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Food Chemistry **52** (1995) 187–192
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0308-8146/95/$9.50

Analytical Food, Nutritional and Clinical Methods Section

Simultaneous determination of maltose and glucose using a dual-electrode flow injection system

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(Received 12 January 1994; revised version received and accepted 24 May 1994)

A dual-electrode system consisting of a glucose oxidase (GOD) electrode and an amyloglucosidase/glucose oxidase (A/G) electrode in an FIA manifold was constructed to determine maltose and glucose simultaneously in their mixture. Highly active enzyme membranes were acquired. The linear range of the system was 0·3–30 mmol/litre of sugars. The variation coefficients were 3·2% for the GOD electrode and 1·6% for the A/G electrode in measuring the mixture of glucose and maltose. The results made by the dual-electrode system agreed well with those made by Fehling titration. During 20 days' determination, the activities of the enzyme electrodes showed no decay.

INTRODUCTION

Maltose coexists with glucose in food and starch hydrolysate. As a result simultaneous determination of glucose and maltose is of great importance to food industries and fermentation processes. Of the various determination methods available nowadays, enzyme sensors are the most attractive because of their speed, repetition, convenience and low cost. Glucose-sensing enzyme electrodes have been well developed, while maltose-sensing enzyme electrodes have been reported since 1974, most of which combined the enzymatic hydrolysis of maltose with glucose determination by the glucose oxidase (GOD) electrode (Cordonnier *et al.*, 1975; Bertrand *et al.*, 1981; Garton *et al.*, 1989; Kawakami *et al.*, 1992; Varadit *et al.*, 1993). But in this attempt the problem of the interference of coexisting glucose is inevitable. To solve this problem, two methods have been suggested. The first is just to eliminate the coexisting glucose by pretreatment (Dullau *et al.*, 1989). The second is the concurrent use of a bienzyme maltose electrode and a GOD electrode. With the GOD electrode measuring glucose only and the bienzyme electrode measuring the sum of sugars, the concentration of either sugar can be calculated. The principle of this method was first proposed in 1980 (Pfeiffer *et al.*, 1980). But, to the authors' knowledge, no experimental data have been reported to date.

The purpose of this research was to develop a biosensor that can simultaneously determine glucose and maltose in their mixture. We decided on the sequence reactions catalysed by amyloglucosidase and GOD and the dual-electrode system mentioned above. The advantages of this system lie in its simple structure, convenience in operation and replacement of enzyme membranes, minor consumption of enzymes, and possibility of large-scale manufacture.

We selected Fehling titration for the reference method, but we used molar concentrations instead of the conventionally used percentage concentration for calculations to make the results comparable with those made by the dual-electrode system.

MATERIALS AND METHODS

Reagents

Glucose oxidase (GOD, EC 1.1.3.4, Type II, from *Aspergillus niger*) and amyloglucosidase (EC 3.2.1.3, from *Aspergillus niger*, 6000 units/ml) were both from Sigma. All other chemicals were of AR quality and distilled water was used throughout.

Preparation of the electrodes

Preparation of the GOD membrane

Bovine serum albumin (BSA) solution (3 μl of 20% (w/v)), 4 μl of 3 units/μl GOD solution and 2 μl of 2·5% (v/v) glutaraldehyde solution were dropped onto a porous Teflon membrane with a diameter of 2 cm, on

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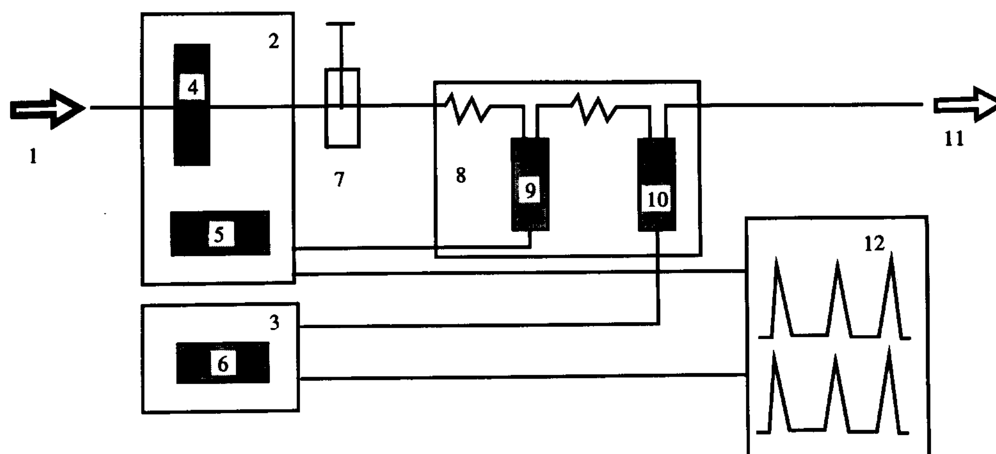

Fig. 1. Schematic diagram of the dual-electrode system: (1) carrier stream; (2) and (3) glucose analysers; (4) peristaltic pump; (5) and (6) screens; (7) sample injection; (8) thermostat; (9) GOD electrode; (10) A/G electrode; (11) waste; (12) chart recorder.

which they were mixed thoroughly and spread on a 7 mm diameter circular area with a fine glass rod. The Teflon membrane was allowed to stand for 30 min at 30°C to form an enzyme membrane on it.

Preparation of the amyloglucosidase and GOD (A/G) membrane

This process was just like the above process to form the GOD membrane, except for the addition of 5 μl of amyloglucosidase and the increased volume of the glutaraldehyde solution, which was 6 μl instead of 2 μl.

Preparation of the enzyme electrodes

The Teflon membrane with immobilised enzyme membrane on it was covered with a dialysis membrane and fixed over the tip of an oxygen electrode to form an enzyme electrode. The enzyme electrodes could be preserved in 30 mmol/litre glucose solution at 4°C for several days without detectable loss of enzyme activities. Preserving of the enzyme electrodes needed further investigation.

Construction of the dual-electrode FIA system

The dual-electrode flow injection analysis system consisted of two glucose analysers (Type GA-1, Wuhan Institute of Virology, Chinese Academy of Sciences), a high-accuracy thermostat (Type GHY-1, Taizhou Electric Equipment Factory), a home-made sample port, and a double-pen chart recorder (Type 3066, Yokogawa Hokushin Electric, Japan) (Fig.1).

Procedure

The carrier stream was pumped through the flow line at a flow rate of 2·86 ml/min. The sample solution was injected into the flow stream when the background current was stabilised. When the sample flowed through the surfaces of the electrodes, consumption of oxygen in the enzymatic reaction generated current drops, the peaks of which were measured for responses. The output of each electrode was processed by the electronic system of a glucose analyser and could be read out on the screen. The amplified analogue signals were surveyed to the chart recorder to draw out the response curves. The sample injection volume was 25 μl. The working temperature was 37 ± 0·05°C. Distilled water was used as the carrier stream except in the pH effect experiment.

Reference method

We used Fehling titration as the reference method. The measurement was carried out according to the conventional procedure (Biochemistry Laboratory, Beijing University, 1979). But in the calculations we used molar concentrations instead of the conventionally used percentage concentrations. Therefore our calculation formula was

$$\text{Reducing sugar (mmol/litre)} = (V' - V) \times 5{\cdot}56 \times N/V_s$$

where V (ml) was the volume of the standard glucose solution used in titrating the sample; V' (ml) was the volume of the standard glucose solution used in titrating the Fehling reagent without the sample; 5·56 mmol/litre was the concentration of the standard glucose solution; N was the dilution multiple of the sample; and V_s (ml) was the volume of the sample solution, which in our experiment was 5 ml.

RESULTS AND DISCUSSION

The role of the dialysis membrane

In the construction of an enzyme electrode for small molecules, a dialysis membrane is usually placed in front of the enzyme membrane to protect the latter from microbes. In maltose determination the dialysis membrane plays a more important role. It keeps starch from the A/G electrode. As a result, the dual-electrode system does not respond to starch, which enables it to be used in determining starch hydrolysate.

Interference of the upstream electrode with the downstream electrode

In preliminary experiments, we determined the interference of the upstream electrode with the response of the

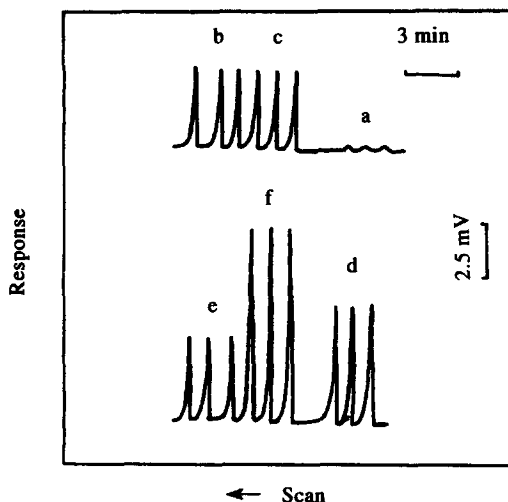

Fig. 2. Typical responses of the dual-electrode system: (a)–(c) GOD electrode to maltose, glucose and their mixture, respectively; (d)–(f) A/G electrode to maltose, glucose and their mixture, respectively.

downstream electrode, and found that whether the upstream oxygen electrode was covered with a GOD–Teflon membrane or with a Teflon membrane without GOD, the responses of the downstream electrode to glucose were unchanged. This means that the response of the downstream electrode was not affected by the enzymatic reaction at the upstream electrode.

Theoretically, the enzymatic reaction at the upstream electrode consumes oxygen and produces hydrogen peroxide, which will interfere with the response of the downstream electrode. But in our system this interference is too small to be detected.

Response curves of the dual-electrode system

Figure 2 shows typical responses of the system to maltose, glucose and their mixture. The samples used were 5 mmol/litre maltose solution, 5 mmol/litre glucose solution and the mixture containing 5 mmol/litre maltose and 5 mmol/litre glucose. The entire response cycle was about 1·2 min.

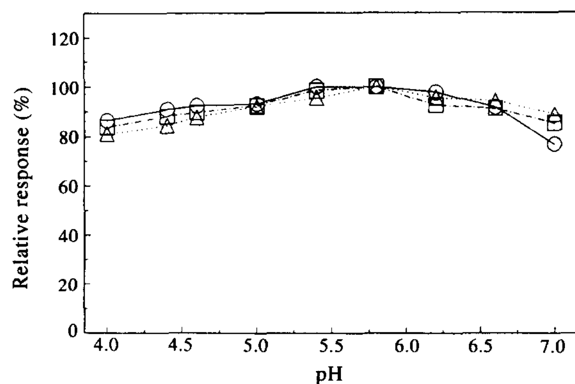

Fig. 3. Effect of pH on the dual-electrode system: (○) A/G electrode to maltose; (△) A/G electrode to glucose; (□) GOD electrode to glucose.

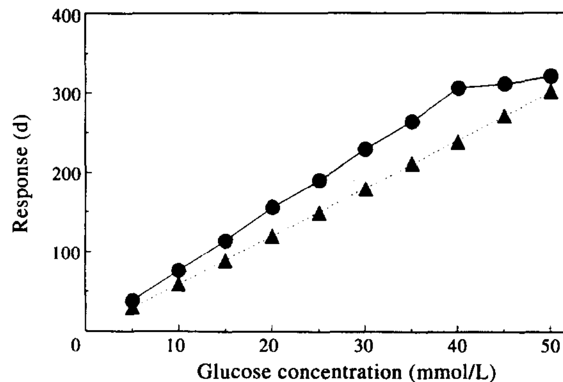

Fig. 4. Calibration graphs of glucose: (●) A/G electrode; (▲) GOD electrode.

Effect of pH on the dual-electrode system

Phosphate buffer solutions (0·01 mmol/litre) and 0·01 mmol/litre acetate buffer solutions with a pH range of 4·0–7·0 were used as carrier stream in succession. The samples were 10 mmol/litre glucose solution and 10 mmol/litre maltose solution. Responses of the dual-electrode system under different pH conditions are shown in Fig. 3, from which we can see that the optimum pH for either substrate was around 5·8 and that under slightly acid conditions the responses were quite constant with pH changes. So we could use distilled water (pH 6·0) as carrier stream. The relative responses in Fig. 3 are based on the response at pH 5·8 as 100%.

Response of the dual-electrode system to glucose, maltose and their mixture

The response of either electrode to glucose solution was proportional to the concentration (Fig. 4). The GOD electrode did not respond to maltose solution, while the response of the A/G electrode to maltose solution was proportional to the concentration (Fig. 5), The response of the GOD electrode to the mixture of glucose and maltose was equal to its response to glucose solution of the same concentration as in the mixture, and the response of A/G electrode to the mixture was the sum of its respective responses to glucose solution and

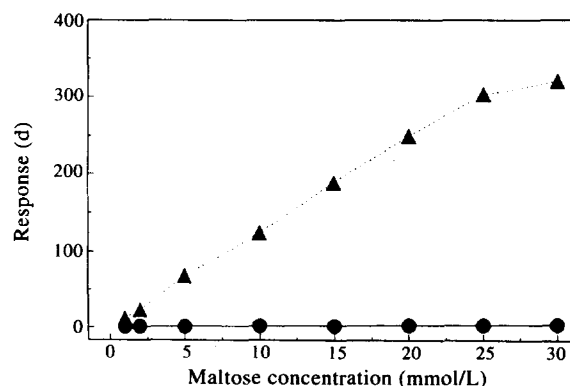

Fig. 5. Calibration graphs of maltose: (●) GOD electrode; (▲) A/G electrode.

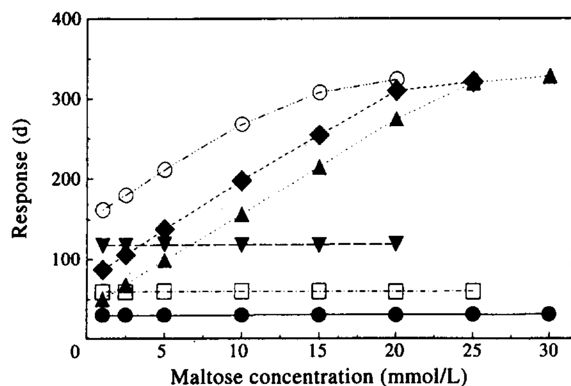

Fig. 6. Response curves of mixtures of maltose and glucose: (▲), (●) responses of A/G electrode and GOD electrode to maltose solution containing 5 mmol/litre glucose, respectively; (♦), (□) responses of A/G electrode and GOD electrode to maltose solution containing 10 mmol/litre glucose, respectively; (○), (▼) responses of A/G electrode and GOD electrode to maltose solution containing 20 mmol/litre glucose, respectively.

maltose solution of the same concentrations as in the mixture (Fig. 6).

Calibration and calculation

The calibration coefficients of the electrodes to glucose and to maltose were determined with standard glucose solution and maltose solution at the beginning of each experiment and should be verified frequently during the experiment. When the mixture of glucose and maltose was injected, the concentration of each sugar could be calculated with the responses of the two electrode by the following formula:

$$C_g = R_g'/A_g'$$
$$C_m = (R_t - A_g \times R_g'/A_g')/A_m$$

where C_m, C_g were the respective concentrations of maltose and glucose in the mixture; R_t, R_g' were the respective responses of the A/G electrode and the GOD electrode; A_g, A_g' were the respective calibration coefficients of the A/G electrode and the GOD electrode to glucose; and A_m was the calibration coefficient of the A/G electrode to maltose.

Recovery of the dual-electrode system

Table 1 shows the recoveries of the dual-electrode system in measuring mixtures of glucose and maltose of various concentrations and proportions.

Linear response range of the dual-electrode system

The linear response range of the dual-electrode system was determined by the response ranges of the oxygen electrodes and the activities of the enzyme membranes. A linear response range of 0·3–30·0 mmol/litre could be easily obtained by suitable sample volume.

Table 1. Recovery of the dual-electrode system

Actual concentrations (mmol/litre)		Calculated concentration (mmol/litre)		Recovery (%)		
Glucose	Maltose	Glucose	Maltose	Glucose	Maltose	All sugars
5·0	1·0	5·0	0·98	100	98	100
5·0	2·5	5·0	2·5	100	100	100
5·0	5·0	5·0	5·0	100	100	100
5·0	10·0	5·0	9·7	100	97	98
5·0	15·0	5·0	14·4	100	96	97
5·0	20·0	5·0	19·4	100	97	98
5·0	25·0	5·2	23·0	104	92	94
5·0	30·0	5·2	23·7	104	79	82
10·0	1·0	9·8	1·0	98	100	99
10·0	2·5	9·8	2·6	98	104	99
10·0	5·0	10·0	5·1	100	102	101
10·0	10·0	10·0	9·9	100	99	100
10·0	15·0	10·0	14·6	100	97	98
10·0	20·0	9·8	19·3	98	96	97
10·0	25·0	10·0	20·1	100	80	86
20·0	1·0	19·7	1·0	98	100	98
20·0	2·5	19·7	2·5	98	100	99
20·0	5·0	19·8	4·9	99	98	99
20·0	10·0	19·7	9·7	98	97	98
20·0	15·0	19·7	13·0	98	87	93
20·0	20·0	19·8	14·2	99	71	85

Precision of the dual-electrode system in measuring the mixture of glucose and maltose

A mixture containing 5 mmol/litre glucose and 5 mmol/litre maltose was used. The measurements were carried out 10 times in succession. Error analyses are made to the response values. The variation coefficients (CV) for the response values are 3·2% for the GOD electrode and 1·6% for the A/G electrode (Table 2).

Determination of reducing sugars in starch hydrolysate using the dual-electrode system and Fehling titration and the comparison between the two methods

Several starch samples were hydrolysed either by acid or by diastasum. The concentrations of maltose and glucose in the hydrolysate were determined by the dual-electrode system, and the concentration of reducing sugars was determined by Fehling titration. The comparison of the results is shown in Table 3, from which we can see that the results made by the dual-electrode system agreed well with those made by Fehling titra-

Table 2. Precision of the dual-electrode system

	GOD electrode	A/G electrode
Response values (d)	32, 30, 31, 31, 32, 31, 31, 33, 33, 33	101, 102, 103, 104, 105, 103, 102, 107 104, 105
Times of measurement	10	10
Mean value (d)	31·7	103·6
Standard error (d)	1·00	1·68
Variation coefficient	3·2%	1·6%

Table 3. Comparison between the results of the dual-electrode system and those of Fehling titration in examining starch hydrolysate

Sample number[a]	Results of the bielectrode system (mmol/litre)			Results of Fehling titration, reducing sugars (mmol/litre)
	Glucose	Maltose	Glucose+maltose	
1	0	1·59	1·59	1·71
2	4·41	0	4·41	4·96
3	4·58	0	4.58	4·57
4	15·9	21·8	37·7	37·5
5	15·9	29·7	45·6	43·9

[a]1, 2·0% soluble starch solution; 2, 0·1% potato starch hydrolysate produced by 6 M hydrochloric acid; 3, +0·1% soluble starch hydrolysate produced by 6 M hydrochloric acid; 4, and 5, 2·0% soluble starch hydrolysates produced by diastasum in different hydrolysing time, each was 10 times diluted before determination.

tion. The correlation coefficient of the two methods is 0·998.

In conventional Fehling titration percentage concentrations are used in calculation. In this way the mole content of reducing sugars (i.e. the amount of the semiacetal hydroxyl group) is converted into the percentage of glucose. Two comparison between Fehling method and the electrode method have been made (Pilloton *et al.*, 1987; Zhou *et al.*, 1990), one determining lactose in milk and the other determining maltose in corn syrup. They both used percentage concentration in their calculations and obtained agreeable results. We think it is because their samples contained mainly one sugar. In our measurement of starch hydrolysate the content of neither glucose nor maltose could be neglected. As a result using percentage concentrations would result in large errors. When using molar concentrations, however, the result of the two methods showed excellent agreement. It is true that in practical use, the content of all reducing sugars is converted into the percentage of glucose by using the percentage concentration of the standard glucose solution in the calculation. This simplification makes no difference if the results are to be compared between themselves, for example, in the fermentation monitoring. But we consider it better to use the actual mole content of reducing sugars when the comparison is to be made between two different methods.

There are no easy specific chemical methods to determine maltose. But maltose can be determined together with all other reducing sugars by reactions of the hemiacetal hydroxyl group. Of the various methods to determine reducing sugars, Fehling titration is widely used because of its accurate results and independence of apparatus and the standard curve. But like all other chemical titrations, it demands critically on operation and cannot be incorporated in on-line monitoring system. To date, there is no chemical method that is fit for on-line determination of reducing sugars.

In contrast to the reducing sugar determination methods, not only can the dual-electrode flow injection system be easily incorporated in on-line monitoring, but it can also provide rapid, repetitive and accurate results with simple operation. It requires 75–150 μl sample solution and less than 4 min to measure a sample, in comparison with the 15 ml sample solution and the 20 min required by Fehling titration, if each sample is measured three times. Above all, it can simultaneously determine the respective concentrations of glucose and maltose, which are not easily obtained by other methods.

Stability of the enzyme electrodes

The dual-electrode system was used in succession for 20 days, during which period over 1000 measurements were carried out and neither the GOD electrode nor the A/G electrode showed any decay of activity. The long-term stability of the dual-electrode system needs further investigation.

CONCLUSION

In comparison with the reported attempts to determine maltose by bioelectronic methods, our dual-electrode system is more suitable for practical use. It can determine the total amount of reducing sugars in starch hydrolysate, which has not been attained by any previous sensors but is of great value to fermentations using starch hydrolysate as a substrate.

We are now making efforts to improve the system for on-line monitoring and controlling in industry processes.

ACKNOWLEDGEMENTS

The authors thank Mr Wei-Ping Hu and Mr Zhi-Ping Zhang for valuable discussions. The assistance of Miss Xiao-Mei Zhang on electronics is gratefully appreciated. The research was granted by the Chinese State Planning Commission and the Chinese Academy of Sciences.

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