AMPEROMETRIC DETERMINATION OF POTASSIUM AND OF UREASE ACTIVITY IN PLANT MATERIALS

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Abstract

Determination of potassium and urease activity of locally found plant seeds have been made. The membrane-stabilized water-nitrobenzene interface was used as an amperometric sensor. The electroanalytical techniques employed were cyclic voltammetry, ac-voltammetry and chronoamperometry.

The sensor was calibrated by the standard addition method. The detection of 0.025 mmol l^{-1} of potassium ion as well as ammonium ion was possible. Since the sodium ion content of the seed powders investigated was extremely low, no interference of sodium ion was observed. Several plant seeds have been screened for urease activity. The transfer of ammonium ion produced by enzymatic hydrolysis of urea was followed by the sensor.

Three new sources of enzyme urease have been found, namely, Kil (*Lagenaria siceraria*), Berbera (*Millitia ferruginea*), and Gibto (*Lupinus albus*).

1.Introduction

Over the years there has been a steady growth in the use of enzymes as selective and sensitive biotransducers and there is a wide field of possible applications [1,2]. Among the enzymes that have been used in analytical systems, urease is one of the best known and extensively studied [3].

Urea is a byproduct which is often monitored in blood and urine to provide some indication of kidney failure [4].The monitoring procedures of this substance usually involves the enzyme urease in combination with different transducers [4-8].

Urease (E.C. 3.5.1.5) occurs in some 200 species of bacteria, in many moulds and in a large number of higher plants, especially in beans . Among the higher plants the Jack bean (*Canavalia ensiformis*), Squash (*Curcurbita maxima*), watermelon (*Citrulus vulgaris*) and Soy bean (*Soja hispida*) are the most known [9]. However, the most frequently used commerciall sources of urease are Jack beans.The activity of urease has been determined by measuring the ammount of ammonia or carbon dioxide released from urea over a fixed interval of time. The techniques include Nesslerization [10], titration of the ammonia [11], manometric determination of carbon dioxide [12] and pH measurement [13].

In this project several Ethiopian plants were screened for urease activity and three of them have been shown to contain urease in the seeds and may be used as a source of the enzyme. These are Kil (*Lagenaria siceraria*), Berbera (*Millitia ferruginia*) , and Gibto (*Lupinus albus*).The activity measurement was based on the facilitated ammonium ion transfer across the membrane stabilised interface between two immiscible electrolytes. In this method, the current due to the facilitated transfer of hydrophilic ions like potassium and ammonium ions from an aqueous to an organic phase is proportional to the ion concentration and can be measured. The neutral molecules which facilitate this transfer of ions are usually referred to as ionophores.

2 LITERATURE REVIEW

Urease (urea amidohydrolase, E.C.3.5.1.5) has a cherished place in the enzymologist's heart, since it was responsible for the deathblow to the proposition that the protein was merely a carrier of the catalytic species [14]. Nonetheless, the enzyme remains ill-understood and is deserving of a detailed and intensive mechanistic attack.

Sumner described urease as "absolutely specific", but two additional substrates, hydroxyurea and dihydroxyurea, have been found for the enzyme [15,16].

The enzyme is probably the more remarkable for its enormous efficiency. It has a k_{cat} for the hydrolysis of urea which is about two orders of magnitude greater than the k_{cat} for any peptidase in the hydrolysis of other carboxamides [17].

The enzyme urease has been found to have a molecular weight of 480,000 [18]. The isoelectronic point of this enzyme has been determined to be at pH 5.1 [19]. The K_m value of urease has been reported to be in the order of 1.05×10^{-2} to 4×10^{-2} M [20] and its turnover number is 460,000 [9].

Laidler *et al* [21,22] carried out kinetic studies of urea hydrolysis by urease and reported the heat of activation as 12.5 kcal and the activation entropy as 7.5 eu respectively. In their postulated urea-urease-water complex, the urease was assumed to displace the water reversibly at high concentration of substrate.

The presence of thiol groups in the enzyme urease appears first to have been described by Summner and Poland, who titrated crystalline urease from Jack bean in 1933 [23]. Although native urease contains many thiol groups [24,25], absolute quantification became possible only with the secure evaluation of the subunit molecular weight (Mr = 96000) and other properties of the enzyme [26].

A variety of evidences indicate that urease contains one unique cysteine residue per sub unit [27,28] and that it becomes inactive when this residue is covalently modified. As a result, this thiol group is considered essential. However, there are inessential thiol groups of five cysteine residues per sub unit of urease, which are more reactive than the unique residue, but whose covalent modification does not affect the enzyme activity [23,5].

Inspite of extensive investigations, the chemical structure of this enzyme has not been well defined because of its large molecular weight. Bailey and Boulter [29] found a single N-terminal methionine residue and a single C-terminal sequence of -Tyr-Leu-Phe-. Mamiya *et al.* reported the partial aminoacid sequence of peptides obtained from urease, but the aminoacid sequence around the active center was not identified. However, the sub unit structure of urease is considered to be hexameric [25].

The aminoacid sequence of the peptide containing the active center of Jack bean urease was determined by Sakaguchi *et al* [30] to be Phe-Glu- Pro- Gly-Asp- Cyt-Asn-Ser-Thr-Phe-Lys. But they observed two kinds of sub units having different aminoacid sequence around the cysteine residue in the active center of urease. All in all, many research groups have studied the aminoacid sequence of urease but the complete sequence has not yet been obtained.

Dixion *et al* [31] reported that urease is a nickel metalloenzyme and contains two tightly bound Ni(ll) ions per one sub unit.

As earlier mentioned, urease has essential sulfhydril groups in the cysteine residue whose covalent modification cause inactivation of the enzyme .The more stable inactivation is due to the reaction of the -SH groups with certain heavy metal ions to form a "mercaptide".

Ambrose *et al* [27] have made an elaborate study of the inactivation of urease by silver ions. The inhibition by cadmium has been shown by Schmidt [32]. As reported in the literature the enzyme urease is highly sensitive to trace quantities of metal ions. Shaw has shown that the

order of toxicity of a series of metal ions to urease is well correlated with the insolubility of their sulfides [33]. Such inactivation can presumably be reactivated by reaction with -SH compounds such as glutathione or cysteine [34].

No conclusive evidence exists that urease requires activators for its functioning, as do certain other enzymes. However, Fasman and Niemann concluded that phosphate ions activate this enzyme whereas the sodium and potassium ions inhibits the enzyme [35]. The hydrolysis product, ammonium ion, also inhibit the enzyme urease [21].

The inhibition of enzymes was extensively studied using structural analogs of the substrate. The inhibition of urease by urea analogs, sulfamide, acetamide and thiourea has been reported [36].

The enzyme urease is involved in different forms of electrochemical transducers to produce different types of urea sensor. All measurements are based on the hydrolysis products of the urea-urease reaction leading to an indirect determination of urea by the biosensors.

All electrochemical sensors fall into one of two categories [37], those based on potentiometric measurements or on amperometric measurements. The analytical application of urease will be discussed in these two categories.

Potentiometric transducers utilize the pH change caused by the hydrolysis products ammonia and carbon dioxide, or the response of electrodes to the ammonium ion concentration. Urease may be employed as a liquid reagent, or immobilized and incorporated in the electrode [38].

Guilbault and Montalvo [39] made the first urea bioelectrodes by immobilizing urease on a glass ammonium ion-selective electrode. The electrode had an enhanced sensitivity to other ions like potassium and sodium. Later on, they constructed three types of electrodes [40]. One approach was to cover a glass electrode with acrylammide gel-immobilized urease. In the second type, a cellophane film was added over the enzyme layer, and for the third type, the

enzyme gel was sandwiched between two cellophane films. The presence of cellophane coatings had little effect on the response times, but the stability of these electrodes was prolonged owing to the prevention of the leaching out of urease by the cellophane film.

In order to enhance the selectivity Guilbault and Hrabankova [41] added ion-exchanger resins to their samples and later, Guilbault and Nagy [42] used a nonactin based ammonium ion selective electrode in which the nonactin was embedded in a silicone rubber matrix. Gil *et al* [43] used urease covalently bound on ammonium-selective potentiometric membranes as part of a disposable probe.

The ammonia gas sensor has also been employed in determining urea and was found completely free from ion interference. Rogers and Pool [44] used soluble enzyme while Anfalt *et al* [45] used immobilized urease directly onto the gas permeable membrane of the electrode using glutaraldehyde as a cross-linking agent. An alternative ammonia sensitive electrode is the air-gap electrode [46] which completely avoids protein contamination as the electrode interface is only in contact with the liberated gaseous ammonia.

Potentiometric electrodes have been used to determine pH changes resulting from the reaction of urea and urease. Nilson *et al* [47] used physically immobilized urease in their analysis . Koncki *et al* [48] have also studied different ways of preparation of gels as a matrix for physical immobilization of urease on the sensing surface of glass pH electrode and recommended the enzyme entrapment within a gel prepared in situ on the glass electrode surface. Eggenstein *et al* [4] recently have reported a disposable urea biosensor based on double matrix membrane technology .

Amperometric transducers are also employed and studied as a urea sensor. Gauilbault and Seo [49]have described an ammperometric transducer based on NADH-dependent coupled enzyme as urea sensor . The change in current was monitored based on the reaction of the

ammonium ion produced by the urease-urea hydrolysis with 2-oxoglutarate in the presence of NADH using glutamate dehydrogenase. The sensor was stable for two weeks and could be used for about 50 assays. It was applied for the determination of urea in fertilizers and clinical analysis.

The application of an amperometric ammonium ion sensor has been reported by Senda and Yamamoto [50]. Urease was immobilized on the sensor and the faciltated ammonium ion transfer to nitrobenzene, in which the organic phase contained dibenzo-18-crown-6 and tetrabutyl ammonium tetraphenyl borate as supporting electrolyte, was monitored. The current response of the biosensor was measured by pulse amperometry. The sensor had a life time of more than three weeks. The same voltammetric sensor was also used as urea sensor using dissolved enzyme urease by T. Taddesse [51] in which the organic phase contain ethylvioletteraphenylborate as supporting electrolyte.

3. THEORETICAL PRINCIPLES OF ENZYMATIC ANALYSIS

3.1. Enzymes

Enzymes are proteins that catalyse biological reactions. Similar to other catalysts, enzymes increase the rate of chemical reaction by reducing the activation barrier of a reaction.

Since the kinetic barrier is lowered by the same amount for both the forward and the reverse reactions, an enzyme equally accelerates the forward and the reverse reactions. Hence, the free energy levels of the substrate and the product remains unaffected by the enzyme action so that the over all equilibrium of the reaction is not affected.

Besides greatly increasing the velocity, enzymes have the special property of specificity which makes them very useful in bioanalytical methods. An individual enzyme will catalyse a specific type of reaction with a unique substrate or group of related substrates [52].

Basically the enzymes are all proteins, but they may be associated with non protein substances (known as coenzymes or prosthetic groups) that are essential to the action of the enzyme. For a number of enzymes the evidence is that the catalytic activity is due to a relatively small region of the protein molecule; this region is usually referred to as the active centre.

Enzymologists use special terms in discussing enzymes. The molecule undergoing reaction is called the substrate and the complete reaction mixture is called a digest. The active site of an enzyme is the part of the enzyme molecule in which the substrate binds and catalysis occurs to give products.

3.2. Kinetics of enzyme actions

Enzymes are not passive surfaces on which reactions take place but rather, are complex molecular machines that operate through a great diversity of mechanisms. The

mechanisms are frequently very complex; a number of elementary steps usually take place, each one of which involves rather complicated interactions between several groups on the enzyme and substrate molecules.

Michaelis and Menten found that the rate or velocity v of catalysis by enzymes varies with substrate concentration. The velocity increases with an increase in substrate concentration until a substrate level is reached, beyond which further additions of substrate does not increase the rate. At this point the rate becomes constant and maximum (v_{max}) as shown in Fig. 1.

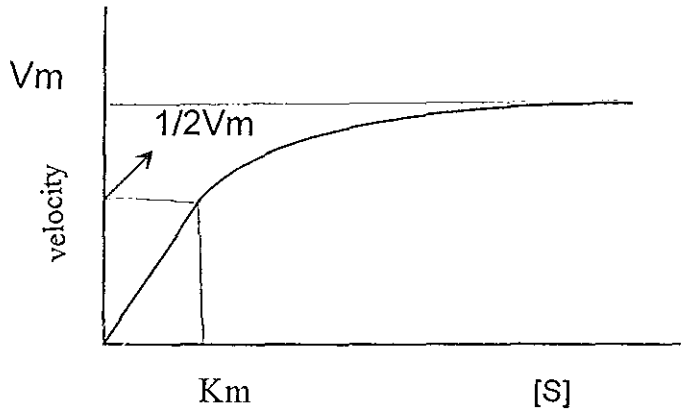


Fig.1. The relationship of substrate concentration and the rate of enzymatic reaction

Michaelis and Menton proposed a simple mechanism to explain the kinetics. In this model, the enzyme E combines with the substrate S to form an enzyme-substrate complex ES. The ES complex can either dissociate back to E and S or proceed to form a product P. This may be expressed as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1)$$

where k_1 and k_{-1} are the forward and backward rate constants for complex formation and k_2 is the rate constant for complex decomposition into product.

According to the above model, when the substrate concentration becomes high enough to entirely convert the enzyme to the complex ES, the second step of the reaction becomes rate limiting. The general expression for the velocity (rate) of this reaction is then

$$v = \frac{d[P]}{dt} = k_2[ES] \qquad (2)$$

The overall rate of production of *[ES]* is the difference between the rate of the elementary reactions leading to its formation and those resulting in its decompositon:

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1}[ES] + k_2[ES]) \quad (3)$$

This equation cannot be explicitly integrated , however, without simplifying assumptions. As illustrated in Figure 2, the rate of synthesis of ES must equal its rate of consumption over most of the course of reaction ; that is, [ES] maintains a steady state. Therefore,

$$\frac{d[ES]}{dt} = 0 \qquad (4)$$

In order to be of use, kinetic expressions for overall reactions must be formulated in terms of experimentally measurable quantities. The total enzyme concentration $[E]_o$ is given by:

$$[E]_o = [E] + [ES] \qquad (5)$$

Combining eqn (3) with the steady-state assumption, eqn (4) , and substituting eqn. (5) yields :

$$[ES] = \frac{k_1[E]_0[S]}{k_{-1} + k_2 + k_1[S]} \qquad (6)$$

Introducing the Michaelis constant K_M as:

$$\frac{k_{-1} + k_2}{k_1} = Km \qquad (7)$$

and further rearrangement of eqn (6) gives

$$[ES] = \frac{[E]_o[S]}{K_m + [S]} \qquad (8)$$

The initial velocity of the reaction can then be expressed as

$$v = \left(\frac{d[P]}{dt}\right) = k_2[ES] = \frac{k_2[E]_o[S]}{K_m + [S]} \qquad (9)$$

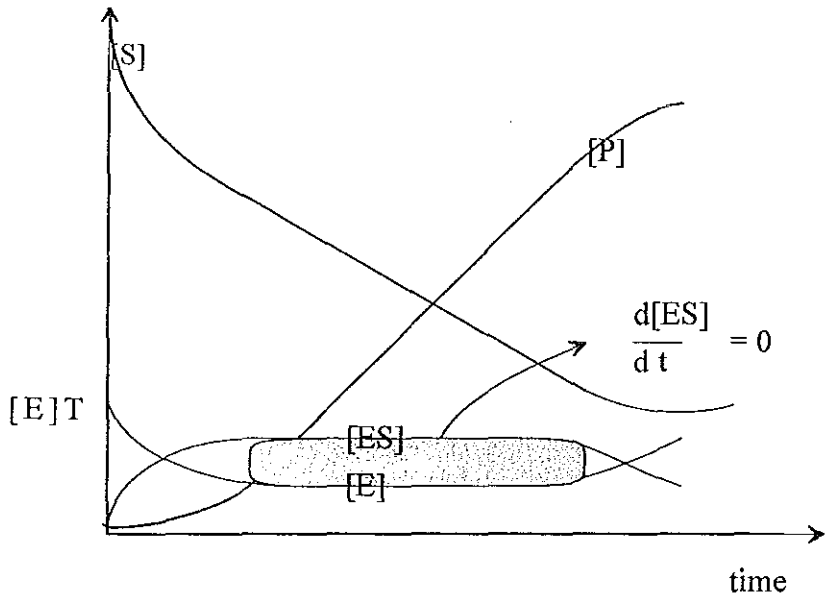


Fig.2. The progress curves for the components of a simple Michaelis- M enten reaction [53].

There are two important points to note from the rate expression. First, at lower substrate concentrations, when $[S] << K_m$, the rate is directly proportional to the substrate concentration and inversely proportional to the Michaelis constant. Second, at high substrate concentrations when $[S] >> K_M$, the rate reaches its maximum velocity given by:

$$v_{max} = k_2[E]_o \qquad (10)$$

Combining (9) and (10) we therefore obtain

$$v = \frac{v_{max}[S]}{K_m + [S]} \qquad (11)$$

This expression, the Michaelis-Menten equation, is the one the basic equation of enzyme kinetics. It describes a hyperbolic plot such as plotted in figure 1.

3.3. Determination of the catalytic activity of enzymes

The catalytic activity of an enzyme is measured in terms of the rate of the reaction catalysed. The activity of an enzyme is the amount of reaction that a certain amount of enzyme will produce in a specified period of time.The activity is determined by measuring

the amount of product produced or the amount of substrate used up per unit of time under high concentrations or saturating conditions of substrate. The activity is essentially the measurement of the initial velocity under conditions that make it the maximum velocity. The activity should be proportional to the amount of enzyme added.

Enzymes are usually assayed at their optimal conditions. In an enzyme assay it is critical that the substrate concentration be saturating during the entire period the reaction is sampled. The concentration of substrate must be at least 20 K_m [52] in which 95 % of the maximum rate is attained so that the amount of product or substrate measured is linear over the period the reaction is sampled. The activity of the enzyme is then obtained as the slope of the linear part of the plot of concentration of products or substrate versus time.

The activity of an enzyme is usually accomplished by performing a chemical analysis for the product or substrate. Unfortunately, an easily measured product is not always produced. It may still be possible, however, to assay the enzyme by coupling the reaction with another enzyme that can convert one of the products into another substance that can be easily measured. Many coupled assays have also been developed using coenzymes.

3.4 Analysis with enzymes [54]

Reactions involving enzymes may be represented in a simplified form by

$$\text{Substrate} \xrightarrow{\text{enzyme}} \text{Product}$$

Methods of analysis involving enzymes may be designed to measure either the substrate or the enzyme activity, and the sensor may be used to detect either one of the products or, less usually, the substrate. By combining the high selectivity of enzymatic reactions many excellent analytical methods may be developed. The construction and working principles of enzyme electrodes have been described in the literature [54-56].

Four essentially different types of procedure for performing these analyses may be distinguished.

1. Single-stage procedure:- The substrate and enzyme are mixed together and allowed either a standard time to react or sufficient time for the reaction to reach completion. The sensor immersed in the solution then indicates the concentration of one of the reaction products or the decrease in substrate concentration.

2. Enzyme electrode procedure :- The enzyme for substrate analysis is immobilised in a thin layer, often gelcoated onto the surface of the sensor. The coated sensor is immersed in the sample and the change is noted. A reaction occurs only within the coating and at the coating-sample interface.

3. Double-stage procedure :- This procedure is similar to procedure 1 except that the reaction and measurement stages are separated. For example, the reaction may be conducted at one fixed pH and the pH is then changed to a second value before the measurement is made to facilitate the measurement. That is, conditions are selected to have a desired reaction and conditions altered in order to carry out the measurements.

4. Mutiple-stage procedure :- If the product of an enzymatic reaction is not itself detectable by a given sensor, additional reactions, enzymatic or otherwise, may be performed to generate a detectable species.

Thus, for any particular enzyme-catalysed reaction, a large variety of analytical methods and procedures may be used; they will not all be equally satisfactory and the best must be chosen for the analysis at hand.

3. 5. Biosensors

Biosensors are defined as bioanalytical chemical sensors which convert chemical reactions into electronic signals [57,58]. They generally consist of two major components : a biological recognition element which provides the specificity and selectivity of the

measurement and a physical transducer which translates the biological event into an electronic signals [59].

Many different types of biosensors have been developed, and the majority of the biosensor concepts are based on the combination of enzymes with classical sensors such as photometers, amperometric or potentiometric electrodes, gas electrodes or thermistors.

3.5.1 Fundamental aspects of biosensor operation [60]

Although a wide variety and an ever increasing number of sensing methods have been employed in biosensors, some fundamental principles are generally common to all. The first is the biological component which may catalyse chemical reactions or specifically binds the analyte. The biological component, either enzymes, micro-organisms, antibodies or receptors provide specificity. Second, sensing will generally take place at a surface and transport to this surface prior to reaction will frequently be an important factor. Third, steady-state conditions, in which the sensor signal is dependent upon a reaction proceeding at a constant rate, will often be encountered. The kinetics of transport coupled with surface reactions will therefore be important. Even where a true equilibrium measurement is to be made, the rate of approach to equilibrium will be important in determining the response-time.

4.DIFFERENTIAL PULSE AND CHRONOAMPEROMETRIC TECHNIQUES[61]

Differential Pulse Time Base (DPTB) and Time Base (TB) are chronoamperometric techniques which measure the current as a function of time. These techniques are used for amperometric titrations, amperometric sensors, flow cells, etc. The variation between the two techniques is the potential wave forms used.

The potential time functions for the two techniques are shown in Fig. 3 and Fig. 4.

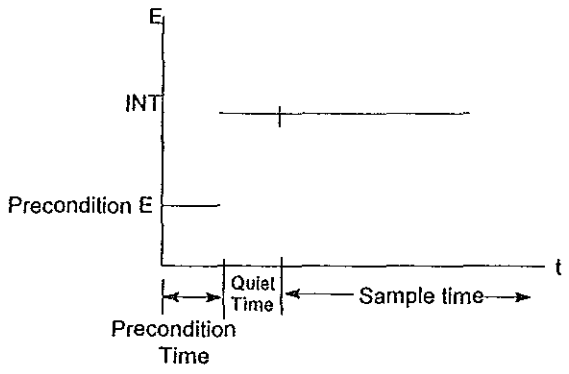


Fig. 3. Potential excitation waveform for TB

As shown in Fig. 3, a constant potential is applied to the sensor throughout the experiment. A preconditioning potential can also be applied prior to the experiment, but this option was not utilised in our experiments.

The other form of time base technique is the differential pulse time base (DPTB). Here, the potential excitation id done by a sequence of pulses of constant amplitude as shown in Fig. 4. In DPTB, the current is sampled just before the pulse and at the end of the pulse so that there is effective discrimination against the background current. The current difference that is displayed which enhances the sensitivity of the method.

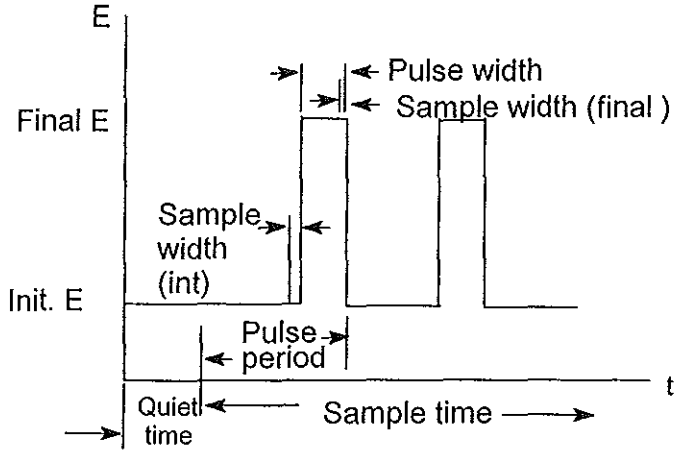


Fig.4. Potential excitation wave form for DPTP

In this research both techniques have been applied to investigate ion transfer across the membrane stabilized ITIES. The potential applied in the TB was set to a point where the ion to be determined is transferred from the aqueous phase to the organic phase. Thus a continuous transfer of ions occurs over the time of the experiment which at prolonged time may change the composition of the organic phase due to the enrichment of transferred analyte ions. This disadvantage is partially overcome by the DPTB since ion transfer from water to the organic phase occurs only at the "final potential " and the "initial potential " is chosen thus the ions are transferred back from the organic phase to water.

5. EXPERIMENTAL

Nitrobenzene (Fluka) was purified by washing with sulfuric acid, sodium hydroxide (20%) and finally with distilled water until neutrality prior to use. Tetradodecylammoniumtetrakis(4-chlorophenyl)borate (TDoAPClTPB) (Fluka), dibenzo-18-crown-6 (DB-18-C-6) (Merck), $Li_2SO_4.H_2O$ (Merck), KCl (Aldrich), NH_4Cl (BDH), Urea (Hopkin and Wiliams), $MgSO_4$ (BDH), petroleum ether (Riedel de Haen) were used as obtained commercially. The aqueous solutions were prepared with double-distilled water.

The electrochemical measurements were carried out with a four electrode potentiostat interface connected to the electrochemical analyzer BAS100W (Bioanalytical Systems, Inc., USA).

The setup of the electrochemical cell is shown in Fig. 5. Ag/AgCl electrodes served as reference electrodes in the aqueous and in the organic phase. The IR drop was compensated manually to a point before the potentiostat starts oscillating.

TDoAPClTPB (10 mmol / l) was the supporting electrolyte in the organic phase, which also contained 10 mmol / l DB-18-C-6. A 5 mmolar solutions of Li_2SO_4 or $MgSO_4$ was the supporting electrolyte in the aqueous phase.

The nitrobenzene interface was stabilized by a hydrophilic dialysis membrane TP20 (Reichelt Chemietechnik, Germany). The membrane was swollen in water prior to mounting on the amperometric sensor. The thickness of the swollen membrane was 40 μm.
Plant seeds collected from different parts of Ethiopia. were soaked in water to easer the removal of their hulls. After removing the hulls the cotyledon was grounded with an agate mortar. The powder was defatted with petroleum ether (40-60°C). The defatted powder was air dried and used as such in the experiments.

Standard solutions of potassium chloride or ammonium chloride were used for the calibration of the sensor. The electroanalytical techniques utilized for the investigation were cyclic dc-voltammetry, chronoamperometry and pulsed amperometry. The aqueous phase was stirred during the measurements.

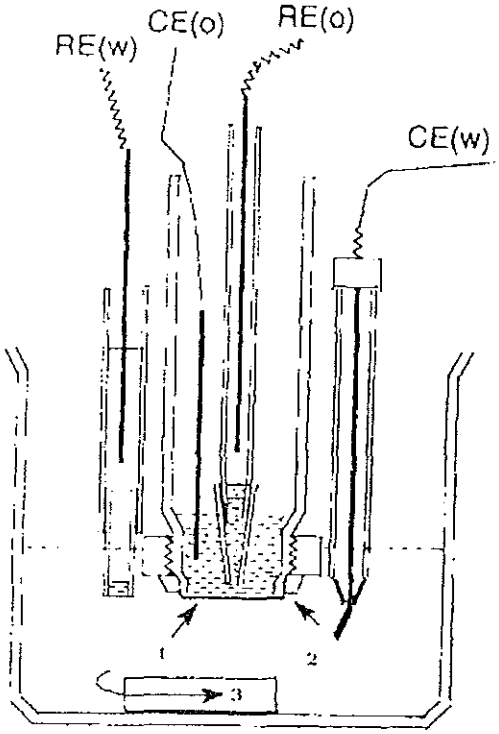


Fig. 5 Schematic diagram of the electrochemical cell
1: Dialysis membrane ; 2: membrane holder ; 3: stirring bar
RE(o,w): Reference electrodes organic phase and aqueous phase
CE(o,w): Counter electrodes organic phase and aqueous phase

All Experiments were carried out at laboratory temperature (20 ± 3 ° C).

6. RESULT AND DISCUSSIONS

6.1 Voltammetric investigation

Fig. 6 shows the dc cyclic voltammogram obtained at the membrane stabilized water nitrobenzene in the presence of the supporting electrolytes and DB-18-C-6.

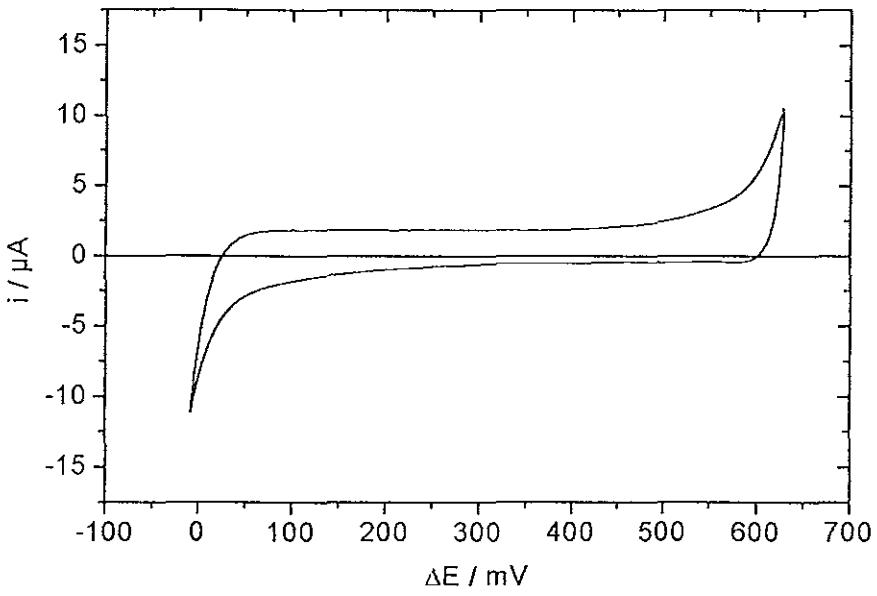


Fig. 6 Cyclic voltammogram at the membrane stabilized water/nitrobenzene interface.
organic phase: 10 mmol/l TDoAPClTPB + 10 mmol/l DB-18-C-6
aqueous phase: 5 mmol/l Li_2SO_4 ; sweep rate: 25 mV/s

There was no change in the voltammogram when Li_2SO_4 was replaced by $MgSO_4$. The polarization range seen in the voltammogram was rather wide and no influence due to complex formation of Li^{2+} or Mg^{2+} with DB-18-C-6 was observed. Since this work is concerned with the determination of the activity of urease based on the detection of ammonium ion formed and the estimation of endogenous potassium in the plant seeds, the voltammetric behavior of the two ions was studied in order to get information about the selectivity of the method. The selectivity is shown by the separation of the half-wave potentials of the ion transfer. Fig. 7 shows voltammograms obtained in the presence of potassium and ammonium ion in the aqueous phase.

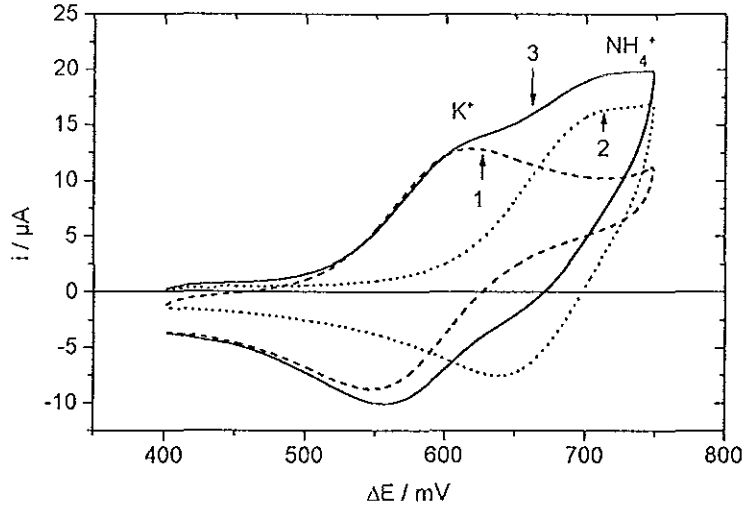


Fig .7 Cyclic voltammograms at the membrane stabilized water nitrobenzene interface
H_2O: 5 mmol / l Li_2SO_4 ; NB: 10 mmol / l TDoAPClTPB + 10 mmol / l DB-18-C-6)
1: 0.1 mmol K^+ / l ; 2: 0.1 mmol NH_4^+ / l ; 3: 0.1 mmol K^+ + 0.1 mmol NH_4^+ / l in H_2O
sweep rate: 25 mV/s

Corresponding ac-voltammograms are shown in Fig. 8. Although ac-voltammetry gives a better resolution, the peak height is seriously influenced by the setting of the IR-compensation and thus less suited for a quantitative determination of the ions.

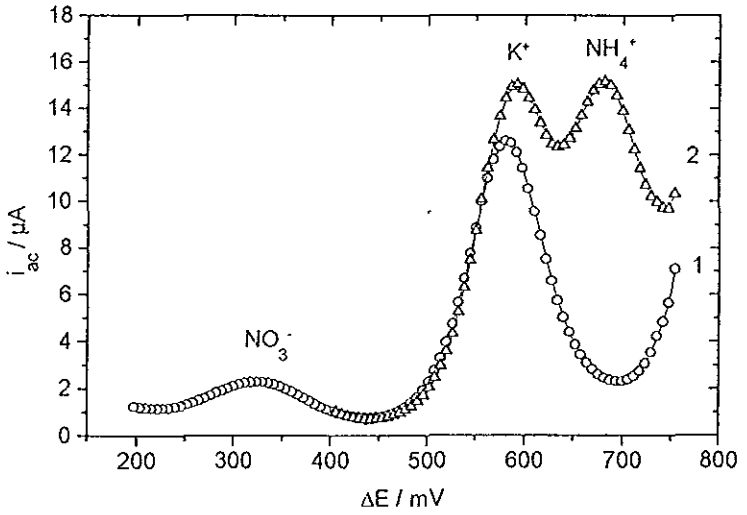


Fig. 8 Ac-voltammograms at the membrane stabilized water/nitrobenzene interface
ac-amplitude: 5 mV ; *f*: 35 Hz other conditions as in Fig. 7
1: mmol / l KNO_3 ; 2: mmol / l KNO_3 + mmol / l NH_4Cl

The current peaks due to the facilitated transfer of K^+ ($\Delta E_{1/2}$ = 583 mV ; i_p = 12.6 μA) and NH_4^+ ($\Delta E_{1/2}$ = 684 mV ; i_p = 12.7 μA) are separated by about 100 mV which is sufficient for a simultaneous determination of the two ions. The ac-current peak indicating the transfer of NO_3^- ($\Delta E_{1/2}$ = 325 mV ; i_p = 2.3 μA) is about six times less than the current peak for the facilitated transfer of the of the cations. Since the concentrations of the ions are the same and the diffusion coefficients in an aqueous solution are not too different ($D_{(NO_3^-)}$ = 1.91 x 10^{-5} cm^2 s^{-1} and $D_{(K^+)}$ = 1.96 x 10^{-5} cm^2 s^{-1}) this behavior can only be explained by assuming a diffusion coefficient for nitrate 2.5 times less than that of potassium ion within the membrane. Although no further investigations were carried out to elucidated this difference we suppose that negatively charged functional groups of the membrane are responsible for this behavior.

6.2 Voltammetric screening of plant materials for urease activity

Cyclic voltammetry was utilized to screen the seeds of the selected plants for urease activity. The selection of the plants was mainly random but somewhat systematically concentrated on plants endemic in Ethiopia. The species studied are compiled in Table 1.

The screening was performed by suspending 50 mg seed powder in 50 ml of the well-stirred aqueous phase. Cyclic voltammograms were recorded throughout the experiment. The dissolution of endogenous potassium from the seed powder could be followed by the increase of the current peak due to the transfer of the potassium ion. When the potassium peak was constant for ten cycles the substrate urea was added. The appearance of the ammonium ion peak after the addition of urea indicated the presence of urease in the seed powder under investigation. Fig. 9 shows the steps of the test. Soybean powder was used in this experiment. Only the steady state voltammograms are shown in the diagram. The separation of the current peaks was the same as that obtained with the mixtures of

ammonium and potassium (see Fig. 7) which proves the formation of ammonium ion by the enzymatic hydrolysis of urea. The test results are compiled in Table 1.

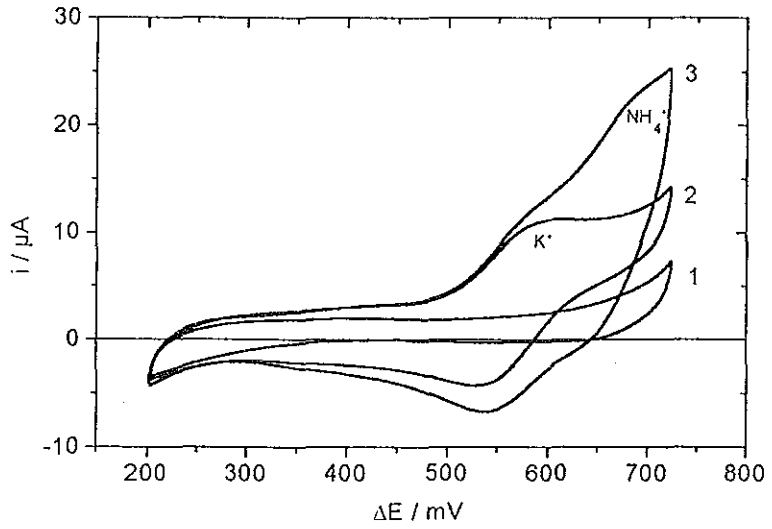
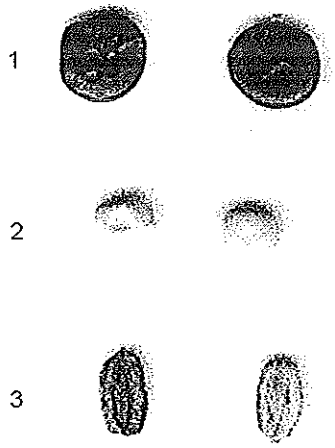


Fig. 9 Cyclic voltammograms at the membrane stabilized water nitrobenzene interface
1: H2O: 5 mmol / l Li_2SO_4 , NB: 10 mmol / l TDoAPClTPB + 10 mmol / l DB-18-C-6)
2: after addition of 50 mg soybean powder to the aqueous phase
3: as 2 after addition of 50 µl 0.1 molar urea solution to the aqueous phase
sweep rate: 25 mV / s

Table 1 Seed powders of plants screened and test results.

Vernacular Name	Scientific Name	Place of Sampling	Test Result
Watermelon	Citrullus vulgaris	Zuway	positive
Soybean	Soja hispida	Addis Ababa	positive
Berbera	Millitia ferugenia	Addis Ababa	positive
Abish	Trigonella foenum-graecum	Addis Ababa	negative
Kil	Lagenaria siceraria	Muger	positive
Tef	Erogrostis tef	Addis Ababa	negative
Gibto	Lupinus albus	Debre Markos	positive

When the preliminary test was positive, the enzymatic activity of the seed powder and its potassium content were determined. The seed of the plants which show enzymatic activity are shown below.



1: Berbera; 2: Gibto; 3: Kil

6.3 Calibration of the sensor

The quantitative determination of potassium and ammonium can be performed by different electroanalytical techniques and is based on the proportionality of the current (due to the facilitated ion transfer) to concentration. Fig. 10a and 10b show cyclic voltammograms for different concentrations of potassium and ammonium ions respectively.

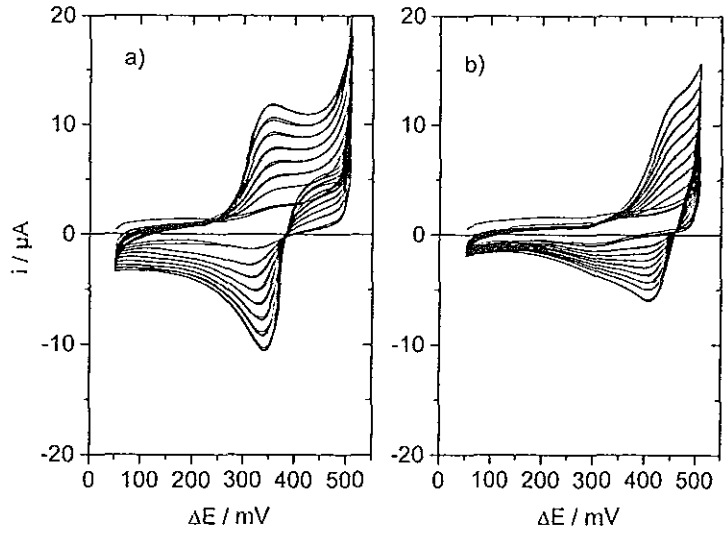


Fig. 10 Cyclic voltammograms at the membrane stabilized water/nitrobenzene interface at increasing concentration of a) K^+ and b) NH_4^+. The concentration change between the cycles is 2 x 10^{-5} mol / l starting from supporting electrolytes only.

The concentration dependence of the peak currents is obvious. Other electroanalytical techniques may also be employed for the determination of the ions. As an example, the concentration dependence of the current peak due to the transfer of potassium ion obtained by Osteryoung square-wave voltammetry is shown in Fig. 11.

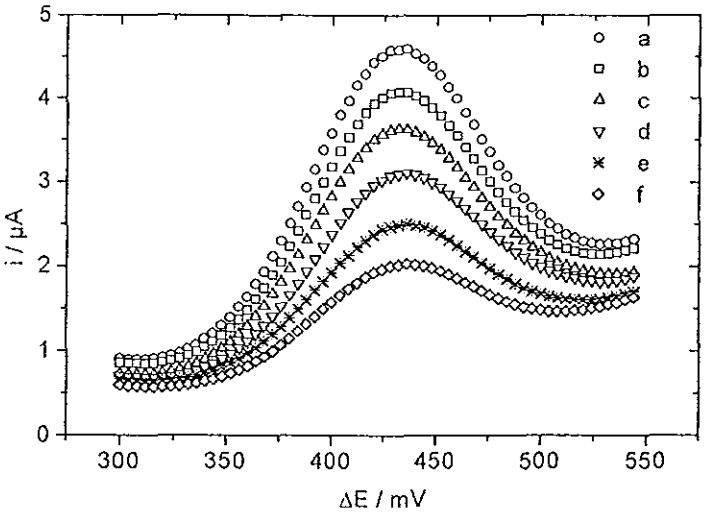


Fig. 11 Osteryoung square-wave voltammograms at the membrane stabilized water/nitrobenzene interface at different potassium ion concentrations.
C_K / mmol l^{-1} : a = 0.15; b = 0.125; c = 0.1; d = 0.075; e = 0.05 and f = 0.025.

Osteryoung square-wave voltammetry is like ac-voltammetry and the other pulse methods somewhat more influenced by the IR-compensation setting than cyclic voltammetry and therefore less suited for routine analysis. On the other hand we were interested in the time dependence of concentrations in order to determine the enzymatic activity of the seed powders. Thus chronoamperometric measurements were easier for the evaluation of results. One requirement for this type of measurement is to keep the thickness of the diffusion layer constant. This can be achieved by stirring the analyte. In order to minimize errors arising from differences in cell arrangement and in cell geometry, which in turn will change the hydrodynamic conditions at the sensor, the standard addition method was chosen for the determination of the sensitivity of the sensor and for the quantitative determination of the ion under study. The working potentials for chronoamperometry and pulsed

amperometry were chosen to come as close as possible to values corresponding to the limiting current range of the investigated ion transfer. There were no problems in the determination of potassium ion but the transfer of ammonium ion occurs at the positive end of the polarization window. In order to prevent a pronounced transfer of the ions of the supporting electrolytes which might shorten the lifetime of the membrane, the potential has to be chosen somewhat negative to the limiting current range. This compromise leads necessarily to a decrease in sensitivity for the determination of the ammonium ion. This is shown in Fig. 12 which shows the response of the sensor to the addition of potassium at two different potentials.

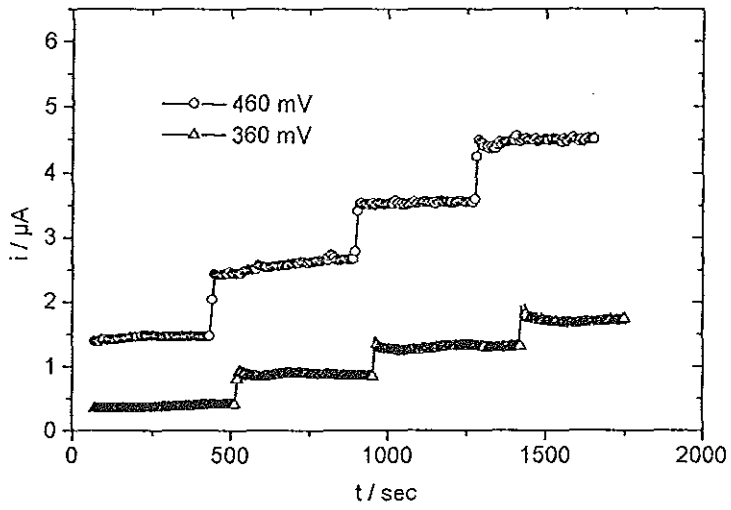


Fig. 12 Sensor response at different potentials to concentration steps of 2 x 10^{-5} mol / l K^+

Changing the potential from 360 mV to 460 mV doubles the sensitivity of the sensor from about 25 mA l mol^{-1} to 50 mA l mol^{-1} .

Pulsed amperometry has the advantage of higher sensitivity. also the ions which were transferred at positive potentials were transferred back to the aqueous phase at the second potential which had to be chosen sufficiently negative. Fig 13 shows the sensor response to ammonium ion obtained with pulsed amperometry.

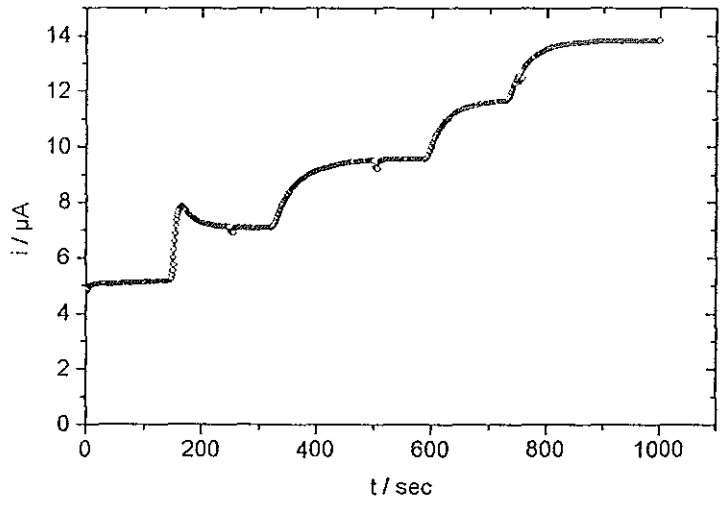


Fig. 13 Pulse amperometric sensor response to concentration steps of 2 x 10^{-5} mol / l NH_4^+ E_{in} = 150 mV ; E_{fin} = 460 mV

A calibration curve for the amperometric determination of potassium ion is shown in Fig. 14.

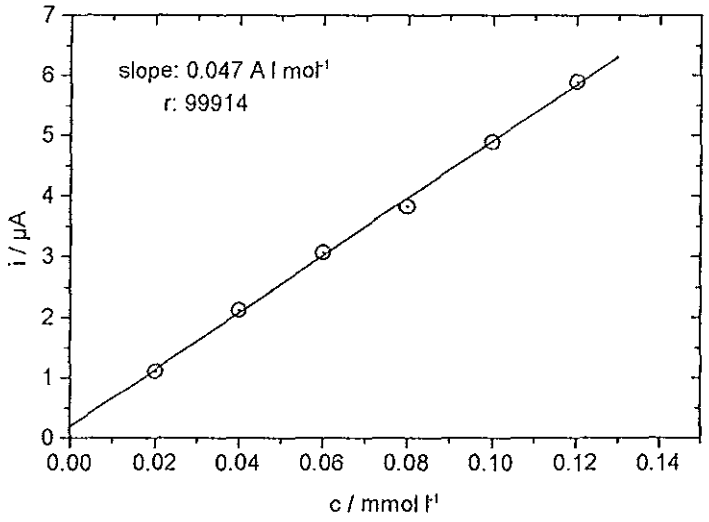


Fig. 14 Amperometric calibration curve for potassium ion . E_{appl} = 460 mV

Fig 15 shows a calibration curve for the pulsed amperometric determination of ammonium ion.

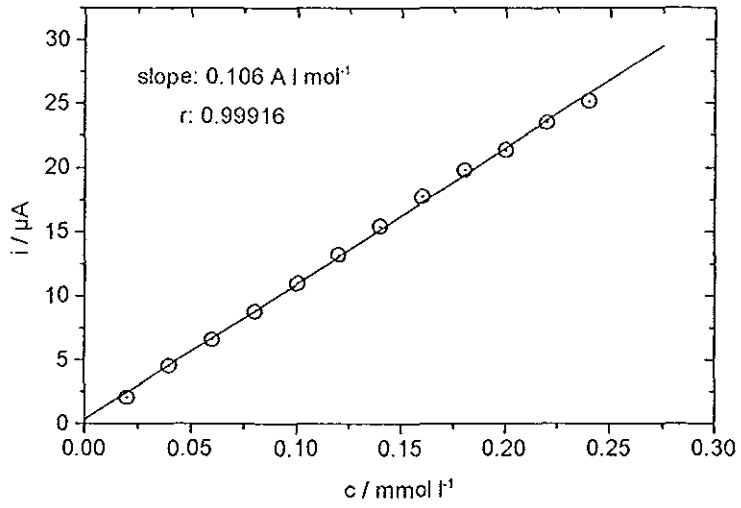


Fig. 15 Calibration curve for ammonium ion obtained by pulsed amperometry. Potentials as given in Fig. 13.

The sensitivity of the method depends on the potentials chosen and cannot be considered as a constant. However the calibration experiments have shown that the method is suitable for the determination of the potassium content and for the determination of ammonium ion as well.

6.4 Estimation of the potassium content in plant seeds

The amount of potassium in the seeds was estimated based on the two amperometric methods which will henceforth be referred to as TB for amperometry and DPTB for pulsed amperometry. The standard addition method was employed for calibration. Standard solutions of KCl were added before and after the addition of the sample. This is shown in Fig.16a and 16b taking Berbera (*Millitia ferugenia*) as an example.

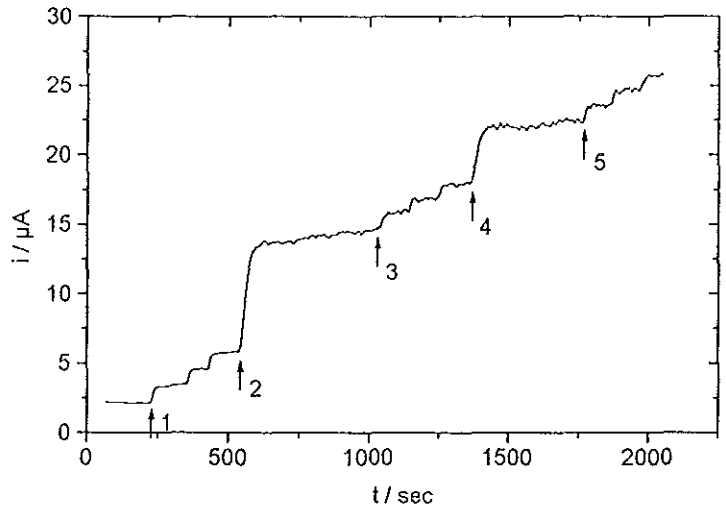


Fig 16 a

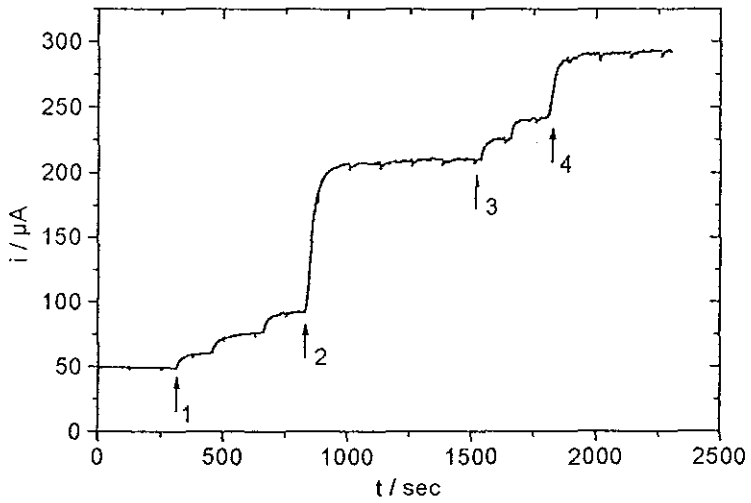


Fig. 16 b

Fig 16 Amperometric determination of the content of endogenous potassium in Berbera (*Millitia ferugenia*)
a) TB: $E_{appl.}$ = 535 mV; 1, 3, 5: standard additions of 25 µl 0.1 mol l^{-1} KCl ; 2: 50 mg and 4: 25 mg Berbera seed powder added.

b) DPTB: E_{init} = 100 mV ; E_{final} = 535 mV ; Pulse Period = 500 ms ; Pulse Width = 250 ms ; Sample Width = 100 ms; 1,3: standard additions of 25 µl 0.1 mol l^{-1} KCl ; 2: 50 mg and 3: 25 mg Berbera seed powder added.

The results are compiled in Table 2.

Table 2 Summary of potassium determination .

Sample	Method	mg of Sample	i / μA	S_{amp} / mA l mol^{-1}	%K		standard deviation
						Average	
Watermelon	DPTB	50	74.98	230	1.27	1.27	0.03
		25	44.4	286	1.29		
		25	40.8	256	1.24		
	TB	50	7.82	24.65	1.24		
		25	4.2	24.65	1.32		
Soybean	DPTB	50	144.47	397.8	1.42	1.35	0.6
	TB	50	10	30.5	1.3		
		25	4.34	25.25	1.34		
Berbera	DPTB	50	156.64	396.23	1.54	1.55	0.04
		50	118.88	320	1.5		
	TB	50	8.85	22.3	1.55		
		25	4.44	21.3	1.6		
Kil	DPTB	50	11.25	20.35	2.15	2.05	0.22
		50	192.04	340	2.2		
	TB	50	15.43	34.4	1.8		
Gibto	DPTB	50	78.52	354	0.87	0.9	0.09
		25	39.3	397	0.8		
	TB	50	5.04	23.2	0.85		
		25	3.42	24.6	1		
		25	3.03	23.3	1		

6.5 Determination of the urease activity of seed powder

The catalytic activity of urease is mostly expressed in Sumner units (SU) . 1 SU is defined as the amount of enzyme that liberates 1 mg of ammonia nitrogen in five minutes at 20^0 C. Thus the following relation holds:

1 SU = 1 mg N / 5 min. = 71.4 μmol N / 5 min.

The sensitivity S_{amp} in A l mol^{-1} of the sensor for ammonium ion was determined by standard addition. The enzymatic activity of the plant sample was then determined by monitoring the current change after the addition of urea. In order to avoid the influences of the inhibition effect of the product (NH_4^+) on the activity determination the current after 300 s (i_{300s}) was not measured directly but was obtained by extrapolation from the slope of

the current - time response immediately after the urea addition. The activity in SU is calculated according to

$$SU = \frac{C_{N(300s)} V_{aq}}{71.4} 10^6 = \frac{i_{300s} V_{aq}}{71.4\, S_{amp}} 10^6$$

where V_{aq} is the volume of the aqueous phase in liter, $C_{N(300s)}$ is the concentration of nitrogen in mol / l obtained from the extrapolated value of i_{300s} , and S_{amp} is the sensitivity of the sensor. The urease activity was determined for those plant materials which have shown positive response in a preliminary test. As an example the activity determination for Kil (*Lagenaria Siceraria*) is shown in Fig. 17a) and 17b)

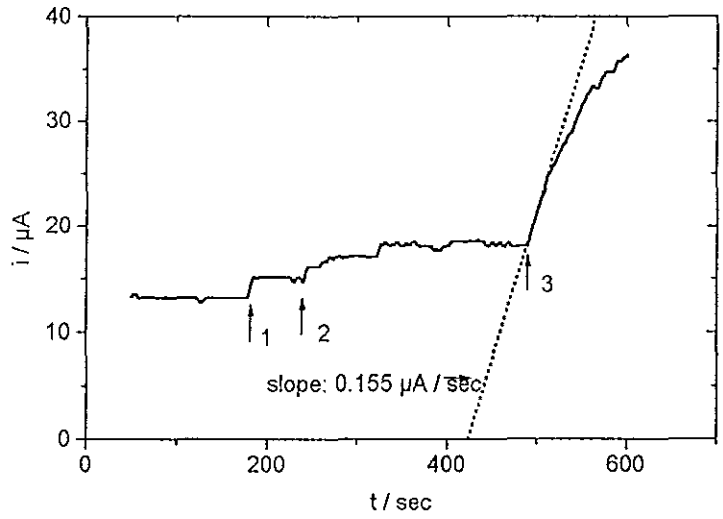


Fig 17 a

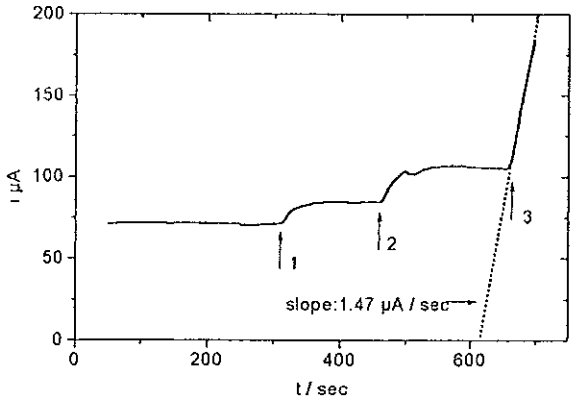


Fig. 17 b

Fig. 17 Urease activity determination for Kil (*Lagenaria Siceraria*). 50 mg seed powder added to 50 ml 5 mmol l^{-1} $MgSO_4$
a) TB: $E_{appl.}$ = 750 mV; 1,2,3: Standard addition of 50 µl 0.1 mol l^{-1} NH_4Cl; 4: Addition of 0.2 mol l^{-1} urea.
b) DPTB: E_{init} = 250 mV; E_{final} = 790 mV; Pulse Period = 1 s ; Pulse Width = 500 ms; Sample Width = 200 ms; 1,2: Standard addition of 50 µl 0.1 mol l^{-1} NH_4Cl ; 3: Addition of 0.2 mol l^{-1} urea.

The results of the urease activity determination are compiled in Table 3.

Table 3 Data summary of activity determination .

Sample	Method	i_{300} / µA	S_{amp} / mA l mol^{-1}	Activity (SU)		
					Average	SU/g of solid
Watermelon	DPTB	704.4	88.6	5.76	5.97	119.4
	TB	159.6	18.4	6.18		
Soybean	DPTB	80.4	90.15	0.63	0.65	13
	TB	13.1	13.6	0.66		
Jack bean	TB	251.6	27.9	6.6	6.7	134
	TB	194.4	20	6.8		
Kil	DPTB	443.05	208.8	1.52	1.53	30.6
	TB	46.5	21.4	1.55		
Berbera	DPTB	99.15	132.5	0.54	0.53	10.6
	TB	8.2	10.95	0.53		
Gibto	DPTB	52.1	236	0.15	0.14	2.8
	TB	5.2	25	0.14		

The enzymatic activity given in SU in Table 3 can be converted into intertnational units by multiplying the former with a factor of 14.28.

7. CONCLUSION

The quantitative determination of urea in clinical and agricultural samples is of great practical importance.The invention of biosensors has efficiently facilitated this determination. The main drawback, however, is that enzymes are expensive and cannot be synthesized so far.To find an alternative to this problem, local plants were screened for the presence of the enzyme urease, and three new plant seeds, namely, Kil, Berbera and Gibto, which are endemic to Ethiopia, have been found to contain the enzyme. In addition to these, experiments were also done on Soybean and Watermelon seeds.

In the determination of the activity and the potassium content of the plant seeds, the pulse and chronoamperometry techniques were used. During the analysis all plant seed powders were defatted with petroleum ether in order to increase the solubility of the enzyme in the aqueous solution so as to determine the maximum activity.

With regard to the order of the activity of the plant seeds, the activity decreases in the order Watermelon , Kil, Soy bean, Berbera, Gibto. The order of potassium content has also been determined to decrease in the order of Kil, Berbera, Soy bean, Watermelon, Gibto.

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