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Determination by the Enzyme Thermistor of Cellobiose Formed on Degradation of Cellulose

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Abstract

A calorimetric assay procedure for the determination of cellobiose has been developed. The cellobiose is hydrolyzed by β-glucosidase and the glucose formed is measured calorimetrically by an enzyme thermistor containing co-immobilized glucose oxidase and catalase. The system was optimized with regard to the arrangement of the enzymes, the pH-dependence of the separate enzymic steps, and of the total system. By placing the β-glucosidase in a precolumn that could be switched in and out of the flow through the enzyme thermistor, both cellobiose and glucose present in the sample could be determined. The performance with standard solutions and with crude samples from cellulose degradation experiments was investigated.

Index Entries: Calorimetric assay, of cellobiose; assay, calorimetric, of cellobiose; enzyme thermistor, for cellobiose; cellobiose, enzyme thermistor for; thermistor, enzyme, for cellobiose; immobilized glucose oxidase; glucose oxidase, immobilized; oxidase immobilized glucose; catalase, immobilized; immobilized catalase; beta-glucosidase; cellulose, determination of cellobiose formed on degradation of.

Introduction

There is a growing interest in the utilization of waste cellulose as a renewable resource for enzymic or microbial sugar production for food purposes or as microbial substrate. In the study and control of the degradation of cellulose, it is of value to

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be able to follow the concentration of various metabolites such as cellobiose. It should preferably be possible to carry out its determination in conjunction with the degradation process and with a minimum of pretreatment of the sample.

Methods currently used for cellobiose determination are based on ion exchange chromatography (*1*), high performance liquid chromatography (*2*), or gas–liquid chromatography (*3*). A direct spectrophotometric method based on enzymic determination can also be used (*4*). Existing methods are generally less well-suited to use in routine applications, in continuous analysis or on multiple samples, especially when the assay has to be performed on crude solutions because of complexity of equipment and procedures, and in situations where their lack of specificity would compromise results.

Higher specificity may be gained by using enzymic methods. After hydrolysis of the cellobiose with β-glucosidase (cellobiase), the glucose formed can be determined by various enzymic methods (*4*). The enzyme thermistor (*5*) has proved very useful for this type of analysis since it is unaffected by the optical properties of the sample and, therefore, can operate on crude solutions. Furthermore, it is highly specific because of the enzyme used and may easily be incorporated in automatic and continuous systems.

The method described here is based on the enzymic degradation of cellobiose by β-glucosidase and the subsequent determination of the glucose produced by an enzyme thermistor equipped with co-immobilized glucose oxidase and catalase. The immobilized β-glucosidase was placed in a precolumn that could be switched in and out of the flow to the enzyme thermistor unit. Thus, both glucose and cellobiose present in the sample could be determined. When the β-glucosidase column was omitted, the glucose present initially in the sample was determined, and when the column was switched into the flow, the glucose formed by the degradation of cellobiose was also included. From these two glucose values the cellobiose concentration was easily calculated.

Materials and Methods

β-Glucosidase (EC 3.2.1.21, from sweet almonds) 40 units/mg (salicin, 25°C) was obtained from Boehringer Mannheim, W. Germany. Glucose oxidase (EC 1.1.3.4, from *Aspergillus Niger*, type V, 200 units/mg) and catalase (EC 1.11.1.6., cat. no. C-100, 30,000 units/mg) were purchased from Sigma Chemical Company (St. Louis, Mo., USA).

Salicin and cellobiose were from Merck Chemical Co. (Darmstadt, W. Germany). All other chemicals used were common analytical grade reagents.

Controlled pore glass, CPG 10-700, 80/120 mesh, mean pore diameter 729 Å (Electro-Nucleonics) was obtained from BDH Chemicals, England.

The split-flow enzyme thermistor used for glucose determinations was the same as that described in references *5–7*. It contained columns 7 cm in length with a diameter of 0.4 cm. The enzyme column was filled with immobilized glucose oxidase/catalase and the reference column with albumin coupled to controlled pore glass, giving the same protein concentration in both columns. Experiments were

also carried out with immobilized β-glucosidase present in the enzyme thermistor column, either alone or combined with glucose/catalase, the latter occupying the upper part of the column. The flow rate through each column was 0.8 mL/min and the waterbath temperature was 27.0° C during all experiments. The precolumn containing 1–2 mL of the β-glucosidase preparation was made of glass tubing, 4–5 mm in diameter. This was connected to the enzyme thermistor through a six-port valve (Rheodyne, Berkeley, CA, USA) for selection of either the precolumn or a bypass loop consisting of a piece of teflon tubing (50–100 cm long, 0.8 mm id) included to give flow resistance as does the column when it is switched in (Fig. 1). By this arrangement a constant flow through the enzyme thermistor was obtained, and simultaneously the flow through the precolumn was uninterrupted.

For continuous flow dialysis we used a Technicon 24-in. dialyzer (Technicon Instruments Corporation, New York, NY, USA) equipped with a premount dialysis membrane, type C. The dialyzer unit was incorporated into the flow line before the enzyme thermistor pump as shown in Fig. 1.

Enzyme thermistor determinations were carried out in 0.05*M* sodium citrate + 0.1*M* sodium phosphate, pH 5.5, with sodium azide or thymol as bacteriostatic agents. Optimum pH studies were performed in this buffer and in 0.1*M* sodium phosphate buffer, but without bacteriostatic agents.

Enzymes were coupled to aminopropyl-derivatized controlled pore glass after activation with glutaraldehyde as a couplying agent (*8*). β-Glucosidase (4 mL of a suspension containing 20 mg protein) was dialyzed against 0.2*M* potassium phosphate, pH 7.4, and added to 1.5 mL of sedimented, activated glass beads. The slurry was gently agitated in the cold for 48 h and then the preparation was extensively washed in the above citrate–phosphate buffer.

To the activated glass beads (650 mg) were added 2 mg of glucose oxidase and 1 mg of catalase in 2 mL of 0.1*M* potassium phosphate buffer, pH 7.0. After gently shaking the suspension overnight, 0.3 mL of 1*M* ethanolamine-HCL, pH 7.0, was added and incubated for 6 h at room temperature; the preparation was then thoroughly washed with the above buffer.

Determination of cellobiose and glucose concentrations in various fermentation broths was performed at the Gesellschaft für Biotechnologische Forschung (GBF),

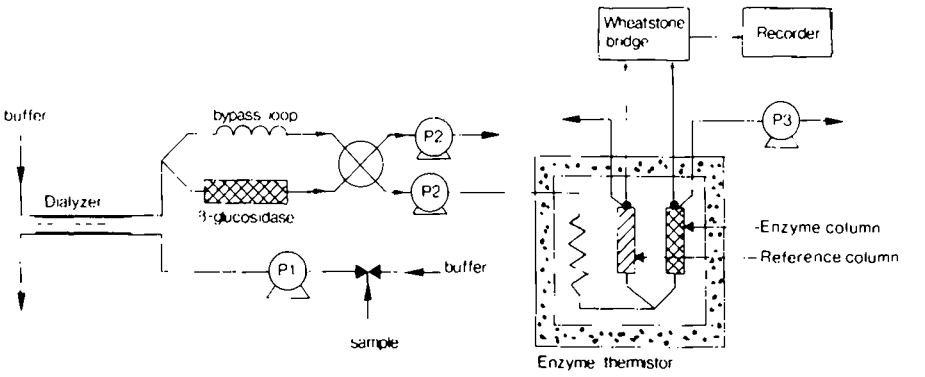


Fig. 1. Experimental arrangement including a split-flow enzyme thermistor. The dialysis unit was omitted in some experiments.

Braunschweig-Stoeckheim, W. Germany. Some experiments were also performed with lyophilized fermentation solutions obtained from GBF.

Results and Discussion

It became clear at an early stage in this investigation that the heat generated in the β-glucosidase reaction was insufficient for practically useful cellobiose determinations. Therefore it was considered necessary to use the above enzyme sequence.

The enzymes used in the cellobiose assay could be arranged in two different ways. The complete reaction sequence comprises the following steps:

$$(1)\ \text{Cellobiose} + H_2O \xrightarrow{\beta\text{-glucosidase}} 2\ \text{glucose}$$

$$(2)\ \text{Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{glucono-}\delta\text{-lactone} + H_2O_2$$

$$(3)\ H_2O_2 \xrightarrow{\text{catalase}} H_2O + (\tfrac{1}{2})\ O_2$$

The β-glucosidase could be contained in the enzyme thermistor column or it could be applied in a precolumn to the enzyme thermistor and in the latter instance higher enzymic activity could be used because a larger column could be employed. Since gluconolactone is a strong inhibitor of β-glucosidase (9), it is necessary to keep this enzyme separate from the glucose oxidase. In the enzyme thermistor column this was achieved by placing the β-glucosidase in the lower part of the column and using a nylon net to separate it from the glucose oxidase/catalase preparation. With the preparations of CPG-bound enzymes that were used a proportion of 5:2 in volume (β-glucosidase: glucose oxidase/catalase) gave optimal sensitivity.

Placing the β-glucosidase preparation directly in the enzyme thermistor leads to a simpler experimental arrangement. However, since the specific activity of the β-glucosidase available was low, it proved advantageous to use a precolumn, which could be made much larger than the enzyme thermistor column, thereby increasing the degree of cellobiose conversion. Furthermore, the use of a separate β-glucosidase column facilitates the analysis of samples containing glucose as well as cellobiose and with this arrangement the glucose concentration in the sample is easily determined by bypassing the precolumn. The cellobiose concentration is obtained from the difference in the glucose values registered with and without the precolumn.

pH Optimum

Since the enzymes used all have different pH optima for maximum activity, the pH dependence of the various reactions were studied in the following situations to find the best reaction conditions for the complete system:

(a) Using β-glucosidase only in the enzyme thermistor column the pH dependence of the first step was studied with salicin and cellobiose as substrates.

(b) Using glucose as substrate the pH dependence of the glucose oxidase/catalase-catalyzed reactions were studied with the enzyme thermistor charged with these enzymes.

(c) Using glucose oxidase/catalase in the enzyme thermistor and β-glucosidase in a precolumn the pH dependence of the complete system was studied with cellobiose as substrate.

(d) Using β-glucosidase and glucose oxidase/catalase in the proportion 5:2 in the enzyme thermistor column.

The results of these experiments are summarized in Fig. 2. The pH-optimum for the β-glucosidase reaction (Fig. 2a) was found to be very broad (pH 4–6) with both salicin and cellobiose as substrates, while the pH optimum for the glucose oxidase/catalase system was in the range of pH 6.5 (Fig. 2b). For the complete enzyme assembly (Fig. 2c) a broad maximum was obtained around pH 5 regardless of whether the immobilized β-glucosidase was placed inside (d) or before (c) the enzyme thermistor. This is about half a pH-unit higher than for the pure β-glucosidase reaction.

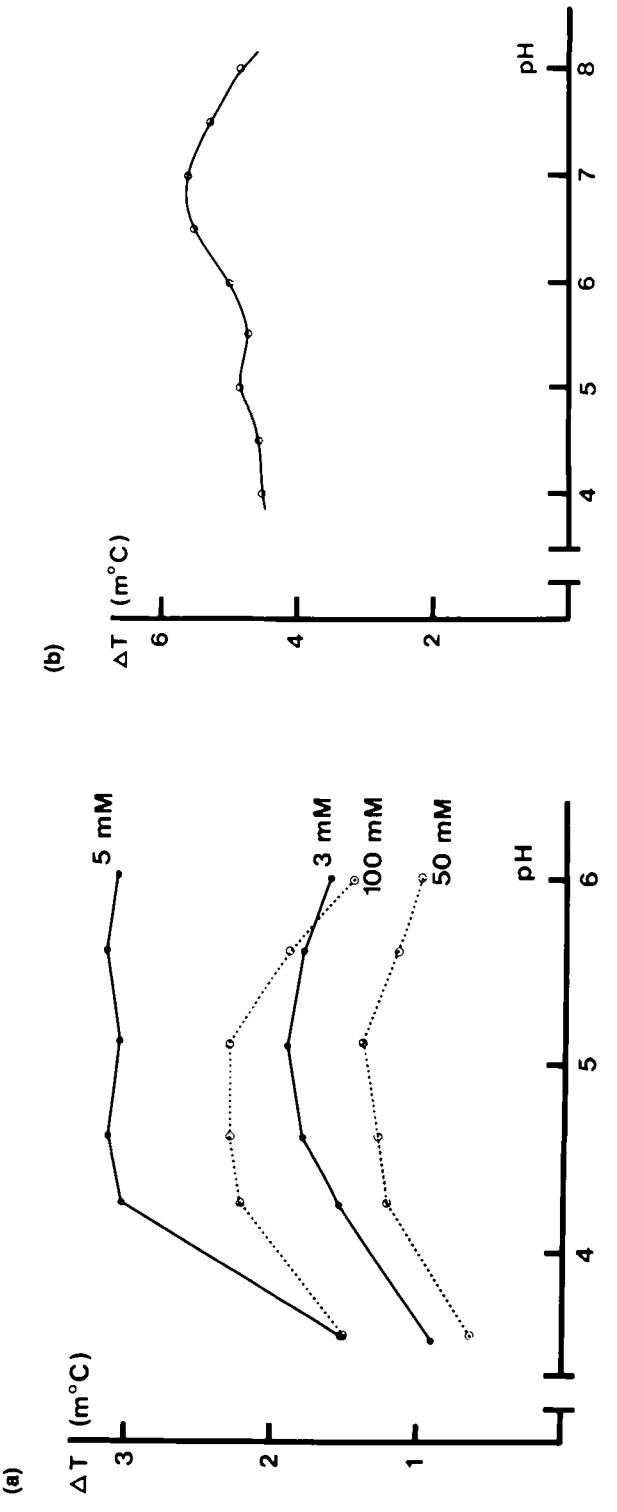
In situation (d) the pH dependence was studied in 0.1*M* Na-phosphate as well as in 0.1*M* Na-phosphate + 0.05*M* Na-citrate, the latter buffer having better buffering capacity at lower pH. The same pH-activity profiles were obtained, but a larger reaction heat was observed with the pure Na-phosphate buffer.

It should be noted that the pH activity profiles for two- or multistep enzyme systems arranged as discussed here do not show the true pH dependence of the enzyme sequence as such, but a merely operational pH profile of the entire system (*10*). It is known from earlier studies that proximity of sequential enzymes with separate pH optima may influence the pH profile of the whole co-immobilized enzyme sequence (*11*). Likewise, it was shown that local production or consumption of protons by one enzyme may severely influence the pH activity profiles of other proximal enzymes reacting simultaneously (*11*). Furthermore, on studying heavily loaded enzyme matrices, as often is the case in analytical applications, it must be realized that the characteristics measured relate only to the operating fraction of all enzyme molecules. The reaction rate will in such cases be strictly dependent upon diffusion of substrate. A system with such an excess of latent catalytic capacity will behave as if the observed catalytic activity was almost insensitive to changes in the external medium. On changing pH so that the activity of the enzyme molecules exposed to substrate decreases, the heretofore redundant enzyme molecules will now be exposed to substrate, resulting in a constant conversion of substrate (*12*). The observed broadened pH activity profile of the system may be explained by the above factors, among which the last probably plays the most important role.

Most of the experiments described here have been carried out with a buffer system of 0.1*M* Na-phosphate + 0.05*M* Na-citrate, pH 5.5, containing thymol as bacteriostatic agent.

Standard Curves

The temperature response of the β-glucosidase and glucose oxidase/catalase reactions was measured separately and in combination using aqueous standard solutions of various substrates. A linear relationship between temperature signal and glucose concentration was obtained with a glucose oxidase/catalase enzyme thermistor up to a glucose concentration of 0.7 m*M* (Fig. 3a). At higher concentrations the curve loses linearity because of oxygen deficiency. The standard curve


(a)
ΔT (m°C)
3
2
1
5 mM
3 mM
100 mM
50 mM
pH
4
5
6
(b)
ΔT (m°C)
6
4
2
pH
4
5
6
7
8

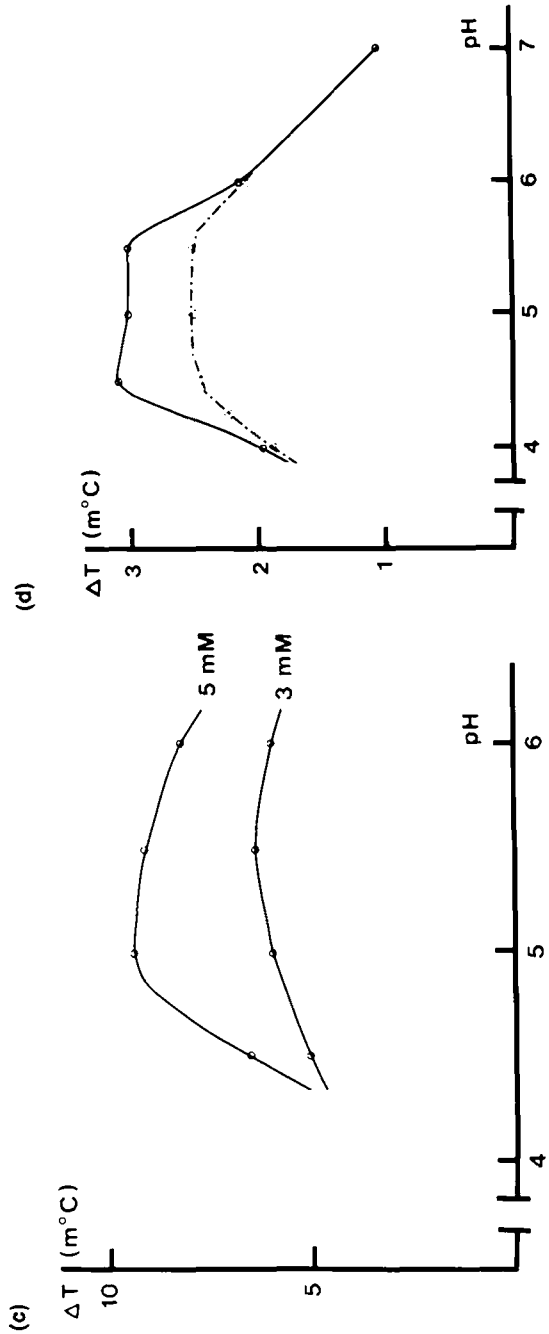
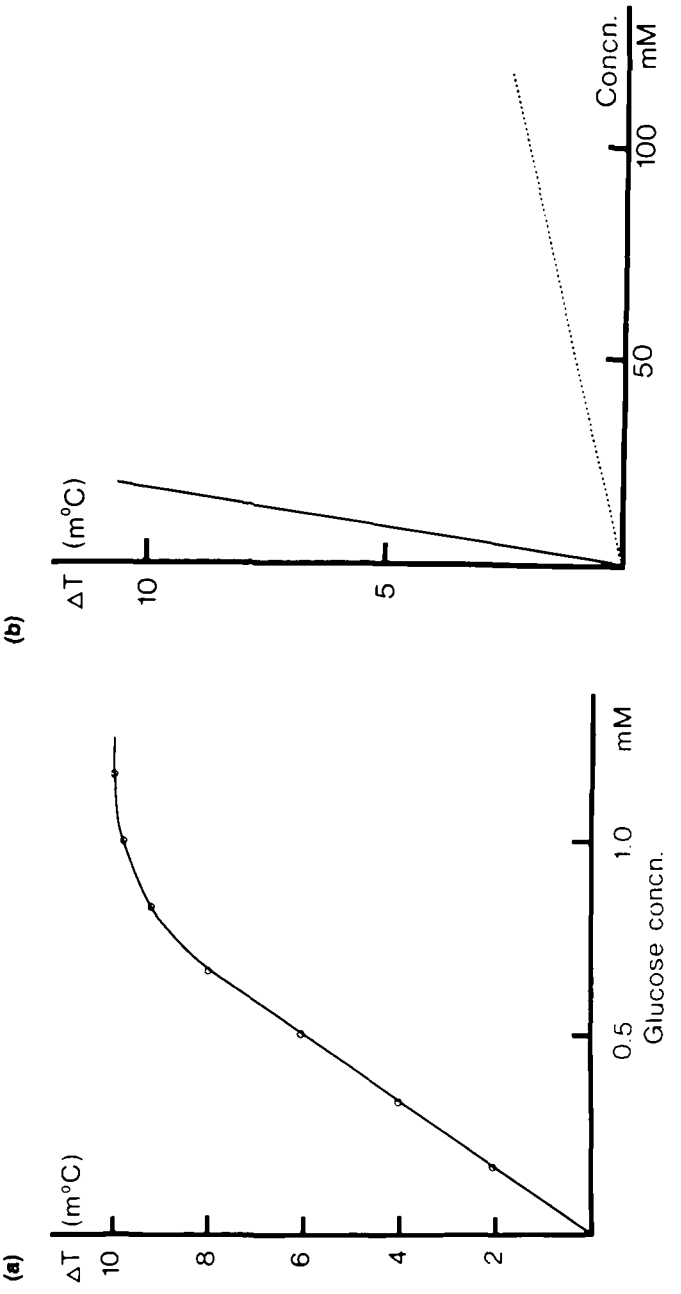


Fig. 2. pH Activity profiles registered as ΔT of (a) immobilized β-glucosidase using cellobiose (----) and salicin (——) as substrate; (b) co-immobilized glucose oxidase and catalase with 0.6 m*M* glucose as substrate; (c) a precolumn containing immobilized β-glucosidase and glucose oxidase/catalase in the enzyme thermistor. The substrate concentrations were 3 and 5 m*M* cellobiose; (d) immobilized β-glucosidase and co-immobilized glucose oxidase/catalase sequentially arranged in the column in the proportions 5:2. The substrate was 1.0 m*M* cellobiose and the buffer was 0.1 *M* Na-phosphate buffer (⊙) and/or 0.05 *M* Na-citrate/0.1 *M* Na-phosphate buffer (△). The buffer used in a–c was 0.1 *M* Na-phosphate and the sample pulse length was 1 min in all cases.


(a)
ΔT (m°C)
10
8
6
4
2
0.5
1.0
mM
Glucose concn.
(b)
ΔT (m°C)
10
5
50
100
mM
Concn.

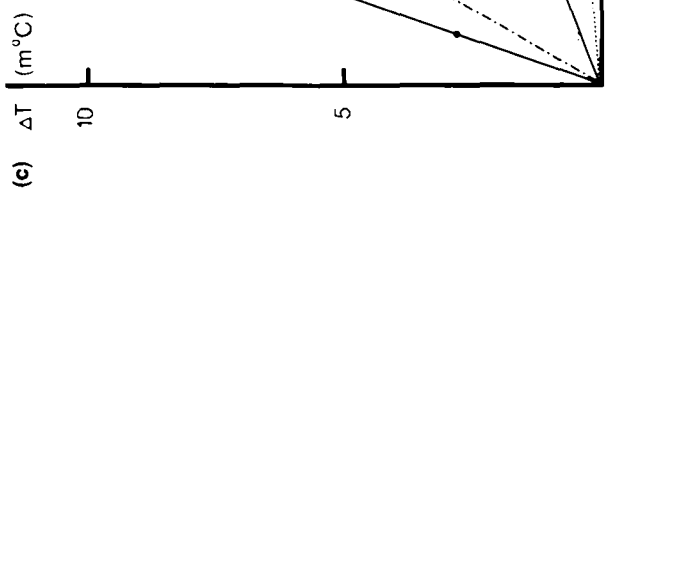


Fig. 3. Standard curves obtained with the following arrangements: (a) glucose oxidase/catalase thermistor; (b) β-glucosidase thermistor with aqueous standards of salicin (——) and cellobiose (----); (c) precolumn with β-glucosidase and glucose oxidase/catalase thermistor. Cellobiose in the concentration range 0–10 m*M* (—o—); cellobiose in the range 0–1 m*M* (—o—). Shown for comparison are the response curves for glucose with this system (----) and for cellobiose on a β-glucosidase thermistor (····). The buffer was 0.1 *M* Na-phosphate + 0.05 *M* Na-citrate, pH 5.5, and the sample pulse length was 1 min in all experiments.

was obtained with 1 mL glucose pulses; similar curves are obtained if larger sample pulses are used (*6*). No temperature response was observed when cellobiose (≤ 100 m*M*) was introduced as sample in glucose oxidase/catalase-containing enzyme thermistor. This is important for the discrimination between glucose and cellobiose when they are simultaneously present in the sample.

Linear correlation between the temperature signal and substrate concentration was also obtained with β-glucosidase thermistor both with cellobiose and salicin as substrates (Fig. 3b). Cellobiose was applied in concentrations up to 100 m*M* and salicin up to 10 m*M*. It can be seen, as already mentioned, that the hydrolysis of cellobiose is accompanied by very limited heat production. Salicin, on the other hand, appears to be a much better substrate than is cellobiose and its reaction is much more exothermic. We also investigated the behaviour of the β-glucosidase thermistor with glucose samples. No reaction was obtained with the enzyme purchased from Boehringer, whereas enzyme preparations from other companies were generally found to contain considerable amounts of glucose oxidase.

Figure 3c shows the temperature response for the complete thermistor system. A precolumn containing β-glucosidase was applied before the enzyme thermistor that contained glucose oxidase/catalase. There is good linearity for cellobiose concentrations below 3–4 m*M*, but at higher values the limited linear range of the glucose oxidase/catalase system determines the shape of the curve. The standard curve for glucose samples applied to the same system is shown for comparison. Also shown in Fig. 3c is a temperature response curve for cellobiose obtained with β-glucosidase only in the enzyme thermistor to give an idea of the high gain in sensitivity that is obtained by introducing the glucose oxidase/catalase step.

Pulse Length

The temperature peaks will be broader and lower when the enzyme thermistor is preceded by the β-glucosidase column because of the longer flow path. For glucose samples run through the enzyme thermistor with and without the precolumn the difference in temperature response was about 10% at a pulse length of 1 min; at longer pulses, however, the difference was less. In order to get a constant response for glucose, a piece of capillary teflon tubing of suitable length was mounted so that it could be switched into the flow path by a valve when the β-glucosidase column was not used (see Fig. 1).

The measurements described so far have been carried out with 1-min pulses of substrate. This time is too short to lead to thermal steady state especially when a precolumn is used. Much higher sensitivities can thus be obtained if longer substrate pulses are applied (*13*). Therefore, a pulse length of at least 5 min was used when the sample supply allowed it. There is one important advantage that follows from the use of a long pulse—that is, when there are substances present in the sample that could give rise to unspecific heat effects, such as dilution and solvation heats, etc., these effects are normally noticeable only at the ends of a sample "plug." Thus, if the sample pulse is long enough, the signal observed in the middle part of the heat signal will represent the true heat of the reaction. Use of a reference column as in the split-flow enzyme thermistor (*7*) used here can also eliminate this problem although at the expense of a more complex apparatus.

Enzyme Thermistor Measurement of Fermentation Solutions

Samples were taken from laboratory to pilot plant-scale fermentations of newspaper-waste or avicel-cellulose using various microorganisms with and without cellulytic enzymes added. Such samples contained so much particulate matter that a pretreatment step had to be made before analysis and usually a low-speed centrifugation step (5 min at 1000*g*) was employed. To avoid unspecific heat signals owing to dilution heat and similar effects, it is important that the sample in enzyme thermistor analysis has the same buffer composition as the perfusing medium that is continuously pumped through the unit. In this case, the perfusing buffer had a composition resembling that of the culture medium as far as inorganic components were concerned.

A continuous dialyzer incorporated in the system (Fig. 1) eliminated all unspecific effects and at the same time cut off larger molecules and microorganisms that might affect the performance of the enzyme thermistor. The amount of low molecular weight components crossing the dialysis membrane was found to be in the order of 5–15%. Glucose and cellobiose had different permeabilities and typical values were 11% for glucose and 8% for cellobiose. There were some variations in these figures, so that calibration had to be performed regularly, preferably daily.

Alternatively, the samples could be diluted 5–10 fold (which is about the same or lower dilution as that obtained in the dialyzer, before introducing them into the enzyme thermistor) and here a useful measure was to pass the samples through a sterile filter to remove particles and microorganisms.

The use of the dialyzer increased the pulse length required for thermal steady state somewhat, but there was no difference in temperature deviation for 5 and 10 min pulses and 3 min pulses gave only 10% lower values, so that this was not of any practical importance. Figure 4 shows standard curves for cellobiose obtained

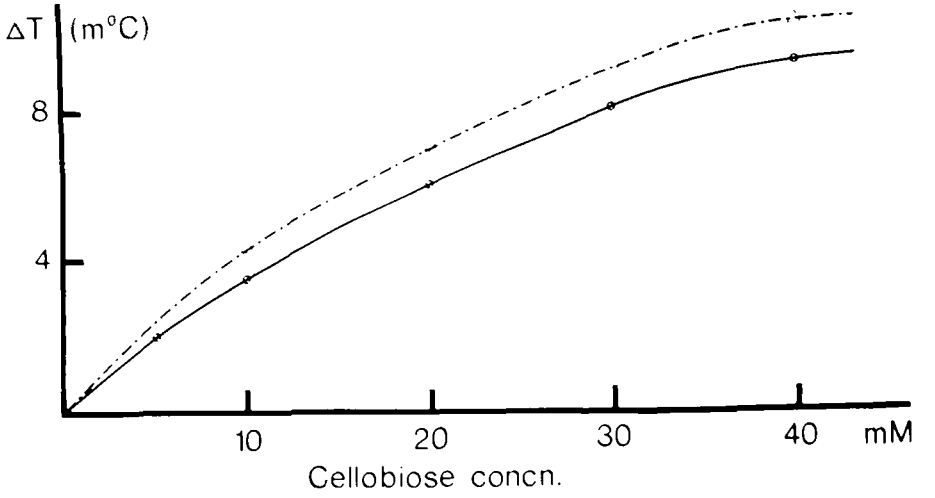


Fig. 4. Response curve for the complete assembly including the dialyzer. Two different pulse lengths were used: (··Δ··) 10 min and (—o—) 3 min. The buffer was 0.1 *M* Na-phosphate + 0.05 *M* Na-citrate, pH 5.5.

with 3 and 10 min pulses. The relationship between sample concentration and temperature deviation appears to be logarithmic when the dialysis step is included and the curves obtained have a logarithmic shape. When the temperature response versus the logarithm of cellobiose concentration was plotted, a straight line was obtained (Fig. 5); similar results were obtained for glucose samples.

Figure 6 shows the recorder trace of an experiment in which both cellobiose and glucose were measured. First, the glucose signal is recorded and then the precolumn is switched into the sample flow and the temperature rose further corresponding to the glucose formed from cellobiose. In this experiment the β-glucosidase column was bypassed again with the sample flow for an additional 5 min, but the normal procedure was to go back to buffer at this point and allow the thermistor response to resume the base line. The total time required for sample measurement could be further reduced by carrying out a steady state reading for glucose only (5 min) and having the β-glucosidase switched in just for a short pulse (1 min) although steady state measurements give better resolution and higher accuracy.

The different permeabilities of glucose and cellobiose in the dialyzer and the incomplete conversion of cellobiose to glucose both imply that separate standards have to be run for glucose and cellobiose. In Fig. 7, standard curves for glucose and for cellobiose are plotted together with a standard curve for cellobiose samples also containing 0.1 m*M* glucose. Since this experiment was performed by a split-flow enzyme thermistor without a dialyzer, these standard curves are straight lines. By varying the cellobiose concentration at different glucose concentrations, it was found that the heat signal corresponding to a certain cellobiose concentration was the same irrespective of the level of the background glucose as long as the total glucose concentration was within the linear region of the standard curve for the glucose oxidase/catalase reactions. Thus, only two standard curves were required,

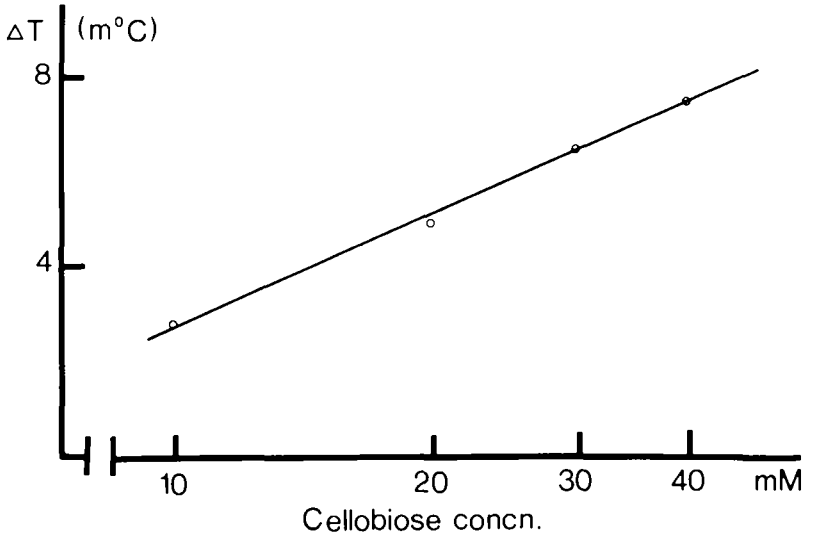


Fig. 5. Thermal response as a function of cellobiose concentration. The concentration scale is logarithmic. The pulse length was 10 min.

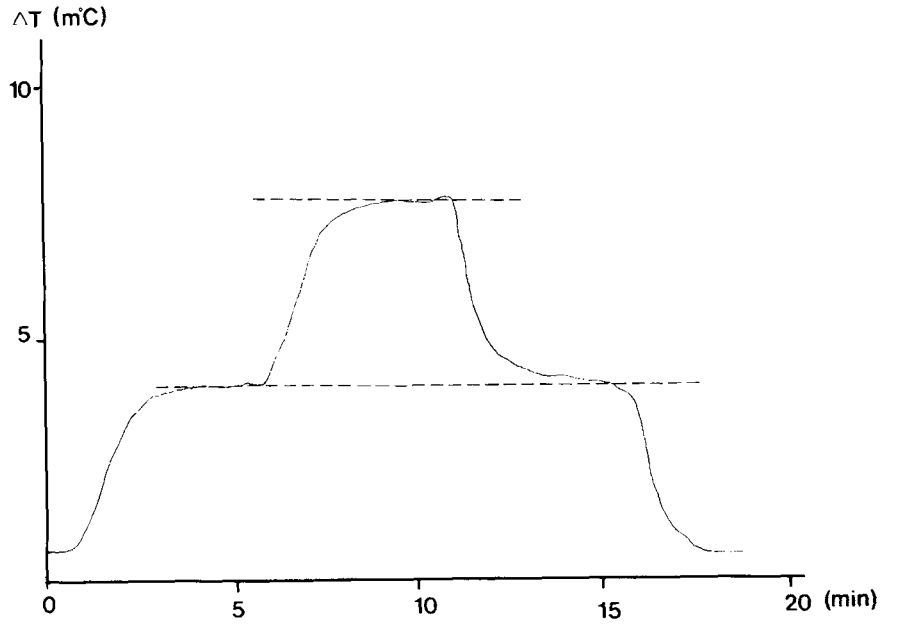


Fig. 6. Temperature recording from a typical experiment with a sample containing both glucose and cellobiose. The β-glucosidase column was connected for 5 min and the total sample introduction time was 15 min.

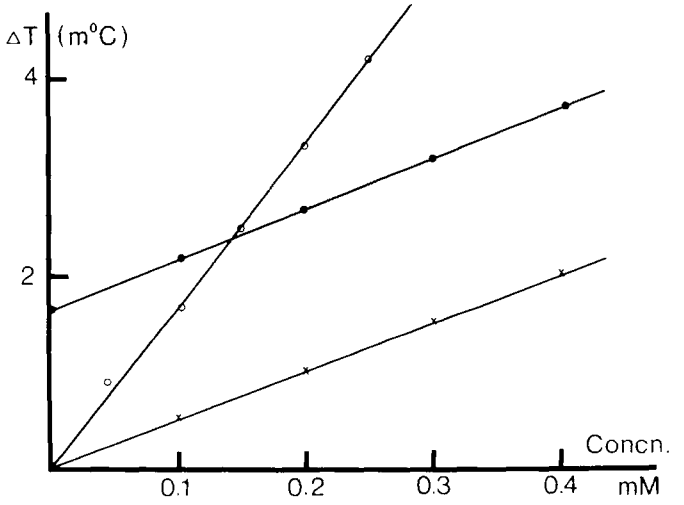


Fig. 7. Standard curves from a fermentation broth standard containing both glucose and cellobiose. Glucose response at steady state (—o—) and overall response with initial glucose (0.1m*M*) plus glucose from cellobiose hydrolysis (—●—) using the β-glucosidase precolumn for 3 min. As reference is shown a calibration curve for cellobiose alone (—x—).

one for the background glucose and one for the glucose generated from cellobiose.

We carried out preliminary tests on fermentation solutions both with and without the dialyzer and the lowest cellobiose concentration that could be determined with an accuracy of ± 10% was 0.1 m*M* without dialyzer and 0.5 m*M* with it, but in

the first case the system was more susceptible to clogging. The operational stability of the complete system was very good if the samples were purified (i.e., passed through sterile filter) or if the dialyzer was used. There was a slight decrease in activity during the first week of daily use, but after that the activity remained constant for several weeks.

Owing to the complex composition of the samples, the precision of the method was less than that reported earlier for glucose (*5*), although generally the within-day precision was better than ± 5% (RSD). Furthermore, the addition of known glucose and cellobiose concentrations to fermentation solutions were accounted for by 98–102%.

Concluding Remarks

A general problem in multistep enzymic analysis is the optimization of the reaction conditions, especially where the enzymes have different pH optima. As is seen from the results presented here, the pH activity profile of a system containing more than one enzyme is influenced by the activity of each separate enzyme. As discussed elsewhere (*11*) the pH activity profile of a co-immobilized enzyme system can, within certain limits, be varied at will by changing the ratio between the participating individual enzyme activities. Where the individual enzymes have clearly separated pH optima, an optimization of the system by changing the ratio of the individual enzymes may be advantageous. We found empirically that a ratio of 2:5 between glucose oxidase/catalase and β-glucosidase, measured as the volume of CPG-bound enzyme (corresponding to a ratio of 0.013:1 in applied activity), was sufficient when all the enzymes were placed in the enzyme thermistor column. It was, however, found that a much higher sensitivity was obtained by placing the β-glucosidase outside the thermistor unit and using a maximum amount of this enzyme bound to CPG.

Another point worth consideration is the treatment of the sample prior to analysis. The use of a dialyzer is in many respects a highly satisfactory solution, since it efficiently eliminates components that may interfere with the analysis and at the same time automatically brings the sample into the correct perfusing medium; it can also be used continuously for on-line measurements. It may, however, be difficult to obtain constant permeability of glucose and cellobiose over longer periods of time since different components have different permeabilities. This explains why a careful calibration with both glucose and cellobiose standards was carried out in this study.

The other method for treating a crude sample used here is to dilute it. This is a simpler procedure and it gives a sample of approximately the correct buffer composition and consequently the sample does not cause any unspecific heat effects. Provided that the sample does not contain too much particulate matter or if a filter or a centrifugation step are used and the dilution factor is reasonably high, the CPG-preparation can last a long time (several weeks of daily use) before clogging. An alternative procedure, if not too large amounts of enzymes are required, is to use chemically modified nylon tubing as an enzyme support (*14, 15*) since this support

is superior to CPG in its ability to withstand clogging in crude sample solutions although the sensitivity is lower.

Enzymic specific methods for cellobiose determination which can be used for continuous measurements such as the one discussed here, are of great interest in studies of enzymic and microbial cellulose degradation, especially in studies of mixed culture fermentations and in work with free cellulases. Most of the currently used methods are either complex and time-consuming, requiring sophisticated equipment, or unspecific, or difficult to use continuously. The present method is simple and the equipment required is less complicated than, e.g., that in an HPLC apparatus. The sensitivity is quite sufficient for many studies and in addition to determination of cellobiose, the amount of glucose present in the sample can also be obtained. However, in fermentations of cellulose, the cellobiose concentration may very well be below 0.1 m*M* and, therefore, the sensitivity of this method is too low. Work is, however, in progress to increase the sensitivity of the method; primarily we are attempting to achieve a more complete hydrolysis of the cellobiose.

Acknowledgments

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