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RAPID AND SENSITIVE, COLORIMETRIC DETERMINATION OF THE ANOMERS OF D-GLUCOSE WITH D-GLUCOSE OXIDASE, PEROXIDASE, AND MUTAROTASE

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ABSTRACT

A modification, utilising mutarotase, of an enzymic, colorimetric system for determining D-glucose with D-glucose oxidase, peroxidase, and ABTS was satisfactory for the assay of the anomers of D-glucose in aqueous solution The time required for a single assay is ~10 min, and the lower limit is 0 4 μg of D-glucose The method is applicable to the anomer analysis of D-glucose released by enzymic hydrolysis of D-glucosides

INTRODUCTION

Assays of the anomers of D-glucose based on the specificity of D-glucose oxidase (β-D-glucose oxygen oxidoreductase, EC 1 1 3 4) for β-D-glucose have involved D-glucose oxidase–peroxidase and manual colorimetry (30 min for a single assay[1]) or complex, automated colorimetry[2] We have used D-glucose oxidase and a polarographic oxygen electrode with or without mutarotase (aldose 1-epimerase, EC 5 1 3 3) which catalyzes the interconversion of D-glucose anomers[3–5], an assay time of 5 min was possible[4,5] We now describe a modification of this method which utilises D-glucose oxidase, peroxidase, and mutarotase, and provides a rapid and sensitive assay

MATERIALS AND METHODS

Crude D-glucose oxidase from *Penicillium amagasakiense*, kindly supplied by Nagase & Co Ltd , Osaka, Japan, when partially purified as described previously[5], was free from catalase and mutarotase, and had a specific activity of 180 1 u /mg Mutarotase, partially purified from hog-kidney cortex according to the method of Okuda and Miwa[5], had a specific activity[6] of 500 units/mg One unit of our mutarotase assay[6] corresponds to ~35 units of the method of Bailey *et al* [7] Horseradish perox-

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idase (145 units/mg), α-D-glucosidase (α-D-glucoside glucohydrolase, EC 3 2 1 20) from yeast, and β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3 2 1 21) from almonds were commercial preparations

Pure α- and β-D-glucopyranose were prepared by using the method[8] for preparing α- and β-D-glucose-*1-t* The anomeric purity was >99% as shown by polarography (oxygen electrode)[4 5] 2,2'-Diazobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Boehringer

Treatment of samples — In order to decrease the rate of mutarotation of D-glucose, and to eliminate interfering materials such as hemoglobin, catalase, and glucosidases, samples were added to 20 vol of ice-cold chloroform–methanol (10 1), shaken vigorously, and then centrifuged for 2 min at 1,700 *g* The supernatant (assay solution) was stored at ~0°, and used for the assay of the anomers of D-glucose For aqueous solutions of D-glucose (up to at least 500 mg/dl), recovery from the supernatant solution was complete When 1 ml of an aqueous solution of D-glucose was treated by this procedure, 1 65 ml of supernatant solution was obtained This dilution ratio (1 0/1 65) was employed in calculating the total amounts of D-glucose in aqueous samples

Determination of the anomers of D-*glucose* — 0 02M Sodium phosphate buffer (pH 6 0, 1 ml) containing ABTS (1 mg/ml), D-glucose oxidase (10 μl, 8 mg/ml), and horseradish peroxidase (10 μl, 1 1 mg/ml) were added to a cuvette (10-mm path-length) When 5–150 μl of an assay solution (containing 0 4–3 μg of D-glucose) was added, only β-D-glucose reacted, and the ABTS was oxidized by the hydrogen peroxide generated The methanol present did not interfere with colour development, which was recorded using a Hitachi Perkin–Elmer Double Beam Spectrophotometer (Model 124) at 420 nm When the colour development (*A*, due to β-D-glucose) had ceased, mutarotase (2 μl, 2,000 units/ml) was added (Fig 1), this caused instantaneous α→β conversion, and total colour development (*B*, equivalent to the total D-glucose)

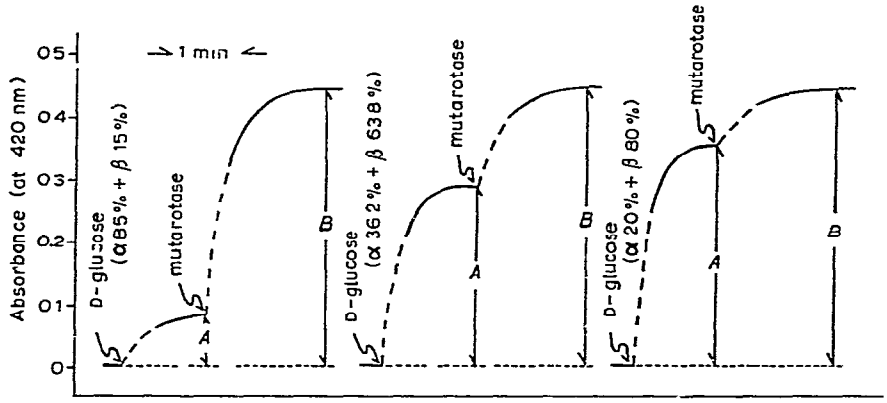

Fig 1 Tracings of the colour development in the anomer analyses of the assay solution containing 0 1 mg/ml of D-glucose (16-μl aliquot) The dotted lines represent those parts not actually recorded because of the mixing of the solution in a cuvette

at 420 nm was determined The percentages of α- and β-D-glucose in the assay solution are $100(B-A)/B$ and $100A/B$, respectively

Enzymolysis — A 0 25M solution (100 μl) of substrate in 0 02M phosphate buffer (pH 6 5) or 0 02M citrate buffer (pH 4 5) was added to the same buffers (40 μl) containing 1 25 units of α- or β-D-glucosidase After incubation for 1 min at 25° (for α-D-glucosidase) or 37° (for β-D-glucosidase), the reaction mixture was treated with chloroform–methanol as described above, and an aliquot (50 μl) of the resulting solution was assayed for the anomers of D-glucose

RESULTS AND DISCUSSION

The rate of mutarotation of D-glucose in an assay solution at ~0° was negligible during at least 1 h Although the treatment of samples with chloroform–methanol caused dilution (×1 65), this slowed mutarotation during the storage of samples, and denatured enzymes and proteins present in reaction mixtures of enzymolysis

Assay for the β anomer of an equilibrated, aqueous solution of D-glucose gave a mean and standard deviation for 21 values of 64 1 ±0 74%, a value which agrees well with that obtained by polarimetry[9]

As the total absorbance at 420 nm was directly proportional to the amount of D-glucose up to 3 μg, aliquots of assay solutions should contain <3 μg

The reliability of this assay was assessed by using standard aqueous solutions (0 1 g/l) of D-glucose freshly prepared from pure α- and β-D-glucose and mixtures thereof (Table I) Whereas the percentages of α-D-glucose obtained for α-abundant solutions were slightly lower than the theoretical values, those for β-abundant solutions were accurate

TABLE I

ANOMER ANALYSIS OF STANDARD D-GLUCOSE

Weight of anomer (mg)		*Anomers (%)*			
		Calc		*Found*[a]	
α	β	α	β	α	β
100	0	>99 0	<1 0	97 0	3 0
85	15	85	15	82 6	17 4
		36 2[b]	63 8[b]	35 9	64 1
20	80	20	80	19 5	80 5
10	90	10	90	10 6	89 4
0	100	<1 0	>99 0	1 0	99 0

[a]Average of duplicate determinations Analyzed within 30 min of the preparation of assay solutions
[b]Value for an equilibrated, aqueous solution of D-glucose

The assay was applied to the D-glucose produced by the hydrolysis of various D-glucosides with α- and β-D-glucosidases Each D-glucoside was hydrolysed to give

D-glucose of the same configuration (Table II) (*cf.* refs 10 and 11) Semenza *et al* [12] identified the anomeric form of D-glucose released by rabbit-intestinal sucrase with the D-glucose oxidase–peroxidase reagent[13], but they could not determine the proportion of each anomer in the reaction mixture

TABLE II

ANOMER ANALYSIS OF D-GLUCOSE PRODUCED BY ENZYMOLYSIS OF VARIOUS D-GLUCOSIDES

Enzyme	*Substrate*	*Anomers (%)*[a]	
		α	β
α-D-Glucosidase	Phenyl α-D-glucopyranoside	96 0	4 0
β-D-Glucosidase	Phenyl β-D-glucopyranoside	1 0	99 0
	Methyl β-D-glucopyranoside	2 0	98 0
	Salicin	2 3	97 7

[a]Average of duplicate determinations

The colorimetric assay for the anomers of D-glucose described herein is rapid (10 min) and accurate, and is the most sensitive of the methods reported hitherto using D-glucose oxidase It is applicable to whole blood[14] and plasma, but not to tissues and organs, because of the presence of certain reducing substances which probably influence the peroxidase reaction

o-Dianisidine[13] and 4-aminoantipyrine (with phenol)[15] were tested as chromogens instead of ABTS in the assay Although *o*-dianisidine gave accurate results, it is a carcinogen and the colour intensity is decreased to one seventh 4-Aminoantipyrine did not give satisfactory results, because of the rapid decrease of the colour intensity

REFERENCES

1 A S KESTON AND R BRANDT, *Anal Biochem*, 6 (1963) 461–467
2 J B HILL, *J Appl Physiol*, 20 (1965) 749–754
3 J OKUDA AND I MIWA *Anal Biochem*, 39 (1971) 387–394
4 J OKUDA AND I MIWA, *Anal Biochem*, 43 (1971) 312–315
5 J OKUDA AND I MIWA *Methods Biochem Anal*, 21 (1973) 155–189
6 I MIWA AND J OKUDA, *J Biochem (Tokyo)* 75 (1974) 1177–1179
7 J M BAILEY, P G PENTCHEV, AND J WOO, *Biochim Biophys Acta*, 94 (1965) 124–129
8 I MIWA, J OKUDA, H NIKI, AND A NIKI, *J Biochem (Tokyo)*, 78 (1975) 1109–1111
9 W PIGMAN AND H S ISBELL, *Adv Carbohydr Chem*, 23 (1968) 11–57
10 F. W FARRISH AND E T REESE, *Carbohydr Res*, 3 (1967) 424–429
11 D E EVELEIGH AND A S PERLIN, *Carbohydr Res*, 10 (1969) 87–95
12 G SEMENZA, H -CH CURTIUS, O RAUNHARDT, P HORE, AND M MULLER, *Carbohydr Res*, 10 (1969) 417–428
13 A ST G HUGGETT AND D A NIXON, *Lancet*, 273 (1957) 368–370
14 I MIWA, J OKUDA, K MAEDA, AND G OKUDA, *Clin Chim Acta*, 37 (1972) 538–540
15 P TRINDER, *Ann Clin Biochem*, 6 (1969) 24–27