THE COUPLING OF CELLOBIASE AND PEROXIDASE BY GLUCOSE OXIDASE*

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Youatt (1958) has described a method for estimating cellobiase activity that uses the coupled glucose oxidase–catalase system to determine manometrically the amount of glucose liberated from cellobiose by the enzyme. The high specificity of glucose oxidase makes it the reagent of choice for the otherwise difficult determination of glucose in the presence of excess cellobiose. The hydrogen peroxide produced by glucose oxidase can, however, also be demonstrated qualitatively by the use of peroxidase and both Eyer, Linzenmeier, and von Schrader (1957) and Huggett and Nixon (1957) have described sensitive quantitative glucose oxidase–peroxidase systems for determining glucose as such.

The most easily available source of a powerful peroxidase was the serum from *Ficus macrophylla* latex (Jermyn and Thomas 1954) which has accordingly been used, suitably diluted, throughout this work.

Rationale

Youatt (1958) has shown pH 5·4 to be the optimal pH for the activity of a system containing glucose oxidase and the cellobiase of *Stachybotrys atra*. The reaction between peroxidase and substrate at this pH must meet the following conditions:

(1) It must be sensitive to very small concentrations of H_2O_2 since the peroxide formed in the system is the result of two sequential reactions, the rate of one of which (cellobiose hydrolysis) cannot be increased at will.

(2) It must give optical densities at least approximately proportional to H_2O_2 concentration.

(3) It must give a stable colour.

About 100 potential substrates were tested. Condition (1) eliminated all but four substances as not giving substantial reactions with 10^{-4}M H_2O_2 in pH 5·4 McIlvaine buffer at 28°C. These were *o*- and *p*-phenylenediamine, *o*-aminophenol, and *p*-diethylaminoaniline. Condition (2) eliminated the last of these.

The products from all three had their highest adsorption peaks in acid solution but the optical density of the *o*-aminophenol solutions continued to change rapidly under these conditions, both in the presence and absence of enzyme. This left the phenylenediamines as possible substrates of which the *o*-isomer was chosen as giving the highest adsorption maximum and an oxidation product (phenazine) of known structure and stability. The final acidification used to attain the maximum optical density also had the effect of stopping all enzymic actions.

Hydrogen peroxide (10^{-5}–10^{-4}M H_2O_2) reacted completely within a minute or two with *o*-phenylenediamine at the concentration finally used, *c.* 0·02%, in the

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presence of peroxidase. When the reaction was allowed to proceed for 5 min, and the mixture then adjusted below pH 1 with HCl, the final optical densities showed a linear dependence on H_2O_2 concentration and the method would be practicable for estimation of traces of peroxide.

The concentration of glucose oxidase to be used was fixed by the arbitrary criterion that, when coupled to peroxidase and *o*-phenylenediamine in the presence of 10^{-4}M glucose, the system should develop an optical density of about unity within 10 min, i.e. the glucose was almost completely oxidized in this time. This amount of glucose, in its turn, can be produced from cellobiose in a few minutes by cellobiase in concentrations that can be readily attained (Youatt 1958).

When an attempt was made to extend the system thus developed by adding the cellobiose–cellobiase step, two difficulties were encountered. In the absence of added cellobiose, the system of three enzymes was found to cause a rapid development of colour in a solution of *o*-phenylenediamine although no combination of two of them would give this effect. Since all three preparations (snail-gut cellobiase, fungal glucose oxidase, *Ficus* peroxidase) were unpurified, it appeared that some coupling of components was taking place to give an amine oxidase system. Such oxidases usually depend on a heavy metal ion, often copper, for activity, and a variety of complexing agents was therefore tested to see whether they would eliminate the effect. Cysteine was found to be highly effective for this purpose, not only eliminating the amine oxidase effect but also much retarding the slow non-specific discoloration of *o*-phenylenediamine solution. The peroxidase activity was only slightly affected by 0·002% cysteine.

Addition of cellobiose to the system without cellobiase also leads to colour development. This phenomenon occurred at much the same rate for a variety of cellobiose samples and does not appear to be due in any great degree to the random contamination of cellobiose by glucose. Keilin and Hartree (1948) cite the comparative rates of oxidation of D-glucose and cellobiose by "notatin" at 0·05M substrate as 100 to 0·09 but comparative Michaelis constants are not given. At the concentrations (*c.* 10^{-3}M) needed to give maximum rates with *S. atra* cellobiase, the rate of cellobiose oxidation by glucose oxidase was found to be strongly dependent on substrate concentration. The cellobiose concentration finally adopted in the assay (1·0 mg/ml) was chosen as the best compromise between the need to maximize one process and minimize the other.

Reagents

(i) *Buffer.*—McIlvaine buffer, pH 5·4.

(ii) *Peroxidase.*—The serum produced by high-speed centrifugation of *F. macrophylla* latex is divided into 1-ml lots which are stored at −20°C until required; one such lot is diluted to 50 ml with buffer. The diluted solution (PO) keeps its activity for some days in the refrigerator.

(iii) *Glucose Oxidase.*—600 mg of glucose oxidase (Sigma Chemical Co., U.S.A.) is suspended in 20 ml of buffer and the solution cleared by filtration. The filtrate (GO) can be stored a day or two in the refrigerator.

(iv) o-*Phenylenediamine Hydrochloride* (*OPD*).—A single recrystallization from concentrated hydrochloric acid after decolorization with charcoal gives a white crystalline product. After washing with the acid and a short drying in the oven, this white hydrochloride can be stored indefinitely in the dark at −20°C without visible deterioration.

(v) *OPD Solution.*—100 mg of *o*-phenylenediamine hydrochloride and 10 mg of commercial L-cysteine hydrochloride are dissolved in 50 ml of buffer. This solution must be prepared afresh daily.

(vi) *Cellobiose Solution.*—400 mg of cellobiose dissolved in 100 ml of buffer.

Method

The following solutions (in ml) are added to the tubes in any convenient order, A and B serving for a series of assays:

A		B		C		D	
General Blank		Cellobiose Blank		Enzyme Blank		Assay	
OPD	0·5	OPD	0·5	OPD	0·5	OPD	0·5
PO	0·5	PO	0·5	PO	0·5	PO	0·5
GO	0·5	GO	0·5	GO	0·5	GO	0·5
Buffer	1·5	Buffer	0·5	Sample	0·5	Sample	0·5
				Buffer	1·0		

The tubes are equilibrated for 10 min at 28°C, and 1 ml of separately equilibrated cellobiose solution is then added to each of tubes B and D. After 40 min further incubation, 2 ml of 0·5N HCl is added to all tubes. The optical densities of the contents are read at 471 μ against a water blank and (D−C−B+A) measures the enzyme activity. Blank B eliminates the cellobiose oxidase effect, and blank C any residual amine oxidase effect. The colours are moderately stable for some hours, any slow increase in colour in the assay samples being compensated for by a corresponding increase in the blanks.

Measured "enzyme activity" is in general not a linear function of enzyme concentration and a calibration curve is needed to convert optical density into enzyme units. As a result of variations in kinetics this calibration curve will not be identical for cellobiases from different sources. Figure 1 shows the results obtained with cellobiases from snail gut and *S. atra.*

The variation in the kinetics of the system with the source of cellobiase and its non-linearity with varying substrate concentration is inherent in the competition for cellobiose of two enzymes, one fixed (glucose oxidase), and one of varying concentration and affinity (cellobiase). In default of exact knowledge of the properties of the enzymes, the use of the system for enzymic assay has therefore to be calibrated on an empirical basis.

Attempts to assay more dilute enzyme samples by increasing the cellobiose concentration or extending the reaction time lead to much greater rises in the optical density of the "cellobiose blank" than of the assay mixture, e.g. a reaction time of

60 min or a cellobiose concentration of 1% treble the blank but raise the enzyme activity only about 75%. The enzyme activity is thus measured as the difference between two large optical densities and accuracy is much reduced. Higher enzyme concentrations may be assayed by lowering the cellobiose concentration or the reaction time (t), or both, the measured enzyme activity of a given sample being approximately proportional to t^2 and [cellobiose]$^{\frac{1}{2}}$. A new calibration curve must be prepared and correlated with the old one.

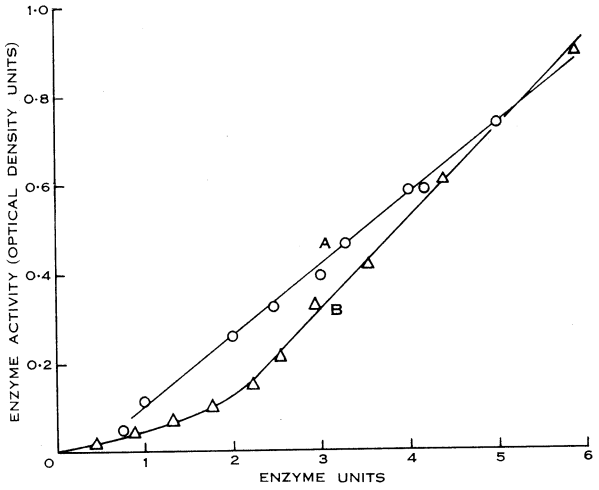

Fig. 1.—Calibration curves for the cellobiases of (A) snail gut and (*B*) *Stachybotrys atra* under the standard conditions. The values of the "enzyme units" have been adjusted to give approximately equal enzyme activities at 5 enzyme units.

The tediousness of the necessary calibrations gives the present method little or no advantage over the manometric method for the accurate determination of cellobiase activities. It has, however, proved very useful in practice for the rapid assessment of relative activities in large numbers of culture samples with enzyme activities at all levels from very high to very low.

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