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A Kinetic Method for the Direct Determination of Cellobiose Hydrolysis by β-Glucosidases

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The *Somogyi—Nelson* colorimetric method is applied in a new manner which is more suitable for following the kinetics of cellobiose hydrolysis catalyzed by β-glucosidase (EC 3.2.1.21). The *Somogyi—Nelson* colour reagent, which is a mixture of the solutions of the reagent of *Somogyi* and that of *Nelson* in a volume ratio of 1 : 1, is added to the enzyme-substrate solution at the very start of the reaction. The colour reagent reacts with the product (*D*-glucose). Under the reaction conditions (0.1 *M* acetate buffer, $pH = 5.0$ and temperature 37 °C) the colour reagent does not affect the enzyme activity. The method excludes any inhibition of the product, owing to the continuous removal of the latter by the colour reagent. The method suggested has been applied to monitor cellobiose hydrolysis with β-glucosidase, contained in four cellulase enzyme preparations from various fungal sources. The values of the *Michaelis* parameters (*Km, V*) were determined.

(Keywords: β-Glucosidase; Cellobiase; Cellobiose hydrolysis; β-Glucosidase kinetics; Cellobiase kinetics)

Eine kinetische Methode zur Verfolgung der Hydrolyse von Cellobiose durch β-Glucosidasen

Die kolorimetrische Methode nach *Somogyi* und *Nelson* wird nach einem neuen Verfahren zur Verfolgung der Kinetik der hydrolytischen Spaltung von Cellobiose, katalysiert durch β-Glucosidase (EC 3.2.1.21), angewandt. Das Farbreagenz nach *Somogyi* und *Nelson* (Mischung der Reagenzien von *Somogyi* und *Nelson* im Volumenverhältnis 1 : 1) wird der Enzym-Substrat-Lösung zu Beginn der Reaktion hinzugefügt. Das Farbreagenz tritt mit der *D*-Glukose in Reaktion, wobei unter den gegebenen Reaktionsbedingungen (0,1 *M* Azetatpuffer, $pH = 5{,}0$ und 37 °C) die Enzymaktivität nicht beeinflußt wird. Die entwickelte Methode wurde zur Verfolgung der Hydrolyse von Cellobiose durch β-

Glucosidasen, die in vier Enzympräparaten aus verschiedenen Pilzstämmen enthalten waren, angewandt. Es wurden die *Michaelis*-Parameter (*Km, V*) bestimmt.

Introduction

β-glucosidase (EC 3.2.1.21) or cellobiase is an enzyme which catalyzes the hydrolysis of a cellobiose molecule to give two molecules of *D*-glucose[1]. This enzyme is a component of the cellulase enzyme complex, which catalyzes the hydrolysis of native cellulose to *D*-glucose[2–7].

The methods and approaches used until now for the study of the hydrolytic kinetics of the system cellobiose—β-glucosidase are of the one-point type and are based chiefly on the measurement of the concentration of the *D*-glucose appearing as a final reaction product. The wide use of the method of *Somogyi*[8,9]—*Nelson*[10] proves difficult to apply for kinetic studies, owing to the following major shortcomings: 1) the method requires heating and subsequent cooling; 2) it is of the one-point type and when once implemented, the solution cannot serve for further kinetic investigations; and 3) the repeated implementation of the method for plotting a kinetic curve requires a long time.

The *Somogyi—Nelson* arsenomolybdate method is applied in a new manner, as discribed in the present paper, which makes possible the direct monitoring of the kinetics of cellobiose hydrolysis by β-glucosidase.

Materials and Methods

Enzymes and Substrates

As sources of the enzyme β-glucosidase we have used the commercial cellulase preparations: Cellulase („E. Merck", FRG) from *Oxyporus* species (E_1), Cellulase („Kiowa Hakko Co.", Japan) from *Trichoderma* species (E_2), Luizym („Luitpoldwerk", FRG) from *Aspergillus oryzae* (E_3) and Celluzym („Nagase & Co.", Japan) from *Aspergillus usani* (E_4). All other chemicals used were from commercial sources and were of analytical grade.

Measurement of Reducing Power

The standard procedure of the *Somogyi—Nelson* method is as follows:

In a test tube (19 × 185 mm) 1 ml of the solution of *D*-glucose (0.010 to 0.100 g/l) in 0.1 *M* acetate buffer was mixed with 1 ml of *Somogyi*'s alkaline copper reagent. The test tubes were covered with glass marbles and heated for 20 min in boiling water. After cooling in running water, 1 ml of *Nelson*'s arsenomolybdate reagent was added and stirred. The resultant solutions were diluted to 25 ml with water and their absorbances were read at 37 °C and 760 nm in a 1 cm cell with a Specord UV-VIS spectrophotometer („Carl Zeiss", DDR). A blank had the same composition, with the exception of *D*-glucose.

Kinetic Measurements

The enzyme-substrate reaction was carried out and followed in a 1 cm cell (total volume of 2.5 ml) at 37 °C in the spectrophotometer in the following manner:

the buffered solutions (0.1 *M* acetate buffer, $pH = 5{,}0$) of the β-glucosidase and cellobiose were mixed together and the solution obtained was mixed with 0.30 ml (2% of the total volume) of *Somogyi—Nelson* colour reagent, which is a mixture of the solutions of the reagent of *Somogyi* and that of *Nelson* taken in a volume ratio of 1 : 1. A control had the same composition, with the exception of the enzyme. The continuous change in the absorption of the sample was automatically recorded at 760 nm at different time intervals ($0 \leqslant t \leqslant 4$ h). The time which was required to record a single curve was 1 min.

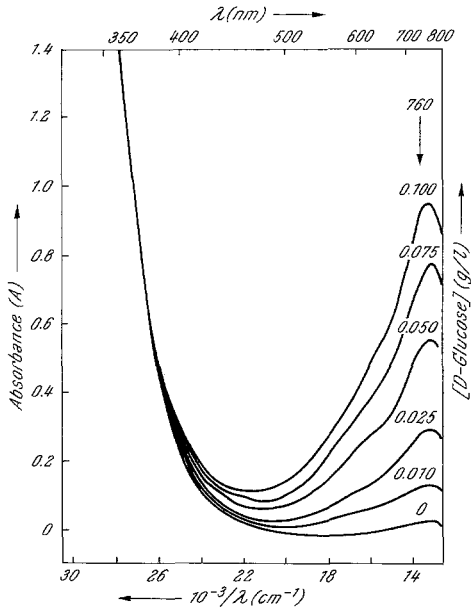

Fig. 1. Absorption spectra from 350 to 800 nm of the colouring developed with *Somogyi—Nelson* reagent (unmodified method) at different concentration of *D*-glucose (0.010, 0.025, 0.050, 0.075, and 0.100 g/l), which were diluted 25 fold

Results

The recorded absorption spectra for the colouring produced from five glucose solutions when the unmodified method of *Somogyi-Nelson* is used, are presented in Fig. 1. The relation between the absorbance change (ΔA) at 760 nm and the molar concentrations of *D*-glucose is shown in Fig. 2. The molar absorption coefficient (ε) determined from this figure is $\varepsilon_{760} = 4.5 \cdot 10^4$ ($M^{-1}\,\mathrm{cm}^{-1}$).

Fig. 3 represents the absorption spectra of solutions at various times ($0 \leqslant t \leqslant 4$ h) of reaction after the beginning of the reaction. These solutions were obtained by mixing β-glucosidase, cellobiose and the

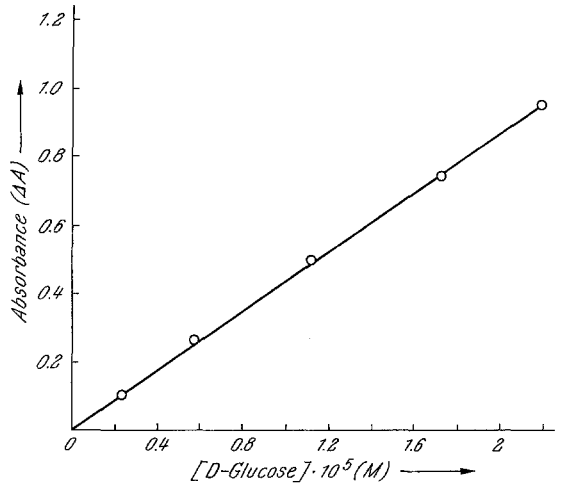

Fig. 2. Calibration curve of *D*-glucose determined by the unmodified *Somogyi—Nelson* method at 760 nm

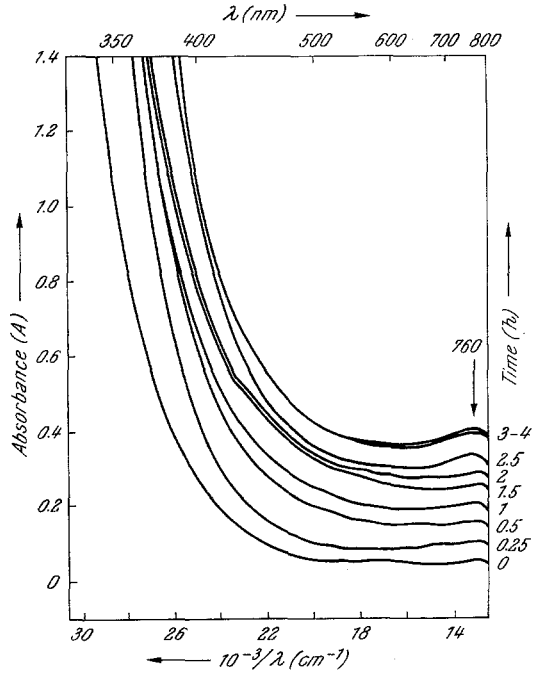

Fig. 3. Absorption spectra of the colouring developed with the suggested method at various times (0 to 4 h) of the reaction mixture of β-glucosidase from *Oxyporus sp.* (0.40 g/l), cellobiose (0.10 g/l) and colour reagent (0.05 ml)

colour reagent. The absorbance reaches a stationary value after 3 h. The change in the absorbance (ΔA), determined at 760 nm, is

$$\Delta A = A_t - A_0 = \varepsilon_{760}[P]_t d, \quad (1)$$

where A_0 and A_t are the absorbance of a given solution at an initial and an arbitrary moment of the reaction time; d is the length of the optical path in centimeters; ε_{760} is the molar absorption coefficient; and $[P]_t$ is the concentration of the accumulated reaction product (*D*-glucose).

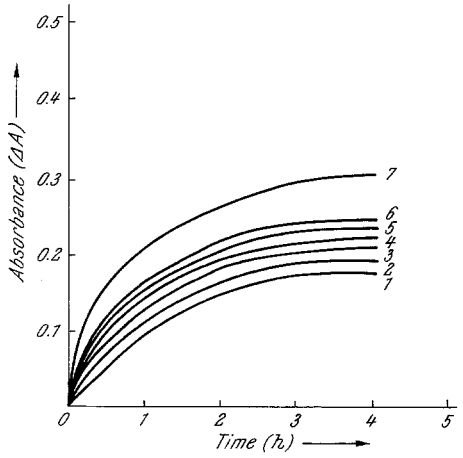

Fig. 4. Kinetic curves of the reaction β-glucosidase—cellobiose at different substrate concentrations (g/l): 1–0.02, 2–0.04, 3–0.06, 4–0.08, 5–0.10, 6–0.12, and 7–0.20; enzyme concentration 0.40 g/l

The initial reaction rates (v_0):

$$v_0 = \left(\frac{d[P]}{dt}\right)_{t=0} = \frac{1}{\varepsilon_{760} d}\left(\frac{d[\Delta A]}{dt}\right)_{t=0} \quad (2)$$

were determined from the kinetic curves (Fig. 4), obtained at different concentrations of substrate $[S]_0$ and at a constant enzyme $[E]_w$ concentration.

Fig. 5 shows *Lineweaver—Burk*[11] plots of data from the degradations of cellobiose with the four β-glucosidase preparations from various fungal sources. The determined values for *Km* and *V* from these data are presented in Table 1.

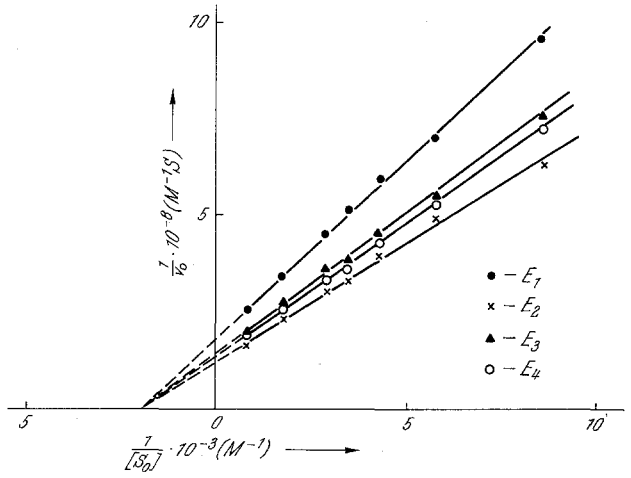

Fig. 5. *Lineweaver—Burk* plots for four β-glucosidases: E_1 (*Oxyporus sp.*), E_2 (*Trichoderma sp.*), E_3 (*Aspergillus oryzae*), and E_4 (*Aspergillus usami*)

Table 1. *Michaelis parameters for β-glucosidases from various fungal sources*

$E_{Nr.}$	Enzyme preparations	Fungal producers	*Km* (m*M*)	*V* (n*M* s^{-1})
E_1	Cellulase („E. Merck", FRG)	*Oxyporus sp.*	0.5	5.40
E_2	Cellulase („Kiowa Hakko Co.", Japan)	*Trichoderma sp.*	0.5	8.35
E_3	Luizym („Luitpoldwerk", FRG)	*Aspergillus oryzae oryzae*	0.5	7.15
E_4	Celluzym („Nagase & Co.", Japan)	*Aspergillus*	0.5	7.40

Discussion

The intensive investigations of the enzyme β-glucosidase or cellobiase have been connected lately with the role and the significance which it plays in the process of the cellulose enzyme hydrolysis[2–6,12].

In the case of the method suggested the colour reagent is introduced into the enzyme—substrate solution at the beginning of the reaction.

Further experiments show that the reagent thus applied: 1) does not affect enzyme activity; 2) does not change the *pH* value of the enzyme—substrate solution; 3) the optimal volume ratio between the *Somogyi* and *Nelson* reagents is 1 : 1; and 4) continuous interaction with the reaction product leads to the removal of the latter from the reaction and thus makes product inhibition impossible, which is otherwise typical for that reaction[4,13]. The method does not require heating and subsequent cooling. It is easily reproduced, simple and convenient to perform and demands a relatively short time to obtain a kinetic curve. The error is smaller than 8%.

With the proposed new method a very interesting fact, connected with the mechanism of action of the colour reagent, was observed. We found that the colour reagent does not react with free reducing sugars (glucose, cellobiose etc.) at 37 °C. For such determinations only the unmodified method of *Somogyi—Nelson* can be used. In the case of enzyme hydrolysis of cellobiose, the colour reagent interacts with the newly formed glucose molecules at 37 °C. "Reactive forms of glucose" could be a possible explanation for this reaction.

The comparative study of four cellulase preparations of different fungal sources, containing the enzyme β-glucosidase or cellobiase, shows (cf. Table 1) that the maximal reaction rates differ but the *Michaelis* constant has identical values.

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