hydrolyses of linamarin by linamarase, thereby reducing the concentration of free cyanide ion available for sensing.

It was observed that the sensor had a limited lifetime with complete loss of response after approximately 8 days. A lifetime of several weeks would be preferable for an eventual electrode product to be cost effective. A similar trend in electrode lifetime was reported by Tan *et al.*[14] in which the potential drift in the response is attributed to electrode poisoning as CN^- ions irreversibly displace I^- ions on the surface; Tan *et al.* also reported however, that their electrode was regenerated completely however by polishing the surface of the electrode.

The results here are encouraging in that a miniature composite ISE and enzyme-based linamarin sensor has been proposed and shown to be effective. However, because of the limited response slope and limited stability for the linamarin sensor, further study is merited to improve performance. This could be done by increasing the concentration of linamarin in the alginate, exploring alternatives to alginate, and adjusting the ratio of enzyme containing gel to AgI/Ag_2S. Also, an alternative approach can be explored in which the enzyme is immobilized on a carbon paste and deposited on the surface of the

AgI/Ag_2S pellet as was done in enzyme amperometric electrodes developed by our group in the past. [18,19]. This would allow us to concentrate enzyme in a thin layer thereby providing a highly active zone that will produce a high concentration of CN^-. Because of the relatively short diffusion path to the pellet surface CN^- will displace larger amounts of I^- ion and responses with larger voltages are anticipated. The disadvantage of this approach is that it would be more challenging to regenerate the electrode though we could assess electrode life and weigh the merits of disposable electrodes.

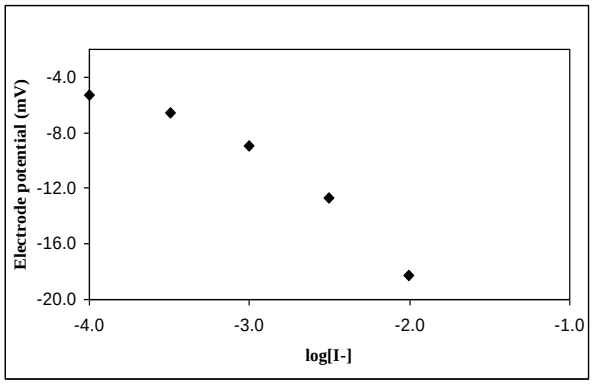

Figure 4: Average Response of Biosensor in Iodide Solutions.

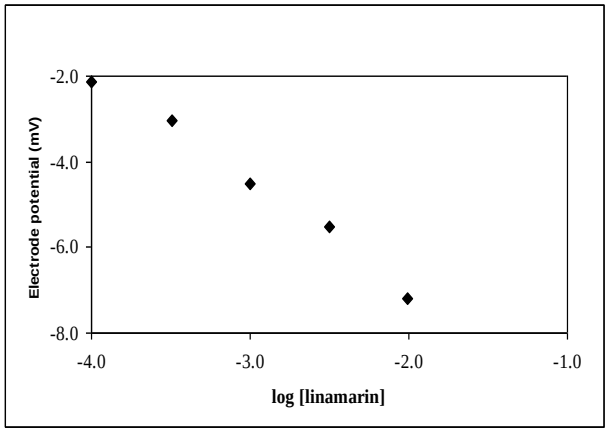

Figure 5: Response of sensor to linamarin.

CONCLUSIONS

An electrochemical biosensor for cyanide determination in cassava was developed using the enzyme linamarase entrapped in alginate beads and consolidated in a composite matrix with an electroactive silver salt mixture. The analytical characteristics of this sensor, including linear range and lower detection limit have been described. This type of biosensor offers an added advantage of easy preparation, manipulation, and modification. Various system modifications have been proposed for further study in order to optimize the sensor performance.

ACKNOWLEDGEMENTS

The authors acknowledge the Fulbright Program sponsored by the U.S. Council for International Exchange of Scholars which supported Prof. B.J. Van Wie during the academic year 2007 – 2008 which fostered the collaboration. We further acknowledge the Washington State University College of Engineering machine shop personnel for fabrication of the pellet press used to make the ion selective electrode pellets. We are grateful to Washington State University office staff who made special efforts in purchasing and shipping supplies for the research. We also acknowledge the late Mr.Alistair F. Smith, former Chief Technologist, Department of Chemistry, Ahmadu Bello University who provided technical expertise in repair of equipment and provision of chemicals for the research.

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Received: 23/02/2008

Accepted: 10/08/2008