

Combination of Gold-Modified Electrode and α -Amyloglucosidase for Simultaneous Determination of Starch and Glucose

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ABSTRACT

The electrocatalytic activity of copper-modified gold electrodes has been investigated for the simultaneous detection of glucose and starch using α -amyloglucosidase in solution. The amperometric response of glucose at the modified gold electrode was monitored for different copper loadings (0.04, 0.1, 0.2, and 0.3 mg cm⁻²), that of 0.2 mg cm⁻² deposited on the electrode surface being chosen for further investigation. The surface characteristics were confirmed by the electrochemical impedance spectra. The oxidation of glucose and starch was studied by linear sweep voltammetry (LSV) in order to determine the best applied potential for measuring

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glucose in the presence of starch, a value of +0.4 V vs. SCE being selected. The glucose detection limit at +0.4 V is equal to 0.64 mg L^{-1} in the absence of starch. The same detection limit was found after adding 200 mg L^{-1} starch to the cell. The properties of the copper-deposited films were investigated in 0.15 M NaOH solution for the determination of α -amylglucosidase enzyme activity. The enzymatic reaction conditions were optimized. Conversion of starch into glucose was found to be around 89% by using α -amylglucosidase (2970 mUI) at 55°C for 30 min.

Key Words: Gold-modified electrode; Copper; Starch; Glucose; α -Amyloglucosidase.

1. INTRODUCTION

A large-scale starch-processing industry has emerged in the last century. Starch-converting enzymes are used in a number of industrial applications in food and biotechnology industries to provide different products.

Different methods are employed for the measurement of starch or its hydrolysates such as HPLC^[1] and calorimetry.^[2] However, these methods are complicated. Consequently, analytical methods for analyzing sugar with selectivity, sensitivity, and not time-consuming need to be established.

With this in mind, several enzymatic techniques have been proposed for the investigation of starch. A calorimetric method based on the determination of glucose produced by starch hydrolysis has been reported.^[3] More recently developed methods rely on the ability of the enzymes α -amylase and α -amylglucosidase to degrade starch to glucose.^[4] Flow-injection systems with immobilized enzymes have been developed for the simultaneous determination of starch and glucose.^[5] Other authors used multi-enzyme electrodes for the determination of starch and glucose.^[6,7]

The chemical modification of electrodes with metal particles has had large interest in the electroanalysis of carbohydrates^[8,9] because of their importance in beverages and foods. Regarding saccharides, until now, modified electrodes have been used only for the determination of mono- and disaccharides,^[10,11] using the electrochemical modification of electrodes with copper species.

The aim of this work was to ascertain whether the electrocatalytic activity of gold electrodes modified with copper could be exploited for the amperometric detection of α -amylglucosidase enzyme activity and for starch determination.

Results achieved, investigating the applicability of copper-modified gold electrodes to starch analysis and to the influence of different copper loadings on the electro-oxidation of glucose in alkaline solution in the absence and presence of starch, are described. For starch analysis, starch was subjected



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to enzymatic hydrolysis using α -amylglucosidase also called glucoamylase, which is an exo-acting amylase that hydrolyses consecutive 1,4- α bonds and 1,6- α bonds to yield glucose monomers.^[12] The unmodified and modified gold electrodes were characterized by electrochemical impedance.

2. EXPERIMENTAL**2.1. Reagents and Preparation of Solutions****Reagents**

Lyophilized α -amylglucosidase (EC. 3.2.1.3. from *Aspergillus niger* 99 units mg^{-1}) was purchased from Fluka (Biochemica 10113). Glucose was from Sigma, and starch from AnalaR, sodium acetate, acetic acid, sodium hydroxide and ethanol were obtained from Riedel-de-Haën. Copper(II) sulphate was from Surechem Products Ltd.

All reagents were of analytical grade and Millipore ultrapure water was used throughout in solution preparation.

Starch Solution

A 0.2% (w/v) starch solution was prepared in 0.15 M NaOH and adjusted to pH 4.6 with acetic acid for use as enzyme substrate for α -amylglucosidase. Electrochemical measurements were carried out using starch solution [2% (w/v)] prepared in 0.15 M NaOH.

Solution of α -Amylglucosidase

Enzyme solutions were prepared by dissolving 4 mg mL^{-1} of lyophilized α -amylglucosidase in acetate buffer (0.01 M $\text{CH}_3\text{COONa}-\text{CH}_3\text{COOH}$, pH 4.6).

Starch Sample Preparation

Six samples [chick pea (1), lentil (2), wheat flour (3), rice (4), garden peas (5), broad bean (6)] were purchased from a local market and analyzed with the proposed method.

Starchy samples were prepared according to the AFNOR method V52A.^[13] Sample of 0.25 g was dissolved in 50 mL of 40% ethanol, stirred for 20 min then centrifuged during 5 min. The aqueous phase was removed using a Pasteur pipette. The residue was washed with 25 mL of 40% ethanol and



centrifuged. The aqueous phase was removed, and the experiment was repeated at least one more time.

The final residue was dispersed using 2 mL of distilled water and 50 mL of 0.5 M NaOH. The solution was maintained for 30 min under stirring. The solution pH was adjusted to 4.6–4.8 with acetic acid.

2.2. Instrumentation

The gold disc electrode, area 0.0134 cm^2 , was purchased from BioAnalytical Systems (BAS). The three-electrode system consisted of the gold-working electrode, a stainless steel rod-auxiliary electrode, and SCE as reference.

An μ Autolab2 potentiostat/galvanostat (Ecochemie, The Netherlands) running on GPES version 4.9 software was used for fixed potential electro-deposition of copper, linear sweep voltammetry (LSV) and cyclic voltammetry (CV) conditioning. LSV experiments were performed in the potential range 0.0–1.0 V in 0.15 M NaOH solution at a scan rate of 100 mV sec^{-1} . Chronoamperometry at fixed potentials (+0.3, 0.4, 0.5 V vs. SCE) was used to record the glucose signal in 0.15 M NaOH.

Impedance spectra were recorded with a Solartron 1286 electrochemical interface coupled to a Solartron 1250 frequency response analyzer (Solartron Analytical, UK). Frequencies were scanned from 65 kHz down to 0.1 Hz, five steps per frequency decade, 10 mV rms perturbations, at 0.0 V vs. SCE. ZPlot software was used to record the spectra and ZView for equivalent circuit analysis.

2.3. Modified Gold Electrode Preparation

The gold electrode surface was prepared by polishing successively with 6 and 1 μm diamond spray on a microcloth, after previous cleaning by sonication in hydrochloric acid [18% (w/w)] to remove any copper particles from the bare electrode.

The electrochemical deposition of copper on gold was carried out at -0.3 V vs. SCE in 50 mM CuSO_4 solution, and the chronoamperometric profile was monitored. Deposition was continued until a chosen value of charge had been passed, equivalent to deposited masses of 0.04, 0.1, 0.2, or 0.3 mg cm^{-2} . Following this, the modified electrode was conditioned by continuous cycling between -0.3 and $+0.8\text{ V}$ at 100 mV sec^{-1} in 0.2 M NaOH.



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The copper loading in moles, n_{Cu} , is related to the charge, Q , consumed during the electrodeposition through

$$n_{\text{Cu}} = \frac{Q}{2F}$$

where $F = 96,485 \text{ C mol}^{-1}$ is the Faraday constant. The final copper mass, m_{Cu} , deposited is

$$m_{\text{Cu}} = 63.5n_{\text{Cu}}$$

where 63.5 is the atomic weight of copper.

2.4. Enzymatic Assay

Starch hydrolysis was investigated using different amounts of α -amylglucosidase. Reaction solutions were prepared by mixing different enzyme volumes (4 mg mL^{-1}) plus $15 \mu\text{L}$ of 0.2% solution of starch, or of the starch-containing sample, in 0.9 mL of acetate buffer for 6 min at 65°C . Then, the enzymatic reaction was stopped by the addition of $100 \mu\text{L}$ of 2 M NaOH. The total volume (1 mL) was injected into a cell containing 10 mL of 0.15 M NaOH, and the resulting signal was recorded.

The starch content is calculated according to the measured amount of glucose from the enzymatic hydrolysis. Total glucose is multiplied by 0.9 to determine total starch; this calculation accounts for the difference in molecular weight between individual glucose units and glucose polymers.^[13,14]

3. RESULTS AND DISCUSSION**3.1. Effect of the Copper Loading on the Electro-oxidation of Glucose**

The influence of the mass of copper electrodeposited on gold electrode on the glucose oxidation signal was studied amperometrically at 0.3 V vs. SCE. The bare gold electrodes were modified with 0.04, 0.1, 0.2, and 0.3 mg cm^{-2} copper. The results obtained with the various modified electrodes are illustrated in Fig. 1; the error bars represent the uncertainty for three determinations.

It can be seen that the current due to the oxidation of glucose is directly related to the copper loading at the gold electrode. Experimental data show that the glucose signal increases with increasing amount of deposited copper up to 0.2 mg cm^{-2} . A uniform distribution of copper particles (high roughness



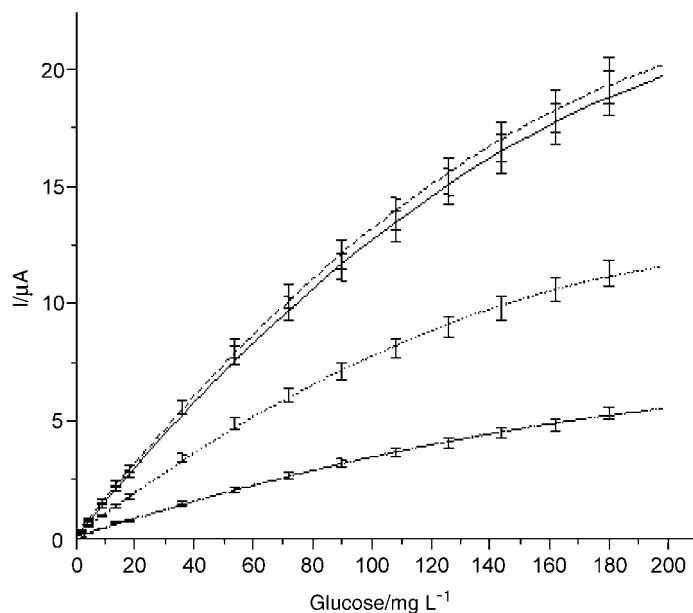


Figure 1. Dependence of glucose oxidation at 0.3 V (0.15 M NaOH) on gold electrode modified with copper loading: (---) 0.3 mg cm^{-2} , (—) 0.2 mg cm^{-2} , (.....) 0.1 mg cm^{-2} , and (-·-) 0.04 mg cm^{-2} .

factor) is probably responsible for the high electrocatalytic activity of the sensing electrode.

Data from the linear range of the calibration plot are given in Table 1. The limit of detection (LOD) was evaluated from the linear range through

$$\text{LOD} = 3 * \frac{\text{SD}}{b}$$

Table 1. Data from linear range of glucose calibration plot.

Deposited copper (mg cm^{-2})	Linear range (mg L^{-1})	Slope ($\mu\text{A L mg}^{-1}$)	Detection limit (mg L^{-1})
0.04	1.8–90	0.267	1.63
0.1	1.8–54	0.206	0.60
0.2	1.8–72	0.250	0.48
0.3	1.8–72	0.173	0.30



where SD is the standard deviation and b is the slope. The lowest detection limits were obtained at high copper loadings.

Since the oxidation current does not increase further for copper loadings higher than 0.2 mg cm^{-2} , modification of the gold electrode was carried out by depositing 0.2 mg cm^{-2} in all subsequent studies.

3.2. Impedance Characterization

Impedance spectra were recorded for the bare and copper-modified electrodes in solutions of 0.15 M NaOH and $0.15 \text{ M NaOH} + 72 \text{ mg L}^{-1}$ glucose, in order to investigate the changes to the electrode surface processes and interfacial region caused by the copper film as well as by the presence of glucose. Complex plane spectra obtained at 0.0 V vs. SCE are shown in Fig. 2, and the results of electrical equivalent circuit fitting are in Table 2. The equivalent circuit comprised of the cell resistance R_{Ω} in series with a parallel combination of a resistance R_p and a constant phase element (CPE), modeled as a non-ideal capacitor of capacity C and roughness factor α . The cell resistance, R_{Ω} , is almost constant under all conditions, except for the highest copper loading, both in the absence and presence of glucose.

In the solution without glucose, the deposition of copper reduces the resistance, R_p , compared to the bare gold electrode and increases the capacitance by a factor of three, but the surface roughness is essentially unchanged. The presence of the copper microparticles, therefore, has a significant effect on the interfacial region. For the highest loading of 0.3 mg cm^{-2} , the capacity increases even further, although this is not reflected in increased response to glucose (see previous section).

Although the form of the spectra is slightly different in the presence of glucose, the general conclusions are the same, as can be seen from Table 2. The main difference is in the values of R_p , which are significantly reduced, the capacity and roughness values being unchanged. This suggests that even at 0.0 V , there is some interaction (charge transfer process) occurring between glucose and the electrode. This could be due to the formation of Cu-chelates with glucose.^[15,16] It is also confirmed that the copper microparticles have a catalytic effect.

3.3. LSV of Glucose and Starch

The electro-oxidation of glucose and starch was studied by LSV in order to determine the best applied potential for measuring glucose in the presence of starch, see Fig. 3. The voltammograms obtained showed that the oxidation of glucose begins at $+0.07 \text{ V}$ vs. SCE; a maximum is reached at $+0.52 \text{ V}$. The



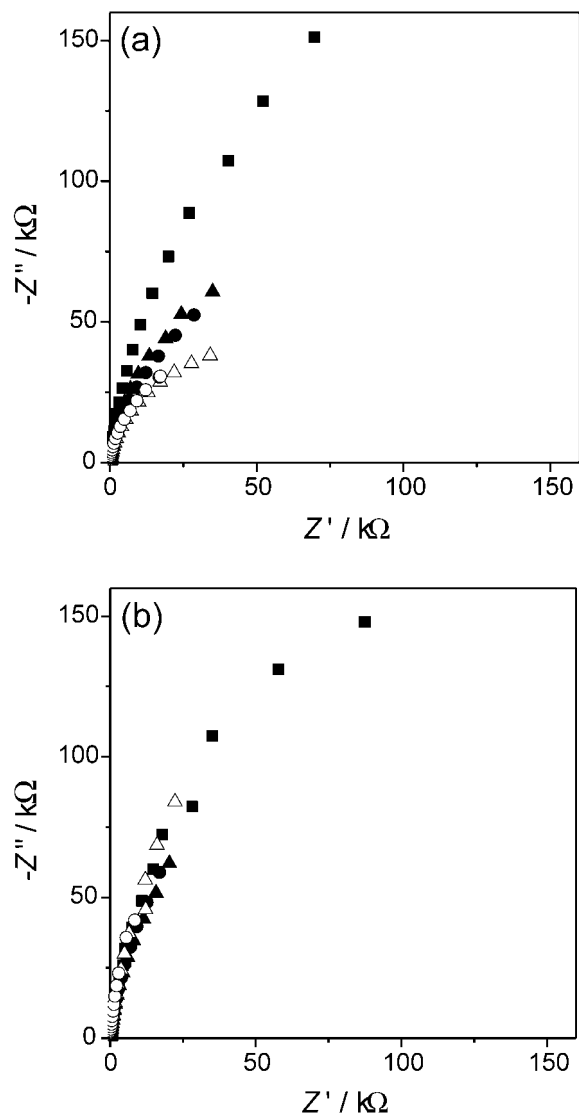


Figure 2. Complex plane impedance plots at the gold electrode unmodified (■) and modified with copper film (●, 0.04 mg cm⁻²; ▲, 0.1 mg cm⁻²; △, 0.2 mg cm⁻²; ○, 0.3 mg cm⁻²) in 0.15 M NaOH containing (a) 72 mg L⁻¹ glucose and (b) free glucose at 0.0 V vs. SCE.



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Table 2. Analysis of impedance data in 0.15 M NaOH at 0.0 V vs. SCE at the unmodified gold electrode and modified with different amounts of copper film.

	Cu deposited (mg cm^{-2})				
	0.0	0.04	0.1	0.2	0.3
(a) Without glucose					
R_{Ω} ($\Omega \text{ cm}^2$)	4.1	3.9	3.8	4.3	2.8
C ($\mu\text{F cm}^{-2}$)	8.1	24.7	21.1	17.6	34.4
α	0.93	0.93	0.95	0.93	0.95
R_p ($\text{k}\Omega \text{ cm}^2$)	58.8	39.2	33.4	69.4	50.4
(b) Containing 72 mg L^{-1} glucose					
R_{Ω} ($\Omega \text{ cm}^2$)	4.2	3.7	3.8	3.7	2.8
C ($\mu\text{F cm}^{-2}$)	8.1	21.2	18.1	20.3	36.7
α	0.93	0.93	0.95	0.91	0.95
R_p ($\text{k}\Omega \text{ cm}^2$)	63.9	18.9	20.7	11.6	10.5

data obtained are in agreement with previous work:^[10,17] starch oxidation begins at +0.32 V and reaches a maximum at +0.62 V.

A value of +0.40 V seems to be an appropriate potential for the determination of starch and α -amylglucosidase activity. At this potential, the current

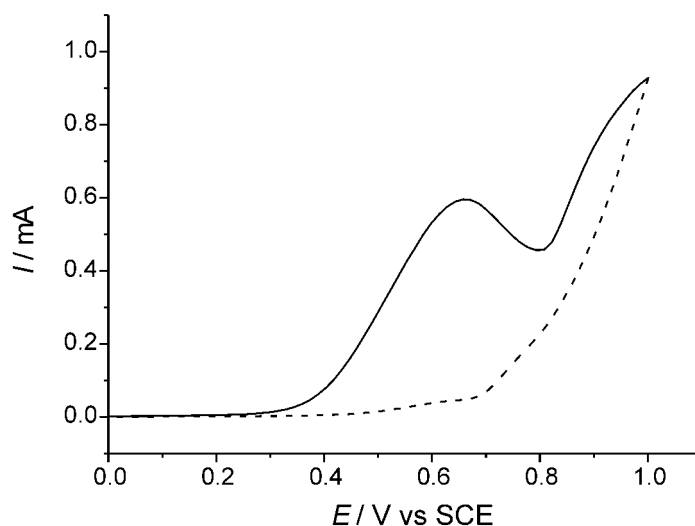


Figure 3. Linear sweep voltammograms at gold electrode modified by copper film (0.2 mg cm^{-2}) in 0.15 M NaOH [(—) glucose 3.6 g L^{-1} , (----) starch 400 mg L^{-1} .]



due to starch is very small compared with the glucose signal, which is more than half of its maximum value.

3.4. Effect of Fixed Applied Potential on the Electrooxidation of Glucose

Different fixed potentials—0.3, 0.4, 0.5 V vs. SCE—for the oxidation of glucose at the copper-modified gold electrode were investigated. The corresponding glucose calibration curves are reported in Fig. 4.

From 0.3 to 0.4 V, the glucose response increases considerably. However, at 0.5 V, the current increases only slightly. Table 3 reports the linear range and detection limit for glucose oxidation at each applied potential and shows that the detection limit is not affected by applying higher potentials whereas the upper limit of the linear range increases from 72 to 180 mg L⁻¹.

Taking into consideration the LSV results and these at fixed potential, a potential of +0.4 V was selected for enzyme activity investigations and starch analysis.

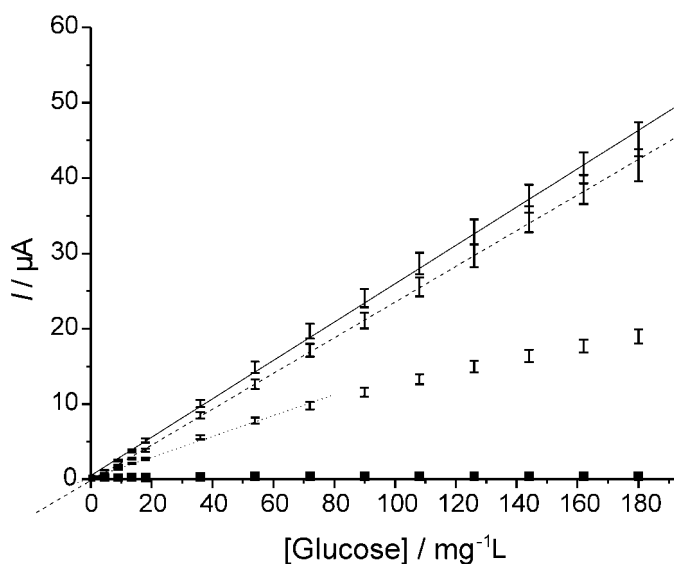


Figure 4. Glucose calibration curves using gold modified electrode (0.15 M NaOH, CuSO₄ 0.2 mg cm⁻²): at (·····) 0.3 V, (—) 0.4 V, (----) 0.5 V, and (■) starch response.



Table 3. Calibration curve data from glucose calibration plots.

<i>E</i> (V) vs. SCE	Linear range (mg L ⁻¹)	Slope (nA L mg ⁻¹)	Detection limit (mg L ⁻¹)
0.3	1.8–72	24.93	0.48
0.4	1.8–180	288.27	0.64
0.5	1.8–180	213.28	1.18

3.5. Enzymatic Activity and Starch Hydrolysis Studies

Enzymatic starch hydrolysis reactions were carried out using different α -amylglucosidase concentrations at 65°C for 6 min, Fig. 5. Starch conversion into glucose increases with increasing enzyme activity, but is not complete even using a large amount of the enzyme. The maximum starch hydrolysis (73%) is obtained using 5400 mUI of α -amylglucosidase.

The effect of the temperature on the extent of starch hydrolysis was then studied using 2970 mUI of α -amylglucosidase. Different enzymatic experiments were done at 18°C, 37°C, 55°C, 65°C using standard starch solution. Figure 6 shows that starch conversion into glucose increases with increasing temperature—89% of starch hydrolysis was reached at 55°C and 65°C for

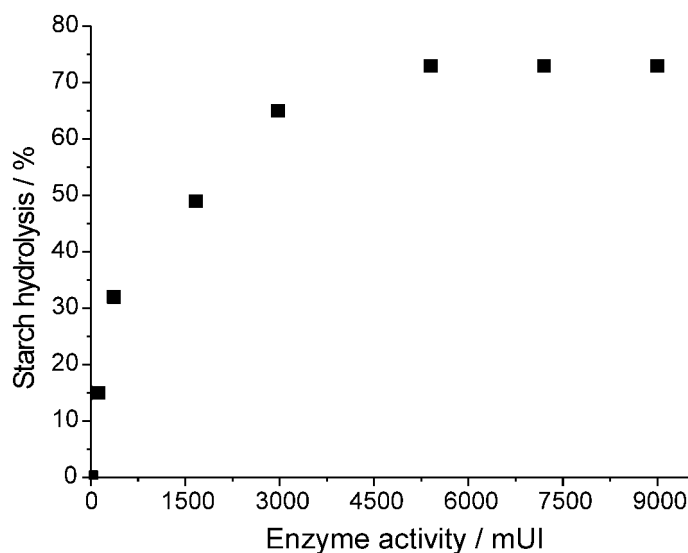


Figure 5. Dependence of starch hydrolysis on enzyme activity (6 min at 65°C).



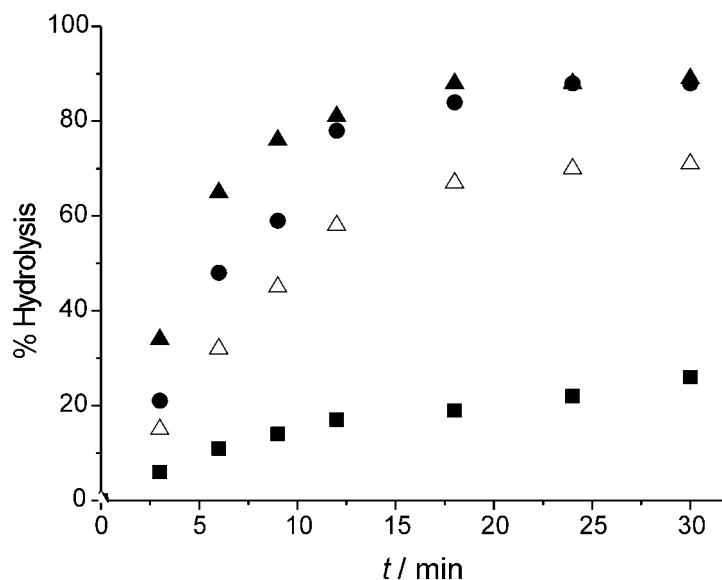


Figure 6. Dependence of starch hydrolysis on the reaction temperature (■) 18°C; (△) 37°C; (●) 55°C; (▲) 65°C, using 2970 mUI of α -amylglucosidase.

24 and 18 min, respectively. At 18°C, this percentage conversion is only achieved after 24-hr reaction time.

The results obtained are in agreement with previous studies.^[18] Starch hydrolysis could be achieved using α -amylglucosidase alone for long reaction times or combining with other enzymes such as α -amylase.

3.6. Analysis of Real Samples and Recovery Test

The modified electrode was applied to the measurement of real samples containing starch by using a standard addition method for the evaluation of the glucose arising from enzymatic hydrolysis with α -amylglucosidase. The results obtained are summarized in Table 4. The data showed that the recoveries ranged 95.8–103.2%. The starch content of the six samples is also reported in Table 4.

These results provide sufficient evidence of the feasibility of the AFNOR method^[13] for the pre-treatment of samples analyzed by the amperometric proposed method.

These samples were also incubated with α -amylglucosidase at 55°C for 4 hr to ascertain the maximum conversion of starch to glucose (89%).



Table 4. Recovery study results and starch content in starch-containing samples, incubated with α -amylglucosidase at 55°C for 30 min.

Sample	Glucose added (mg L ⁻¹)	Glucose found (mg L ⁻¹)	Recovery (%)	Starch content (%)
1	36	35.6 ± 0.7	98.8	49.9 ± 1.1
2	63	65.0 ± 1.0	103.2	45.6 ± 0.7
3	12	11.5 ± 0.65	95.8	74.4 ± 1.3
4	45	44.0 ± 1.3	97.8	84.7 ± 1.7
5	25	25.7 ± 0.7	102.8	37.5 ± 0.4
6	18	17.4 ± 0.35	96.7	86.6 ± 2.0

The result (data not shown) confirmed that the maximum starch conversion to glucose by α -amylglucosidase hydrolysis is obtained under the optimized conditions (55°C, 30 min).

Starch-containing samples were also measured with the proposed method. The reaction mixtures were incubated at 55°C for 30 min.

4. CONCLUSIONS

The enzyme α -amylglucosidase has been used in combination with a copper-modified electrode to measure starch and glucose. The procedure consists of two steps: enzymatic conversion of starch to glucose and electro-oxidation of the product on the modified electrode surface.

Different parameters have been investigated, namely the amount of copper microparticles deposited on the bare gold electrode and the applied potential for glucose oxidation in order to avoid interference from oxidation of starch. Reaction temperature and time, and concentrations of enzyme and substrate have also been studied.

The optimal conditions for starch hydrolysis were found to be 2970 mUI α -amylglucosidase, 55°C, 30 min. The starch conversion to glucose reaches 89% in these conditions. Glucose electro-oxidation is done at 0.4 V using 0.2 mg cm⁻² copper deposited on the gold electrode. Although this potential is lower than that necessary to reach the maximum glucose oxidation current, it is highly reproducible and avoids interference from di- and polysaccharides. Additionally, the proposed method seems to be advantageous since it is low time consuming, and with a low detection limit in the micromolar range.

This system has been developed with one enzyme, which is relatively cheap and easily available. It has been successfully applied to the analysis of real starch samples, with recoveries close to 100%.



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